ARTICLES

FA Composition of Cholesteryl Esters and Phospholipids in Maternal Plasma During Pregnancy and at Delivery and in Cord Plasma at Birth

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ABSTRACT: The purpose of this study was to assess the FA composition of both cholesteryl esters (CE) and phospholipids (PL) in maternal plasma during pregnancy and at delivery and in umbilical plasma at birth. A longitudinal study of 32 normal pregnant women was carried out with three cutoff points during pregnancy (first, second, and third trimester) and at delivery. Few significant differences occurred in the FA profile of maternal CE: 18:1n-9 increased, 18:2n-6 dropped slightly, and 18:3n-3 decreased with progressing gestation. In maternal PL, long-chain highly unsaturated FA concentrations dropped and were replaced by saturated FA as gestation progressed. Additionally, changes in saturated FA in PL occurred: Shorter-chain 16:0 was higher whereas longer-chain 18:0 was lower at delivery compared to early pregnancy. The FA profile of umbilical venous plasma was strikingly different from that of maternal plasma at delivery. Cord plasma CE contained more saturated and monounsaturated FA than maternal CE. The polyunsaturates 18:2n-6 and 18:3n-3 are lower in umbilical CE than in maternal CE whereas 20:4n-6 and 22:6n-3 are twice as high in umbilical CE. Cord plasma PL have a higher content of long-chain highly unsaturated FA than maternal plasma PL at delivery. In contrast to maternal plasma PL, 16:0 was lower and longer-chain saturated FA were higher in cord plasma PL. The FA profile of umbilical plasma at birth shows preferential accumulation of 20:4n-6 and 22:6n-3, with low concentrations of 18:2n-6 and 18:3n-3 in CE and PL, indicating a preferential supply of the fetus with long-chain highly unsaturated FA needed for fetal development.

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The two most important families of long-chain FA for human beings are the n-6 and n-3 families. Linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) can be desaturated and elongated to form long-chain PUFA, which play a major role in the development of new life as important structural components of cell membrane phospholipids (PL) (1,2). During pregnancy, accretion of maternal, placental, and fetal tissue occurs. Therefore, the requirement for PUFA is high for pregnant women and the developing fetus. Arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3) are important structural FA in neural tissue such as the brain and retina (3,4). The FA 18:2n-6, 18:3n-3, 20:4n-6, and 22:6n-3 are conditionally indispensable FA for fetuses, and pregnant and lactating women (5). Cunnane (5) introduced the classification of conditionally indispensable and conditionally dispensable FA instead of EFA (5).

Several authors have analyzed the FA composition of maternal plasma PL throughout pregnancy and of umbilical plasma PL at birth (6–12). Few studies have described the FA composition of cholesteryl esters (CE) in maternal and umbilical plasma at delivery (13–16). The FA composition of maternal plasma CE on three different occasions during the course of pregnancy and at delivery has, to our knowledge, never been described before.

In a previous study we reported a number of changes in the composition and the calculated mean melting point (MMP) of FA in plasma PL throughout pregnancy in a small study population (n = 16) (7). We found that the MMP of maternal PL was significantly higher at delivery compared to mid-gestation due to a loss of highly unsaturated FA, which were replaced by saturated FA (SFA). In addition, changes in SFA occurred: The content of 16:0, with a lower melting point (MP), was higher while 18:0, with a higher MP, was lower at delivery (7). In contrast to maternal plasma, 16:0 was lower in umbilical plasma while the longer chain SFA were higher, tending to raise the overall MMP.

The aim of the present work was to confirm previous findings in a larger study population and to assess whether similar changes occur in the FA profile of plasma CE. Furthermore, the relationship between the FA in the PL and the CE fractions was investigated.

SUBJECTS AND METHODS

Study population. Healthy pregnant women attending the Department of Gynecology of Ghent University Hospital, Belgium, were asked to cooperate in this study. All pregnant volunteers signed a written informed consent form, approved by the ethics committee of Ghent University Hospital. Inclusion criteria were: singleton pregnancy, nullipara, term delivery (38–42 wk), normotensive (diastolic blood pressure below 90 mm Hg). We excluded women (i) who were diagnosed with gestational diabetes mellitus; (ii) who had signs of proteinuria; and (iii) who suffered from renal or cardiovascular disease. We did not exclude women who delivered through a Caesarean section (n = 2) because no important differences in the maternal FA composition of serum PL was found between mothers who had a normal vaginal delivery and mothers who

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Abbreviations: CE, cholesteryl esters; HUFA, highly unsaturated FA; MCL, mean chain length; MMP, mean melting point; MP, melting point; MUFA, monounsaturated FA; PL, phospholipids; SFA, saturated FA.

had a Caesarean section (16). From the 39 pregnant women who entered the study, 34 completed the study. Five women failed to finish the study owing to lack of motivation. Two pregnant women delivered preterm and were excluded from the study. The study population thus consisted of 32 healthy pregnant women and their neonates (13 girls and 19 boys).

Maternal venous blood samples were obtained thrice during the course of pregnancy: (i) between 5 and 14 wk of gestation (median 12 wk), (ii) between 20 and 24 wk (median 22 wk), and (iii) between 29 and 37 wk (median 32 wk) and (iv) at delivery (median 39.5 wk). Umbilical venous blood was collected immediately after the cord had been clamped. Blood was collected in EDTA-containing Vacutainer tubes (Belliver Industrial Estate, Plymouth, United Kingdom) and temporarily stored at 6°C. Within 24 h of collection, plasma was separated from blood cells by centrifugation ($600 \times g$ for 5 min at 4°C) and stored in plastic tubes under nitrogen at –80°C until further analysis.

FA analysis of plasma CE and PL. All samples of a given mother–infant pair were analyzed simultaneously. Lipids were extracted from 1 mL plasma according to a modified Folch *et al.* extraction with methanol/chloroform (1:2) (17). The lipids were separated by TLC on rhodamine-impregnated silica gel plates using petroleum ether (b.p. 60–80°C; Merck Belgolab, Overijse, Belgium)/acetone 85:15 as mobile phase (18). The CE and PL fractions were scraped off and the FA converted into methyl esters by transesterification with 2 mL of a mixture of methanol/benzene/HCl (aqueous, 12 N) (80:20:5) (19). After cooling and adding 2 mL of water, FAME were extracted with petroleum ether (b.p. 40–60°C), evaporated to dryness under a nitrogen flow at a temperature not exceeding 40°C, and analyzed by temperature-programmed capillary GC (Varian Model 3500) on a 25 m × 250 μm (length × i.d.) × 0.2 μm df Silar 10C column (19). The injection and detection temperatures were set at 285°C. The starting temperature of the column was 150°C, which was increased to 240°C after 3 min at a rate of 2°C/min. The carrier gas was nitrogen with a flow of 25 cm/s. Peak identification was performed by spiking with authentic standards (Sigma-Aldrich, Bornem, Belgium). Peak integration and calculation of the percent composition was performed electronically with a Varian Model 4290 integrator. The coefficient of intra-assay variation of the entire method of FA analysis was less then 5%.

The results are expressed as weight percentage (wt%) of total FA. Twenty-six different FA with chain lengths between 14 and 24 carbon atoms were identified. The sum of all the SFA (∑SFA); the monounsaturated FA (∑MUFA); the PUFA (∑PUFA); the long-chain highly unsaturated FA (∑HUFA = FA with 20 or more carbon atoms and with at least three double bonds); ∑n-3; ∑n-6; and ∑*trans* FA were calculated and are reported together with the individual FA. The mean melting point (MMP, °C) (sum of the mole fraction multiplied by the MP for each FA) and the mean chain length (MCL) (sum of the mole fraction multiplied by the number of carbon atoms in the FA) of the plasma lipids were assessed (7).

Statistical analysis. Values are reported as mean and SD in parentheses. The normality of distribution was ascertained with the Kolmogorov–Smirnov test. The FA (wt%) that had a skewed distribution were log-transformed for the statistical analyses of these variables. Group mean differences were assessed by means of ANOVA. Repeated-measures ANOVA was used to test for significant differences in the FA composition of maternal serum PL and CE during the course of pregnancy. The ANOVA model included only a time factor (FA data thrice during the course of pregnancy and shortly after delivery). A paired Student's *t*-test was performed for maternal-umbilical FA comparisons at delivery and birth. In order to avoid type 2 errors, due to multiple comparisons, a value of $P < 0.005$ was taken as the criterion of significance. The degree of association was calculated using the Spearman rank correlation. The data were analyzed using both SPPS (version 10.0 for Windows; SPSS Inc., Chicago, IL) (20) and the MedCalc statistical program (version 6; MedCalc Software, Mariakerke, Belgium) (21). For 4 of our 32 subjects, we were unable to obtain a complete set of matching plasma PL or CE data for maternal and umbilical blood samples. This accounts for the various sample sizes in Tables 1 and 2. However, all repeated-measures ANOVA or paired *t*-tests were made with matching samples.

RESULTS

Clinical characteristics. The mean age of the mothers (n = 32) at delivery was 29 yr (range 21–41 yr). The mean body mass index of the women before pregnancy was 23.6 (range 17.6–35.6). All the infants were born healthy with a mean birth weight of 3155 g (range 2300–4020 g) and a mean crown–heel length of 50.2 cm (range 48–52 cm).

The FA composition of maternal plasma CE and PL (wt%) during the course of pregnancy and at delivery and from umbilical plasma shortly after birth are summarized in Tables 1 and 2.

FA composition of maternal plasma lipid classes. (i) CE. The fraction of the individual SFA remained stable in maternal CE during pregnancy. The major monounsaturate, oleic acid (18:1n-9), and as a consequence ∑MUFA, significantly increased with progressing gestation. Linoleic acid with more than 50 wt% of the FA was the major FA of the CE fraction. The acid 18:2n-6 slightly decreased with progressing gestation. On the other hand, α-linolenic acid increased slightly with progressing gestation and reached a maximum during the third trimester. ∑PUFA significantly decreased in maternal CE during pregnancy. None of the long-chain highly unsaturated acids changed significantly in maternal CE. When we compare maternal values from the first trimester with delivery values, more significant differences were found in CE: 20:4n-6, 20:5n-3, ∑n-6, and ∑HUFA were significantly ($P <$ 0.001) lower at delivery. Neither the MMP nor the MCL of the FA in maternal plasma CE changed significantly with progressing gestation.

(ii) PL. ∑HUFA significantly decreased while ∑SFA significantly increased during gestation. ∑PUFA slightly

TABLE 1
Composition (wt% of total FA) of FA in CE Isolated from Maternal Venous Plasma During the Course of Pregnancy and at Delivery and from Umbilical Venous Plasma at Birth[a]: Mean and SD

	Trimester I (n = 32)		Trimester II (n = 28)		Trimester III (n = 29)		Delivery (n = 30)		RM ANOVA	Umbilical cord (n = 28)		Paired t-test
CE	Mean	SD	Mean	SD	Mean	SD	Mean	SD	(P)	Mean	SD	(P)
14:0	0.84	0.24	0.83	0.24	0.77	0.20	0.77	0.28	0.03	1.20	0.58	0.001‡
15:0	0.24	0.05	0.22	0.08	0.22	0.09	0.29	0.41	0.73	0.43	0.30	0.04
16:0	11.73	1.34	11.83	1.31	11.74	1.32	12.04	1.93	0.18	19.63	3.23	0.0001‡
17:0	0.15	0.12	0.13	0.19	0.13	0.17	0.13	0.13	0.32	0.80	1.32	0.01
18:0	0.97	0.72	0.82	0.50	0.83	0.71	0.70	0.45	0.30	3.92	2.63	0.0001‡
∑SFA	14.28	2.22	14.11	1.91	14.00	2.36	14.29	2.82	0.05	27.05	6.03	0.0001‡
16:1n-9	0.35	0.17	0.33	0.15	0.30	0.16	0.38	0.32	0.59	1.20	1.07	0.0001‡
16:1n-7	2.62	0.90	2.84	1.46	3.00	1.16	3.93	1.66	0.04	5.49	2.55	0.02
18:1n-9	15.96	2.24	16.65	2.37	17.31	2.34	18.58	2.31	0.0001*	24.28	6.29	0.0001‡
18:1n-7	0.70	0.55	0.62	0.46	0.55	0.52	0.58	0.59	0.28	1.95	1.15	0.0001‡
∑MUFA	19.82	2.75	20.56	3.44	21.35	2.92	23.72	3.26	0.0001*	33.89	7.12	0.0001‡
16:2n-6	0.09	0.09	0.08	0.10	0.10	0.10	0.11	0.13	0.62	0.42	0.54	0.007
18:2n-6	55.00	4.74	54.90	5.06	54.68	4.54	52.44	5.97	0.01	20.18	6.66	0.0001‡
18:3n-6	0.37	0.27	0.28	0.25	0.27	0.28	0.41	0.29	0.20	0.41	0.35	0.99
20:3n-6	0.74	0.24	0.79	0.21	0.79	0.17	0.74	0.19	0.45	1.37	0.86	0.001‡
20:4n-6	6.79	1.62	6.42	1.30	5.87	1.16	5.59	1.50	0.05	11.31	4.18	0.0001‡
∑n-6	63.09	4.37	62.53	4.78	61.84	3.92	59.45	5.55	0.01	34.27	7.50	0.0001‡
18:3n-3	0.55	0.25	0.63	0.23	0.69	0.19	0.62	0.14	0.009	0.26	0.25	0.0001‡
18:4n-3	ND		ND		ND		ND		ND	0.25	0.26	0.0001‡
20:5n-3	0.59	0.32	0.63	0.50	0.47	0.24	0.38	0.26	0.02	0.41	0.33	0.80
22:5n-3	0.09	0.13	0.09	0.12	0.11	0.12	0.13	0.19	0.42	0.23	0.23	0.10
22:6n-3	0.76	0.33	0.80	0.27	0.74	0.33	0.61	0.35	0.09	1.24	1.12	0.01
∑n-3	2.01	0.73	2.18	0.75	2.04	0.50	1.75	0.63	0.04	2.40	1.27	0.04
20:3n-9	ND		ND		ND		ND		ND	0.21	0.14	0.0001‡
∑PUFA	65.15	4.10	64.73	4.47	63.91	3.82	61.23	5.45	0.005*	36.88	8.03	0.0001‡
∑HUFA	9.09	1.83	8.79	1.69	8.08	1.35	7.58	1.98	0.03	15.22	5.63	0.0001‡
∑*trans* FA	0.47	0.21	0.43	0.19	0.49	0.26	0.50	0.22	0.67	1.08	1.01	0.007
MMP (°C)	5.36	1.88	5.43	1.61	5.81	1.79	6.42	2.49	0.21	14.84	5.00	0.0001‡
MCL	17.82	0.05	17.80	0.06	17.79	0.04	17.76	0.09	0.04	17.67	0.22	0.04

[a]RM, repeated measurements; ND, not detectable; ∑SFA, sum of the saturated FA; ∑MUFA, sum of the monounsaturated FA; ∑PUFA, sum of PUFA; ∑HUFA, sum of the long-chain highly unsaturated FA (FA with 20 or more carbon atoms and with at least three double bonds); ∑*trans* FA, sum of the *trans* FA;MMP, mean melting point (sum of the mole fraction multiplied by the melting point for each FA); MCL, mean chain length (sum of the mole fraction multiplied by the number of carbon atoms in the FA).
*Significantly different according to repeated measurements ANOVA (maternal values during pregnancy); ‡significantly different according to paired Students' t-test (comparison between maternal values at delivery and umbilical values at birth).

decreased and ∑MUFA slightly increased throughout gestation, but significance was not reached. Palmitic acid (16:0) and stearic acid (18:0), the two major saturates, changed significantly but in opposite directions: 16:0 increased while 18:0 fell during pregnancy. The long-chain 24:0 also dropped significantly during gestation. Few of the individual n-6 or n-3 FA differed significantly with progressing gestation. Arachidonic acid (20:4n-6) declined, but linoleic acid remained stable. In the series of n-3 FA, only 22:5n-3 significantly dropped during pregnancy. When we compare maternal values from the first trimester with delivery values, more significant differences were found in PL: 20:4n-6, 20:5n-3, and 22:5n-3 were significantly ($P < 0.001$) lower at delivery. The MMP of the FA of maternal plasma PL significantly rose with progressing gestation and their MCL significantly decreased.

Maternal-umbilical FA comparisons at delivery and birth. (i) CE. ∑SFA and ∑MUFA were significantly higher in cord plasma CE compared to maternal plasma CE. The two major saturates, 16:0 and 18:0, and oleic acid were much higher in umbilical CE. ∑PUFA were significantly lower (36.9 vs. 61.2 wt%), whereas ∑HUFA were significantly higher in cord plasma CE. The only two FA that were lower in umbilical CE compared to maternal CE were 18:2n-6 and 18:3n-3. Linoleic acid was extremely low in cord plasma (20.2 vs. 52.4 wt%). Arachidonic acid, on the other hand, was twice as high in the neonate as in the mother (11.3 vs. 5.6 wt%). Similarly, 22:6n-3 was also twice as high in cord plasma (1.24 vs. 0.61 wt%). Mead acid (20:3n-9), the marker of combined linoleic and α-linolenic acid deficiency, was significantly higher in the neonate. The MMP of the FA in umbilical plasma CE was significantly higher than the MMP of the maternal plasma CE-associated FA.

(ii) PL. As we found in the CE fraction, ∑SFA and ∑HUFA were significantly higher in umbilical PL compared to maternal values. ∑MUFA and ∑PUFA did not differ between maternal and umbilical plasma PL. The SFA content of umbilical PL was signifcantly different from that of maternal PL. Palmitic acid was lower in cord plasma. In contrast, the 18:0

TABLE 2
Composition (wt% of total FA) of FA in PL Isolated from Maternal Venous Plasma During the Course of Pregnancy and at Delivery and from Umbilical Venous Plasma at Birth[a]: Mean and SD

	Trimester I (n = 32)		Trimester II (n = 28)		Trimester III (n = 30)		Delivery (n = 32)		RM ANOVA	Umbilical cord (n = 32)		Paired t-test
PL	Mean	SD	Mean	SD	Mean	SD	Mean	SD	(P)	Mean	SD	(P)
14:0	0.34	0.09	0.40	0.17	0.38	0.09	0.34	0.20	0.28	0.33	0.12	0.87
15:0	0.25	0.14	0.27	0.13	0.23	0.10	0.24	0.25	0.14	0.18	0.10	0.14
16:0	28.40	1.73	29.77	2.79	30.21	1.03	32.24	2.46	0.0001*	28.69	2.39	0.0001‡
17:0	0.38	0.30	0.47	0.40	0.32	0.13	0.34	0.20	0.17	0.33	0.20	0.85
18:0	11.75	1.01	11.19	1.27	10.58	0.71	9.90	1.04	0.0001*	14.11	2.20	0.0001‡
20:0	0.52	0.10	0.57	0.13	0.56	0.09	0.56	0.14	0.10	0.92	0.20	0.0001‡
22:0	1.54	0.25	1.60	0.28	1.60	0.21	1.48	0.20	0.03	1.73	0.27	0.0001‡
23:0	0.62	0.12	0.66	0.11	0.59	0.17	0.53	0.19	0.02	0.25	0.13	0.0001‡
24:0	1.14	0.19	1.17	0.19	1.08	0.15	0.99	0.16	0.0001*	1.74	0.47	0.0001‡
∑SFA	44.95	1.28	46.10	3.89	45.55	0.99	46.61	3.32	0.001*	48.29	2.50	0.02
16:0 DMA	0.82	0.14	0.73	0.17	0.66	0.11	0.64	0.26	0.0001*	0.98	0.36	0.0001‡
18:0 DMA	0.49	0.28	0.46	0.23	0.36	0.24	0.39	0.16	0.03	0.49	0.28	0.02
∑DMA	1.31	0.34	1.19	0.33	1.02	0.29	1.03	0.31	0.0001*	1.47	0.58	0.0001‡
16:1n-9	0.27	0.11	0.30	0.14	0.27	0.11	0.27	0.15	0.21	0.26	0.11	0.85
16:1n-7	0.41	0.30	0.40	0.19	0.43	0.20	0.54	0.23	0.66	0.60	0.23	0.26
18:1n-9	8.02	1.30	7.83	1.20	8.63	1.17	8.69	1.26	0.54	7.11	1.18	0.0001‡
18:1n-7	1.11	0.35	1.07	0.38	1.10	0.27	1.10	0.37	0.005*	1.92	0.37	0.0001‡
24:1n-9	2.24	0.54	2.20	0.41	2.26	0.39	2.16	0.55	0.72	2.74	0.59	0.0001‡
∑MUFA	12.25	1.52	12.10	1.38	12.92	1.31	12.98	1.23	0.008	12.85	1.22	0.60
18:2n-6	19.90	3.00	19.83	3.04	20.29	2.66	19.56	3.01	0.62	8.85	3.98	0.0001‡
20:2n-6	0.38	0.18	0.36	0.18	0.40	0.13	0.35	0.14	0.08	0.26	0.11	0.0001‡
20:3n-6	3.12	0.66	2.99	0.64	3.12	0.72	3.08	0.58	0.66	4.24	0.84	0.0001‡
20:4n-6	9.76	1.76	8.78	1.49	8.49	1.31	8.70	1.82	0.0001*	14.80	3.33	0.0001‡
22:4n-6	0.28	0.22	0.35	0.17	0.32	0.20	0.30	0.21	0.23	0.67	0.52	0.0003‡
22:5n-6	0.30	0.16	0.33	0.12	0.34	0.15	0.38	0.16	0.006	0.59	0.25	0.0001‡
∑n-6	33.99	2.50	32.84	3.68	33.19	2.43	32.52	3.10	0.12	29.62	2.33	0.0001‡
18:3n-3	0.19	0.08	0.26	0.29	0.26	0.13	0.21	0.14	0.05	0.07	0.08	0.0001‡
20:5n-3	0.70	0.39	0.72	0.52	0.56	0.37	0.49	0.30	0.07	0.36	0.23	0.003‡
22:5n-3	0.85	0.24	0.73	0.21	0.68	0.19	0.62	0.15	0.0001*	0.48	0.17	0.0002‡
22:6n-3	4.93	0.99	5.13	1.16	4.94	1.16	4.73	1.32	0.11	5.95	1.68	0.001‡
∑n-3	6.73	1.47	6.94	1.74	6.52	1.51	6.16	1.66	0.05	7.01	1.81	0.02
20:3n-9	0.07	0.05	0.16	0.26	0.10	0.09	0.11	0.12	0.23	0.28	0.17	0.0001‡
∑PUFA	40.79	2.08	39.93	3.44	39.81	1.76	38.79	3.05	0.007	36.91	2.75	0.01
∑HUFA	20.00	2.55	19.18	2.62	18.55	2.46	18.40	2.94	0.001	27.37	4.73	0.0001‡
∑*trans* FA	0.59	0.28	0.59	0.25	0.60	0.20	0.50	0.22	0.15	0.32	0.17	0.0001‡
MMP (°C)	25.32	1.29	26.35	3.12	26.34	1.16	27.01	2.81	0.0001*	25.90	2.70	0.10
MCL	18.05	0.10	18.00	0.11	17.98	0.08	17.91	0.12	0.0001*	18.25	0.19	0.0001‡

[a]DMA, dimethylacetals; PL, phospholipids; for other abbreviations see Table 1.

content was significantly higher in umbilical plasma. The other long-chain saturates (20:0; 22:0, and 24:0) were also higher in the neonate. The odd-chain 23:0 was lower in the neonate. In contrast to what was found in CE, 18:1n-9 was significantly lower in umbilical plasma PL. Umbilical PL were significantly enriched in all the individual n-6 long-chain highly unsaturated compounds compared to maternal plasma PL. Consistent with their reduced fraction in the CE, 18:2n-6 and 18:3n-3 were significantly lower in umbilical PL. EPA (20:5n-3) and 22:5n-3 were significantly lower in umbilical PL, and 22:6n-3 was significantly higher in cord plasma PL. Mead acid was significantly higher in the neonate compared to the mother. The MMP of the FA in umbilical plasma PL did not differ from that of the maternal plasma PL-associated FA although the MCL of the FA in umbilical plasma PL was significantly higher than that of maternal plasma PL FA.

Relationship between the FA in plasma CE and PL. Strong correlations ($P < 0.0001$) were found between the maternal CE and PL fractions for 18:2n-6 ($r = 0.73$), 20:3n-6 (0.67), 20:4n-6 (0.71), 20:5n-3 (0.79), and 22:6n-3 (0.49). In umbilical plasma the correlations between these FA in the CE and the PL fraction were not so strong: for 18:2n-6 ($r = 0.45$, $P < 0.01$), 20:5n-3 (0.47, $P < 0.01$), and 22:6n-3 (0.36, $P < 0.05$).

DISCUSSION

In this study population very few significant changes in the FA composition of the polyunsaturates with progressing gestation occured: In PL only 20:4n-6, 22:5n-3, and ∑HUFA dropped significantly, and in CE only 18:2n-6 slightly decreased and 18:3n-3 slightly increased. In other longitudinal studies, more significant changes in PUFA composition were

found (6,10). When we compare maternal values from the first trimester with delivery values, more significant differences are found. Our study shows preferential accumulation of 20:4n-6 and 22:6n-3, with low concentrations of 18:2n-6 and 18:3n-3 in fetal plasma CE and PL, indicating a preferential supply of the fetus with HUFA needed for fetal development. These findings are consistent with other reports (6,7,13,16,22). These observations support the hypothesis of placental selectivity for transport of certain FA (23–25). Kuhn and Crawford (24) found that during *in vitro* perfusion of the human placenta, the majority of radiolabeled 20:4n-6 from the maternal circulation was selectively exported to the fetal circulation and incorporated into fetal PL, in contrast to small amounts of 18:2n-6 and 18:3n-3.

It has been established that ∑SFA increases whereas ∑HUFA decreases in maternal plasma PL during pregnancy (7,10). We demonstrated that in maternal plasma PL the loss of HUFA during gestation is accompanied by a shorter MCL of SFA and that the high content of HUFA in umbilical plasma PL is associated with a significantly longer MCL of SFA (7). In this study we confirmed similar changes in the PL fraction. The most remarkable finding in the composition of SFA of maternal plasma PL is the increase in 16:0 together with the decrease in the longer-chain FA 18:0 and 24:0 during gestation. The concentration of HUFA in umbilical plasma PL is much higher than in maternal plasma PL at delivery. Additionally, the composition of SFA in umbilical plasma PL is completely different from maternal plasma PL; 16:0 is lower and the longer-chain FA (18:0, 20:0, 22:0, and 24:0) are much higher. Thus, this study supports our hypothesis that the FA composition of SFA of plasma PL changes in a way to counteract changes in the MCL and consequently in the MMP induced by a changed HUFA composition (7).

The increase in ∑SFA and the decline in ∑HUFA in maternal plasma PL could be related to changes in the dietary intake of FA. However, this is rather unlikely as we found in this study population that the dietary habits remain unaltered during pregnancy (26,27). Neither the amount and type of fat nor the FA composition of the maternal diet changed during pregnancy until 1 mon postpartum, as has been confirmed by others (26–28). We can conclude that in this study population maternal diet cannot be a confounding factor in the plasma FA composition.

Another possible explanation for the observed differences in the maternal plasma FA composition during pregnancy is changes in the maternal hormonal status during gestation. The major pathway for PC synthesis, the Kennedy pathway, preferentially results in the appearance of 16:0 in the *sn*-1 position and 18:2n-6 or 18:1n-9 in the *sn*-2 position. Estrogen enhances an alternative pathway, the Greenberg pathway, resulting in the appearance of more PC with 18:0 in the *sn*-1 position and 20:4n-6 in the *sn*-2 position (29–31). During pregnancy, levels of estrogens and progesterone rise steadily as a result of placental production of these hormones (32). One would expect that the rise in estrogen during pregnancy would result in an increased synthesis of PC along the Greenberg pathway, resulting in an increased ratio of 18:0 over 16:0. On the contrary, an increase in 16:0 together with a decrease in 18:0 and 20:4n-6 with progressing gestation is observed, indicating an enhanced synthesis of PC along the Kennedy pathway in spite of estrogen (7,30,31). Skryten *et al.* (30) suggested subclinical cholestatic changes in the liver during normal pregnancy to explain this discrepancy in PC synthesis. Indeed, intrahepatic cholestasis of pregnancy is well described in humans and is associated with hyperlipidemia during pregnancy (33,34). Cholestasis is characterized by higher levels of 16:0 and lower levels of 18:0 in serum PL (35). Cholestatic conditions enhance the Kennedy pathway (30). Intrahepatic cholestasis of pregnancy generally resolves after delivery (34). The results of our study are in concurrence with the concept of enhanced synthesis along the Kennedy pathway as a result of increase cholestatic influence on liver PC synthesis during pregnancy. Of course, these explanations are rather speculative as we measured neither estrogen levels nor markers for cholestasis. Furthermore, the level of dimethylacetals, which originate from plasmalogens, dropped during pregnancy. Thus, this decrease shows that there is a decrease in plasmalogens relative to diacylphospholipids during gestation. It is not known whether this reflects a change in the relative contribution of both pathways for PL synthesis.

Minor changes were observed in the maternal CE FA composition during pregnancy. The adaptations in the SFA composition to counteract changes in the MCL and MMP found in PL were not confirmed in plasma CE. In maternal CE the loss of linoleic acid during gestation is compensated by an increase in oleic acid. In umbilical plasma, the higher concentration of ∑HUFA is accompanied by considerably higher levels of all the individual SFA (even the shorter-chain 14:0 and 16:0) compared to maternal plasma. In normal, fed, healthy persons, most of the circulating CE are formed in plasma under the action of LCAT (36,37). The esterification takes place in the plasma mainly by transfer of the FA from the *sn*-2 position of PC, the major plasma PL, to the 3-β-OH-group of cholesterol under the influence of LCAT. Human LCAT utilizes the *sn*-2 FA from most PC species (including 16:0–18:1 PC, 16:0–18:2 PC, and 18:1–16:0 PC) (38). In other words, human LCAT preferentially utilizes linoleic acid, which is the predominant FA in CE. However, when the long-chain FA 20:4n-6 and 22:6n-3 are present in *sn*-2 of PC, LCAT prefers the *sn*-1 acyl group. Thus, from 16:0–20:4 PC and 16:0–22:6 PC, the *sn*-1 acyl group is utilized by LCAT, producing 16:0 CE. This mechanism explains why such small amounts of 22:6n-3 are found in CE (38). The substrate preference of LCAT can explain why the FA composition of CE is less influenced by pregnancy than the FA composition of plasma PL.

Striking differences exist between the maternal and umbilical FA profiles of the two plasma lipid classes studied (CE and PL). In agreement with other studies (6,7,13,16,22), we found that the percentage values of 18:2n-6 and 18:3n-3 were markedly lower in cord plasma than in maternal plasma (CE and PL). The long-chain n-3 and n-6 PUFA are markedly higher in cord plasma in the two lipid fractions compared to

maternal plasma. The maternal-umbilical plasma differences are not always consistent for the two lipid fractions (CE and PL). In some cases the differences are even in the opposite direction. Consistent differences were found for 20:3n-6, 20:4n-6, and 22:6n-3 (umbilical values significantly higher than maternal values) and for 18:2n-6 and 18:3n-3 (umbilical values significantly lower than maternal values). Opposite differences were found in the composition of saturated and monounsaturated FA.

In summary, small but significant deviations occurred in PUFA composition of maternal plasma CE and PL during the course of pregnancy. The FA profile of umbilical plasma at birth is very different from maternal values at delivery in the two lipid fractions (CE and PL). This concurs with previous literature findings on the EFA status of the mother during pregnancy and of the neonate at birth.

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Effects of Intermittent Cycle Exercise on Intramyocellular Lipid Use and Recovery

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ABSTRACT: The purpose of this investigation was to compare intramyocellular lipid (IMCL) changes in skeletal muscle in nine moderately trained subjects after 45 min of interval cycling and through 1 h of recovery. The exercise session was continuous with alternating cycling intensity achieving 50 (3 min) and 110% (2 min) of ventilatory threshold. Spectra from the vastus lateralis were acquired before, immediately after, and 60 min following exercise using a 1.5 T Signa whole-body magnet (point-resolved spectroscopy sequence, echo time 60 ms, transverse relaxation time 2000 ms, 128 acquisitions, and 20 mm^3 voxel). Immediately following exercise, IMCL concentration decreased 38% compared to pre-exercise levels ($P < 0.05$). Fitness level and baseline IMCL were not correlated with changes in IMCL following exercise ($P > 0.05$). In the 60-min recovery, IMCL was reduced 30% compared to baseline ($P < 0.05$) and did not recover. In contrast, a nonexercising control group showed no change in IMCL. Our results suggest that IMCL decreased significantly following 45 min of interval cycling, with little recovery in the hour following.

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Recent work with NMR has lead to the identification of two separate lipid compartments in skeletal muscle (1,2). The two compartments include intramyocellular lipid (IMCL) and extramyocellular lipid (EMCL) locations. Although these pools contain predominantly TG [over two-thirds of the total FA in IMCL are thought to consist of three FA (18:0, 18:1, and 16:0)] (2), there are distinct metabolic differences between the locations. IMCL has a rapid turnover, and EMCL is thought to have a slow turnover and serve as a long-term storage depot (3). Differences in turnover may be related to location and metabolic enzyme activity (2). IMCL is stored in droplets (<200 Å radius) close to mitochondria and is associated with enzymes involved with FA esterification, transport, and hydrolysis. On the other hand, EMCL is located in more of an annular compartment oriented along muscle fibers and connective tissue (2).

Given the contribution of IMCL as a fuel source at rest, there has been increased interest in its role during exercise (4). Although much is known about muscle glycogen use during exercise, less is known about IMCL use. Until recently, plasma FFA from adipose stores were thought to be the primary fuel source during rest and mild exercise. However, recent work by Romijn *et al.* (4) and others (5,6) has suggested that FA from IMCL contribute significantly to fuel metabolism during moderate-intensity exercise.

Relatively few studies have used proton magnetic resonance spectroscopy (^{1}H MRS) to measure IMCL following exercise (1,7–11). This technique is noninvasive and has higher reproducibility than traditional biopsy studies (12). Most studies have focused on aerobic exercise, and fewer on anaerobic exercise. Larson-Meyer *et al.* (9) found a 25% decrease in soleus muscle IMCL concentrations in trained female subjects after 2 h of treadmill running at 67% of maximal oxygen uptake (VO_{2max}). Rico-Sanz *et al.* (11) observed a 32 and 19% decrease in IMCL concentration in the tibialis anterior and soleus muscles, respectively, in a group of male distance runners following 90 min of moderate-intensity (64% VO_{2max}) exercise. Krssak *et al.* (8) had trained subjects complete several discontinuous 45-min treadmill bouts to exhaustion at 65–70% VO_{2max} and found a 33% reduction in IMCL concentration in the soleus muscle. Taken together, these studies confirm that, under aerobic conditions with moderate-intensity exercise levels, IMCL is an important and measurable fuel.

In contrast, Rico-Sanz *et al.* (10) found no change in IMCL in a group of male athletes who performed several short sprint bouts with rest periods in between. It is likely that the strictly anaerobic type of exercise involved in this study relied less on lipid for fuel substrate compared with studies that have used longer-duration aerobic exercise in their investigations.

Additionally, little is known regarding whether IMCL is altered in a similar manner in sustained moderate- to high-intensity exercise. This is important because many exercise programs emphasize a combination of aerobic and anaerobic energy systems to improve both explosive and endurance exercise capacity. Fewer studies report recovery kinetics of IMCL following

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Abbreviations: aHSL, adipocyte hormone-sensitive lipase; AU, arbitrary units; BF, body fat; EMCL, extramyocellular lipid; IMCL, intramyocellular lipid; mHSL, muscle hormone-sensitive lipase; ^{1}H MRS, proton magnetic resonance spectroscopy; RER, respiratory exchange ratio; V_E, minute ventilation; VCO_2, carbon dioxide production; VO_2, oxygen consumption; VO_{2max}, maximal oxygen consumption; VT, ventilatory threshold.

exercise-induced perturbation. In one study of moderate exercise (8), after 5 h of recovery, IMCL recovered from 67 to 83% of baseline values.

Therefore, the purpose of the following study was to compare IMCL levels before and after 45 min of interval cycling in moderately trained subjects. Exercise consisted of a combination of anaerobic and aerobic intervals. ^{1}H MRS was used to measure IMCL at rest, following exercise, and 60 min into recovery.

MATERIALS AND METHODS

Nine moderately active, healthy young adult males were recruited from the University of New Mexico student and recreational community. All subjects exercised on average from two to five times per week. Criteria for inclusion in the study included having no cardiovascular or orthopedic limitations that would prevent completion of vigorous exercise. After being informed of the procedures involved and the possible risks associated with each procedure, participants signed an informed consent that was approved by the Human Research Review Committee at the University of New Mexico.

Study design. Subjects were evaluated for VO_{2max} and ventilatory threshold (VT) prior to inclusion in the study. The exercise trial was carried out within 7 d of the preliminary evaluation. To further minimize dietary variation, subjects were instructed to maintain similar eating habits in the week preceding the trial. Twenty-four hours before the exercise trial, subjects were instructed to refrain from exercise and from the consumption of alcohol and caffeine. Three hours before testing, subjects consumed a standard 400-kcal meal composed of 25% fat and 75% complex carbohydrates. The subjects reported to the magnetic resonance laboratory between 8:30 and 10:00 A.M.

After the initial resting ^{1}H MRS of the vastus lateralis, each subject performed 45 min of cycling on a Monark cycle ergometer (Varberg, Sweden). The subjects were placed in the magnet immediately following exercise and spectra were acquired. Additional spectra were obtained 60 min into recovery.

Preliminary metabolic testing. Measurements of VO_{2max}, VT, and body fat percentage (BF%) were conducted at the University of New Mexico Human Performance Laboratory. These measurements characterized the aerobic fitness of each subject and were used to prescribe exercise workloads to match relative exercise intensities for all subjects. VO_{2max} was determined using a cycle ergometer protocol, beginning with a light warm-up followed by progressive incremental workloads starting at 50 watts and increasing by 25 watts each minute. The criteria for test termination included a plateau in oxygen uptake with increasing workload and/or a respiratory exchange ratio (RER) greater than 1.15 (13). Heart rates were monitored using telemetry. Minute ventilation (V_E), oxygen consumption (VO_2), carbon dioxide production (VCO_2), and RER were monitored every 30 s using a Jaeger metabolic cart (Wurzburg, Germany). VT was determined through a series of graphs acquired from metabolic data and included V_E and ventilatory equivalents for (V_E/VO_2 and V_E/VCO_2) vs. workload (14). VT was defined as the point when V_E increased in a nonlinear fashion or when V_E/VO_2 began to rise without a concomitant rise in V_E/VCO_2. VT was verified by two independent investigators and was subsequently used to prescribe the exercise workload during the experimental protocol. BF% was estimated with the equation of Jackson–Pollock (15).

Exercise protocol. The 45-min exercise trial consisted of a 5-min warm-up followed by alternating 3-min intervals at 50% (easy) and 2-min intervals at 110% (hard) of the subject's VT. Therefore, each subject performed eight repetitions of 3 min easy and 2 min hard during the 45-min exercise trial. The exercise trial finished with 2 min of cycling at 110% of the subject's VT. This protocol was designed so that untrained subjects could complete the 45-min exercise protocol in a continuous manner and was part of a larger study.

MRS. ^{1}H MRS spectra were acquired using a 1.5 Tesla SIGNA whole-body imaging system (GE Medical Systems, Milwaukee, WI). High-resolution localizing images were initially acquired. Spectra were recorded from a 20 mm^3 region of interest in the subject's vastus lateralis using a general-purpose flexible extremity coil that was wrapped around the leg at the level of the region of interest. Voxel positions were carefully selected to avoid vascular structures and contamination by gross adipose tissue deposits. Localized proton spectra within muscle were collected using a point-resolved spectroscopy sequence (echo time 60 ms, transverse relaxation time 2000 ms, 128 acquisitions, water suppression and outer volume suppression).

^{1}H MRS was performed on the right vastus lateralis, one-third of the distance between the superior border of the patella and the crest of the ileum. This corresponded to a distance of between 12 and 20 cm superior to the superior margin of the patella. This location was marked on the thigh and used as a landmark for the center of the ^{1}H MRS voxel (Fig. 1). The subject's leg was rotated to be in alignment with the bore of the magnet using the longitudinal magnet alignment light (1).

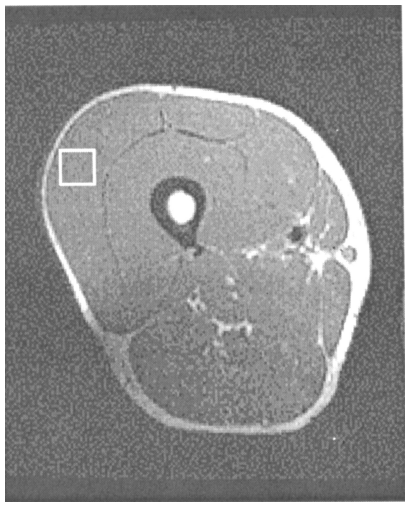

FIG. 1. Cross section of vastus lateralis. Voxel region of interest is highlighted in the scanning area.

To ensure that the leg was placed in the same position in the postexercise scan, anatomical landmarks, alignment lights, and padding were used to ensure relocalization in the same location within the magnet following exercise. The time between the end of exercise and the start of actual ^{1}H MRS data acquisition was approximately 15 min. This time included repositioning the subject in the magnet, acquiring a localizing thigh image, and shimming the voxel for optimal magnetic field homogeneity. Spectra were acquired immediately following and 60 min after exercise cessation.

Fitting of spectra. Spectra were analyzed using the Magnetic Resonance User Interface (MRUI) data analysis package (Leuven, Belgium). Initially, water filtering using the Hankel Lanczos Single Value Decomposition (HLSVD) filtering was performed to remove residual water resonances from the spectrum to flatten the baseline.

Time-domain fitting using Gaussian shapes was then performed on trimethylamine peaks (3.2 ppm), the creatine/phosphocreatine peak (3.03 ppm), the IMCL peak (1.28 ppm), and the EMCL peak (1.4 ppm) by AMARES (Advanced Method for Accurate, Robust, and Efficient Spectral Fitting). The total area under each peak was recorded for subsequent analysis. IMCL was expressed in arbitrary units (AU). In this study, peak fitting for IMCL has a within-subject variability of 6% on repeated scans.

Statistical analysis. Descriptive differences were analyzed with a Student's *t*-test. IMCL changes were tested with a one-way (within) ANOVA with repeated measures. Pearson product-moment correlation coefficients (R) were used to assess relationships between variables. An α level of $P < 0.05$ was considered significant. The Statistical Package for the Social Sciences (SPSS version 10.0; Chicago, IL) was used for all statistical analyses.

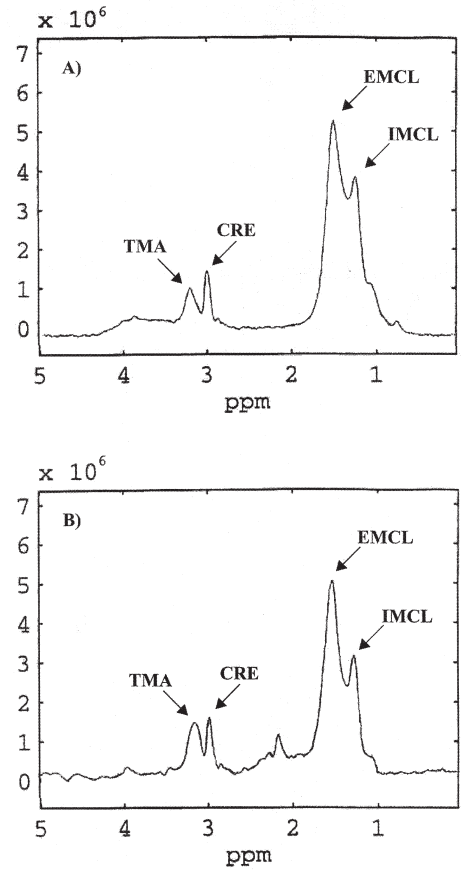

FIG. 2. ^{1}H Magnetic resonance spectroscopy of the vastus lateralis (A) pre-exercise and (B) postexercise following 45 min of continuous interval cycling. Abbreviations: TMA, trimethylammonium-containing compounds; CRE creatine and/or phosphocreatine; EMCL, $(CH_2)_n$ resonance of extramyocellular lipid pool; IMCL, $(CH_2)_n$ resonance of intramyocellular lipid pool.

RESULTS

Subject characteristics. Group characteristics of subjects ($n = 9$) were as follows: age 30.7 ± 4.9 yr; height 175.7 ± 7.5 cm; weight 74.0 ± 6.3 kg; BF% 11.9 ± 6.4; VO_{2max} 58.1 ± 14.9 mL kg min^{-1}; VT 225.0 ± 66.9 watts. Subjects were moderately trained as assessed by their VO_{2max} and self-reported physical activity (16). Body fat analysis showed their average body fat to be 11.9%.

In vivo *^{1}H MRS. In vivo* ^{1}H MRS from the vastus lateralis before and after exercise from one subject are shown in Figure 2. At rest there were no significant associations between IMCL and VO_{2max} ($R = 0.39$, $P = 0.30$), BF% ($R = -0.09$, $P = 0.81$), and VT ($R = 0.54$, $P = 0.13$). Subjects showed a significant decrease in IMCL immediately following exercise ($P < 0.01$). That is, the IMCL, expressed (in AU) as mean ± SEM, for pre-exercise was $(1.89 \pm 0.21) \times 10^7$, for postexercise was $(1.16 \pm 0.23) \times 10^7$, and after 60 min recovery was $(1.31 \pm 0.21) \times 10^7$; the differences between the latter two values and the pre-exercise IMCL value were both significant at $P < 0.05$. This finding was similar when examining the total area under the IMCL curve and quantifying IMCL relative to muscle creatine and H_2O levels ($P < 0.05$). The IMCL decrement immediately after exercise represented a 38% change (Fig. 3). However, there was no relationship between pre-exercise IMCL levels and the amount of IMCL change with exercise ($R = -0.05$, $P = 0.90$). Thus, subjects with greater IMCL levels at baseline did not show any greater change in IMCL levels than those with less IMCL at baseline. To examine IMCL recovery rates, ^{1}H MRS was obtained 60 min into recovery. At this time, IMCL had recovered to 30% of baseline values ($P < 0.05$ compared to baseline). Recovery rates were not greater in subjects who had greater baseline IMCL concentrations ($R = 0.52$, $P = 0.15$). No changes were noted in EMCL (data not shown, $P > 0.05$).

Immediately after exercise an unidentified peak presented at approximately 2.13 ppm (Fig. 1). During the course of recovery, this peak was present but decreased in magnitude over time.

DISCUSSION

This study compared IMCL changes in moderately trained subjects before and after a session of intense intermittent cycle ergometry. IMCL decreased significantly, and the

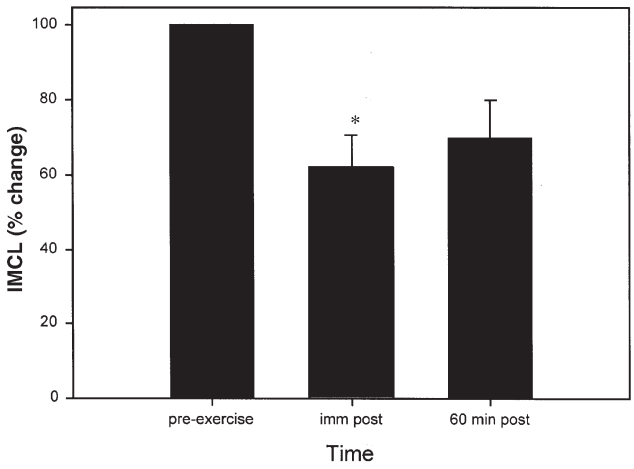

FIG. 3. Percent change in IMCL over time. Key: pre-exercise, immediately before exercise; imm post, immediately after exercise; 60 min post, 60 min after exercise cessation. Error bar represents SEM. Asterisk (*) indicates $P < 0.05$ compared to immediately before exercise.

magnitude of change immediately after exercise was 38% and did not recover significantly in the hour following exercise. No changes were noted for EMCL with exercise.

Until recently, most studies examining IMCL changes with a single bout of exercise have used muscle biopsy techniques. These studies found decreases (6) or no changes (17,18) in IMCL concentrations immediately following exercise. However, the biopsy technique has been criticized for its invasive nature and high variability with repeated sampling (12). For example, Wendling *et al.* (12) found as much as 26% variability in repeated biopsy IMCL sampling with exercise, justifying alternative methods of measurement of muscle lipid.

More recent studies have used ^{1}H MRS to measure IMCL at rest and with exercise. The majority of these studies have been completed with well-trained subjects using prolonged exercise protocols of submaximal workload. Recent work by Larson-Meyer *et al.* (9) studied IMCL changes in well-trained female endurance runners and triathletes following a 2-h treadmill run at 67% of VO_{2max}. IMCL decreased significantly (~25%) in soleus muscle immediately after exercise when subjects consumed either a moderate- or low-fat diet prior to exercise. Similar results were found by Rico-Sanz *et al.* (11) in a group of trained male distance runners following a 90-min treadmill run at 64% of VO_{2max}. A significant decrease in IMCL was found in the tibialis anterior (32%) and soleus (19%) muscles. The authors suggested that muscle fiber composition and muscle oxidative capacity played roles in differences in muscle lipid substrate use with exercise.

Our study is similar to previous studies that find decreases in IMCL in moderately trained subjects immediately following moderate- to high-intensity exercise (8,9,11). When IMCL changes from exercise were correlated with baseline IMCL or VO_{2max}, no significant associations were found. Thus, those subjects with greater baseline values of IMCL, or subjects with higher fitness levels did not show a different response for IMCL during exercise when compared to less fit subjects. To ensure that decrements in IMCL in the exercise treatment were from the exercise session, a resting male control group ($n = 4$) with similar ages, physical activity, and fitness levels were compared. This group completed the same experimental protocol as the exercise group, but instead of exercising for 45 min, this group rested quietly in the laboratory for 45 min. For the control group there were no significant changes in IMCL during the two time points measured (–2.1%) (pre- vs. 45 min post-rest period, $P = 0.90$). However, when the changes over time were compared between groups, the decrease in IMCL was significantly greater for the exercise group ($P < 0.05$). These results suggest that IMCL decreases during exercise and that FA from this depot are a significantly fuel source in skeletal muscle.

Our data are consistent with those of Romijn *et al.* (4), which suggest that IMCL may contribute as much as 50% of the total lipid used as a fuel source during moderate- to high-intensity exercise. It is likely that increased muscle hormone-sensitive lipase (mHSL) contributed to the activation of muscle lipolysis during exercise. Immunoblotting (19) and Northern blotting techniques have found protein and mRNA in muscle similar to that found with adipose tissue hormone-sensitive lipase (aHSL) (20). In adipose tissue, the activity of aHSL is regulated by sympathetic activity (21). During exercise, the plasma catecholamine concentration increases dramatically, inducing greater sympathetic activity (22). Although no blood samples were taken during the current study, it is probable that catecholamine levels were elevated during exercise (23), inducing the mHSL cascade.

Although its significance is not clear, a peak resonating around 2.1 ppm was present immediately after exercise. Previous work suggests that resonances in this area represent acetyl groups, possibly attached to carnitine groups (24). Increased acetylcarnitine formation during exercise could help explain how substrate use switches from predominantly carbohydrates to lipid during exercise (25). Less carnitine available during exercise would decrease FA shuttling into the mitochondria for oxidation, forcing the use of nonlipid fuel sources (26). However, more work is needed in this area before definitive statements can be made regarding the mechanisms controlling lipid use during exercise.

IMCL recovery was evaluated at 60 min after cessation of exercise. At this time, IMCL had recovered to 30% of the initial baseline value. These results are not surprising considering most published studies report that it takes several hours (8) to days (1) to replenish IMCL levels following exercise. Recovery rates of IMCL may be affected by substrate use and availability (4,17). Substrate availability (FFA from plasma TG) is regulated primarily by muscle lipoprotein lipase activity (27), which may not increase until 4 h after exercise (17). These data suggest that other mechanisms may contribute to the small recovery observed in the current investigation. Recent work by Ghou *et al.* (28) suggests that blood glycerol may contribute to IMCL replenishment and that there is a small depot of glycerol kinase in muscle available for FFA re-esterification. However, additional work is needed to clarify

whether FFA is re-esterfied in muscle following short-duration exercise and whether this mechanism is involved.

In conclusion, our results suggest that IMCL significantly decreases in response to interval cycling in moderately trained subjects. Changes in IMCL were not related to baseline IMCL levels or fitness. After 60 min of recovery, IMCL had not changed significantly, suggesting that IMCL does not significantly recover in the hour following exercise. Future work with extended follow-up as well as the study of potential mechanisms that contribute IMCL decrements during exercise is warranted.

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Acute Elevations of Medium- and Long-Chain Fatty Acids Have Different Impacts on Endothelium-Dependent Vasodilation in Humans

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ABSTRACT: It has previously been shown that acute elevation of long-chain fatty acids (LCFA) impairs endothelium-dependent vasodilation (EDV) in humans. In this study, we tested the hypothesis that an elevation of both medium-chain fatty acids (MCFA) and LCFA affects the endothelium differently from LCFA elevation alone. Ten healthy volunteers received an intravenous infusion of Structolipid® (structured TG, MCFA/LCFA ratio 1:1) and heparin for 2 h, while another 10 subjects received an infusion of Intralipid® (LCFA only) and heparin. EDV and endothelium-independent vasodilation (EIDV) were studied in the forearm after local administration of methacholine chloride (2 and 4 μg/min) and sodium nitroprusside (5 and 10 μg/min). Forearm blood flow was determined by venous occlusion plethysmography. Intralipid and heparin increased circulating FA levels from 0.2 ± 0.1 to 1.4 ± 0.5 mmol/L ($P < 0.001$) and reduced EDV by 20% ($P < 0.01$). Although Structolipid and heparin increased circulating FA levels to a similar extent (from 0.4 ± 0.1 to 1.8 ± 0.4 mmol/L after 2 h), EDV was not significantly changed. EIDV increased slightly during both interventions ($P < 0.05$). In conclusion, an acute elevation of LCFA attenuated EDV, whereas an elevation of both MCFA and LCFA did not influence EDV. Thus, FA composition seems to be of importance for EDV in healthy humans.

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Previous studies have demonstrated that an acute elevation of plasma nonesterified fatty acids (NEFA) during infusion of a long-chain TG emulsion (Intralipid®) with heparin impairs endothelium-dependent vasodilation (EDV) in healthy humans (1–4). Intralipid infusion also has been shown to produce elevated levels of malondialdehyde, a widely used but unreliable marker of lipid peroxidation (3,5). Taken together, these observations indicate that the negative effect of NEFA on EDV might be mediated through increased lipid peroxidation. However, a number of different FA exist and might affect EDV in divergent ways. In a recent cross-sectional study of healthy individuals, we found that endothelial vasodilatory function was inversely and independently predicted by the proportion of palmitoleic acid (16:1) in serum cholesterol esters, whereas α-linolenic acid (18:3n-3) in serum phospholipids was positively related to EDV (6). Furthermore, *in vitro* studies have reported that arachidonic acid (20:4n-6) produces EDV in arterial rings (7), and that palmitic acid (16:0) inhibits calcium ionophore-stimulated NO production (8). Interestingly, oleic acid (18:1n-9) has been reported to inhibit the endothelium-dependent response to acetylcholine in rabbit femoral artery rings preconstricted with phenylephrine (9).

Thus, it seems likely that not only NEFA level but also plasma FA composition influences EDV. This statement might be extended to cardiovascular disease, such as coronary heart disease, as epidemiological studies have shown that certain FA may predispose an individual to myocardial infarction, whereas others may be protective (10,11).

The aim of this study was to investigate whether an acute elevation of circulating NEFA with a higher proportion of saturated medium-chain fatty acids (MCFA) influences EDV and lipid peroxidation differently as compared to an acute elevation of long-chain fatty acids (LCFA).

MATERIALS AND METHODS

Subjects. Twenty young, healthy men and women aged 20–30 yr (mean age 23 ± 2 yr, mean body mass index 22.3 ± 2.0 kg/m^2, mean serum cholesterol 4.2 ± 0.7 mmol/L, mean systolic blood pressure 108 ± 12 mm Hg, and mean diastolic blood pressure 70 ± 6 mm Hg), without any known cardiovascular or metabolic disorders, were recruited from the general population of Uppsala, Sweden. Subjects on regular medication, on vitamin supplements at doses exceeding U.S. Food and Drug Administration recommendations, and habitual smokers were not included in the study.

Experimental procedure. The studies began at 8 A.M. after an overnight fast and were carried out with the subjects in supine position. Room temperature was maintained at 20–22°C. An arterial catheter (1.0 mm; Ohmeda, Swindon, United Kingdom) was inserted into the brachial artery for regional infusion of methacholine chloride (Mch, Metakolinklorid; Apoteksbolaget, Umeå, Sweden) and sodium nitroprusside (SNP, Nitropress®; Abbott Labs, Chicago, IL). The arterial catheter was also used for blood sampling. An intravenous catheter was inserted in an antecubital vein for infusion of Intralipid or Structolipid and heparin.

Ten subjects received an intravenous infusion of Structolipid (200 mg/mL; Fresenius Kabi AB, Uppsala, Sweden)

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Abbreviations: COX, cyclooxygenase; EDV, endothelium-dependent vasodilation; EIDV, endothelium-independent vasodilation; FBF, forearm blood flow; LCFA, long-chain fatty acids; MCFA, medium-chain fatty acids; Mch, methacholine chloride; NEFA, nonesterified fatty acids; PG, prostaglandin; RIA, radioimmunoassay; SNP, sodium nitroprusside; TX, thromboxane; VSMC, vascular smooth muscle cell.

and heparin (Heparin; Fresenius Kabi AB) for 2 h. Another 10 subjects received Intralipid (200 mg/mL; Fresenius Kabi) and heparin for the same time period. Structolipid is a lipid emulsion of structured TG in which both MCFA (8–14 carbon atoms) and LCFA (16–20 carbon atoms) have been esterified to the same glycerol molecule in a 1:1 proportion, whereas Intralipid contains 100% long-chain TG. The tocopherol content is 5 mg/L α-tocopherol, 53 mg/L γ-tocopherol, and 18 mg/L β/δ-tocopherol in Structolipid; the corresponding concentrations are 29 mg/L α-tocopherol, 118 mg/L γ-tocopherol, and 38 mg/L β/δ-tocopherol in Intralipid according to Fresenius Kabi AB. Both lipid emulsions were infused at an initial rate of 0.5 mL/kg during 10 min and 90 mL/h thereafter. Thus, the amount of infused lipids was equal in the two groups. The total heparin dose was 340 U during the 2-h period in both groups. Arterial blood samples were drawn at baseline and after 2 h of lipid infusion.

Forearm blood flow (FBF) was measured by venous occlusion plethysmography with mercury-in-Silastic strain gauges (Elektromedicin, Kullavik, Sweden). A venous occlusion pressure of 40 mm Hg was applied proximal to the elbow. The infused dosages were 2 and 4 μg/min for Mch, evaluating EDV, and 5 and 10 μg/min for SNP, evaluating endothelium-independent vasodilation (EIDV). Both vasodilators were given during 5 min for each dose. FBF was measured during the fifth minute of vasodilation and calculated as a mean of five consecutive readings. FBF was expressed as mL/min per 100 mL of forearm volume. Washout periods of 20 min were allowed between drugs. Mch and SNP were administered in one arm; the other arm served as a control for basal FBF. EDV and EIDV were evaluated in random order at baseline and after 2 h of lipid infusion. The endothelial function index, used to evaluate the contribution of the endothelium to the vasodilatory process, was defined as FBF at the highest dose of Mch divided by FBF at the highest dose of SNP.

The short-term (2-h) and long-term (3-wk) reproducibility of FBF during vasodilation with Mch and SNP with this method has a CV of 5–7% (12). We recently found that the highest dose of Mch (4 μg/min) results in a significant increase in forearm venous plasma nitrite and nitrate concentrations in healthy volunteers. The arteriovenous difference in plasma nitrite and nitrate concentrations showed a more than 10-fold increase in venous blood, indicating that Mch mediates vasodilation by an increased NO production (13).

Eight subjects with characteristics similar to those subjected to the main protocol (mean age 25 ± 2 yr, mean body mass index 21.9 ± 1.1 kg/m^2, mean serum cholesterol 4.1 ± 0.6 mmol/L, mean systolic blood pressure 111 ± 12 mm Hg, mean diastolic blood pressure 69 ± 10 mm Hg) were given a slow intravenous saline infusion instead of lipid emulsions to exclude any effects of time or the procedure itself on basal FBF and responses to Mch and SNP (time-control study). EDV and EIDV were measured at baseline and after 2 hr of intravenous saline infusion.

Biochemical analysis. Enzymatic kits were used to determine serum concentrations of TG and cholesterol (IL Test Triglyceride 181610-60 and IL Test Cholesterol 181618-10; Instrumentation Laboratory Co., Lexington, MA) and NEFA (NEFA C 994-75409; Wako Chemicals GmbH, Neuss, Germany) in a Monarch 2000 multicentrifugal analyzer (Instrumentation Laboratory Co.). Serum TG and NEFA levels were adjusted for *in vitro* lipolysis. Whole serum FA composition was analyzed with GLC, as described previously (14). α-, β-, and γ-tocopherol were analyzed with HPLC (15). Serum tocopherol concentrations were divided by the sum of plasma cholesterol and TG concentrations (16). Urine 8-iso-prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), an indicator of lipid peroxidation, was analyzed by a newly developed radioimmunoassay (RIA). In brief, unextracted urinary samples were analyzed for 8-iso-$PGF_{2\alpha}$ by a highly specific and validated RIA at our laboratory as described elsewhere (17). The cross-reactivity of the 8-iso-$PGF_{2\alpha}$ antibody with 15-keto-13,14-dihydro-8-iso-$PGF_{2\alpha}$, 8-iso-$PGF_{2\beta}$, $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, 15-keto-13,14-dihydro-$PGF_{2\alpha}$, thromboxane B_2 (TXB_2), 11β-$PGF_{2\alpha}$, 9β-$PGF_{2\alpha}$, and 8-iso-$PGF_{3\alpha}$, respectively, was 1.7, 9.8, 1.1, 0.01, 0.01, 0.1, 0.03, 1.8, and 0.6%. The detection limit of the assay was about 23 pmol/L. The urinary levels of 8-iso-$PGF_{2\alpha}$ were adjusted for creatinine concentration. This compound was not measured in the first four subjects in the Intralipid group.

Written informed consent was obtained from each participant. The study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association, and was approved by the Human Ethics Committee of Uppsala University.

Statistical analysis. Differences between means were calculated by ANOVA for repeated measurements. Two-tailed *P*-values were used, and $P < 0.05$ was regarded as significant. Data are expressed as mean ± SD unless otherwise specified.

RESULTS

Biochemical analysis. After 2 h of Intralipid and heparin infusion, circulating NEFA levels rose from 0.2 ± 0.1 to 1.4 ± 0.5 mmol/L ($P < 0.001$); 2 h of Structolipid and heparin infusion elevated circulating NEFA levels from 0.4 ± 0.1 to 1.8 ± 0.4 mmol/L ($P < 0.001$). Serum TG increased from 0.8 ± 0.2 to 3.1 ± 1.0 mmol/L ($P < 0.001$) in the Intralipid group, and from 0.7 ± 0.3 to 4.3 ± 1.2 mmol/L ($P < 0.001$) in the Structolipid group. In the Intralipid group, the proportion of PUFA increased from 39 to 52% ($P < 0.001$), and saturated FA decreased from 35 to 25% ($P < 0.001$) after 2 h (Table 1). The proportion of monounsaturates was not significantly altered. In the Structolipid group, there were minor changes in the proportions of saturates (from 34 to 32%, $P < 0.001$) and PUFA (from 40 to 43%, $P < 0.05$) after 2 h. In both groups, linoleic (18:2n-6) and α-linolenic (18:3n-3) acids were the major contributors to the elevated circulating NEFA level.

The lipid-adjusted tocopherol level changed similarly in both groups (Table 2). α-Tocopherol was significantly reduced, but β-tocopherol was unchanged. γ-Tocopherol, on the other hand, increased significantly in both groups. The urine 8-iso-$PGF_{2\alpha}$ level was significantly elevated after 2 h in the Structolipid group but not in the Intralipid group (Table 2).

TABLE 1
FA Composition in Whole Serum at Baseline and After 2 h in the Intralipid® and Structolipid® Groups[a]

	FA composition (%) in the Intralipid group (n = 10)		FA composition (%) in the Structolipid group (n = 10)	
FA	Baseline	2 h	Baseline	2 h
8:0	Not detectable	Not detectable	Not detectable	1.85 ± 2.91
10:0	0.14 ± 0.08	0.06 ± 0.03	0.11 ± 0.06	3.19 ± 1.11***
12:0	0.16 ± 0.07	0.08 ± 0.03*	0.28 ± 0.41	0.22 ± 0.19
14:0	1.47 ± 0.26	0.75 ± 0.19***	1.54 ± 0.69	1.07 ± 0.42*
15:0	0.24 ± 0.04	0.13 ± 0.03***	0.27 ± 0.03	0.18 ± 0.03***
16:0	26.24 ± 1.72	18.45 ± 1.32***	24.12 ± 1.29	19.69 ± 2.11***
16:1	3.05 ± 0.64	1.59 ± 0.42**	3.12 ± 0.59	2.02 ± 0.61***
17:0	0.26 ± 0.02	0.19 ± 0.00*	0.27 ± 0.03	0.20 ± 0.03***
18:0	7.15 ± 0.48	5.35 ± 0.30*	7.32 ± 0.89	5.83 ± 0.59***
18:1	22.57 ± 3.01	21.62 ± 0.91	23.35 ± 1.61	23.35 ± 1.62
18:2n-6	26.23 ± 2.32	40.67 ± 2.24*	26.38 ± 2.44	32.46 ± 2.32***
18:3n-3	1.10 ± 0.19	5.36 ± 0.65***	0.93 ± 0.18	2.77 ± 0.33***
18:3n-6	0.37 ± 0.13	0.18 ± 0.07*	0.40 ± 0.16	0.24 ± 0.09***
20:3n-6	1.81 ± 0.27	0.84 ± 0.26**	1.84 ± 0.47	1.02 ± 0.31***
20:4n-6	5.91 ± 0.57	2.78 ± 0.82***	6.14 ± 0.74	3.39 ± 0.47***
20:5n-3	0.80 ± 0.13	0.37 ± 0.11**	1.00 ± 0.46	0.52 ± 0.22***
22:5n-3	0.40 ± 0.29	0.23 ± 0.16	0.65 ± 0.18	0.43 ± 0.12***
22:6n-3	2.10 ± 0.38	1.33 ± 0.50**	2.24 ± 0.56	1.57 ± 0.31***

[a]Means ± SD are given, *P < 0.05, **P < 0.01, ***P < 0.001 vs. baseline.

Vasodilations with Mch and SNP. Infusions of Intralipid and Structolipid with heparin did not influence basal FBF significantly after 2 h (Table 3). In the Intralipid group, FBF during infusions of Mch at 2 and 4 µg/min was significantly reduced after 2 h, but no significant changes in responses to Mch were seen after 2 h in the Structolipid group (Table 3). FBF during infusions of SNP at 5 and 10 µg/min increased slightly, but significantly, during infusion with both types of lipid emulsions (Table 3). The endothelial function index (FBF at Mch 4 µg/min to FBF at SNP 10 µg/min ratio) decreased significantly in the Intralipid group (from 1.10 ± 0.25 to 0.79 ± 0.08, P < 0.01) but was unchanged in the Structolipid group (Fig. 1).

No changes were observed for basal FBF or forearm responses to SNP and Mch after 2 h in the time-control study (Table 3).

DISCUSSION

The main finding of the present study was that an acute simultaneous elevation of MCFA and LCFA (Structolipid) did not influence EDV, whereas elevation of LCFA alone (Intralipid) diminished EDV, despite similar increments in serum NEFA and TG in both groups.

Acute elevation of circulating NEFA, induced by infusion of Intralipid and heparin, has previously been shown to impair EDV in humans (1–4). Intralipid infusion has also been shown to produce elevated levels of malondialdehyde, a widely used but unreliable marker of lipid peroxidation (3,5). If increased lipid peroxidation *per se* contributes to impaired EDV during Intralipid/heparin infusion, the higher proportion of saturated MCFA in Structolipid would theoretically result in a lower degree of lipid peroxidation than the 100% LCFA in Intralipid. Furthermore, a less negative impact on EDV would be expected after Structolipid/heparin infusion. Indeed, phagocyte-induced lipid peroxidation has been shown to be lower during Structolipid incubation than during incubation with Intralipid *in vitro* (18). However, urine 8-iso-$PGF_{2\alpha}$ increased significantly in the Structolipid group, but no significant elevation of this lipid peroxidation marker was detected in the Intralipid group. Owing to the small sample size of the 8-iso-$PGF_{2\alpha}$ analysis in the Intralipid group, no conclusions

TABLE 2
Creatinine-Adjusted Urinary 8-Iso-prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) Levels and Lipid-Adjusted Tocopherol Levels at Baseline and After 2 h in Both Groups[a]

	Intralipid group (n = 10)		Structolipid group (n = 10)	
	Baseline	2 h	Baseline	2 h
Plasma α-tocopherol (g/mol)	1.56 ± 0.15	0.85 ± 0.9**	1.48 ± 0.24	0.79 ± 0.13***
Plasma β-tocopherol (g/mol)	0.017 ± 0.006	0.019 ± 0.004	0.021 ± 0.005	0.015 ± 0.002
Plasma γ-tocopherol (g/mol)	0.09 ± 0.05	0.33 ± 0.06***	0.10 ± 0.05	0.16 ± 0.03***
Urine 8-iso-$PGF_{2\alpha}$ (nmol/mmol)	0.24 ± 0.05	0.29 ± 0.04	0.35 ± 0.18	0.58 ± 0.39*

[a]Means ± SD are given. *P < 0.05, **P < 0.01, ***P < 0.001 vs. baseline.

TABLE 3
Basal Forearm Blood Flow (FBF) (mL/min per 100 mL) and Vasodilations During Brachial Infusion of Methacholine Chloride (Mch) and Sodium Nitroprusside (SNP) at Baseline and After 2 h of Intravenous Infusion of Intralipid/Heparin, Structolipid/Heparin, and Saline[a] (time-control study)

	Intralipid group (n = 10)		Structolipid group (n = 10)		Time-control study (n = 8)	
	Baseline	2 h	Baseline	2 h	Baseline	2 h
Basal FBF	2.6 ± 1.2	3.0 ± 1.1	4.1 ± 2.1	3.7 ± 1.3	4.1 ± 1.1	4.2 ± 1.2
FBF, Mch 2 μg/min	15.4 ± 5.1	12.4 ± 4.5**	14.6 ± 5.8	16.4 ± 7.7	20.5 ± 7.5	20.6 ± 7.4
FBF, Mch 4 μg/min	18.2 ± 6.2	14.5 ± 4.9**	17.4 ± 6.7	20.2 ± 11.9	25.7 ± 9.0	25.8 ± 9.3
FBF, SNP 5 μg/min	12.8 ± 4.1	15.0 ± 5.3**	14.6 ± 7.7	18.2 ± 7.7**	17.2 ± 4.1	17.9 ± 5.2
FBF, SNP 10 μg/min	16.6 ± 4.2	18.4 ± 5.2**	19.1 ± 8.1	21.1 ± 8.8*	22.1 ± 6.1	22.1 ± 7.7

[a]Means ± SD are given. *$P < 0.05$, **$P < 0.01$ vs. baseline.

can be made about differences in lipid peroxidation between the groups in the present study. However, the fact that EDV was unaffected in the Structolipid group, despite a significantly elevated 8-iso-$PGF_{2\alpha}$ level, suggests that lipid peroxidation was not the mechanism whereby EDV was impaired in the Intralipid group.

Linoleic (18:2n-6) and α-linolenic (18:3n-3) acids constitute about 60% of the FA in Intralipid, but only 40% in Structolipid. Consequently, a quite different plasma FA composition was seen after 2 h in the Structolipid group with a higher proportion of plasma MCFA as compared to the Intralipid group. Furthermore, the proportion of PUFA increased significantly after 2 h in the Intralipid group but not in the Structolipid group. Individual FA have been shown either to enhance or attenuate EDV. Hence, supplementation with EPA (20:5n-3) and DHA (22:6n-3) improved EDV in pigs after 4 wk (19), and EPA was shown to dilate rabbit ear arteries through an endothelium-dependent mechanism (20). We have recently found that EDV is positively related to α-linolenic acid in serum phospholipids in humans (6), and *in vitro* studies have shown that arachidonic acid (20:4n-6) produces EDV (21) but oleic acid (18:1n-9) inhibits EDV in rabbit femoral artery rings (9).

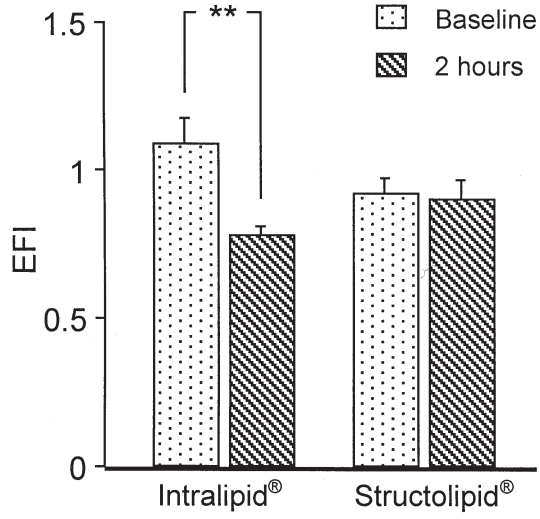

FIG. 1. Endothelial function index (EFI) at baseline and after 2 h of infusion with Intralipid® plus heparin or Structolipid® plus heparin. EFI was calculated as forearm blood flow (FBF) during infusion of methacholine chloride at 4 μg/min divided by FBF during sodium nitroprusside infusion at 10 μg/min. Means ± SEM are given. **$P < 0.01$ vs. baseline.

Although Intralipid lacks 20-carbon FA, its most abundant FA is linoleic acid (52% in Intralipid, 33% in Structolipid), which is converted to arachidonic acid *in vivo*. In the present study, increased availability of arachidonic acid might have stimulated cyclooxygenase (COX)-dependent production of vasoconstricting PG and TX, such as TXA_2 and PGH_2. On the other hand, the COX pathway also is an important source of free radical superoxide anions in endothelial cells (22). Superoxide anion rapidily reacts with NO and forms the powerful oxidant peroxynitrite ($ONOO^-$) (23). Indeed, inhibition of the COX pathway has previously been shown to restore EDV in essential hypertension and to protect EDV during experimental ischemia (24,25). In our own laboratory, we demonstrated that the attenuated EDV observed during Intralipid and heparin infusion in humans could be counteracted by concomitant infusion of the COX inhibitor diclophenac (26). Selective blockers of TXA_2 and PGH_2 and assessment of endothelium-derived superoxide anion production are needed to further clarify the role of these NO antagonists in NEFA-induced endothelial dysfunction.

The elevated γ-tocopherol concentration in both groups is explained by the high content of γ-tocopherol in Intralipid and Structolipid. The decreased α-tocopherol concentration in both groups is most likely due to the adjustment of all tocopherol levels for serum cholesterol and TG concentrations.

Interestingly, 2-h EIDV increased in both the Intralipid and Structolipid groups. This result is in conflict with previous studies, in which acute elevation of plasma NEFA or TG had a negative or no impact on EIDV (1–4,27). SNP donates NO at or inside the vascular smooth muscle cell (VSMC). Hence, inactivation of SNP-derived NO by superoxide anion in the subendothelial space or in the endothelium itself is probably of minor importance or absent even if the generation of superoxide anion is increased. The increased response to SNP seen during acute NEFA elevation could be due to a compensatory augmentation in VSMC sensitivity to NO due to increased breakdown of endothelium-derived NO. To elucidate these issues, future studies need to assess endothelial superoxide

anion production and NO degradation during different FA patterns.

Our findings contradict a recently published study, in which EDV was suppressed both by an intravenously administered physical mixture of medium-chain and long-chain TG (Lipofundin®, 30% EFA) and by Intralipid infusion (4). Differences in heparin and lipid doses, as well as differences between Lipofundin and Structolipid, might explain the discrepancy between the two studies.

In conclusion, an acute elevation of LCFA attenuated EDV, whereas EDV was maintained during simultaneous elevation of both MCFA and LCFA. These findings emphasize that FA composition influences EDV. Thus, in order to understand the pathophysiological or protective effects of FA on endothelial vasodilatory function, it is necessary to investigate FA individually.

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Dietary CLA Decreased Weight Loss and Extended Survival Following the Onset of Kidney Failure in NZB/W F1 Mice

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ABSTRACT: In an earlier study, we showed that feeding CLA immediately after weaning prolonged survival of NZB/W F1 mice after onset of proteinuria. In the present study, the feeding of CLA was delayed until mice had developed proteinuria. Thirty NZB/W F1 mice were fed a regular rodent chow after weaning. Urine samples were collected to detect proteinuria. Once a mouse was proteinuria positive, it was then randomly assigned to a 0.5% CLA supplement semipurified diet or a control diet (supplement 0.5% corn oil). The next proteinuria positive mouse was then assigned to the opposite diet to which the first mouse was assigned. Mice fed the control diet lost 25% more body weight (13.0 g) than mice fed the CLA diet (9.7 g). Moreover, CLA-fed mice survived an average 1.7-fold longer (148 d) than mice fed the control diet (89 d) after the onset of proteinuria. This follow-up study confirmed that dietary CLA had a beneficial effect in the autoimmune NZB/W F1 mouse. In summary, the cachectic symptom of systemic lupus erythematosus was decreased by dietary CLA and survival days were increased over control group.

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CLA was originally found to be an anticarcinogen in ground beef (1). It was later shown to have a wide range of unique physiological activities including anticarcinogenesis (2), antiatherosclerosis (3), and an inducer of changes in body composition associated with increased body protein and water, and decreased body fat .

CLA is a mixture of 18-carbon FA with two double bonds. The difference between linoleic acid and CLA is that linoleic acid has the two double bonds at the n-6 and n-9 positions, whereas the two double bonds of CLA are conjugated, meaning there is only one single bond between the two double bonds. Synthetic CLA is a mixture of isomers of which the *c*9,*t*11 and *t*10,*c*12 CLA isomers are the most abundant. Since it is difficult to purify pure CLA isomer, until recently many CLA studies, particularly in animal feeding studies, were carried out using synthetic CLA containing a mixture of conjugated dienes.

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Abbreviations: COX, cyclooxygenase; IL-2, interleukin-2; LPS, lipopolysaccharide; NZB/W F1, F1 hybrid of New Zealand Black and New Zealand White mice; SLE, systemic lupus erythematosus; TNF, tumor necrosis factor; TX, thromboxane.

Dietary CLA also affects the immune system. Chickens fed CLA had decreased lipopolysaccharide (LPS)-induced cachectic response but increased lymphocyte blastogenesis (5). Increased interleukin-2 (IL-2) production also was reported (6). Production of IgM, IgG, and IgA by splenocytes was increased in CLA-fed rats, but that of IgE was reduced (7). Owing to the immune-enhancing ability of dietary CLA, our lab has been studying the potential risk of CLA on immune hypersensitivity responses. With guinea pigs as a model for type I hypersensitivity, dietary CLA decreased antigen-induced histamine release and reduced or had no effect on tracheal contraction (8). In using a type III immune hypersensitivity model, autoimmune mice were fed CLA after weaning, and it was found that CLA shortened the time necessary for the development of spontaneous autoantibodies and proteinuria. On the other hand, CLA actually prolonged the survival time after the onset of proteinuria, and CLA protected against body weight wasting in F1 hybrid of New Zealand Black and New Zealand White (NZB/W F1) mice associated with the end stage of lupus (9).

In the present study, we tested the effect of feeding CLA on the autoimmune NZB/W F1 mice after development of proteinuria in contrast to feeding CLA prior to development of proteinuria. CLA was again shown to increase survival time as well as to protect against body weight wasting in the end stage of autoimmune NZB/W F1 mice.

MATERIALS AND METHODS

CLA. CLA was a gift from Natural Lipids Inc. (Hovdebygda, Norway) and contained 90% CLA (CLA-90) with the following isomer distribution: 43.5% *t*10,*c*12; 41.9% *c*9,*t*11 and *t*9,*c*11; 1.5% *t*9,*t*11 and *t*10,*t*12; 0.9% *c*9,*c*11; 0.9% *c*10,*c*12. Other FA in CLA-90 were 5.6% oleate, 1.4% palmitate, 0.5% linoleate, 0.4% stearate, and 3.4% unidentified compounds.

Animals. An autoimmune mouse model was used in this study to test the effect of CLA. Thirty weanling female NZB/W F1 mice were purchased from Harlan-Sprague Dawley (Madison, WI). Upon arrival, every three mice were housed in a plastic shoebox cage and fed rodent chow, and placed in a temperature- and humidity-controlled room with a 12-h light/dark cycle. Both feed and water were provided *ad libitum*. After dietary intervention, body weights were recorded weekly.

Diet. Semipurified powdered diet (TD94060, 99% basal mix; Harlan-Teklad, Madison, WI) was mixed with 0.5% oil

(either CLA or corn oil) and 0.5% sucrose by weight, such that the final diet contained either 5.5% corn oil (control diet) or 5% corn oil plus 0.5% CLA (CLA diet). Diets were prepared fresh every other week and stored at 4°C.

Experimental design. Urine samples were collected weekly from all mice to detect proteinuria. If two consecutive proteinuria assays were positive, the mouse was considered as proteinuria positive and randomly assigned to either a CLA diet or control diet. The very next mouse that showed positive proteinuria was then assigned to the opposite diet to which the previous mouse was assigned. This crossover assignment continued until all mice (n = 15 for each diet) were proteinuria positive (in a range of 218 d). Since these NZB/W F1 mice did not develop proteinuria at the same time, the design was intentionally set up as a crossover to let the mice develop the autoimmune symptom (proteinuria) before dietary CLA intervention.

Proteinuria assay. Metabolic cages were used to collect individual urine samples. Weekly urine samples were obtained by placing each mouse in an individual cage for 4 h. Urine samples were used to determined proteinuria by Bio-Rad (Richmond, CA) protein assay reagent. Urine was first diluted four times with water, then 20 μL of a diluted urine sample was added into a 96-well plate along with 200 μL diluted Bio-Rad assay reagent. Plates were shaken gently for 5 min and read at 600 nm with a microtiter plate reader. Duplicate samples and standards were used in all assays, and bovine γ-globulin was used as positive control, and to create a standard curve. The basal level of protein concentration was about 350 μg/mL in these mice before onset of proteinuria. A urine sample with at least 1,000 μg/mL protein was considered as proteinuria positive.

Survival days and body weight loss. Mice were checked daily. If a sick mouse was found to be lethargic or could not move around the cage and eat or drink, the mouse was weighed and euthanized with CO_2 for humane reasons. The survival days were then calculated as the difference between the onset of proteinuria and the day of euthanasia. This end point was considered death since previous results showed that mice do not survive more than a few days once these signs are observed (9). Protocols for animal care and use were approved by the Research Animal Resources Center at the University of Wisconsin–Madison.

Statistical analysis. Body weight and survival days were compared by Student's paired t-test to determine the CLA treatment effect using PROC GLM by Statistical Analysis System (10) computer program version 8.

RESULTS

NZB/W F1 mice lost body weight during the progression of autoimmune disease, and they became emaciated at the end stage of their lives. In the present study, mice fed control diet lost 13.0 g of body weight following the onset of proteinuria. Mice fed CLA lost about 9.7 g of body weight, which was 25% less body weight loss than mice fed the control diet (Fig. 1). Feeding CLA after these mice had already developed proteinuria protected against autoimmune cachectic body weight wasting over control-fed mice.

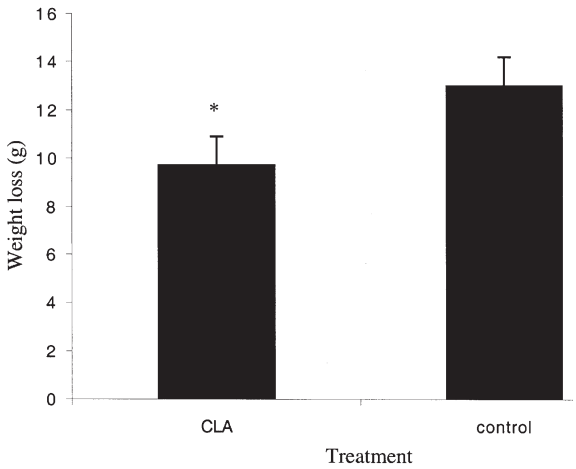

FIG. 1. Effect of dietary CLA on body weight loss between proteinuria and death. Thirty female F1 hybrid of New Zealand Black and New Zealand White (NZB/W F1) mice were fed either CLA or control diet after proteinuria was detected. Two different diets were alternately assigned to mice after they had developed proteinuia. Body weight loss was calculated starting from the day of feeding treatment diet until the mouse died. A mouse was humanely killed if it could not move freely for food and water. Data were presented as mean + SEM. *P < 0.05 when compared to control group.

Moreover, the average number of survival days after the onset of proteinuria for CLA-fed mice was 1.7-fold longer than for mice in control diet group. Mice fed the CLA diet survived an average of 148 d after the onset of proteinuria, as compared to 89 d of survival in mice fed the control diet (Fig. 2). The longer survival for CLA-fed mice over control-fed mice after

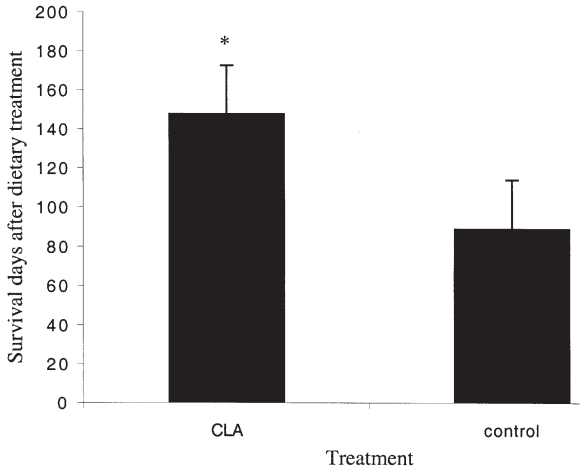

FIG. 2. Effect of dietary CLA on the survival days of NZB/W F1 mice. Thirty female NZB/W F1 mice were fed either CLA or control diet after proteinuria was detected. Two different diets were alternately assigned to mice after they had developed proteinuia. Days of survival were calculated starting from the day of feeding treatment diet until the mouse died. A mouse was humanely killed if it could not move freely for food and water. Data were presented as mean + SEM. *P < 0.05 when compared to control group. For abbreviation see Figure 1.

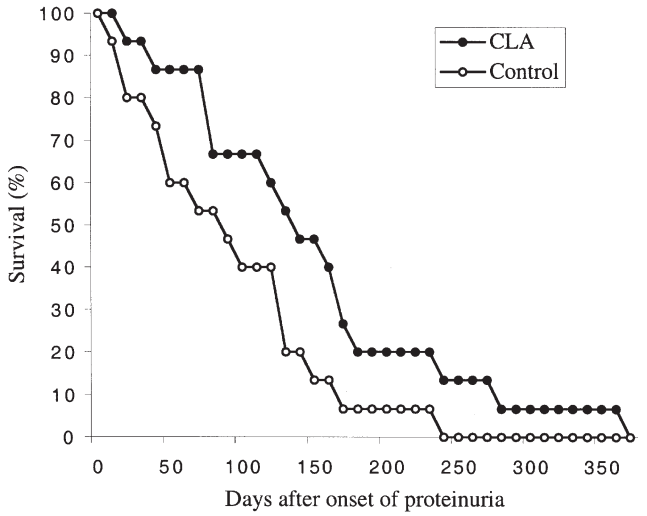

FIG. 3. Effect of dietary CLA on the survival of NZB/W F1 mice. Thirty female NZB/W F1 mice were fed either CLA or control diet after proteinuria was detected. Two different diets (●, CLA feeding; ○, corn oil feeding) were alternately assigned to mice after they had developed proteinuia. For abbreviation see Figure 1.

onset of proteinuria was significant at $P < 0.05$ and was consistent with a previous study (9). The percentage of survival was higher for the CLA group in any time point during the whole feeding period (Fig. 3).

DISCUSSION

Systemic lupus erythematosus (SLE) is an autoimmune disease that is caused by defects in immune regulation resulting in hyperactive T and B lymphocytes. Dietary FA interventions have been shown to regulate lupus in NZB/W F1 mice, a murine model for human SLE. Feeding fish oil as well as decreasing the energy intake delays lupus progression in these mice (11,12). We have demonstrated that feeding NZB/W F1 mice CLA after weaning prolongs survival after the onset of proteinuria. Body weight wasting due to lupus also is reduced when mice are fed CLA (9).

The present study evaluated feeding CLA to NZB/W F1 mice after they had developed proteinuria, a sign of kidney damage presumably caused by autoantibodies. Several reasons prompted us to delay CLA feeding until the onset of proteinuria. CLA has been shown to enhance immunity by increasing lymphocyte blastogenesis (13), macrophage cytotoxicity (5), IL-2 production (14), and delayed-type hypersensitivity response to phytohemagglutinin (13). Moreover, in our previous study, CLA accelerated the autoimmune process in NZB/W F1 mice as evidenced by an earlier appearance of anti-DNA autoantibodies and proteinuria. However, mice fed CLA survived longer after the onset of proteinuria when compared to mice from the control group. In the present study, we tested the effect of CLA on this SLE model after autoimmune symptoms, i.e., proteinuria, had already developed in these mice. Furthermore, by not assigning any dietary treatment to the mice until they were proteinuria positive, we attempted to eliminate the influence of CLA's immunity-modulating effects on the induction of the initial stages of clinical disease. Alternatively, this dietary treatment regime can focus on the effect of CLA after the autoimmune process has already developed in this SLE mouse model. Indeed, in this follow-up study, mice fed the CLA diet lost less body weight than mice fed control diet. One might speculate that the reason for less body weight loss is that mice died faster and so did not have the same opportunity to lose body weight. On the contrary, mice fed CLA actually survived 1.7-fold longer than control-fed mice after onset of proteinuria. Hence, feeding CLA post proteinuria protected the mice against end-stage autoimmune weight loss and improved their survival.

Immune-induced cachexia was decreased in animals fed CLA (5,13,15). CLA-fed mice lost less body weight after endotoxin challenge (13). We recently showed that the level of serum tumor necrosis factor (TNF)-α post-endotoxin injection was decreased by feeding CLA (16). Decreased production of TNF-α upon LPS stimulation may provide an explanation of the mechanism by which CLA may help to protect against body weight wasting. Therefore, these results indicate that macrophages may be a potential target for CLA in the NZB/W F1 mouse model.

CLA may compete with linoleic acid for the same enzymes for chain elongation and desaturation. Feeding 0.5 to 2% CLA had been shown to cause proportional increases of CLA and CLA metabolites in different tissues (17). In fact, *ex vivo* prostaglandin E_2 synthesis was dramatically decreased by feeding CLA in rats or guinea pigs (8,18,19). Furthermore, CLA may interfere with prostanoid synthesis and metabolism pathways (20).

In lupus nephritis, renal thromboxane A_2 (TXA_2) production was increased in NZB/W F1 mice, and TXA_2 inhibitor prolonged the survival of these autoimmune mice (21). In active lupus nephritis patients, TXB_2 (stable end product of TXA_2) production and cyclooxygenase-2 (COX-2) gene expression were elevated in peripheral blood mononuclear cells compared to patients with either the inactive form of the disease or healthy individuals (22). Immunostaining of COX-2 and COX-1 from kidney biopsies showed stronger staining of COX-2 in active lupus nephritis patients than controls, and COX-1 staining was not different (22). In a type I hypersensitivity animal model, CLA-fed guinea pigs showed reduced leukotriene, TX, and prostaglandin profiles in response to antigen challenge *ex vivo* (20). TXB_2 was significantly reduced in lung, trachea, and bladder. The decreased TXB_2 level provided at least one of the possible mechanistic explanations of prolonged survival of NZB/W F1 mice by CLA in the present study.

Immune hypersensitivity can cause collateral damage to the host. Even though CLA has immunity-enhancing activities, recent work (8,9) and observations presented here suggest that CLA may not exacerbate type I and type III immune hypersensitivity responses. To the contrary, CLA may have a beneficial effect in animals undergoing a type I or type III immune hypersensitive reactivity.

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Digestion and Assimilation Features of Dietary DAG in the Rat Small Intestine

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ABSTRACT: Several recent studies have demonstrated that dietary DAG oil rich in 1,3-species suppresses the postprandial increase of serum TAG level and decreases body fat accumulation, compared with TAG oil. To clarify the mechanisms underlying the beneficial effects of DAG, we investigated the metabolic features of DAG in the small intestine with regard to the digestion pathway in the lumen and the TAG-synthesis pathway in the mucosa. When intraduodenally infused as an emulsion, TAG was digested to 1,2-DAG, 2-MAG, and FFA, whereas 1,3-DAG was digested to 1(3)-MAG and FFA. When assessed by the incorporation of [1-^{14}C]linoleic acid in lipids, the mucosal TAG-synthesis was significantly reduced by DAG infusion compared with TAG infusion. However, the mucosal 1,3-DAG synthesis was remarkably increased in the DAG-infused rats. The total amount of mucosal 1,3-DAG was also increased (4.5-fold) after DAG infusion compared with that after TAG infusion. Next, we examined the synthesis pathway of 1,3-DAG. In cultures of the everted intestinal sacs, 1,3-DAG production required the presence of 1-MAG, suggesting that the 1,3-DAG synthesis was due to acylation of 1(3)-MAG in the DAG-infused rats. Furthermore, measurements of DAG acyltransferase activity indicated that 1,3-DAG was little utilized in TAG synthesis. These findings suggest that features of 1,3-DAG digestion and assimilation in the intestine may be responsible for the reduction of the postprandial serum TAG level by dietary DAG.

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DAG, which consists of 1,3-DAG and 1,2(2,3)-DAG, is contained in edible oils at a level of 2–10% (1,2) and is widely consumed in the daily human diet. Recent studies have demonstrated the beneficial effects of DAG with regard to the prevention and management of obesity and hypertriglyceridemia (3–6).

The long-term ingestion of dietary DAG composed mainly of 1,3-DAG decreased both body weight and visceral fat mass in humans, in comparison with ingestion of TAG (3). Moreover, several studies with humans have demonstrated that DAG ingestion reduced postprandial hypertriglyceridemia compared with TAG ingestion (4,5). Since impaired postprandial TAG clearance is associated with visceral obesity (7,8), the inhibition of body fat accumulation by DAG (3) might result from the suppression of the postprandial serum TAG increase (4,5).

Recently, we demonstrated that DAG prevented the accumulation of body weight and fat associated with a high-fat and high-sucrose diet in obesity-prone mice (9). A previous study using rats showed that the rate of lymphatic transport of TAG as chylomicrons was significantly reduced after an intragastric infusion of DAG emulsion compared with that after an infusion of TAG (10). The human and rodent studies demonstrating anti-obesity and hypolipidemic effects of DAG (3–6,9,10) were conducted under conditions in which the FA compositions of DAG and TAG were equivalent; thus, the beneficial effects of DAG are likely attributable to the structural differences between DAG and TAG. Moreover, the absorption efficiency of DAG is comparable to that of TAG (9,11), suggesting that the reduced transport of lymph chylomicrons by dietary DAG was caused by a specific metabolic fate in the small intestine.

It is well known that TAG are digested into 2-MAG and FFA in the intestinal lumen and then resynthesized to TAG in the intestinal mucosal cells. TAG have been shown to be resynthesized in the mucosa mainly *via* the MAG pathway through two steps of enzyme action, the acylation of 2-MAG and the subsequent acylation of 1,2(2,3)-DAG (12,13). However, the metabolic pathway in the small intestine of 1,3-DAG, a major constituent of the dietary DAG oil, has not been clarified.

In the present study, we investigated the metabolic features of DAG in the small intestine. The digestion products in the lumen and metabolic products in the mucosa after intraduodenal infusion of DAG were analyzed in order to elucidate the mechanisms by which dietary DAG lowers the postprandial serum TAG level.

EXPERIMENTAL PROCEDURES

Animals. Male Wistar rats (6 wk old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan) and used at 6 to 10 wk of age (250 to 350 g). The animal experiments were performed with the approval of the Ethics Committee for Experimental Animals of the Kao Corporation.

Materials. Monooleins (MO), *sn*-1 and *sn*-2 species, were purchased from Doosan Serdary Research Laboratories (Kyungki-Do, Korea). [1-^{14}C]Linoleic acid was obtained

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Abbreviations: DGAT, diacylglycerol acyltransferase; DO, dioleoylglycerol; IOD, integrated optical density; MO, monooleoylglycerol; TO, trioleoylglycerol.

TABLE 1
FA Distributions and Compositions of Dietary TAG and DAG (%)

	TAG	DAG
FA distribution		
TAG	98.8	0.4
1,2(2,3)-DAG	0.4	32.6
1,3-DAG	0.8	65.2
MAG	0.0	1.7
FA composition		
16:0	3.8	4.5
16:1	0.4	0.1
18:0	1.3	1.5
18:1	60.5	60.7
18:2	22.3	23.8
18:3	11.6	10.9

from Nycomed Amersham Plc. (Buckinghamshire, England) as a toluene solution, dried under nitrogen gas, and dissolved in ethanol. 1,3-[Carboxyl-^{14}C]Diolein (DO) (99% pure, 13.3 mCi/mmol) was obtained from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan).

"The DAG preparation was enzymatically synthesized by esterifying glycerol with FFA from rapeseed oil and was purified by silica gel chromatography as described previously (10,14,15). Most FA in rapeseed oil exist as TAG, whereas FA in the DAG preparation were recovered mainly in 1,3-DAG (69.5%) and, to a lesser extent, in 1,2(2,3)-DAG (30.9%). The FA composition of the DAG preparation was very similar to that of rapeseed oil."

Intraduodenal infusion. Intraduodenal infusion was performed according to the method of Mansbach and Nevin (16). TAG or DAG emulsions prepared by sonicating 150 mM NaCl, 10 mM Tris (hydroxymethyl)aminomethane, Tris-HCl, pH 7.0, 10 mM taurocholate (Sigma, St. Louis, MO) and 30 mM of TAG or 45 mM of DAG, both of which were esters of 90 mM FFA. Wistar rats, under pentobarbital anesthesia, were operated on through a subcostal incision. A duodenal cannula (Clay Adams PE 50; Becton Dickinson, Parsippany, NJ) was placed about 0.5 cm distal to the pylorus. The tip of the cannula was inserted about 1 cm into the duodenum, which is the level of entry of the common bile duct. The rats were placed in a restraining cage and infused with TAG or DAG emulsion at 4.5 mL/h for 5 h using an injection pump (Harvard Apparatus, South Natick, MA).

In experiment 1, to determine the digestive product in the intestinal lumen, the infusion was stopped and 1 mL of the TAG or DAG emulsion labeled with 3.2×10^6 dpm/mL of [carboxyl-^{14}C]TO (where TO = trioleoylglycerol) or 1,3-[carboxyl-^{14}C]DO, respectively, was injected through the cannula. After the bolus injection, the TAG or DAG emulsion not containing ^{14}C label was again allowed to flow through the cannula at 4.5 mL/h. After the rat was killed by injection of an overdose of pentobarbital (50 mg) into the peritoneal cavity, the proximal 40 cm of the intestine was removed and placed in ice-cold 150 mM NaCl at 10 min after the restart of the infusion. The intestine was opened lengthwise on an ice-cold aluminum plate. It was washed with 20 mL of 150 mM NaCl, and the washing solution was used for analysis of the lipid digestion products. Four rats were used for the TAG infusions, and four for the DAG infusions.

In experiment 2, we determined the TAG-synthesis intermediates in the intestinal mucosal cells. In this experiment, 1 mL of the TAG or DAG emulsion supplemented with [1-^{14}C]linoleic acid (1.6×10^6 dpm/mL) was injected through the cannula. The bolus injection was performed at 5 h after the start of the TAG or DAG infusion, and was followed by infusion of the TAG or DAG emulsion at 4.5 mL/h. The rat was killed by injection of pentobarbital, and the proximal 40 cm of the intestine was removed and placed in ice-cold 150 mM NaCl at 10 min after the restart of the infusion. The intestine was opened lengthwise on an ice-cold aluminum plate. It was washed once with 150 mM NaCl and once with 150 mM NaCl containing 0.2% Triton X-100 and then rinsed twice with 150 mM NaCl. The intestine was placed with its mucosal side up on an ice-cold aluminum plate, and the mucosa was scraped free using a microscope glass slide and homogenized in 10 mL of 150 mM NaCl using a glass-Teflon homogenizer. Six rats were used for the TAG infusions, and six for the DAG infusions.

The washing solution (experiment 1) and the homogenate (experiment 2) were stored at −80°C, and the frozen samples were placed on ice immediately after thawing to prevent hydrolysis of acylglycerols by lipase. Experiments on the rats from the different experimental groups were performed on the same day.

Incubation of intestinal everted sacs with micellar solutions. Micellar solutions of the test lipids were prepared by sonicating 1.2 mM [1-^{14}C]linoleic acid (0.16 Ci/mol), 0.6 mM *sn*-1-MAG or 2-MAG, 8 mM taurocholate, 150 mM NaCl, 10 mM glucose, and 10 mM Tris-HCl (pH 7.0). A FFA micellar solution was prepared by sonicating a solution containing 0.6 mM oleic acid and 0.6 mM glycerol instead of the same concentration of MAG.

The preparation of everted sacs was carried out according to the method of Breckenridge and Kuksis (17). The everted sacs were placed in a 20-mL Erlenmeyer flask containing 5 mL of the micellar solutions (*sn*-1-MO, 2-MO, or FFA micellar solution) equilibrated with O_2. The incubation was carried out for 40 min. At the end of the incubation, the sac was removed from the medium and washed four times as described above. The serosal contents were removed by cutting one end of the sac, and then the mucosal cells were scraped off using microscope glass slides and homogenized in 2 mL of 150 mM NaCl using a glass homogenizer. The homogenates were stored at −80°C. The frozen samples were used for analysis within a week.

DAG acyltransferase (DGAT) assay. The microsome fraction was prepared from the proximal third of the small intestine of rats fed a commercial diet according to the method of Grigor and Bell (18). The DGAT activity was determined by an acyl-CoA generating system (19). Microsomal protein (25 μg) was incubated at 37°C for 3 or 10 min in 200 μL of a reaction mixture containing 100 μM potassium phosphate buffer (pH 7.4), 150 μM DO, 2 mM DTT, 2 mM ATP, 4 mM

$MgCl_2$, 2 mM CoA, and 100 μM [1-^{14}C]linoleic acid (0.5 μCi/mL) complexed to fat-free BSA (60 μM). The incubation was terminated by the addition of 2 mL of chloroform/methanol (2:1, by vol/vol).

Extraction of lipids. Lipids were extracted by the method of Folch *et al.* (20), dried under a stream of nitrogen, and redissolved in chloroform. Measurement of radioactivity was performed using a TOP COUNT microplate scintillation counter (Packard Instrument Co., Meriden, CT).

TLC. The extracted lipids were separated by TLC using a Silica gel 60-precoated high-performance TLC (HPTLC) plate (Merck, Darmstadt, Germany) and hexane/diethyl ether/acetic acid (80:20:1, by vol) as the development solvent. For separation of 1,2(2,3)-DAG and 1,3-DAG, chloroform/acetone (96:4, vol/vol) was used as the development solvent. Separation of isomeric MAG [i.e., 1(3)-MAG and 2-MAG] was carried out using boric acid-impregnated plates (21) and chloroform/acetone (96:4, vol/vol). For preparation of boric acid-impregnated plates, HPTLC plates were soaked overnight in 10% boric acid in methanol and then dried in air; they were then activated at 110°C for 1 h. After development, HPTLC plates were exposed to a Fuji Imaging Plate (Fuji Photo Film Co., Tokyo, Japan) for 1 or 2 d, and the obtained fluorograms were analyzed with a BAS2000 system (Fuji Photo Film).

For quantification of DAG, HPTLC plates were sprayed with 40% sulfuric acid, immediately heated to 180°C to visualize the lipids, and used for densitometry (22). The integrated optical density (IOD) of the lipids was measured using a densitograph software program, Lane & Spot Analyzer (ATTO Co., Tokyo, Japan). Standards for *sn*-1,2-DAG (99% pure, Sigma) and 1,3-DAG (98% pure, Sigma) were applied to the plate, and the calibration curves were constructed by plotting the IOD vs. the amount of lipid loaded. The standard curve was linear between 0.25 and 10 μg, and the coefficient of regression was 0.98 on average. The value of the IOD of the lipid was interpolated on the corresponding calibration curve.

Statistical analyses. All values are expressed as the means ± SD. Significant differences of the means were established using Student's *t*-test at the level of $P < 0.05$.

RESULTS

Identification of digestion products in the intestinal lumen after DAG infusion. When the rats were duodenally infused with [carboxyl-^{14}C]TO, 76.0% of the labeled TO was digested 10 min after infusion (mean, $n = 4$), and 1,2(2,3)-DO (9.9%), 2-MO (28.2%), and oleic acid (36.8%) were generated in the intestinal lumen as the major digestion products (Fig.1). In contrast, when 1,3-[carboxyl-^{14}C]DO was infused, the degree of DO digestion (mean: 79.8%, $n = 4$) was similar to that of TO, and 1(3)-MO (25.6%) and oleic acid (50.6%) were mainly generated as the digestion products in the lumen (Fig. 1).

TAG synthesis in the intestinal mucosa after DAG infusion. Next, we analyzed the lipid metabolites in rat intestinal mucosal cells infused with DAG or TAG. To induce a steady state of digestion, TAG or DAG emulsion was infused into the duodenum for 5 h. Five hours later, the emulsion supplemented with ^{14}C-labeled linoleic acid was infused and then the incorporation of the ^{14}C label into the mucosal lipids was determined. Ten minutes after the ^{14}C-infusion, 59.8 ± 9.1% ($n = 6$) of the ^{14}C was incorporated into the mucosal lipids of the TAG-infused rats, whereas a lower amount of ^{14}C label was incorporated in DAG-infused rats [42.9 ± 7.8% ($n = 6$), $P < 0.001$].

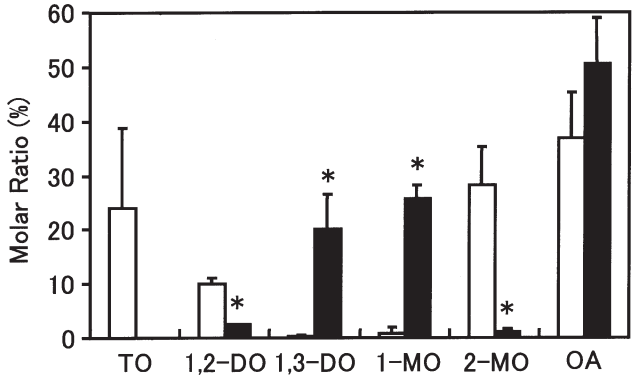

FIG. 1. Analysis of digestion products of [carboxyl-^{14}C]trioleoylglycerol a (TO) and 1,3-[carboxyl-^{14}C]dioleoylglycerol (DO) in the rat intestinal lumen. Rats were intraduodenally injected with a TAG emulsion containing [carboxyl-^{14}C]TO (30 mM TO, 3.2×10^6 dpm; open bar) or a DAG emulsion containing 1,3-[carboxyl-^{14}C]DO (45 mM DO, 3.2×10^6 dpm; solid bar). After 10 min, the molar ratios of metabolites of TO and 1,3-DO produced in the intestinal lumen were determined. MO, monooleolglyceroyl; OA, oleic acid. Data represent the means ± SD; $n = 4$. *, Significantly different from TAG infusion, $P < 0.001$.

Incorporation of the ^{14}C label into the mucosal TAG was decreased in the DAG-infusion group compared with that in the TAG-infusion group. In the TAG-infusion group, 91.5 ± 1.2% of the incorporated radioactivity was detected in TAG, whereas 86.2 ± 1.4% was detected in TAG in the DAG-infusion group (Fig. 2). This finding suggested that DAG digestion products were synthesized less into TAG in the intestinal mucosa compared with TAG digestion products.

Analysis of TAG synthesis intermediates in the intestinal mucosa after DAG infusion. In contrast to the lower incorporation of the ^{14}C label into the mucosal TAG in the DAG-infusion group, a higher proportion of radioactivity was detected in 1,3-DAG, MAG, and FFA in the DAG-infusion group compared with the TAG-infusion group. The radioactivity of 1,3-DAG accounted for 1.1% of the total amount of incorporated radioactivity in the TAG-infusion group, whereas that in the DAG-infusion group accounted for 4.5% (Fig. 2), resulting in a 4.4-fold increase in the ratio ($P < 0.001$).

The incorporation of the label into MAG was also significantly higher in the DAG-infusion group (DAG infusion: 0.4%; TAG infusion: 0.1%) (Fig. 2). Separation of MAG isomers by TLC using boric acid-impregnated plates revealed that 57.3 ± 2.1% of MAG in the mucosal lipids was

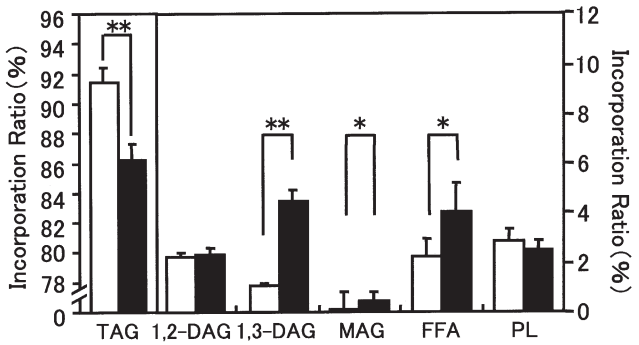

FIG. 2. Incorporation of [1-^{14}C]linoleic acid into lipids in rat intestinal epithelial cells after infusion of DAG or TAG. A lipid emulsion containing 30 mM TAG (open bar) or 45 mM DAG (solid bar) was infused at a rate of 4.5 mL/h into the duodenum in rats. After 5 h, [1-^{14}C]linoleic acid was added to the infusion emulsion, and radioactive FA at a final activity of 2.2×10^6 dpm was injected. At 10 min after the initiation of [1-^{14}C]linoleic acid infusion, the intestinal epithelial cells were collected. The percentages of radioactivity in TAG, DAG, MAG, FFA, and phospholipids (PL) were determined. Data represent the means ± SD; $n = 4$. *$P < 0.01$, **$P < 0.001$.

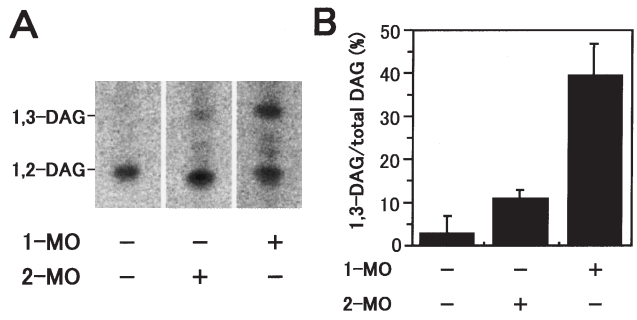

FIG. 4. Incorporation of [1-^{14}C]linoleic acid into 1,3-DAG in epithelial cells of rat intestinal everted sacs. Everted sacs of rat intestine were incubated with [1-^{14}C]linoleic acid in the presence or the absence of *sn*-1-MO (1-MO) and *sn*-2-MO (2-MO). In the case of the incubation without MAG, the medium included glycerol and oleic acid. The extracted lipids were fractionated by TLC using chloroform/acetone (95:4) as the developing solvent. Representative data are shown (A). The percentage of radioactive 1,3-DAG to the total radioactivity of DAG was determined (B). Data represent the means ± SD; $n = 3$. For abbreviation see Figure 1.

1(3)-MAG in the DAG-infusion group. However, 1(3)-MAG was not detected in the mucosal lipids of the TAG-infusion group.

The quantification of DAG revealed that, compared with TAG infusion, a 4.5-fold higher amount of 1,3-DAG was accumulated in the intestinal mucosa after DAG infusion (Fig. 3), whereas the amounts of 1,2(2,3)-DAG were comparable between the TAG- and DAG-infusion groups. The amount of mucosal 1,3-DAG was two-thirds the amount of 1,2(2,3)-DAG in the TAG-infusion group, whereas in the DAG infusion group the amount of mucosal 1,3-DAG was threefold higher than the amount of the 1,2(2,3)-DAG.

Synthesis pathway of 1,3-DAG. To identify the synthesis pathway of 1,3-DAG, everted sacs prepared from rat duodenum were cultured with labeled FFA in the presence or absence of MAG. When the everted intestinal sacs were cultured with [1-^{14}C]linoleic acid in the presence of 1-MO, 1,3-DAG synthesis in the mucosa of the everted sacs accounted for 39.6% of the total DAG synthesis (Fig. 4). In contrast, when incubation of the everted sacs was performed in the presence of 2-MO and labeled linoleic acid, the mucosal 1,3-DAG synthesis accounted for 11.0% of the total DAG synthesis. Moreover, the 1,3-DAG synthesis was only 2.8% of the total DAG synthesis in the absence of MO (a culture with glycerol, oleic acid, and labeled linoleic acid). These findings suggested that 1,3-DAG is produced by the acylation of 1-MAG in the intestinal mucosal cells.

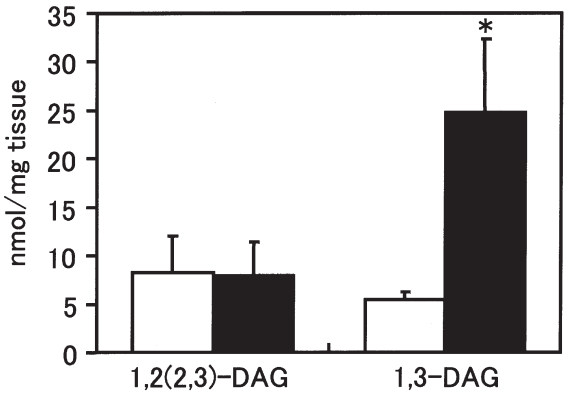

FIG. 3. Analysis of the total amount of 1,2-DAG or 1,3-DAG in rat intestinal epithelial cells after intraduodenal infusion. Rats were intraduodenally infused with TAG (open bar) or DAG (solid bar) as described in the legend for Figure 2. The total amounts of 1,2-DAG and 1,3-DAG in the intestinal epithelial cells were determined as described in the Experimental Procedures section. Data represent the means ± SD; $n = 4$. *Significantly different from TAG infusion, $P < 0.05$.

Utilization of 1,3-DAG for TAG synthesis. Measurement of the DGAT activity in the microsome fraction prepared from the intestinal mucosa revealed that the isomeric 1,3-DO was utilized little as a substrate for TAG synthesis compared with 1,2-DO (Fig. 5). The amount of TAG produced in the presence of 1,3-DO was comparable with that in the absence of DO, and about 1/10 of that in the presence of 1,2-DO. A similar result was obtained when labeled palmitic acid was used instead of labeled linoleic acid (data not shown).

DISCUSSION

In the current study, we clarified the metabolic features of dietary DAG in the small intestine. In the intestinal lumen, TAG was digested mainly to 2-MAG and FFA. In contrast, 1,3-DAG, which is a major constituent of DAG oil, was digested mainly to 1(3)-MAG and FFA. The analysis of lipid metabolites in the intestinal mucosa revealed a lower incorporation of [1-^{14}C]linoleic acid into the mucosal TAG after DAG infusion, suggesting a decreased TAG synthesis in the intestinal mucosal cells after DAG infusion. However, the 1,3-DAG

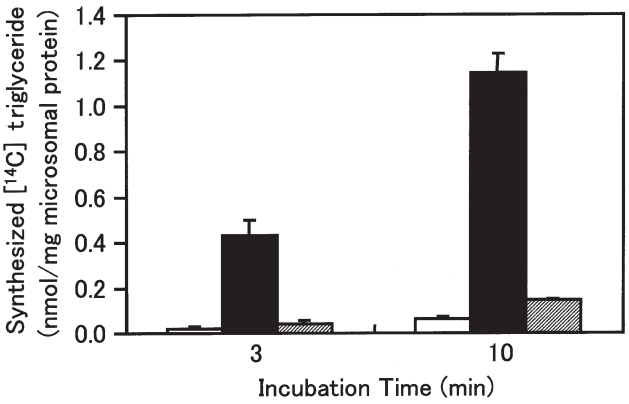

FIG. 5. Utilization of the DAG isomers for TAG synthesis. The microsome fraction (0.5 mg/mL) obtained from the rat intestinal mucosa was incubated with *sn*-1,2-DO (solid bar) or 1,3-DO (hatched bar). The open bar represents the control incubated without DO. Incorporation of ^{14}C label into TAG was determined as described in the Experimental Procedures section. Data represent the means ± SD; $n = 3$. For abbreviation see Figure 1.

synthesis was significantly increased after DAG infusion. The accumulation of 1,3-DAG appeared to be caused by its lower utilization in TAG synthesis. The decreased postprandial serum TAG levels after DAG ingestion demonstrated by previous studies (4,5,14) may be explained, at least in part, by the specific metabolism of DAG in the small intestine.

Intraduodenally infused TO was digested into 1,2(2,3)-DO, 2-MO, and oleic acid at the molar ratio of 1:3:4 in the lumen (Fig. 1). The oleic acid/MO ratio appeared to be considerably smaller than the ratio calculated on the assumption of the equal absorption of oleic acid and MO, suggesting that oleic acid was absorbed more rapidly than MO. Consistent with this idea, Morgan and Borgström (23) reported that FFA was absorbed more efficiently than MAG. On the other hand, the intraduodenally infused 1,3-DO was digested into 1(3)-MO and oleic acid at the molar ratio of 1:2 in the lumen (Fig. 1). As mentioned above, it is plausible that oleic acid is absorbed more rapidly than MO. Furthermore, pancreatic and preduodenal lipases preferentially digest acyl-groups at the *sn*-1 and *sn*-3 positions (24–27). In consideration of the findings, the high oleic acid/MO ratio in the lumen infused with DAG suggests that substantial amounts of 1(3)-MAG were further digested into FFA and glycerol in the lumen after DAG infusion.

When the emulsion containing [1-^{14}C]linoleic acid was intraduodenally injected during DAG infusion, the incorporation of ^{14}C into the mucosal TAG was lower than by the injection during TAG infusion. In contrast, the incorporation of ^{14}C into 1,3-DAG was much higher in the DAG-infusion group (Fig. 2). Moreover, the total amount of 1,3-DAG formation in the intestinal mucosal cells was also increased after DAG infusion (Fig. 3). Furthermore, 1,3-DAG synthesis was prominent (39.6% of total DAG) in the everted intestinal sacs only when incubated with labeled FFA in the presence of 1-MAG. In contrast, the 1,3-DAG synthesis was significantly reduced when the everted sacs were incubated with 2-MAG, and only slight amounts of 1,3-DAG were produced when the sacs were incubated in the absence of both MAG (Fig. 4). These findings suggest that, after intraduodenal infusion of DAG, some of the 1(3)-MAG was absorbed from the lumen into the mucosal cells without hydrolysis and then esterified to 1,3-DAG in the mucosal cells. Substantial amounts of 1,3-DAG were also produced in the mucosal cells after TAG infusion (Figs. 2,3). The 1,3-DAG might be synthesized *via* acylation of 1(3)-MAG generated by the isomerization of 2-MAG.

DGAT is a microsomal enzyme that catalyzes the last step of TAG synthesis (28, 29). The reaction involves the acylation of DAG, which is mainly supplied by esterification of 2-MAG in the intestine after TAG ingestion. So far, two species of DGAT, DGAT-1 and DGAT-2, have been identified, and the small intestine expresses both of them (30,31). In this study, we demonstrated that 1,3-DO was little utilized as a substrate for TAG synthesis in the microsomes prepared from the intestinal mucosa (Fig. 5). The low substrate specificity of mucosal DGAT to 1,3-DAG may be a factor responsible for the accumulation of 1,3-DAG and the decrease in TAG synthesis in the intestinal mucosal cells after DAG infusion.

The difference in the TAG synthesis pathway in the intestinal mucosa could be another important factor for the reduction by dietary DAG of lymphatic chylomicron TAG transport (10). The intestinal mucosal cells assimilate dietary TAG via two pathways: the MAG pathway and the PA pathway (12,13). These pathways account for 80 and 20% of the mucosal TAG synthesis, respectively, under normal conditions (17,32,33). TAG formation *via* the PA pathway occurred in subcellular structures and was distinct from that which occurred *via* the MAG pathway (34,35), and the TAG synthesized *via* the PA pathway were stored in the cytoplasm rather than being directly used for chylomicron assembly (36,37). Therefore, the possible increase in TAG formation *via* the PA pathway may be another factor responsible for the reported reduction of chylomicron release by DAG (10). Detailed studies are required to determine the relative contribution of the MAG and PA pathways to intestinal TAG synthesis after DAG infusion.

In the present study, we clarified the metabolic fate of dietary DAG in the rat small intestine. The features of the digestion and assimilation of 1,3-DAG presented here may be factors responsible for the decrease in postprandial serum TAG levels (4,5,10) and the prevention of body fat accumulation (3,9) by dietary DAG.

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Effects of Dietary Thermoxidized Fats on Expression and Activities of Hepatic Lipogenic Enzymes in Rats

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ABSTRACT: This study was undertaken to investigate the effect of dietary oxidized fats on the relative mRNA concentrations and the activities of fatty acid synthase (FAS) and glucose-6-phosphate dehydrogenase (G6PDH) in the liver of rats treated with vitamin E or selenium. Two experiments with male Sprague-Dawley rats were carried out. The first experiment included eight groups of rats fed diets with either fresh fat or three different types of oxidized fat, prepared by heating at temperatures of 50, 105, or 190°C, over a period of 6 wk. The diets contained either 25 or 250 mg α-tocopherol equivalents per kg. The second experiment included four groups of rats fed diets with fresh fat or oxidized fat, heated at a temperature of 55°C, containing either 70 or 570 μg selenium per kg, over a period of 8 wk. Feeding the diets with oxidized fats led to a significant overall reduction of the relative mRNA concentrations and the activities of FAS and G6PDH in both experiments. The effects of the oxidized fats on mRNA concentrations and activities of these enzymes were independent of the dietary concentrations of vitamin E or selenium. Moreover, in both experiments the rats whose diet contained the oxidized fats had significantly lower concentrations of TG in liver, plasma, and VLDL than the rats whose diet contained fresh fat. The study suggests that oxidized fats contain substances that suppress gene expression of lipogenic enzymes in the liver.

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Oxidized fats as components of heated and deep-fried foods play an important role in the development of various diseases. For instance, increased intake of oxidized fats has been linked to increased coronary artery disease and endothelial dysfunction in humans (1). Oxidized fats are also relevant in the etiology of cancer (2). Lipid peroxidation products in oxidized fats, moreover, affect the metabolism of animals in a variety of ways (3). We were able to show recently that feeding oxidized fats to rats leads to lower TG levels in liver and plasma compared with feeding fresh fat (4,5). The reason for this is still unknown. In a previous study we observed a slight reduction in the activities of several lipogenic enzymes in the liver (4) after feeding a moderately oxidized fat to rats, which led us to suspect that oxidized fats inhibit the expression of lipogenic enzymes in the liver. The precise nature of the relationship between lipid peroxidation products and lipogenic enzymes is still unknown. The present study was therefore set up to investigate the effects of oxidized fats on expression and activities of selected lipogenic enzymes, namely, fatty acid synthase (FAS) and glucose-6-phosphate dehydrogenase (G6PDH), in the liver. To this end we carried out two feeding trials using rats as model animals.

The concentrations of various lipid peroxidation products in heated fats depend on their thermal treatment. Fats heated for a prolonged period at low temperatures mainly contain primary lipid peroxidation products such as peroxides and hydroperoxides. Fats heated at high temperatures, on the other hand, contain high concentrations of secondary lipid peroxidation products such as dimers, trimers, polymers, and cyclic FA. It is known that the physiological effects of primary and secondary lipid peroxidation products differ. Primary lipid peroxidation products are highly toxic when administered parenterally but far less toxic when administered orally, presumably because of their lower absorption rate (6,7). Secondary lipid peroxidation products, on the other hand, are more readily absorbed and therefore highly relevant physiologically (8). We wanted to find out whether fats heated at low temperatures or those heated at high temperatures have greater effects on lipogenic enzymes. In the first experiment we therefore used fats heated at different temperatures.

Many effects of oxidized fats on the metabolism of animals are caused secondarily through oxidative stress induced by feeding oxidized fats and can be mitigated by a higher intake of antioxidants, especially vitamin E (9). We also wanted to find out whether the effects of oxidized fats on lipogenic enzymes can be modified by supplementation with antioxidants. In the first experiment we also varied the intake of vitamin E. In the second experiment the type of fat as well as the selenium supply was varied. Selenium is a constituent of glutathione peroxidase, which plays a key role in the detoxification of organic peroxides (10).

We know that the expression of lipogenic enzymes is influenced by a range of nutritive factors, especially the intake of energy and carbohydrates as well as the intake of PUFA (11,12). To eliminate the effects of different dietary intakes we applied a restrictive feeding regime where all animals received identical amounts of diet within each experiment. To equalize the intake of PUFA, the FA composition of control fats and oxidized fats was standardized by combining different fats. This enabled us to study the effect of oxidized fats

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Abbreviations: FAS, fatty acid synthase; G6PDH, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; POV, peroxide value; PCR, polymerase chain reaction; RT, reverse transcription; SREBP, sterol regulatory element-binding protein.

without interference by differences in the intake of energy and PUFA.

MATERIALS AND METHODS

Animals. Two experiments were carried out with male Sprague-Dawley rats supplied by Charles River (Sulzfeld, Germany). Experiment 1 included 80 rats with an initial body weight of 103 (± 2, SEM) g which were assigned to 8 groups of 10 rats each; Experiment 2 included 40 rats with an initial body weight of 52 (± 1, SEM) g which were assigned to 4 groups of 10 rats each. The animals were kept individually in Macrolon cages in a room maintained at a temperature of 23°C and 50 to 60% RH with lighting from 0600 to 1800 h. All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt.

Diets. Semisynthetic diets were used (Table 1). The composition of the diets was similar in both experiments. The concentration of fat was 100 g per kg in Experiment 1 and 50 g per kg in Experiment 2. The diets contained sufficient amounts of minerals and vitamins based on recommendations by the American Institute of Nutrition (13) for rat diets. The concentrations of vitamin E (Experiment 1) and selenium (Experiment 2) were selected in accordance with the experimental design.

In Experiment 1 fats treated under various conditions were used (see section on preparation of oxidized fats). The vitamin E concentrations of the diets were 25 or 250 mg α-tocopherol equivalents per kg diet. To adjust the dietary vitamin E concentration, we analyzed the initial concentrations of tocopherols in the fats. Based on these values, diets were supplemented individually with all-*rac*-α-tocopheryl acetate (the biopotency of all-*rac*-α-tocopheryl acetate is considered to be 67% that of α-tocopherol).

TABLE 1
Composition of the Basal Experimental Diets (g/kg)

Component	Experiment 1	Experiment 2
Casein	200	200
Cornstarch	300	345
Sugar	298	313
Fat[a]	100	50
Fiber	40	30
Mineral mixture[b]	40	40
Vitamin mixture[c]	20	20
DL-Methionine	2	2

[a]See Materials and Methods section ("Preparation of the oxidized fats").
[b]Mineral mixture supplied the following (per kg diet): 10.0 g of potassium sulfate, 9.8 g of calcium carbonate, 9.4 g of dicalcium phosphate, 3.0 g of sodium chloride, 0.9 g of magnesium oxide, 160 mg of ferrous sulfate hydrate, 51 mg of zinc oxide, 24 mg of manganese oxide, 24 mg of copper sulfate pentahydrate, 0.32 mg of calcium iodate, and 0.33 mg of sodium selenite pentahydrate (Experiment 1 only).
[c]Vitamin mixture supplied the following (per kg diet): 1.34 mg of all-*trans*-retinol, 25 µg of cholecalciferol, 7.5 mg of menadion sodium bisulfite, 5 mg of thiamine hydrochloride, 6 mg of riboflavin, 6 mg of pyridoxine hydrochloride, 15 mg of calcium pantothenate, 30 mg of nicotinic acid, 2 mg of folic acid, 0.2 mg of biotin, and 1,000 mg of choline chloride.

In Experiment 2, the type of fat (fresh fat vs. oxidized fat) and the selenium concentration (low vs. high selenium) were varied. The low-selenium diet contained 70 µg selenium per kg of the dietary components, as initially prepared; high-selenium diets were supplemented with 500 µg selenium per kg diet as sodium selenite pentahydrate (supra-pure quality; Merck, Darmstadt, Germany).

Diets were prepared by mixing the dry components with the fat and water and subsequent freeze-drying. The residual water content of the diet was below 5 g per 100 g of diet.

In both experiments, diets were administered in restricted amounts to standardize the diet intake. The rats were fed once daily at 0800 h. The amount of diet administered was 20% below the amounts of identical diets with fresh fats consumed *ad libitum* by rats in preliminary studies. The daily amount of diet was increased continuously during the experiment from 8.3 g to 17.4 g (Experiment 1) and from 8.0 g to 18.3 g (Experiment 2). In this feeding system, the diet administered was consumed completely by all rats. Thus, all the rats within one experiment consumed identical amounts of diet. Water was freely available from nipple drinkers. The experimental diets were fed for 6 wk in Experiment 1 and for 8 wk in Experiment 2.

Preparation of the oxidized fats. In Experiment 1, a fresh fat consisting of a mixture of sunflower oil and lard (31:69, w/w) and three different types of oxidized fats consisting of mixtures of sunflower oil and lard (1:1, w/w) were used. The first type of oxidized fat was prepared by heating for 38 d at a temperature of 50°C; the second type of oxidized fat was prepared by heating for 81 h at a temperature of 105°C; the third type of oxidized fat was prepared by heating for 24 h at a temperature of 190°C. In Experiment 2, the fresh fat consisted of a mixture of sunflower oil and lard (4:1, w/w). The oxidized fat used in Experiment 2 was prepared by heating sunflower oil at a temperature of 55°C for a period of 42 d. For heat treatment, the fats were filled into quartz glass beakers that were placed into a drying oven set at the intended temperature. Throughout the heating process, air was continuously bubbled through the fats. This treatment caused a loss of PUFA and a complete loss of tocopherols and raised the concentrations of lipid peroxidation products in the fats. We equalized the FA composition of fresh and oxidized fats by varying the ratio of lard to sunflower oil in the mixtures of the fresh fats. The extent of lipid peroxidation was determined by assaying the peroxide value (POV) (14), concentration of TBARS (15), concentration of conjugated dienes (16), acid values (17), the percentage of total polar compounds (18), and the concentration of total carbonyls (19). To determine the concentrations of those lipid peroxidation products of the dietary fats after they have been included in the diets, the fats were extracted from the diets with a mixture of *n*-hexane and isopropanol (3:2, vol/vol according to Ref. 20).

Sample collection. After completion of the feeding periods the rats were starved for 12 h and killed by decapitation under light anesthesia with diethyl ether. Blood was collected into heparinized polyethylene tubes. The liver was excised.

Plasma was obtained by centrifugation of the blood (1,800 × *g*, 10 min, 4°C). Plasma was stored at a temperature of –20°C. Liver samples for RNA isolation and measurement of enzyme activities were frozen in liquid nitrogen and stored at –80°C.

Activities and mRNA concentrations of lipogenic enzymes. Liver tissue was homogenized in 0.25 M sucrose buffer (in 0.1 M phosphate buffer, pH 7.4) using a Potter–Elvehjem homogenizer. Homogenates were centrifuged (105,000 × *g* for 1 h at 4°C) and the supernatants used for enzyme assays. Lipogenic enzymes were assayed by spectrophotometric methods. G6PDH was assayed by the rate of NADP reduction (21), whereas the activity of 6-phosphogluconate dehydrogenase was inhibited by maleimide. Units of G6PDH activity are defined as nmol of NADP reduced per min at 25°C. FAS was determined from the rate of malonyl-CoA-dependent NADPH oxidation (22). Units of FAS activity are defined as nmol of NADPH oxidized per min at a temperature of 25°C.

Reverse transcription polymerase chain reaction (RT-PCR). Expression analysis was done as semiquantitative analysis using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization. Total RNA was isolated from liver tissue by Trizol reagent (Life Technologies, Karlsruhe, Germany) according to the manufacturer's protocol. Synthesis of cDNA and PCR was performed using Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech, Freiburg, Germany) in a Mastercycler Personal (Eppendorf, Hamburg, Germany). The gene-specific primers (synthesized by MWG-Biotech AG, Ebersberg, Germany) and product lengths were:

GAPDH	5′ GCATGGCCTTCCGTGTTCC 3′	337 bp
	5′ GGGTGGTCCAGGGTTTCTTACTC 3′	
G6PDH	5′ CCAGCCTCCACAAGCACCTCAAC 3′	406 bp
	5′AATTAGCCCCCACGACCCTCAGTA 3′	
FAS	5′ GGGCCTGGAGTCTATCATCAACAT 3′	518 bp
	5′ GCGGGTACAGGGCTAAGACAAAA 3′	

RNA (1 µg) was used for cDNA synthesis in a final volume of 50 µL. After inactivation of the reverse transcriptase and the addition of the corresponding primers, the PCR was done with 32 cycles of denaturation (30 s, 95°C), annealing (30 s, 58°C), and elongation (45 s, 72°C). Thus, the reaction was stopped in the exponential range. Ten microliters of the reaction mixture was applied to agarose gel electrophoresis (2% agarose; Serva, Heidelberg, Germany). Digitalized video pictures of ethidium bromide-stained gels were used for quantification (apparatus and software from Syngene, Cambridge, United Kingdom).

TG concentrations in liver, plasma, and VLDL. VLDL were isolated by ultracentrifugation of the plasma (Mikro-Ultrazentrifuge, Sorvall Products, Bad Homburg, Germany) at 900,000 × *g* at 4°C for 1.5 h.

Lipids from the liver were extracted with a mixture of *n*-hexane and isopropanol (3:2, vol/vol), according to Reference 20. An aliquot of the lipid extract was dried, and the lipids were dissolved in a small volume of Triton X-100 (23). Concentrations of TG in liver lipid extract, plasma, and VLDL were determined using an enzymatic reagent kit (Merck, Darmstadt, Germany).

FA composition of dietary fats. The FA composition of the experimental fats was determined by GC. Fats were transmethylated into FAME with trimethylsulfonium hydroxide. FAME were separated by GC using a GC system (HP 5890; Hewlett-Packard, Waldbronn, Germany) fitted with an automatic on-column injector, an FID, and a polar capillary column (FFAP, 30 m, 0.53 mm internal diameter; Macherey-Nagel, Düren, Germany). FAME were detected by flame ionization and identified by comparing their retention times with those of individually purified standards (24).

Tocopherol concentrations in the liver. Concentrations of tocopherols in liver and oils were determined by HPLC using a Hewlett-Packard system (HP 1100) (25). Samples were mixed with 1 mL of 1% pyrogallol solution (in ethanol, absolute) and 150 µL of saturated sodium hydroxide solution. This mixture was heated for 30 min at 70°C, and tocopherols were extracted with *n*-hexane. Individual tocopherols of the extracts were separated isocratically, using a mixture of *n*-hexane and 1,4-dioxane (94:6, vol/vol) as mobile phase and a LiChroCART Si 60 column (5 µm particle size, 250 mm length, 4 mm i.d., Hewlett-Packard), and detected by fluorescence (excitation wavelength: 295 nm; emission wavelength: 330 nm).

Selenium concentration and activity of glutathione peroxidase. Selenium concentrations in the liver and plasma were measured after wet-ashing of liver and plasma samples with a mixture of 65% nitric acid, 30% hydrochloric acid, and 70% perchloric acid (2:1:1, by vol) in an atomic absorption spectrophotometer equipped with a graphite tube system (HGA-500; PerkinElmer, Überlingen, Germany). The activity of glutathione peroxidase in plasma was determined with *t*-butyl hydroperoxide at 25°C according to the method of Paglia and Valentine (26). A unit of enzyme activity is defined as 1 µmol NADPH oxidized per min.

Statistical analysis. Results of both experiments were treated by two-way ANOVA. For statistically significant *F* values, individual means were compared by Fisher's multiple range test. Means were considered significantly different at $P < 0.05$.

RESULTS

Characterization of the experimental fats. Some characteristics of the fats used in Experiment 1 are shown in Table 2. The FA composition in the fresh fat and the three different types of oxidized fats was similar. Before inclusion into the diet, the POV of the oxidized fat treated at 50°C was 500 times higher, that of oxidized fat treated at 105°C was nearly 100 times higher, and that of oxidized fat treated at 190°C was two times higher than that of the fresh fat. Percentages of total polar compounds were similar in all three types of oxidized fats. In the fats treated at 50, 105, and 190°C, they were 20, 22, and 17 times higher, respectively, than in the fresh fat. Inclusion of the fats into the diet increased the POV of all fats.

TABLE 2
Characteristics of the Dietary Fats Used in Experiment 1

Composition	Fresh fat	Oxidized fat 1	Oxidized fat 2	Oxidized fat 3
Sunflower oil (g/100 g fat)	31	50	50	50
Lard (g/100 g fat)	69	50	50	50
Treatment, temperature, time	None	50°C, 38 d	105°C, 81 h	190°C, 24 h
Major FA (g/100 g FA)				
14:0	1.1	0.9	0.9	0.9
16:0	19.2	17.4	17.8	17.5
18:0	11.5	9.8	10.4	10.2
18:1	35.6	36.9	35.6	34.7
18:2n-6	26.1	26.9	26.6	27.2
18:3n-3	0.6	0.3	0.3	0.3
Peroxidation products				
Before inclusion in the diet				
Peroxide value (meq O_2/kg)	1.6	804	150	3.5
Total polar compounds (%)	2.2	43.7	46.8	37.6
Acid value (g KOH/kg)	0.83	2.89	2.20	2.01
Total carbonyls (meq/kg)	<3	430	426	365
After inclusion in the diet				
Peroxide value (meq O_2/kg)	4.5	918	231	39
TBARS (mmol/kg)	0.01	21.9	2.24	0.18
Conjugated dienes (μmol/kg)	17.3	349	310	178

The acid value of the three oxidized fats was similar, being 2.5–3.5 times higher than that of the fresh fat. The concentration of total carbonyls was also similar in the three types of oxidized fat, being at least 100 times higher than in the fresh fat. After inclusion into the diet, the POV of oxidized fats treated at 50, 105, and 190°C were 200, 50 and 9 times higher, respectively, than that of the fresh fat. TBARS levels rose even more sharply, with concentrations 2000, 200, and 20 times higher in oxidized fats treated at 50, 105, and 190°C, respectively, than in the fresh fat. The concentrations of conjugated dienes were similar in oxidized fats treated at 50 and 105°C, being nearly two times higher than in the oxidized fat treated at 190°C and 20 times higher than in the fresh fat.

Some characteristics of the fat used in Experiment 2 are shown in Table 3. FA compositions of the fresh fat and that of the oxidized fat were similar. Before inclusion into the diet, the POV of the oxidized fat was 200 times higher than that of the fresh fat; the concentrations of TBARS were similar in both fats. The percentage of total polar compounds of the oxidized fat was 15 times higher than that of the fresh fat. After the oxidized fat had been included in the diet, its POV was slightly reduced, whereas the concentration of TBARS showed a 70-fold increase compared with the level before inclusion into the diet. In the fresh fat, POV and TBARS concentrations were also higher after inclusion into the diet than before. After inclusion into the diets, POV, TBARS, and acid value of the oxidized fat were 21, 36, and 2.5 times higher, respectively, than those of fresh fat.

Diet intake and body weight gain of the rats. The diet intake of the rats in each of the two experiments was identical. In the first experiment the diet intake averaged 14.3 g per day over the entire period, in the second experiment it was 16.9 g per day. In Experiment 1, body weight gains differed little between the eight groups. Feeding the oxidized fats heated at 50 or 190°C led to a slight reduction in body weight gains at the low dietary vitamin E concentration (Table 4). In the rats with the high vitamin E intake none of the three oxidized fats led to reduced weight gains. In Experiment 2 weight gains were similar in all four groups (Table 5).

Tocopherol concentration in the liver (Experiment 1). As was to be expected, the rats whose diets contained high vitamin E concentrations had distinctly higher concentrations of α-tocopherol in the liver than rats whose diets contained the low vitamin E concentration (Table 4). The feeding of all three oxidized fats led to a marked reduction of tocopherol concentrations in the liver, both in the high and the low vitamin E group.

Selenium status (Experiment 2). Selenium concentrations in liver and plasma as well as the activity of glutathione

TABLE 3
Characteristics of the Dietary Fats Used in Experiment 2

Composition	Fresh fat	Oxidized fat[a]
Sunflower oil (g/100 g fat)	80	100
Lard (g/100 g fat)	20	0
Major FA (g/100 g FA)		
16:0	10.6	7.4
18:0	6.7	4.7
18:1	25.4	26.4
18:2n-6	54.1	54.1
Peroxidation products		
Before inclusion in the diet		
Peroxide value (meq O_2/kg)	4.0	810
TBARS (mmol/kg)	0.27	0.34
Total polar compounds (%)	2.8	40.6
After inclusion in the diet		
Peroxide value (meq O_2/kg)	35.5	754
TBARS (mmol/kg)	0.67	24.2
Acid value (g KOH/kg)	0.57	1.44

[a]Prepared by heating at a temperature of 55°C for 42 d.

TABLE 4
Body Weights, Hepatic Tocopherol Concentrations, Hepatic Lipogenic Enzymes, and Concentrations of TG and Total Cholesterol in Liver, Plasma, and VLDL of Rats Fed Fresh Fat or Various Types of Oxidized Fats at Two Different Dietary Vitamin E Concentrations (Experiment 1)[a]

Fat type	Fresh fat[b]		Oxidized fat 1[c]		Oxidized fat 2[c]		Oxidized fat 3[c]		Pooled	Results of ANOVA
Treatment (temperature, time)	—		50°C, 38 d		105°C, 81 h		190°C, 24 h		SEM	Significant effect of
Vitamin E (mg α-tocopherol equivalents/kg)	25	250	25	250	25	250	25	250		($P < 0.05$)
Body weight (g)										
Initial	103	104	103	103	102	103	101	103	2	—
Final	388[d]	378[d,e]	364[f]	374[e,f]	376[d,e]	385[d]	362[f]	382[d,e]	4	Fat, vitamin E
Daily body weight gain (g)	6.77[d]	6.52[d,e]	6.21[f]	6.45[e,f]	6.54[d,e]	6.73[d]	6.21[f]	6.66[d,e]	0.06	Fat, vitamin E
Total tocopherols										
Liver (nmol/g)	26.1[f]	160[d]	20.1[f]	84.1[e]	18.6[f]	88.9[e]	16.1[f]	91.3[e]	7.0	Fat, vitamin E, fat × vitamin E
Lipogenic enzymes										
FA synthase										
Activity (U/mg protein)	6.7[d]	6.2[d,e]	4.4[e,f]	3.6[f]	4.9[e]	4.9[e]	5.1[e]	4.7[e]	0.42	Fat
mRNA (% of GAPDH)	162[d]	159[d]	97[e]	107[e]	94[e]	94[e]	99[e]	92[e]	11	Fat
Glucose-6-phosphate dehydrogenase										
Activity (U/mg protein)	77[d]	61[d,e]	53[e,f]	42[f]	54[e,f]	48[f]	67[d,e]	70[d,e]	4	Fat, vitamin E
mRNA (% of GAPDH)	118[d,e]	128[d]	101[d,e]	80[e]	97[e]	110[d,e]	87[e]	98[e]	9	Fat
TG										
Liver (μmol/g)	10.1[d]	9.3[e]	6.0[f]	6.8[f]	7.7[e,f]	6.5[f]	6.9[f]	6.7[f]	0.3	Fat
Plasma (mmol/L)	2.58[d]	1.67[e]	0.83[f]	0.64[f]	0.96[f]	0.81[f]	0.63[f]	0.91[f]	0.33	Fat, vitamin E, fat × vitamin E
VLDL (mmol/L)	2.34[d]	1.44[e]	0.68[f,g]	0.50[g]	0.80[f]	0.67[f,g]	0.48[g]	0.74[f,g]	0.09	Fat, vitamin E, fat × vitamin E
Total cholesterol										
Liver (μmol/g)	6.89[d]	7.13[d]	4.62[f]	5.19[e]	5.40[e]	5.14[e,f]	5.20[e]	5.49[e]	0.26	Fat
Plasma (mmol/L)	1.72[e,f]	1.86[e]	1.56[f]	1.66[e,f]	1.74[e,f]	2.13[d]	1.71[e,f]	1.84[e]	0.09	Fat, vitamin E
VLDL (mmol/L)	0.35[d]	0.20[e]	0.08[f]	0.06[f]	0.08[f]	0.08[f]	0.05[f]	0.08[f]	0.03	Fat

[a]Results are means, $n = 10$ for each treatment group. Values not sharing the same superscript letters are significantly different at $P < 0.05$ determined by Fisher's least significant difference test.
[b]Sunflower oil and lard (31:69, w/w).
[c]Sunflower oil and lard (1:1, w/w). GADPH, glyceraldehyde-3-phosphate dehydrogenase.

peroxidase in the plasma were distinctly higher in the rats on the high-selenium diet than in the rats on the low-selenium diet (Table 5). Feeding oxidized fat led to a reduction of the selenium concentration and a reduction of the glutathione peroxidase activity in the plasma, irrespective of the dietary selenium concentration. The selenium concentration in the liver was reduced in the rats on the low-selenium diet as a result of feeding the oxidized fat.

Relative mRNA concentrations and activities of FAS and G6PDH (Experiments 1, 2). In Experiment 1 the feeding of all three oxidized fats led to a statistically significant reduction of the mRNA concentration and the activity of FAS, irrespective of the dietary vitamin E concentration (Table 4). mRNA concentrations and activities of FAS were similar within the groups fed the three different oxidized fats. Regarding the mRNA concentrations and activities of G6PDH, significant differences between the treatment groups whose diets contained fresh fats and those whose diets contained oxidized fats were found only very occasionally. Two-way ANOVA did, however, show significant differences in the mRNA concentration between the rats whose diet contained fresh fat and those whose diets contained fats heated at 50 or 190°C. The relative mRNA concentrations, taking the average of both vitamin E concentrations, were (in % of GAPDH): fresh fat, 121[a]; oxidized fat heated at 50°C, 92[b]; oxidized fat heated at 105°C, 104[a,b]; oxidized fat heated at 190°C, 93[b] (pooled SEM, 9; values not sharing the same superscript letters are significantly different, $P < 0.05$). The activity of G6PDH differed significantly between the groups fed the fresh fat and the groups on the diets containing fats heated at 50 or 105°C. The activities of G6PDH, taking the average of both vitamin E concentrations, were (in U/mg protein): fresh fat, 69[a]; oxidized fat heated at 50°C, 47[b]; oxidized fat heated at 105°C, 51[b]; oxidized fat heated at 190°C, 68[a] (pooled SEM, 4; values not sharing the same superscript letters are significantly different, $P < 0.05$). There were no interactions between the type of fat and the dietary vitamin E concentration regarding the activities and the mRNA concentrations of both enzymes.

In Experiment 2, feeding the oxidized fat led to a significant reduction in the relative mRNA concentrations and the activities of FAS and G6PDH, irrespective of the dietary selenium concentration (Table 5). There were no significant interactions between the type of fat and the dietary selenium concentration regarding the mRNA concentrations and the activities of both enzymes.

Concentrations of TG in liver, plasma, and VLDL. Feeding the oxidized fats led to a reduction of the TG concentra-

TABLE 5
Body Weights, Parameters of the Selenium Status, Hepatic Lipogenic Enzymes, and Concentrations of TG and Total Cholesterol in Liver, Plasma, and VLDL of Rats Fed Fresh or Oxidized Fat at Two Different Dietary Selenium Concentrations (Experiment 2)[a]

Fat type	Fresh fat[b]		Oxidized fat[c]		Pooled	Results of ANOVA
Selenium (μg/kg)	70	570	70	570	SEM	Significant effect of ($P < 0.05$)
Body weight (g)						
Initial	51	52	52	55	1	—
Final	331	326	331	332	3	—
Daily body weight gain (g)	5.00	4.89	4.98	4.94	0.05	—
Selenium						
Liver (nmol/g)	26.1[e]	42.3[d]	17.7[f]	42.7[d]	1.2	Fat, selenium, fat × selenium
Plasma (μmol/L)	6.35[e]	7.84[d]	4.68[f]	6.96[e]	0.25	Fat, selenium, fat × selenium
Plasma glutathione peroxidase (U/mg protein)	2.91[e,f]	4.78[d]	2.35[f]	3.61[e]	0.34	Fat, selenium
Lipogenic enzymes	9.92	9.48	10.6	9.28	0.96	—
FA synthase						
Activity (U/mg protein)	7.1[d]	7.7[d]	5.1[e]	3.8[e]	0.7	Fat
mRNA (% of GAPDH)	121[d]	104[d]	59[e]	43[e]	9	Fat
Glucose-6-phosphate dehydrogenase						
Activity (U/mg protein)	52[d]	54[d]	32[e]	39[e]	6	Fat
mRNA (% of GAPDH)	73[d]	62[d]	28[e]	29[e]	8	Fat
TG						
Liver (μmol/g)	20.0[d]	17.0[d,e]	15.9[e]	14.0[e]	1.4	Fat
Plasma (mmol/L)	2.01[d]	1.79[d]	0.69[e]	0.50[e]	0.17	Fat
VLDL (mmol/L)	1.63[d]	1.33[d]	0.32[e]	0.24[e]	0.13	Fat
Total cholesterol						
Liver (μmol/g)	8.3	7.3	7.2	6.5	0.6	Fat
Plasma (mmol/L)	1.45	1.53	1.48	1.47	0.09	—
VLDL (mmol/L)	0.23[d]	0.27[d]	0.04[e]	0.03[e]	0.03	Fat

[a]Results are means, $n = 10$ for each treatment group. Values not sharing the same superscript letters are significantly different at $P < 0.05$ determined by Fisher's least significant difference test.
[b]Sunflower oil and lard (4:1, w/w).
[c]Sunflower oil, heated at a temperature of 55°C for 42 d. For abbreviation see Table 4.

tion in liver, plasma, and VLDL in both experiments. In Experiment 1, significant interactions were observed between the type of fat and the dietary vitamin E content regarding the concentration of TG in plasma and VLDL (Table 4). In rats fed the diet containing fresh fat, increasing the vitamin E content from 25 to 250 mg α-tocopherol equivalents per kg led to a significant reduction of the TG concentrations in plasma and VLDL. After feeding the three oxidized fats, on the other hand, the TG concentrations in plasma and VLDL were independent of the dietary vitamin E concentration. Within the groups whose diets contained the three oxidized fats, the TG concentrations in liver, plasma, and VLDL were similar. In Experiment 2, the effect of the oxidized fat on the TG concentrations in liver, plasma, and VLDL was independent of the rats' selenium intake (Table 5).

Concentrations of total cholesterol in liver, plasma, and VLDL. Feeding diets with oxidized fats led to a reduction in the concentration of total cholesterol in liver and VLDL in both experiments when compared with feeding diets with fresh fats. The concentration of total cholesterol in plasma was not influenced by feeding diets with oxidized fats in both experiments. In Experiment 1, the effect of the oxidized fat on cholesterol concentrations in liver and VLDL was independent of the vitamin E intake of the rats (Table 4). Within the groups whose diets contained the three different types of oxidized fat, cholesterol concentrations in liver, plasma, and VLDL were similar. In Experiment 2, the effect of the oxidized fat on the concentration of total cholesterol in liver and VLDL was independent of the selenium intake of the rats (Table 5).

DISCUSSION

Characteristics of the fats used. When investigating the effects of oxidized fats, special consideration must be given to the treatment of the fat because the formation of primary and secondary lipid peroxidation products depends on the conditions during treatment. The present study included fats treated at a low temperature over a long time period and others treated at a high temperature for a short time. A definite characterization of the concentrations of primary and secondary lipid peroxidation products in thermally treated fats is not easy because of the large number of different products, most of which are unstable and decompose during heating and storage (27). To provide a rough assessment of the fats used, we measured the POV, the concentrations of conjugated dienes, TBARS, and total carbonyls, the acid value; and the percentage of total polar compounds in the fats. The fat treated for a long period at a low temperature had a high POV, and high concentrations of TBARS and conjugated dienes. We can

therefore assume that the diets with fats treated at low temperatures had high concentrations of primary lipid peroxidation products. By contrast, the fat treated at a high temperature had a much lower POV and lower concentrations of TBARS and dienes than the fat treated at the lower temperature. This might be due to the fact that a large proportion of the relatively volatile lipid peroxidation products is stripped from the fat by air at the high temperature, and a virtual steady state is reached between their production and removal from the system. The percentages of polar compounds were high in both types of fat, those treated at low temperatures, and those treated at high temperatures. As these compounds are formed primarily during the latter stages of lipid peroxidation, it is assumed that the concentrations of secondary lipid peroxidation products were high in all the oxidized fats used in this study.

The observation that feeding the oxidized fats had no marked effect on body weights of the rats indicates that the fats used in our study were moderately oxidized compared to those used in other studies and did not produce generalized toxic effects in the animals. In several other studies, feeding diets with oxidized oils reduced animal growth due to oxidative stress and reduced nutrient digestibility (9,28,29). The only occasion when a small reduction in body weight gains occurred due to the oxidized fat was in Experiment 1 when the dietary vitamin E concentration was relatively low. Since an increase in the dietary vitamin E concentration from 25 to 250 mg α-tocopherol equivalents per kg prevented growth reduction, we assume that the growth depression might be attributed to oxidative stress induced by the oxidized fat.

Effects of oxidized fats on relative mRNA concentrations and activities of FAS and G6PDH in the liver. This study shows that feeding oxidized fats lowers the activity of FAS and G6PDH in the liver, confirming a previous study where feeding oxidized fats reduced the activities of the lipogenic enzymes FAS, acetyl-CoA carboxylase, and ATP-citrate lyase in the liver (5). Although the present study was only concerned with investigating FAS and G6PDH, feeding oxidized fats probably results in a general reduction of all lipogenic enzymes. It is known from several studies that the regulation of lipogenic enzymes is coordinated and that they respond in a similar manner to dietetic manipulation, e.g., through PUFA (11,30). Since the mRNA concentrations of FAS and GAPDH were also reduced, we suspect that feeding oxidized fats suppresses gene expression of these enzymes. The observation that the effect of the oxidized fats was independent of both the vitamin E and the selenium supply suggests that this effect is not due to oxidative stress. Rather, we suspect that certain constituents of oxidized fats, which are formed during heating, lead to reduced expression of lipogenic enzymes in the liver. The observation made in the first experiment that the activity of G6PDH in the group receiving the fat heated at 190°C was not reduced relative to the control group, might suggest that primary lipid peroxidation products, whose concentrations were lower in this fat than in the fats heated at 50 or 105°C, could play a major role in the suppression of lipogenic enzymes. Cyclic FA might also be involved. It was shown recently that cyclic FA, which are constituents of heated fats, lead to the suppression of other lipid-synthesizing enzymes in rats, namely Δ9-desaturase and phosphatidate phosphohydrolase (31). Further studies with isolated substances are needed to find out which specific substances are responsible for the suppression of lipogenic enzymes.

So far we do not know which mechanism underlies the inhibition of lipogenic enzymes through lipid peroxidation products. Recent studies have shown that the sterol regulatory element-binding protein-1 (SREBP-1) plays a key role in the regulation of lipogenic enzymes and cholesterol synthesis (32,33). That not only mRNA concentrations and activities of lipogenic enzymes but also the concentrations of cholesterol in the liver were reduced by feeding oxidized fats suggests that some lipid peroxidation products influence the expression of SREBP-1. Further studies are needed to elucidate a potential link between oxidized fats and the expression or activation of SREBP.

Effects of oxidized fats on concentrations of TG and total cholesterol in liver, plasma, and VLDL. In agreement with previous studies (4,5) we were able to show that feeding oxidized fats leads to a marked reduction in the TG concentrations in liver, plasma, and VLDL. Based on the activities of FAS and G6PDH, it is assumed that this effect is primarily due to inhibition of lipogenesis through a reduced expression of lipogenic enzymes. A link between the activity of the hepatic lipogenic enzymes and the TG concentrations in liver, plasma, and VLDL is plausible. Beside lipogenesis, the extent of β-oxidation is another key factor in determining the concentration of TG in liver and plasma. It was shown recently that cyclic FA, which are also constituents of fats heated at high temperatures, lead to a proliferation of peroxisomes and an increased rate of β-oxidation by activating the peroxisome proliferator activated receptor α (31). It is therefore conceivable that the reduced concentrations of TG found in the liver, and especially in plasma and VLDL, after feeding oxidized fats are also due to increased β-oxidation.

The reduced concentrations of cholesterol in liver and VLDL in rats fed oxidized fats could be due to various reasons. Likely, feeding of oxidized fats also reduced hepatic cholesterol synthesis. Earlier studies in rats also demonstrated that feeding diets with oxidized fats reduces the concentrations of cholesterol in the liver (5,34). The reason for this is not known. However, oxysterols, which are endogenously formed from cholesterol in the state of oxidative stress, could be involved in this phenomenon. It has been shown that oxysterols lower the rate of hepatic cholesterol synthesis by inhibiting the activity of 3-hydroxy 3-methyl glutaryl-CoA reductase and reduce the concentrations of VLDL lipids in plasma (35,36). More research is needed to shed light on this point.

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Pyloric Ceca Are Significant Sites of Newly Synthesized 22:6n-3 in Rainbow Trout *(Oncorhynchus mykiss)*

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ABSTRACT: In this pulse-chase study, rainbow trout fed a diet containing deuterated (D_5) (17,17,18,18,18)-18:3n-3 ethyl ester accumulated D_5-22:6n-3 in pyloric ceca to a greater extent than in liver 2 d post-dose. The ratio of newly synthesized D_5-22:6n-3 in ceca to that in liver 2 d after feeding D_5-18:3n-3 was 4.7 ± 1.2 when expressed as per mg tissue and 5.2 ± 2.4 when expressed as per mg protein. The amount of D_5-22:6n-3 in ceca then declined whereas that in liver and blood increased, with the ratio of ceca to liver falling to 1.7 and 1.4, respectively, by day 5 and approaching unity by day 9. A crude cecal mucosa fraction contained 123 ± 50 ng D_5-22:6n-3/mg protein/mg D_5-18:3n-3 eaten 2 d after feeding the tracer, compared with 35 ± 21 ng D_5-22:6n-3/mg protein/mg D_5-18:3n-3 eaten in liver. Three days later the amount in cecal mucosa had fallen by one-third and that in liver had increased threefold. Most of the D_5-18:3n-3 was catabolized very rapidly. The ratio of D_5-18:3n-3 to 21:4n-6 (a relatively inert FA marker) in the diet was 4.0, but this fell to 0.30 in ceca and *ca.* 0.8 in liver, blood, and whole carcass one day after feeding. These results indicate that ceca are active in the synthesis of 22:6n-3 and the oxidation of 18:3n-3.

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The liver is thought to be the main region of PUFA biosynthesis in vertebrates, including fish (1,2), although in some species brain and retina can perform some or all of the necessary steps (1,3). Hepatocytes and liver subcellular fractions have been used to elucidate the sequence of desaturation and elongation of precursor C_{18} PUFA to the final C_{20} and C_{22} functional end products of the n-3 FA pathway (4,5). However, it is difficult to extrapolate the results obtained from such studies to the whole animal. The use of stable isotope-labeled FA and GC–MS has permitted *in vivo* studies of PUFA metabolism to be carried out in a number of species including humans (reviewed by Emken, Ref. 6). Information on the rates of 22:6n-3 biosynthesis is crucial to our understanding of the bioequivalence of 18:3n-3 and 22:6n-3 and the adequacy of n-3 PUFA intakes in human diets.

Fish are the best source of long-chain n-3 PUFA (20:5n-3 and 22:6n-3) for human nutrition. Marine fish, and especially oily species such as mackerel, tuna, herring, and salmon, are the most enriched sources of these FA (7). However, most marine fish are unable to synthesize these FA, obtaining them preformed from their diet, ultimately from the phytoplankton *via* zooplankton and smaller fish (7). Many freshwater fish contain high levels of 22:6n-3 in tissue lipids as well and can synthesize 22:6n-3 from 18:3n-3 (2). We recently confirmed this directly in rainbow trout using deuterated (D_5)-18:3n-3 as substrate with identification and quantitation of product FA by GC–negative chemical ionization–MS (8). In that study we noted an appreciable accumulation of newly formed D_5-22:6n-3 in the whole body (excluding liver, brain, and eyes) at early time points post-dose of the tracer. Here we show that in rainbow trout the midsection of the gut containing the ceca incorporate newly synthesized 22:6n-3 more rapidly and to higher concentrations than other tissues, indicating that this tissue is active in 22:6n-3 synthesis.

MATERIALS AND METHODS

Chemicals. Chloroform, methanol, ethanol, isohexane, and diethyl ether were HPLC-grade from Fisher (Loughborough, Leicestershire, United Kingdom). Diisopropylamine, anhydrous acetonitrile, and pentafluorobenzyl bromide were obtained from Aldrich (Gillingham, Dorset, United Kingdom). D_5(17,17,18,18,18)-linolenic acid was purchased from Cambridge Isotope Laboratories (Andover, MA) as the FA ethyl ester (FAEE). Linseed oil was from ICN (Basingstoke, Hampshire, United Kingdom), and refined olive oil was from Tesco supermarkets. High-oleic acid sunflower oil was a gift from Croda Chemicals (Goole, United Kingdom). Fish meal was from Biomar (Grangemouth, United Kingdom). All other chemicals were from Sigma (Poole, Dorset, United Kingdom).

Synthesis of 21:4n-6. Heneicosatetraenoic acid (Δ6,9,12,15-21:4) ethyl ester was prepared by a one-carbon addition to 20:4n-6 FA (9). The product was obtained in 51.2% yield and was 98.9% pure by GC and GC–MS of the FAME and pentafluorobenzyl ester (see later).

Fish and diets. Rainbow trout approximately 2 g in size were obtained from a commercial hatchery and kept in a running freshwater aquarium at ambient temperature (3.5 to 16.5°C) on a 14 h/10 h light/dark cycle. Fish were fed a diet based on casein and a blend of vegetable oils containing predominantly oleic acid with 18:2n-6 and 18:3n-3 at approximately 1% each. This was done to maximize 22:6n-3 synthesis and satisfy the EFA requirements of the fish. The full composition of the diet was (g/kg): vitamin-free casein, 480;

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Abbreviations: D_n, deuterated; FAEE, fatty acid ethyl ester; tri23:0, tritricosanoyl glycerol.

starch, 150; fish meal, 50; mineral mix, 47; vitamin mix, 10; arginine, 4; methionine, 3; cystine, 2; leucine, 4; orange G, 1; α-cellulose, 138.6; blended vegetable oil, 110; and antioxidant mix, 0.4. The composition of the mineral mix, vitamin mix, and antioxidant mix were described previously (10). The final diet provided 50% crude protein and 11% oil, blended to give 0.99% 18:2n-6, 1.02% 18:3n-3, and 0.12% highly unsaturated FA (20:5n-3 and 22:6n-3) from the fish meal, which was added to make the diet palatable and readily accepted by the fish. The remaining FA were predominantly 16:0 (1.02%) and 18:1n-9 (7.18%). The fish were fed this diet for at least 25 wk before starting the experiments.

Preparation of labeled diet. A small portion of diet containing D_5-18:3n-3 FAEE and 21:4n-6 FAEE was prepared as follows. An oil sample containing 10 mg D_5-18:3n-3 FAEE, 2.5 mg 21:4n-6 FAEE, 153 mg of high-oleic acid sunflower oil, and 61 μg antioxidant was dissolved in 0.82 mL isohexane, and 1.335 g of dry diet mix was added. The isohexane was then removed at 37°C under nitrogen and the diet was desiccated *in vacuo* for 18 h. The diet was mixed thoroughly, 0.95 mL water added, and it was mixed to a stiff paste. This was extruded through a 1-mL disposable syringe, dried at room temperature for 2–3 h, and cut into 3–4-mm lengths. The diet was stored under argon at −20°C and was used within 3 d.

Experimental protocol. Groups of 10 to 21 fish were acclimated in a 100-L circular tank with running water for at least 4 d before starting an experiment. They were then fed the labeled diet, all of which was observed to be eaten. The fish were then fed the normal unlabeled diet daily and sampled at intervals of up to 15 d post-dose. Various tissues were then removed for measurement of D_5-22:6n-3. The temperature ranged from 9.0 to 16.0°C during the experiments.

Fish were anesthetized with MS 222 (ethyl 3-aminobenzoate methane sulfonate) and bled from the tail vein into a weighed vial containing heparinized saline (100 U/mL of 0.9% NaCl). The fish were weighed and individual tissues were dissected for analysis. Fat was trimmed from the gut and ceca and either analyzed separately or added to the remaining carcass. Ceca refer to the whole midsection of the gut between the points where the first and last cecum branch from the main gut. A crude cecal mucosal fraction was prepared by cutting individual cecum from the main gut and firmly squeezing the cecal sac from the closed end with smooth-jawed forceps to extrude the contents and cells forming the gut epithelia. Portions of liver, cecal mucosa, and blood were taken for protein determination by the method of Lowry *et al.* (11). At short time points after feeding the tracer, the gut contents were removed by cutting the gut longitudinally, and these were analyzed separately. Samples were homogenized in chloroform/methanol (2:1, vol/vol) using a Potter™ or Ultraturrax™ homogenizer, and an extract by the method of Folch *et al.* (12) was prepared. Tritricosanoyl glycerol (tri23:0) standard was added to each tissue sample before homogenization. The amount of tri23:0 standard added varied with the weight of the fish and the lipid content of the respective tissues, and was judged by previous experience to maintain the correct proportion of 23:0 to deuterated FA and correct loading of 23:0 on the gas chromatograph–mass spectrometer. For example, for a 15-g fish the amounts of tri23:0 were as follows: liver, 2.5 μg; ceca, 5.0 μg; cecal mucosa, 2.5 μg; blood, 0.5 μg; visceral fat, 100 μg; and carcass, 250 μg. Samples were kept on ice under nitrogen during workup and were stored at −20°C under argon.

Quantification of FA. One milligram of total lipid was saponified with 2 mL of 0.1 M KOH in 95% (vol/vol) ethanol under nitrogen for 1 h at 78°C. Nonsaponifiable material was removed by extracting with isohexane/diethyl ether (2:1, vol/vol), the aqueous phase was acidified, and FFA were extracted with diethyl ether. Pentafluorobenzyl esters were then prepared from 100 μg FFA using acetonitrile/diisopropylamine/pentafluorobenzyl bromide (1000:10:1, by vol) at 60°C for 30 min under nitrogen (13). Excess reagent and solvent were removed under nitrogen, and samples were dissolved in isohexane and stored at −20°C under argon until analysis.

Calibration standards of individual FA (18:3n-3, 21:4n-6, and 22:6n-3) with 23:0 were prepared by varying the amount of unknown FA while keeping the 23:0 constant and plotting the peak area ratio against the mass ratio of the different FA. Sample volumes for analysis were adjusted such that the amount of 23:0 injected onto the gas chromatograph–mass spectrometer was constant. Pentafluorobenzyl esters were chromatographed and quantified on a Fisons MD 800 gas chromatograph–mass spectrometer fitted with an on-column injector and a Chrompack CP-Wax 52CB column (30 m × 0.32 mm i.d., 0.25-μm film thickness; Burke Analytical, Alva, Clackmannanshire, United Kingdom) using helium as carrier gas (column head pressure, 7 psi) and running in negative chemical ionization mode with methane as reagent gas (pressure, 7 psi). The temperature program was 80–190°C at 40°C/min, 190–240°C at 1.5°C/min, then 240°C for 10 min. FA were identified by selective ion scanning for the required masses using a dwell time of 80 ms and a cycle time of 20 ms, and were quantified by reference to the appropriate FA calibration curve.

Analysis of results. Mean values at different times or between tissues were compared using a one-way ANOVA with Tukey's multiple comparison test or by Student's *t*-test, depending on the number of samples (Prism statistical package; GraphPad Software Inc., San Diego, CA).

RESULTS

Sections of the gut were analyzed for newly synthesized D_5-22:6n-3 1, 2, and 3 d after feeding D_5-18:3n-3 and were compared with liver (Table 1). No significant difference was found between the values at days 1, 2, and 3, so the data were pooled. Ceca and liver each accounted for approximately one-fourth of the recovered D_5-22:6n-3, with the stomach and hind gut (posterior to the ceca) accounting for 0.5 and 1.0% of newly synthesized 22:6n-3, respectively. Liver and ceca were at least an order of magnitude more enriched in D_5-22:6n-3 on a per mg tissue basis than hind gut and stomach.

TABLE 1
Deposition of Newly Synthesized D_5-22:6n-3 in Liver and Gut Tissues[a]

Tissue	Amount of D_5-22:6n-3 deposited		
	ng D_5-22:6/mg tissue/mg D_5-18:3 eaten	ng D_5-22:6/mg lipid/mg D_5-18:3 eaten	% of total
Liver	22.2 ± 11.5	737.4 ± 399.0	24.4 ± 10.5
Stomach	0.37 ± 0.27	7.30 ± 4.86	0.5 ± 0.2
Ceca	14.6 ± 10.9	84.9 ± 75.2	23.2 ± 14.8
Hind gut	1.97 ± 2.17	1.79 ± 2.78	1.0 ± 0.8

[a]Values are mean ± SD. Data from three fish each from days 1, 2, and 3 after feeding deuterated (D_5)-18:3n-3. Data were pooled as there was no significant difference between days (n = 9). Mean weight of fish was 18.6 ± 3.8 g.

In a second experiment the deposition of newly synthesized D_5-22:6n-3 was examined in ceca and liver over a longer time course, up to 15 d post-feeding (Table 2). Ceca contained 28.7 ± 27.1 ng D_5-22:6n-3/mg tissue/mg D_5-18:3n-3 eaten at day 2 compared with 7.0 ± 6.4 ng D_5-22:6n-3/mg tissue/mg D_5-18:3n-3 eaten in liver. The value in ceca then fell slowly, whereas that in liver increased to about 16 ng D_5-22:6n-3/mg tissue/mg D_5-18:3n-3 eaten at days 5 and 9 before falling slightly. The values in the two tissues approached parity by day 15. A similar pattern was found when the data were expressed as ng D_5-22:6n-3/mg protein/mg D_5-18:3n-3 eaten. The large scatter in the data, particularly at day 2, means the changes were not significant. This was probably because of variability in the amount of food eaten and the kinetics of digestion in different fish at short times after feeding. However, when the data were expressed as a ratio of D_5-22:6n-3 deposited in the tissues of individual fish at different times, the data became highly significant. The ratio of newly formed D_5-22:6n-3 in ceca compared to liver was 4.7 at day 2 on a per mg tissue basis and 5.2 on a per mg protein basis, then fell to 1.67 and 1.40, respectively, by day 5 and to unity by day 15 (Table 2).

Some fish were grown to *ca.* 60 g in weight so that the formation of D_5-22:6n-3 in ceca could be examined in more detail (Table 3). In this group of fish, cecal mucosal scrapings contained 9.4 ± 4.3 ng D_5-22:6n-3/mg tissue/mg D_5-18:3n-3 eaten compared to 6.4 ± 4.8 ng D_5-22:6n-3/mg tissue/mg D_5-18:3n-3 eaten in liver at day 2, then fell to 6.5 ± 3.9 ng D_5-22:6n-3/mg tissue/mg D_5-18:3n-3 eaten at day 5. In contrast, liver increased to 23.3 ± 21.7 ng D_5-22:6n-3/mg tissue/mg D_5-18:3n-3 eaten after this time. On a per mg protein basis, mucosal scrapings accumulated some 3.5 times (122.7 vs. 35.0 ng D_5-22:6n-3) more than liver at day 2 (Table 3), but by day 5, the amount in liver had increased and that in mucosal scrapings had decreased so that liver contained 1.3 times more D_5-22:6n-3 than did mucosal scrapings.

The ratio of D_5-18:3n-3 to 21:4n-6 decreased rapidly during passage through the gut and into tissue lipids (Table 4). The ratio of D_5-18:3n-3 to 21:4n-6 in the labeled diet was 4.0 in all experiments. This fell in the gut contents (stomach and cecal contents) from 2.24 at day 1 to 0.47 at day 3 post-feeding. In ceca this ratio was already 0.30 at day 1 and fell further to 0.12 at day 3. In contrast, blood, liver, and carcass had D_5-18:3n-3 to 21:4n-6 ratios of about 0.8 at day 1 and fell to 0.25 by day 3 in blood and liver but remaining virtually unchanged (0.7) in carcass.

The concentration of D_5-18:3n-3 in blood declined very sharply after feeding (Table 5). Although the scatter in this data is large at day 2 for all FA and for D_5-22:6n-3 at all times, it is apparent that D_5-18:3n-3 was metabolized differently from the C_{20} and C_{22} PUFA. Although the concentration of D_5-22:6n-3 remained approximately constant from days 2 to 15, the concentration of D_5-18:3n-3 dropped an order of magnitude by day 5 and a further order of magnitude by day 15. In contrast, the blood concentration of 21:4n-6 fell by half from day 2 to day 5 then remained constant.

The rate of D_5-22:6n-3 synthesis for fish sampled between days 5 and 9 during these experiments was 4.88 ± 2.47 μg D_5-22:6n-3/g fish/mg D_5-18:3n-3 eaten/7 d (n = 10) for 15.6-g fish, and 1.84 ± 0.81 μg D_5-22:6n-3/g fish/mg D_5-18:3n-3 eaten/7 d (n = 6) for the larger 60.1-g fish. The recovery of D_5-22:6n-3 in the whole carcass was related to the concentration of D_5-22:6n-3 in blood with all fish analyzed between days 5 and 9 post-feeding, giving a correlation of 0.8417 (slope of 7.74) for whole-body D_5-22:6n-3 (μg) compared to ng D_5-22:6n-3/mg protein in blood.

DISCUSSION

The results clearly show that ceca accumulated substantially more D_5-22:6n-3 than liver at time points immediately after feeding the tracer and that this decreased rapidly, whereas the amounts in liver increased. Ceca contained 4.7 times more D_5-22:6n-3 than liver on a per mg tissue basis and 5.2 times

TABLE 2
Comparison of D_5-22:6n-3 Deposition in Liver and Ceca at Intervals Post-Dose of D_5-18:3n-3[a]

Time (d)	Number of fish	ng D_5-22:6n-3/mg tissue/mg D_5-18:3n-3 eaten		ng D_5-22:6n-3/mg protein/mg D_5-18:3n-3 eaten		Ceca/liver ratios	
		Ceca	Liver	Ceca	Liver	per mg tissue	per mg protein
2	5	28.70 ± 27.15	6.96 ± 6.38	186.0 ± 164.2	47.2 ± 46.3	4.69 ± 1.22[a]	5.18 ± 2.39[a]
5	6	23.08 ± 10.49	16.10 ± 12.50	132.6 ± 62.3	93.1 ± 48.7	1.67 ± 0.55[b]	1.40 ± 1.23[b]
9	4	21.38 ± 11.20	16.47 ± 8.01	106.2 ± 51.1	99.1 ± 41.8	1.29 ± 0.25[b]	1.06 ± 0.16[b]
15	6	12.23 ± 4.77	12.06 ± 5.60	56.7 ± 21.7	59.1 ± 29.8	1.07 ± 0.27[b]	0.93 ± 0.55[b]

[a]Values are mean ± SD. One-way ANOVA gave differences in ceca/liver ratios of $P < 0.0001$ per mg tissue and $P = 0.0003$ per mg protein. Within columns, values with different superscripts are different at $P < 0.01$. Mean weight of fish was 15.6 ± 3.8 g. For other abbreviation see Table 1.

TABLE 3
Comparisons of Newly Synthesized D_5-22:6n-3 in Liver and Cecal Fractions 2 and 5 d Post-feeding[a]

	Time (d)	Liver	Cecal mucosa
ng D_5-22:6n-3/mg tissue/mg D_5-18:3n-3 eaten			
	2	6.35 ± 4.80	9.39 ± 4.26
	5	23.25 ± 21.68	6.53 ± 3.94
ng D_5-22:6n-3/mg protein/mg D_5-18:3n-3 eaten			
	2	35.0 ± 21.0[a]	122.7 ± 50.4[b]
	5	109.5 ± 99.7	83.2 ± 46.4

[a]Values are mean ± SD, $n = 4$ at day 2 and $n = 6$ at day 5. The values marked with different superscripts are different at $P = 0.029$ (*t*-test). Mean weight of fish was 60.1 ± 11.3 g. For other abbreviation see Table 1.

more on a per mg protein basis 2 d after feeding D_5-18:3n-3. At later times, the amount in ceca decreased while that in liver increased so that the ratio approached unity by day 9 after feeding. Analysis of sections of digestive tract showed that this was specific to ceca and not to stomach or hind gut, the sections anterior and posterior to the midsection holding the ceca. The crude mucosal fraction contained 3.5 times more newly synthesized D_5-22:6n-3 than liver 2 d post-dose. This fraction must have contained some undigested material, and a cleaner preparation of gut epithelial cells would probably have shown an even greater enrichment in D_5-22:6n-3. The concentration of D_5-22:6n-3 in blood reflected the pattern in liver much more closely, peaking at day 9 (in this study) and day 14 (see Ref. 8). It is therefore very unlikely that transport of newly synthesized D_5-22:6n-3 from liver to ceca accounted for the accumulation of D_5-22:6n-3 in ceca 2 d after feeding the tracer. The kinetics are compelling that ceca are major sites of 22:6n-3 synthesis in fish and may be at least five times more active than liver.

It is difficult to firmly conclude on the relative contributions of ceca and liver to whole-body accumulation of 22:6n-3 without knowing the availability of substrate to the different tissues over time or the concentration of pathway enzymes and their kinetics in the two tissues. In this pulse-chase experiment, it is likely that D_5-18:3n-3 was depleted rapidly in ceca, as the ratio of D_5-18:3n-3 to 21:4n-6 was lowest in ceca at all time points 1, 2, and 3 d after feeding the tracer. The blood concentration of D_5-18:3n-3 fell rapidly also, so substrate available to liver dropped quickly. The gut epithelial cells had the greatest exposure to D_5-18:3n-3; therefore, it is reasonable to suppose that the Δ6-desaturase, the first enzyme in the pathway, would be maximally active. However, there are seven sequential steps of desaturation and elongation followed by a final chain-shortening reaction, all steps at which the flux of FA through the pathway could be modulated. It is possible also that different isoforms of the various enzymes can be found in the two tissues with different K_m and/or V_{max}. In liver the pathway in fish can be induced (5), but that may not be the case in ceca.

A further complication is that the number of ceca in individual fish is quite variable (14). In rainbow trout the number of ceca appears to be precociously fixed once the alevin reaches 3.5 to 4 cm (14) and typically varies between 35 and 70 (15), although up to 147 have been found (16). Fish with higher numbers of ceca showed better food conversion ratios than those with fewer ceca (16). However, Ulla and Gjedrem (15) concluded that the variation in the number and length of ceca is not of great importance for either fat or protein digestibility in rainbow trout. The results here would suggest that fish with larger numbers of ceca may be more efficient at synthesizing 22:6n-3 than those with fewer ceca. The cell structure of the cecum is similar to that in small intestine (17), and ceca are thought to be an adaptation for increasing intestinal surface area without increasing the length or thickness of the intestine (17). However, this study showed that ceca accumulated newly synthesized D_5-22:6n-3 at a much higher concentration than hind gut, suggesting that these parts of the gut are not identical.

TABLE 4
Change in Ratio of D_5-18:3n-3 to 21:4n-6 in Different Tissues with Time[a]

Time (d)	Gut contents	Ceca	Blood	Liver	Carcass
1	2.24 ± 0.57[a,x]	0.30 ± 0.11[b]	0.84 ± 0.52[b]	0.85 ± 0.40[b,x]	0.81 ± 0.45[b]
2	1.25 ± 0.16[y,x]	0.19 ± 0.11[b]	0.26 ± 0.10[b]	0.20 ± 0.09[b,y]	0.71 ± 0.39[c]
3	0.47 ± 0.09[z]	0.12 ± 0.10[a]	0.24 ± 0.16	0.25 ± 0.09[y]	0.73 ± 0.50[b]

[a]Values are mean ± SD ($n = 4$ at each time). The ratio of D_5-18:3n-3 to 21:4n-6 in the diet was 4.0. One-way ANOVA gave differences of $P = 0.0002$ for gut contents vs. time, $P = 0.043$ for blood vs. time, and $P = 0.0073$ for liver vs. time; tissues differed at $P = 0.0003$ on day 1, at $P < 0.0001$ on day 2, and at $P = 0.024$ on day 3. Values with different superscripts a to c across rows and x to z down columns differed at $P < 0.05$. For abbreviation see Table 1.

TABLE 5
FA Concentrations in Blood at Intervals After Feeding the Labeled Diet[a]

		ng FA/mg protein/mg D_5-18:3n-3 eaten		
Time (d)	No. of fish	D_5-22:6n-3	D_5-18:3n-3	21:4n-6
2	5	5.85 ± 5.90	2.67 ± 2.89	2.23 ± 2.34
5	6	4.84 ± 2.03	0.15 ± 0.10	0.94 ± 0.23
9	4	10.25 ± 11.58	0.12 ± 0.08	1.08 ± 0.50
15	6	6.65 ± 3.39	0.02 ± 0.01	1.11 ± 0.33

[a]Values are mean ± SD. For abbreviation see Table 1.

The experimental protocol assumed that FA administered as FAEE are digested and absorbed similarly to those in TAG. In salmon it was shown that although ethyl esters of PUFA were less well absorbed than TAG or FFA, no FAEE could be detected in feces, indicating complete hydrolysis (18). Linolenic acid was absorbed equally as well as TAG or FFA (18). We therefore believe that the D_5-18:3n-3 was absorbed and metabolized similarly to the bulk of 18:3n-3 in the diet, which was as TAG.

The cecal preparation used here was not sterile. There are several reports of gut bacteria from a number of species of fish being able to synthesize 20:5n-3 and 22:6n-3 (19). However, we do not believe that gut bacteria are responsible for the synthesis of 22:6n-3 described here for several reasons. A large biomass of bacteria of the appropriate species would be required in the fish intestine to account for the amount of material synthesized here. For the gut flora to be supplying 22:6n-3 to the fish, either the bacteria must secrete PUFA or the bacteria must be ingested by the fish. Both these scenarios seem unlikely. Recent evidence showed that microorganisms may be using the polyketide pathway for PUFA synthesis (20). This pathway inserts double bonds into the nascent acyl chain and has a different series of FA intermediates from the classical desaturase pathway. Linolenic acid is not on this pathway, and it is therefore unlikely that it could be used as a substrate for PUFA biosynthesis by organisms using this pathway.

A consistent finding during all our experiments using this technique has been that the recovery of D_5-18:3n-3 is much lower than that of 21:4n-6, suggesting a very active catabolism of 18:3n-3. The changing ratio of D_5-18:3n-3 to 21:4n-6 during passage through the gut and into tissue lipids confirms this. In the experiments described here, the recovery of D_5-18:3n-3 as linolenate was 13.5 (18.6-g fish up to 3 d), 4.8 (60.1-g fish up to 5 d), and 3.8% (15.6-g fish up to 15 d), respectively, whereas the recovery of 21:4n-6 was 58.2, 81.8, and 25.2%. Over a 5-wk time course in fish up to 10.4 g weight, the recovery of D_5-18:3n-3 was 1.18% and of 21:4n-6 was 57.6% (8). Further experiments using this approach have recovered 10–20 times more 21:4n-6 than D_5-18:3n-3 (Bell, M.V., unpublished work). Therefore, a large catabolism of 18:3n-3 occurs even under conditions where there is a dietary restriction on 22:6n-3. The diet used here was not sufficient to maintain the whole-body concentration of 22:6n-3, even though 18:3n-3 was present at 1% of the diet and a small amount of 22:6n-3 was present in the fish meal (8). Oxidation of 18:3n-3 occurred rapidly: The blood concentration of D_5-18:3n-3 dropped sharply between days 2 and 5 post-dose, and the ratio of D_5-18:3n-3 to 21:4n-6 in ceca fell from 4.0 (in the diet) to 0.30 24 h post-dose. Red muscle, liver, and heart are generally regarded as the most important tissues for FA oxidation in fish (2), but this study suggests that the gut is active in the β-oxidation of linolenate also.

It is becoming clear that β-oxidation of 18:3n-3 (and 18:2n-6) is the main metabolic fate of these FA (reviewed by Cunnane, Ref. 21). Substantial carbon recycling of 18:2n-6 into *de novo* lipogenesis occurred in rat liver even under conditions of extreme linoleate deficiency (22), and pregnant and lactating rhesus monkeys recycled 18:2n-6, 18:3n-3, and 22:6n-3 into saturated and monounsaturated FA (23). It was concluded that the majority of dietary linoleate in growing rats was β-oxidized or stored in visceral fat (24), and it seems probable that this is the fate of dietary linolenate also. In human subjects plasma 18:3n-3 had a half-life of 1 h, and only 0.2% was destined for synthesis of 22:6n-3 (25). Most substrate FA thus appears to be oxidized even under dietary restrictions, and it seems surprising that some control mechanism has not evolved to spare 18:3n-3 and 18:2n-6 from β-oxidation under such conditions. The bioequivalence of 18:3n-3 and 22:6n-3 and the requirement for 22:6n-3, the functional EFA of the n-3 pathway, at different stages during development are still undefined in the majority of species.

The rates of whole-body accumulation of D_5-22:6n-3 in this study were 4.9 ± 2.5 and 1.8 ± 0.8 μg D_5-22:6n-3/g fish/mg D_5-18:3n-3 eaten/7 d for 15.6- and 60.1-g fish, respectively. This corresponds to a percentage conversion of 8.6 ± 4.2 and 7.5 ± 4.9% over 5 d and 5–9 d, respectively, in these two groups of fish. These rates are similar to that reported in Bell *et al.* (8) of 3.8 ± 0.8 μg D_5-22:6n-3/g fish/mg D_5-18:3n-3 eaten/7 d measured over a similar time period. The possibility that fish lose the capability to synthesize 22:6n-3 as they grow is currently being investigated. Not surprisingly, the blood concentration of 22:6n-3 and the whole-body content of D_5-22:6n-3 were correlated, even in fish of different sizes, when measured over the same time period (8). Thus, it might be possible to identify individuals most active in 22:6n-3 synthesis from a blood sample.

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Characterization of Glycerophosphocholine Phosphodiesterase Activity and Phosphatidylcholine Biosynthesis in Cultured Retinal Microcapillary Pericytes. Effect of Adenosine and Endothelin-1

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ABSTRACT: In pericytes from bovine retina, the enzyme glycerophosphocholine phosphodiesterase, catalyzing the hydrolysis of *sn*-glycero-3-phosphocholine to glycero-3-phosphate and choline, has been characterized with respect to pH optimum, metal ion dependence, K_m, inhibitors, and subcellular localization. In these cells, the natural substrate *sn*-glycero-3-phosphocholine was present at relatively high concentration (6.4 ± 1.2 nmol/mg protein), and the EDTA-sensitive phosphodiesterase activity was also found to be markedly high (9.80 ± 1.5 nmol/min/mg protein) compared to that estimated in liver and brain (1–3 nmol/min/mg protein) or in renal epithelial cell culture (0.27 nmol/min/mg protein). The reaction conditions were in general agreement with those found earlier in brain and other tissues. The majority of the enzyme specific activity was located in the plasma membrane, whereas a minor part was present in the microsomal fraction. The physiological significance of the high catabolic phosphodiesterase activity in these cells may be related to the transfer, followed by deacylation, of lysophosphatidylcholine from the bloodstream to nervous tissue. In addition, capillary pericytes in culture were able to incorporate ^{3}H-choline rapidly into choline-containing soluble phosphorylated intermediates and into phosphatidylcholine. To find a positive and negative effector on phosphatidylcholine formation, adenosine, an important intercellular mediator in the retina in response to alterations in oxygen delivery, and endothelin-1, a potent paracrine mediator present at the blood–brain and blood–retina barrier, were tested. The cells cultured for 1 or 24 h in a medium containing adenosine at concentrations of 10^{-6} and 10^{-4} M showed significant reduction in ^{3}H-choline incorporation compared to control cultures, whereas endothelin-1, at a concentration of 10 and 100 nM, caused stimulation of phosphatidylcholine biosynthesis. These findings provide evidence that both agonists may modulate phosphatidylcholine metabolism in pericytes.

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Our previous studies concerned the transport across the blood–retina and blood–brain barrier of plasma lysophosphatidylcholine (lyso-PtdCho), a transport form to tissues, and especially the brain, of both choline and unsaturated FA (1). We were stimulated to investigate whether endothelial cells (EC) and pericytes, which organize together the physical barrier between blood and tissues in microvessels, may deacylate lyso-PtdCho to *sn*-glycero-3-phosphocholine (GroPCho), and how high the potential is for these cells to catabolize this last phosphorylated intermediate to liberate free choline.

We first demonstrated a marked presence of glycerophosphocholine phosphodiesterase (*sn*-glycero-3-phosphocholine glycerophosphohydrolase, GroPChoPDE, EC 3.1.4.2) in bovine brain microvessel preparations (2). This suggested investigating the cellular origin, whether endothelial or pericytal, of this enzyme activity related to the last step of phosphatidylcholine (PtdCho) degradation. Therefore, in the present study, experiments were performed to measure the activity in cultured pericytes and endothelial cells, both isolated from bovine retina microcapillaries. We found that the enzyme activity tested was high in pericytes (9.80 + 1.5 nmol/min/mg protein) and was present to a much lesser extent in EC (0.39 + 0.08 nmol/min/mg protein). In view of this result, the elevated GroPChoPDE activity we had previously measured in brain microvessels (2) could be attributed to the pericyte component. The consequence of this finding was to promote our interest in expanding the study of such highly expressed GroPChoPDE in this cell type; here we further characterize its enzymatic parameters and subcellular localization.

GroPChoPDE activity is enriched in rat kidney (40 nmol/min/mg protein) (3), and it is present at lower levels of activity in renal epithelial cell culture (0.27 nmol/min/mg protein) (4) and in many organs including brain (1–3 nmol/min/mg protein) (3–7), but not in myelin (8). Among brain subcellular fractions, microsomes contain the greatest amount (6). A glycerylphosphorylcholine diesterase, secreted by epithelial cells in the rat uterus during preovulatory estrogen surge, has been purified and characterized (9); native glycoprotein is a trimer of 105 kDa.

At the same time, we tested for the presence of some enzymes involved in PtdCho biosynthesis, degradation, and remodeling in pericytes, for which no studies have been done. Indeed, their physiological function and biochemical characteristics are not yet clearly identified. Because they contain contractile elements (muscle actin) similar to those present in the vascular smooth muscle cells, a role as modulators of microcapillary dilation has been proposed (10).

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Abbreviations: CDP-Cho, cytidine diphosphocholine; CT, CTP:phosphocholine cytidylyltransferase; EC, endothelial cells; ET-1, endothelin 1; γ-GPT, γ-glutamyltranspeptidase; GroPCho, *sn*-glycero-3-phosphocholine; GroPChoPDE, glycerophosphocholine phosphodiesterase; lyso-PtdCho, lysophosphatidylcholine; PCho, phosphorylcholine; PLA_2, phospholipase A_2; PtdCho, phosphatidylcholine; SN, supernatant.

A growing body of evidence indicates that adenosine is an important intercellular mediator in the retina, where it plays a major role in response to alterations in oxygen delivery, critically mediating blood flow changes in response to ischemia, hypoxia, and hyperglycemia. Neverthless, a limited number of adenosine effects have been demonstrated in retina pericytes, despite their putative importance. Inasmuch as adenosine is a potent vasodilator in the retinal microvasculature (11), we hypothesized that, by a direct action on these cells, it could cause cell metabolic derangement in an autocrine or paracrine manner. Membrane phospholipids could be the target.

Endothelin-1 (ET-1) is a potent paracrine mediator present at the blood–brain and blood–retina barrier (12). Capillary pericytes are target cells for this endothelium-derived agonist (13). Addition of ET-1 to pericyte cultures induces marked changes in the cell morphology that are associated with a reorganization of F-actin and intermediate filaments. Recently, it has been demonstrated that ET-1 induces changes in the physiology of retinal pericytes (14). Responsiveness of pericytes to ET-1 has only been studied with respect to calcium early release from intracellular stores (15), stimulation of DAG and inositol triphosphate (IP_3) levels, and protein kinase C (PKC) activity. The findings reported in our paper provide support for the concept that changes in PtdCho synthesis, preceding phenotypic changes and increase or decrease in DNA synthesis, are endothelin- and adenosine-inducible.

MATERIALS AND METHODS

Culture of retinal pericytes. Primary cultures of microcapillary pericytes were established from bovine retinas dissected from freshly slaughtered cattle. The retinas were homogenized in minimum essential medium and filtered through an 85-μm nylon sieve. The trapped microvessels were digested in PBS, pH 7.4, containing 1 mg/mL collagenase-dispase and 0.5% BSA for 60 min at 37°C. The homogenate was centrifuged (1,000 × *g*, 2 min), and the resuspended pellet was plated in DMEM, supplemented with 20% fetal calf serum, and transferred to 60-mm tissue culture petri dishes coated with skin porcine gelatin. The culture medium was DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. After allowing 2–3 h for cell attachment, the media were removed and replaced with the same medium. When the primary cells reached confluence, they were subcultured in 60-mm plastic petri dishes and incubated with the standard medium. The majority of cells cultivated exhibited an irregular and stellate morphology. Typically, growth was not contact inhibited, and in some areas of the culture dishes the cells formed multilayers. The retinal pericytes were characterized by negative staining for factor-VIII-related antigen, positive staining for smooth muscle α-actin monoclonal antigen (Sigma), low presence of γ-glutamyltranspeptidase (γ-GPT) activity, and morphological features including absence of contact inhibition. Cells from the second to sixth subcultures were used for the experiments.

Retinal pericyte-cell membrane preparation. Crude bovine pericyte-cell membranes were prepared by rinsing confluent cells twice with ice-cold PBS, in 100 cm^2 flasks (50–60 × 10^6) placed on ice, scraping the flasks with a rubber policeman, and pelleting the collected cells at 800 × *g* for 5 min at 4°C. The pellet was then resuspended in 4 mL ice-cold buffer composed of 10 mM Tris, pH 7.5, 250 mM sucrose, 1 mM DTT, and the following protease inhibitors: leupeptin (2.5 μg/mL), pepstatin (0.5 μg/mL), aprotinin (0.5 μg/mL), soybean trypsin inhibitor (30 μg/mL), and PMSF (0.2 mM). The cells were disrupted by nitrogen cavitation, leaving the nuclei intact, in a Kontes mini-bomb cell disruption chamber with 35–50 atm (500–700 psi) of N_2 for 15 min at 0–4°C, and the lysate was centrifuged at 800 × *g* for 5 min *(procedure a)*. The supernatant (SN) was removed, and the pellet was resuspended in 2 mL lysis buffer and sedimented as above. The resulting pellet was resuspended in lysis buffer and designated as the crude nuclear fraction. The SN was spun at 10,000 × *g* at 4°C for 30 min to obtain the mitochondrial and plasma membrane fraction. The SN obtained at this stage was further spun at 105,000 × *g* for 20 min in a Beckman Optima TL ultracentrifuge. The microsomal pellet was resuspended in buffer without sucrose and re-spun at 105,000 × *g* for 20 min. The resuspended crude membrane fraction and the concentrated cytosol obtained after the last centrifugation were used immediately for enzyme assays.

Alternatively, for some experiments in which the presence of plasma membrane activities was at issue, subcellular fractions (cytosols and plasma membranes) were obtained on self-forming Percoll gradients (16) *(procedure b)*. Briefly, the SN (3 mL) was applied to the top of 9.0 mL 40% Percoll solution at pH 9.3. A gradient was formed, and fractions were separated during centrifugation in a Beckman L8-80 ultracentrifuge using a 70.1 Ti rotor at 100,000 × *g* for 10 min. The plasma membrane fraction was removed by a Pasteur pipette and washed by diluting fivefold in enzyme assay medium, followed by centrifugation at 10,000 × *g* for 5 min. The pellet containing plasma membrane was finally resuspended in 200 μL of enzyme assay medium. Protein was determined in individual fractions and cell lysate by reaction with fluorescamine using BSA as standard. The characterization of membrane fractions by classical marker enzyme assays was conducted as previously described (17). Lactate dehydrogenase was assayed in 0.05 M sodium phosphate buffer (pH 7.5) containing 0.15 mM NADH and 5 mM sodium pyruvate, in a final volume of 1.0 mL.

Enzyme assays. Microsomal glycerophosphorylcholine phosphodiesterase (EC 3.1.4.2) was measured as described previously (2,18). The inhibitors to which the enzyme was exposed were EDTA and L-α-glycero-3-phosphate, under concentrations described in Table 2. Enzyme K_m was estimated from Lineweaver–Burke plots.

The buffers used for the study of pH effects were: pH 6.0–6.5, 100 mM sodium acetate/acetic acid; pH 7–8, 100 mM HEPES/sodium hydroxide; and pH 8.5–10, 100 mM glycine/sodium hydroxide.

TABLE 1
Marker Enzymes, GroPCho Cell Content, and Characteristics of Glycerophosphocholine Phosphodiesterase (GroPChoPDE) Activity of Retinal Pericytes in Culture[a]

Alkaline phosphatase (nmol/min/mg protein)	10.0 ± 3.0
γ-Glutamyltranspeptidase (nmol/min/mg protein)	40.2 ± 7.3
GroPCho (nmol/mg protein)	6.4 ± 1.2
GroPChoPDE pH optimum	8.5
K_m	0.31 mM
Metal activation	Mg^{2+}, Ca^{2+}

[a]Assay conditions are described in the Materials and Methods section. Homogenates of confluent cell cultures (2 mg protein/mL) were incubated in appropriate buffer with assay ingredients at 37°C for the allotted times. Values are means ± SD. GroPCho, *sn*-glycero-3-phosphocholine.

Alkaline phosphatase was determined using *p*-nitrophenylphosphate as substrate (19). GroPCho is not hydrolyzed in significant amounts by this enzyme activity (20). γ-GPT was determined by the procedure of Orlowski and Meister (21).

Choline incorporation. Twenty-four hours prior to the labeling procedure, confluent cells in 60-mm culture dishes were incubated with serum-free medium. Serum-starved cultures were washed and incubated with labeling medium containing 10 μM choline and 5 μCi/dish of [Me-^{3}H]choline (Amersham Intl., Buckinghamshire, England) for 4 h at 37°C and in 5% CO_2/95% air. The incubations were then stopped by putting dishes on ice and removing the labeling medium. Cell layers were rapidly washed three times with ice-cold, fresh HBSS buffer containing 1 mM choline, and then covered with methanol. Pericytes were scraped while in cold methanol and transferred to a Teflon screw-capped glass test tube. Then the suspension was added with chloroform to reach a solvent ratio of 1:2. The cells were extracted once more with a mixture of 1 mL $CHCl_3$/CH_3OH (2:1,vol/vol). The SN lipid extracts, obtained after centrifugation, were pooled with the first organic extracts. The pellet was further extracted twice with 20% ethanol in water. The water/ethanol mixtures were retained. After the lipid extract had been taken to dryness under N_2, it was washed by the procedure of Folch *et al.* (22). The upper aqueous phase was removed and combined with the water/ethanol washings to give a totally water-soluble extract. This was concentrated under vacuum in a centrifugal evaporator and then taken to dryness. The residue was dissolved in 20–40 μL of distilled water and used to measure the radioactivity distribution of choline derivatives and the size of the pool of GroPCho. Choline-containing water-soluble compounds were resolved by TLC on precoated Silica gel 60 thin-layer plates (Merck, Darmstadt, Germany), and chromatographed for 3 h at room temperature with methanol/0.5% NaCl/conc. NH_4OH (50:50:5, by vol) as previously described (23). Radioactivity distribution on the plate was measured using a Berthold digital autoradiograph. The segment of the TLC plates containing GroPCho in association with phosphorylcholine (PCho) (R_f between 0.30 and 0.60, fraction II), identified by comparison with standards, was scraped into a centrifuge tube and extracted from the silica gel with three 1-mL washes with 50% ethanol in water. The pooled washings were concentrated under vacuum in a centrifugal evaporator and analyzed. The recovery of silica gel extraction was about 82%. In aliquots of extracted fraction II, radioactivity associated to GroPCho was determined after hydrolysis with 2 N HCl for 20 min, neutralization with NaOH, evaporation to dryness, and extraction of labeled choline from the salts with ethanol. Lipid extracts were fractionated in phospholipid classes on Silica gel 60 thin-layer chromatoplates with $CHCl_3$/MeOH/CH_3COOH/H_2O (65:45:1:4, by vol) as developing solvent, and visualized with I_2 vapor. Radioactivity distribution on the plate was measured using a Berthold digital autoradiograph.

In testing the effect of adenosine and ET-1, 24 h prior to adding the effectors and pulse labeling with ^{3}H-choline, confluent cells in 60-mm culture dishes were incubated with serum-free medium. The pericytes were then cultured in a 37°C incubator for 1 or 24 h in the same medium containing adenosine at 10^{-4} and 10^{-6} M concentrations, or human ET-1 (Sigma) at 10 and 100 nM concentrations. Subsequently, [Me-^{3}H]choline (5 μCi/60 mm dish) dissolved in a serum-free medium containing cold choline was added to each dish to obtain a final concentration of 10 μM choline. After reincubation for 4 h in the presence of effectors, cells were rinsed three times with ice-cooled phosphate saline buffer and harvested as above. Pericytes incubated for the indicated periods in the same medium without adenosine or ET-1 were used as controls.

Pool size of GroPCho. GroPCho content was determined in perchloric acid cell extracts by a combined enzymatic and chemical method that employs GroPCho:choline phosphodiesterase from mold to catalyze production of choline from GroPCho, and then measures the choline by using choline oxidase, peroxidase, phenol, and 4-aminoantipyrine to produce a red dye (18). One milliliter of 5% perchloric acid was added to 6 × 35-mm dishes and the cells were scraped with a rubber policeman, transferred to Eppendorf microtubes, and sonicated. Acid-insoluble material was centrifuged, and perchloric acid present in the supernatant was precipitated by the addition of an equimolar concentration of potassium acetate and centrifugation. An 0.8-mL aliquot of the supernatant was dried under vacuum, and the dried extracts were routinely dissolved in 0.1 mL of 2.5 mM Tris-HCl buffer (pH 8.0). One unit of GroPChoPDE (Sigma) was added, and the mixture was incubated at 37°C for 30 min. The reaction mixture was frozen, then lyophilized, and the residue was extracted with 70% ethanol. The ethanol in the extracted samples was evaporated under N_2, and the liberated choline in the residue was determined enzymatically.

Other enzymes of PtdCho metabolism. Enzyme activities were determined after homogenization of cells with 10 mM Tris-HCl, pH 7.4, in a Potter–Elvehjem motor-driven apparatus. Protein determinations were conducted according to Lowry *et al.* (24). All enzyme determinations were performed under optimal conditions. Acyl-CoA:1-acyl-*sn*-glycero-3-phosphocholine *O*-acyltransferase (EC 2.3.1.23) was assayed using 25 μM [1-^{14}C]oleoyl-CoA (Amersham Intl.) and 50 μM lyso-PtdCho as substrates, as previously described (17). CTP:phosphocholine cytidylyltransferase (CT) (EC 2.7.7.15)

was assayed radiometrically according to Jamil and Vance (25), using phospho[Me-^{14}C]choline (Amersham Intl.) as substrate (1.5 mM). Microsomal choline phosphotransferase (EC 2.7.7.2) was measured according to Sipione *et al.* (2).

Confluent cells grown on 60-mm dishes were stimulated or incubated with agonists. At the indicated times, treated and control cells were collected and cytosolic phospholipase A_2 (PLA_2) activity was assayed with 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine as substrate, following the procedure described previously (23).

RESULTS

It should be pointed out that, unlike in capillary endothelial cells and under conditions used here, very low alkaline phosphatase activity was found (Table 1) compared to that measured in isolated microcapillaries, 205.3 ± 37 nmol/min/mg protein. In a previous study, this enzyme was not detected in retinal pericytes at subconfluent or confluent densities, but was expressed in areas of high cell densities, such as multilayers and nodules (26). Another enzyme activity, γ-GPT, which has been described as being highly present in cerebral pericytes (27,28), was also found to be low (Table 1), compared to the value measured in brain pericytes, 79.1 nmol/min/mg protein (29). This is in agreement with the observation that γ-GPT activity in retinal microvessels is about 4% of that in brain microvessels, 8.5 ± 1.5 vs. 185 ± 39 nmol/min/mg protein (30).

The pH-activity curve (6.5–9.5) for the hydrolysis of GroPCho showed maximal activity at about pH 8.5 (data not shown). This result was in agreement with that of Webster *et al.* (31) and Baldwin and Cornatzer (3), who studied the influence of pH on the activity of this diesterase in rat brain and kidney.

The study of the sensitivity of GroPChoPDE in cell homogenates, dialyzed for 24 h, to metal ions shows that Mg^{2+} and Ca^{2+} had a significant activating effect (Table 2). EDTA (1 mM) caused more than 80% inhibition. The enzyme was not affected by the presence of 2 mM NaF, 3 mM *N*-ethylmaleimide, 0.1 mM *p*-chloromercuribenzoate and treatment with 0.2% trypsin (Table 2), in agreement with the notion that sulfhydryl groups do not appear to be required for activity.

The influence of substrate concentration on enzymatic activity was also examined. The calculated Michaelis constant appears to be approximately 0.31 mM (Table 1), in agreement with the value of 0.6 mM reported by Abra and Quinn (5), and lower than the value of 2.7 mM obtained by Webster *et al.* (31) for rat brain homogenate.

GroPCho content in the pericytes was 6.4 + 1.2 nmol/mg protein (Table 1), whereas endogenous choline contributed 4.6 + 0.9 nmol/mg protein.

Table 3 shows the distribution of GroPChoPDE among the various subcellular fractions of pericytes. GroPChoPDE activity was enriched twofold in the microsomes compared to the starting homogenate. The enrichment ratio of this enzyme in the cytosol is only 0.43 on total homogenate, suggesting that the enzyme may not be physiologically associated with this fraction. The GroPChoPDE specific activity of plasma membrane is 2.7-fold greater than the original homogenate, suggesting its abundant presence in this particulate fraction. Nuclear and mitochondrial fractions showed some activity, probably due to plasma membrane contamination during homogenization and fractionation procedures. When tested for putative marker enzymes, isolated microsomes were enriched approximately fivefold over total homogenate in NADPH-cytochrome c reductase, whereas no enrichment in 5′-nucleotidase was observed (Table 3). The plasma membrane fraction isolated by Percoll procedure was enriched roughly twofold over homogenate in its putative marker 5′-nucleotidase, whereas the enrichment in NADPH-cytochrome c reductase was about twofold. As expected, the cytosol fraction was enriched threefold in lactate dehydrogenase, and no enrichment of this enzyme was found in any of the membrane fractions (data not shown).

Table 4 shows five selected enzyme activities related to *de novo* synthesis and remodeling of PtdCho, measured in pericyte cultures and compared to those estimated in bovine retina endothelial cells. Our results provide the first direct measurement of such activities contributing to homeostasis of membrane PtdCho levels in both microcapillary cell types.

TABLE 2
Effect of Inhibitors and Various Cations on GroPChoPDE Activity of Retinal Pericytes in Culture

Enzyme preparation	Activity (nmol/min/mg protein)
No additions	9.80 ± 1.5
Cells in FCS-free medium (24 h)	9.80 ± 1.9
No Mg^{2+} ions	6.80 ± 0.6
EDTA (1 mM)	1.20 ± 0.3*
N-Ethylmaleimide (3 mM)	6.80 ± 0.9
p-Cl-mercuribenzoate (0.1 mM)	7.50 ± 0.6
NaF (2 mM)	9.60 ± 1.3
Trypsin (0.2%)	6.00 ± 1.0
GroPChoPDE[b]	5.11
GroPChoPDE[c]	0.22
GroPChoPDE[c] + Ca^{2+} (1 mM)	2.45
GroPChoPDE[c] + Zn^{2+} (1 mM)	4.66
GroPChoPDE[c] + Mg^{2+} (1 mM)	3.88
GroPChoPDE[b] + Cu^{2+} (1 mM)	2.44
PM-GroPChoPDE[d]	19.50
PM-GroPChoPDE[d] + PLC	13.31

[a]Assay conditions are as described in the Materials and Methods section. Homogenates of confluent cell cultures (2 mg protein/mL) were incubated in the appropriate buffer with assay ingredients at 37°C for the allotted times. Values are means ± SD. A comparison with enzyme activities in immortalized endothelial cells (32) and bovine retina endothelial cells in culture, isolated and grown by the procedure of Stitt *et al.* (33), was made. The values were: GroPChoPDE, 0.39 ± 0.08 and 0.38 nmol/min/mg protein (mean of two experiments with different cell cultures), respectively.
[b]In nondialyzed pericyte homogenates, after 3 d at 4°C, the enzyme activity was measured in an incubation medium deprived of Mg^{2+} ions.
[c]In dialyzed pericyte homogenates, after 3 d at 4°C, the enzyme activity was measured in an incubation medium deprived of Mg^{2+} ions.
[d]The enzyme activity was measured in plasma membrane preparations (PM), incubated with phospholipase C from *Bacillus cereus* for 30 min at 37°C, as described in the Materials and Methods section. FCS, fetal calf serum; for other abbreviation see Table 1. In enzyme preparations *b,c,* and *d,* values are the mean of two independent experiments.

TABLE 3
Subcellular Distribution of GroPChoPDE and Marker Enzyme Activities Isolated After Differential Centrifugation of Pericyte Homogenate[a]

Fraction	GroPChoPDE[a]	NADPH-cyt c red.*	5′-Nucleotidase**
Homogenate	7.80	0.72	12.4
Nuclear pellet	7.17 (0.92)	0.56 (0.77)	6.42 (0.51)
Mitochondrial fraction	8.01 (1.0)	1.16 (1.6)	3.13 (0.25)
Microsomal fraction	11.9 (1.5)	3.72 (5.2)	7.31 (0.59)
Plasma membranes	21.1 (2.7)	1.28 (1.8)	22.8 (1.8)
Cytosol	3.40 (0.43)	0.22 (0.30)	15.0 (1.2)

[a]Pericytes were grown at confluence on 100-mm culture dishes, harvested, and homogenized by nitrogen cavitation in 10 mM Tris-HCl (pH 7.5) containing 0.25 mM sucrose. Enzyme activities are in nmol/min/mg protein (*) or mmol/h/mg protein (**), and were measured at room temperature; values represent the averages of two experiments. Numbers in parentheses are the enrichment ratios, i.e., specific activity of fraction/specific activity of homogenate. All subcellular fractions were prepared as described in the Materials and Methods section.

Between them, no values showed significant differences and all were in agreement with those found previously in cultures of quiescent cells of various origins.

After 4 h, incubation of untreated cells in the presence of ^{3}H-choline resulted in labeling of several water-soluble compounds (19.5%) and PtdCho (80.5%). The ratio of ^{3}H-activity in phosphocholine and PtdCho in the cells was about 1 to 5 (data not shown). TLC of the water-soluble compounds yielded four fractions, and the labeling distribution (mean analysis of two experiments) was: choline 2.5%, PCho 86.5%, GroPCho 7.7%, and CDP-choline 3.3% of the aqueous-phase radioactivity. The radioactivity incorporated into the large pool of phosphocholine was much higher than that in choline and CDP-choline, in agreement with the notion that the rate-limiting step of PtdCho *de novo* biosynthesis occurs at the conversion of phosphocholine to CDP-choline, a reaction catalyzed by CT. This result agrees with *in vitro* CT estimates, which in pericytes have some of the lowest activities of the cytidine pathway enzymes. Among phospholipids, PtdCho was the only ^{3}H-labeled compound.

As shown in Figure 1, 1 μM adenosine treatment of the cells for 1 h was responsible for an increase in ^{3}H-choline

TABLE 4
Enzyme Activities of Phospholipid Metabolism of Retinal Pericytes and Endothelial Cells (EC) in Culture

Enzyme	Specific activity	
	Pericytes	EC
Choline kinase	1.24 ± 0.21	1.48 ± 0.30
CTP:phosphocholine cytidylyltransferase	0.54 ± 0.15	0.38 ± 0.08
Choline phosphotransferase	0.20 ± 0.04	0.41 ± 0.18
Acyltransferase	1.02 ± 0.08	1.01 ± 0.14
Cytosolic PLA_2*	3.29 ± 0.46	6.60 ± 0.73

[a]Assay conditions are described in the Materials and Methods section. Homogenates of confluent cell cultures (2 mg protein/mL) were incubated at 37°C for the allotted times in the appropriate buffer with assay ingredients. Values, expressed as nmol/min/mg protein, are means ± SD. To determine enzyme activities in endothelial cells (EC), immortalized GP8.39 line was used (32). (*) Cytosolic phospholipase (PLA_2) activity is reported as pmol/min/mg protein. For comparison, results obtained in bovine retina EC, isolated and grown following the procedure of Stitt *et al.* (33) are also reported as means of two experiments with different cell cultures: CTP:phosphocholine cytidylyltransferase, 0.89; choline phosphotransferase, 0.45; acyltransferase, 0.60 nmol/min/mg protein.

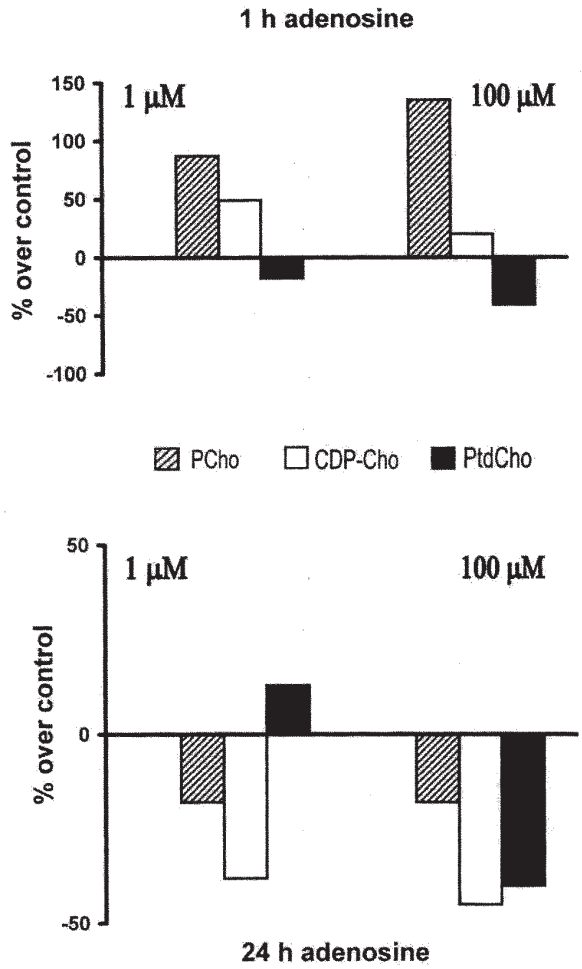

FIG. 1. Effect of adenosine on [Me-^{3}H]choline incorporation into retina pericyte cultures. Twenty-four hours before the addition of the effector, pericytes were maintained in starvation with serum-free medium and then cultured for 1 and 24 h in the same medium containing the agonist. Subsequently, cells were treated with labeled choline (10 μM, 5 μCi/60 mm dish) for 4 h in the presence of adenosine (1 and 100 μM, final concentration). The incubations were then stopped by rinsing cell layers with ice-cold phosphate buffer and scraping them with a rubber policeman for the quantitative radioactive determinations. Bars represent the percentage of incorporation over control cultures (mean of three separate experiments). PCho, phosphorylcholine; CDP-Cho, cytidine diphosphocholine; PtdCho, phosphatidylcholine.

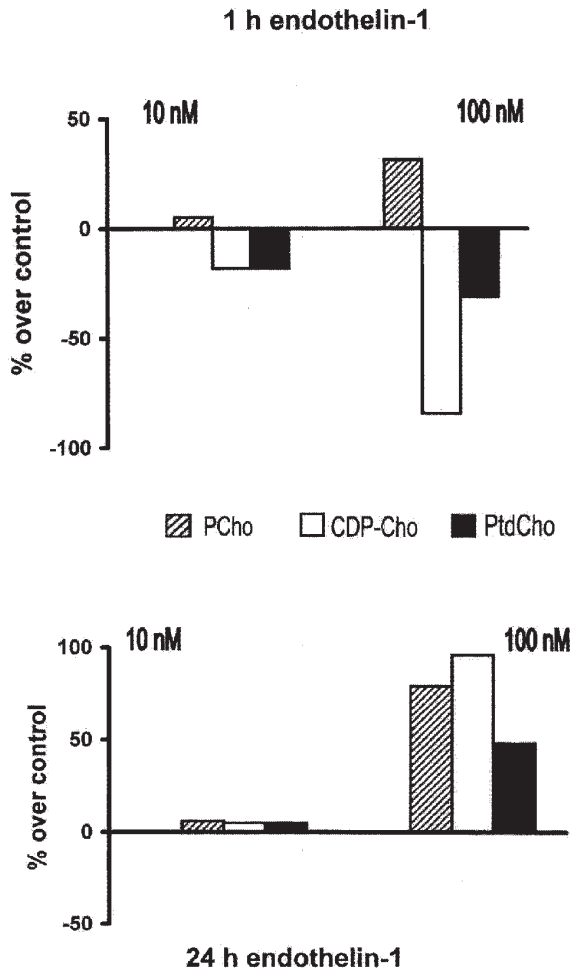

FIG. 2. Effect of endothelin-1 on [Me-^{3}H]choline incorporation into retina pericyte cultures. Experimental conditions are similar to those in Figure 1. Final concentrations of endothelin-1 were 10 and 100 nM. Bars represent the percentage of incorporation over control cultures (mean of three separate experiments). For abbreviations see Figure 1.

incorporation into the intermediates PCho and cytidine diphosphocholine (CDP-Cho), compared to the control, and for a concomitant decrease in ^{3}H-choline incorporation into PtdCho by 18%. Addition of 100 μM adenosine caused a clear increase in amount of ^{3}H-phosphocholine present in cell extracts after 1 h incubation while causing a decrease in ^{3}H-choline incorporation into PtdCho by 41%. ^{3}H-CDP-Cho was a minor component of the choline-compound pool in the cells, and its level was increased only slightly (20%) in cells treated with 100 μM adenosine for 1 h. When adenosine treatment was prolonged for 24 h, the effect of 1 and 100 μM adenosine was very different compared to 1 h treatment since ^{3}H-choline incorporation into the intermediates of the *de novo* pathway decreased, with the exception of PtdCho (+13%).

As shown in Figure 2, 10 and 100 nM ET-1 treatment for 1 h produced a slight increase in ^{3}H-choline incorporation into PCho by 5 and 31%, respectively, and a decrease into PtdCho and CDP-Cho. When ET-1 treatment was prolonged for 24 h, addition of 10 nM ET-1 had no effect on ^{3}H-choline incorporation into all three intermediates. Addition of 100 nM ET-1 increased ^{3}H-choline incorporation into PCho by 79%, CDP-Cho by 96%, and PtdCho by 48%.

DISCUSSION

This study presents three novel findings. First, we show that GroPChoPDE, which hydrolyzes glycerophosphocholine to glycerophosphate and free choline, is highly expressed in retina microcapillary pericytes, suggesting a main role in the last step of PtdCho catabolism. Second, in the same cell type we estimate some enzyme activities involved in PtdCho synthesis, degradation, and remodeling, comparing them with those present in retina microcapillary EC as well as in immortalized brain microcapillary EC. The values observed in EC were quite similar to those found in pericytes, with the exception of GroPChoPDE. Third, we provide evidence that adenosine and ET-1 are able to modulate PtdCho *de novo* synthesis in retina pericytes, suggesting their involvement in the control of blood–brain and blood–retina barrier functions (Fig. 3).

Pericytes are intramural vascular cells which, in early development, participate in vasculogenesis together with EC. Although normally not in contact with flowing blood, pericytes are uniquely situated so that, after vascular injury, they may participate in intravascular processes such as thrombosis, hemostasis, diabetic microangiopathy, and, perhaps, inflammatory events (23). In the retina and brain microvasculature, normal and pathological function of pericytes has certainly been underestimated and remains to be elucidated. In retina pericytes, oxidative stress is able to exacerbate the cytotoxic effect induced by LPS alone, as has been found in many other cell types (23).

Since the earliest reports on phosphodiesterase activity, the physiological role of this catabolic enzyme has remained unclear. GroPCho is the major water-soluble form of choline ester in brain and retina of vertebrates and invertebrates (34). We agree with the idea of Spanner and Ansell (6) suggesting that one component contributing to the origin of this large pool may derive from the relevant amount of plasma lyso-PtdCho, which is able to cross the blood–brain barrier easily (1). Inside the microvascular cells, GroPChoPDE at high specific activity may well be responsible for the release of

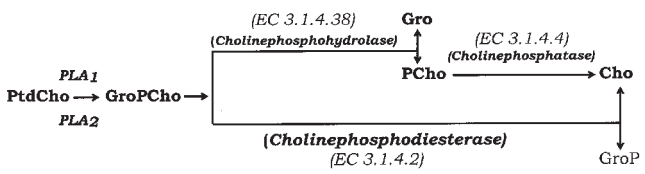

FIG. 3. Enzyme activities involved in PtdCho and glycerophosphocholine degradation. GroPCho, *sn*-glycero-3-phosphocholine; Gro, glycerol; PCho, phosphocholine; Cho, choline; GroP, glycerophosphate; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2. For other abbreviations see Figure 1.

choline from choline lysocompounds massively transported across the endothelium and pericytes, and rapidly deacylated. As in other tissues, GroPChoPDE may serve as a source of choline and *sn*-glycero-3-phosphate from the end product of PtdCho catabolism, allowing slow recycling of the building blocks in the *de novo* synthesis of lipids (Figs. 1,2). It has also been postulated that GroPChoPDE in the brain may contribute to maintenance of normal choline and/or acetylcholine homeostasis (7). Whatever the case, the presence of GroPChoPDE in microvessels, particularly in pericytes, discovered here must be interpreted in light of the barrier function of retina and brain microvascular cells.

The discovery of such high GroPChoPDE activity in pericytes induced us to extend our investigations on this enzyme in order to determine its biochemical characteristics and to understand the meaning of its high expression in pericytes. Our data indicated that GroPChoPDE is not inhibited by thiolic agents, suggesting that, in performing enzymatic activity, a sulfhydryl functional group is not involved. Exposure to trypsin had no effect on the enzyme activity, indicating that the catalytic domain is not easily accessible to proteolytic digestion.

We also investigated the effect of two agonists, adenosine and ET-1, on PtdCho biosynthesis in retinal pericytes. Adenosine is a vasodilator acting as a metabolic messenger carrying information on the intracellular metabolism to extracellular-facing receptors of the same and adjacent cells. It accumulates locally in response to hypoxia *via* dephosphorylation of accumulated AMP (35). In vascular endothelial cells, NO production is significantly increased by adenosine *via* a receptor-mediated mechanism. In addition, adenosine is known to stimulate angiogenesis and EC proliferation (36,37). Adenosine has a direct inhibitory effect on retinal pericyte growth (37), as it does on smooth muscle cells (38). This effect is probably mediated either by primary cAMP production and protein kinase A activation or by rapid accumulation of S-adenosylhomocysteine (40). Extracellular adenosine itself causes DNA damage and is responsible for the apoptotic effect of ATP on pulmonary artery EC by reducing the methylation index of macromolecules (41). Ecto-5′-nucleotidase is expressed by pericytes, indicating that adenosine may be formed extracellularly from nucleotides and cAMP (42).

Cultured pericytes were utilized in the present study to allow direct measurement of PtdCho metabolism in response to adenosine administration. In agreement with the antimitogenic action of adenosine on pericytes, we found that addition of adenosine (at 100 μM) decreased ^{3}H-choline incorporation into PtdCho transiently and 24 h after addition. The inhibition of membranous PtdCho synthesis may be a secondary event in response to an increase in Ca^{2+} intracellular concentration and, at least partially, to an activation of the PLA_2 pathway. We speculate that extracellular adenosine or ATP, released by activated platelets and cells undergoing cytolysis, causes damage to vascular cells and thereby exacerbates injury during angiogenic repair.

Evidence has been obtained that ET-1, a potent vasoconstrictor peptide with local hormonal action, also synthesized and secreted by mitogenic astroglia (43), retinal, and cerebrovascular endothelial cells (44,45), stimulates proliferation and causes contraction of pericytes, which are able to express endothelin receptors and may have a muscle-like function in the microvasculature (46–48). Retinal capillary pericytes are believed to regulate retinal blood flow at the microvascular level (10).

From these data we conclude that the action of such an agonist may be associated with specific alterations in pericyte PtdCho metabolism. In agreement with the mitogenic activity of ET-1 on pericytes, we found that addition of ET-1 (at 100 nM) has a stimulating effect on PtdCho synthesis 24 h after addition (Fig. 2). The slight inhibitory effect seen at 1 h may be associated with transient PtdCho hydrolysis induced by endothelin through the phospholipase C and D signaling pathways generating lipid second messengers (49). The ability of pericytes to be sensitive to ET-1 action suggests their potential and contributive role either in endothelin-mediated vasoconstriction of retina, brain, and coronary vasculature or in disease states due to altered activity of the endothelin system.

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Dual Action of Neutral Sphingomyelinase on Rat Hepatocytes: Activation of Cholesteryl Ester Metabolism and Biliary Cholesterol Secretion and Inhibition of VLDL Secretion

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ABSTRACT: To address the role of cell membrane neutral sphingomyelinase (EC 3.1.4.12; SMase) in the regulation of cholesterol metabolism in the liver parenchymal cell, we examined the effect of exogenous neutral SMase on the metabolism of cholesteryl esters and the secretion of VLDL and biliary lipids in isolated rat hepatocytes. We show that treatment of hepatocytes with SMase (20 mU/mL) resulted in the intracellular buildup of cholesteryl esters, increased ACAT (EC 2.3.1.26) activity without affecting the ACAT2 mRNA level, and increased cytosolic and microsomal cholesteryl ester hydrolase (EC 3.1.1.13) activity. This was accompanied by increases in the secretion of biliary bile acid, phospholipid, and cholesterol and in increased cholesterol 7α-hydroxylase (EC 1.14.13.17) activity and levels of mRNA, as well as decreased levels of apoB mRNA and a decreased secretion of VLDL apoB (apoB-48, ~45%; apoB-100, ~32%) and lipids (~55%). Moreover, the VLDL particles secreted had an abnormal size and lipid composition; they were larger than controls, were relatively enriched in cholesteryl ester, and depleted in TG and cholesterol. Cell-permeable ceramides did not replicate any of the reported effects. These findings demonstrate that the increased cholesteryl ester turnover, oversecretion of biliary cholesterol and bile acids, and undersecretion of VLDL cholesterol and particles are concerted responses of the primary hepatocytes to exogenous neutral SMase brought about by regulation at several levels. We suggest that plasma membrane neutral SMase may have a specific, ceramide-independent effect in the regulation of cholesterol output pathways in hepatocytes.

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Plasma membrane neutral sphingomyelinase (EC 3.1.4.12; SMase) is a member of the SMase family that catalyzes the hydrolysis of plasma membrane sphingomyelin to ceramide and phosphocholine (reviewed in Ref. 1). This enzyme seems to be activated by a variety of physiologically relevant molecules involved in cellular growth regulation, differentiation, and apoptosis (programmed cell death), such as tumor necrosis factor-α (TNF-α); the phenomenon can also be reproduced by exogenous neutral SMase (1,2). Neutral SMase has been implicated in the regulation of cholesterol homeostasis in mammalian cells. It is fairly well established that the activation of endogenous neutral SMase or treatment of cells with neutral SMase not only cleaves sphingomyelin but also causes a measurable fraction of cellular cholesterol to translocate from the plasma membrane to the endoplasmic reticulum (ER) (3,4). There it expands a regulatory pool that downregulates cholesterol synthesis (4) and its rate-limiting enzyme HMG-CoA reductase (EC 1.1.1.34) (5) and upregulates cholesterol esterification and its controlling enzyme ACAT (EC 2.3.1.26) (4). Studies have usually been performed on cells other than hepatocytes that have a cholesterol metabolism that is strictly controlled by regulatory feedback (i.e., see Ref. 6). The function of cell membrane neutral SMase in the regulation of cholesterol metabolism in hepatocytes remains to be elucidated. However, recent evidence, demonstrating that TNF-α-mediated neutral SMase activation in cultured human hepatoma cells stimulates the proteolytic cleavage of sterol regulatory element-binding protein (SREBP) in a sterol-independent manner (7), suggests that the hepatocytes may elicit specific responses to SMase activation.

Cholesterol metabolism in the hepatocytes is not only complex but also unique. It involves (i) two input pathways, cholesterol synthesis and lipoprotein uptake; (ii) the reversible conversion of cholesterol to cholesteryl esters (CE); (iii) and two output routes, the conversion of cholesterol to bile acids and biliary secretion and the secretion of VLDL into circulation. Esterification of cholesterol is mediated by (i) ACAT1, an enzyme that is widely distributed in tissues (8) and allosterically regulated by cholesterol (9), and (ii) ACAT2, which is expressed predominantly in the intestine and liver (10) and which is involved in the secretion of lipoprotein CE (11). CE accumulate in lipid droplets in the cytoplasm, from which they can either be incorporated into VLDL (12) or undergo hydrolysis by the CE hydrolase (EC 3.1.1.13; CEH) isoforms localized in the cytosol (cCEH) (13) and ER (erCEH) (14). Secretion of VLDL requires the coordinated synthesis and assembly of apolipoprotein B (apoB), TG, free cholesterol (FC), CE, and phospholipid. ApoB-100 and apoB-48 are the major structural proteins of rat VLDL

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Abbreviations: ApoB, apolipoprotein B; cCEH, cytosolic cholesteryl ester hydrolase; CE, cholesteryl ester; CEH, cholesteryl ester hydrolase; C_2-ceramide, D-*erythro*-*N*-hexanoylsphingosine; C_6-ceramide, D-*erythro*-*N*-acetylsphingosine; CYP7A, cholesterol 7α-hydroxylase; ER, endoplasmic reticulum; erCEH, endoplasmic reticulum cholesteryl ester hydrolase; FC, free cholesterol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTP, microsomal TG transfer protein; SCAP, SREBP cleavage-activating protein; SMase, sphingomyelinase; SREBP, sterol regulatory element-binding protein; TNF-α, tumor necrosis factor-α.

(15), and both apoproteins are absolutely dependent on lipidation and microsomal TG transfer protein (MTP) activity (16,17) to fold into their native form and be secreted. In fact, the cellular availability of TG (18,19), CE (13,20–24), and PC (25) regulates VLDL secretion. The synthesis and secretion of bile acid together with the excretion of cholesterol into the bile constitutes the major route for the disposal of cholesterol. Bile acid synthesis is primarily initiated by 7α-hydroxylation of cholesterol catalyzed by the rate-limiting cholesterol 7α-hydroxylase (EC 1.14.13.17; CYP7A), an enzyme that is exclusively localized in the liver parenchymal cell (26) and is transcriptionally regulated by the cellular ratio of cholesterol to bile acids (27).

To address the role of cell membrane neutral SMase in the regulation of cholesterol metabolism in the liver parenchymal cell, we explored the effect of exogenous neutral SMase on the metabolism of CE and the liver-specific processes for exportation of cholesterol in primary rat hepatocytes in suspension. The remarkable fact that the ER membrane contains only small amounts of cholesterol (3) while being the site of cholesterol synthesis (5), esterification (8), and regulation (28,29) is accentuated in the hepatocyte, where the ER is critical for cholesterol output. Proteins essential for bile acid synthesis (26), VLDL formation (16,17), and CE hydrolysis (14) are located in the ER of the hepatocyte; therefore, they may be expected to be particularly sensitive to increases in ER cholesterol levels. We report that exogenous neutral SMase, and not cell-permeable ceramides, activates CE turnover. It causes a flow of cholesterol that supplies substrate for bile acid synthesis and expands a regulatory pool that leads to upregulation of CYP7A mRNA and the secretion of biliary lipid, and it leads to downregulation of apoB mRNA and the secretion of VLDL apoB and lipid. The relevance of these findings remains to be explored. However, our observations suggest a role for plasma membrane neutral SMase activity in regulating cholesterol homeostasis in hepatocytes independent of the generation of ceramide.

MATERIALS AND METHODS

Animals and chemicals. Female Sprague-Dawley rats (~200 g) were kept on a standard low-fat pellet diet, allowed access to food and water *ad libitum*, and housed in a temperature- (22°C) and light-controlled room (lights on from 1600 to 0400) for at least 1 wk before the experiment. Animals were anesthetized with sodium pentobarbital (60 mg/kg body weight) in the middle of the dark cycle. Animals were given humane care in compliance with institutional guidelines. Radiochemicals were obtained from Amersham Biosciences (Bucks, United Kingdom). Collagenase A was from Roche Molecular Biochemicals (Germany). FA-free BSA, Cab-O-Sil (particle size 0.011 μm), neutral SMase from *Staphylococcus aureus*, D-*erythro-N*-acetylsphingosine (C_2-ceramide), and D-*erythro-N*-hexanoylsphingosine (C_6-ceramide) were obtained from Sigma Chemical Co. (St. Louis, MO). The cDNA for mouse ACAT2 and for rat apoB and CYP7A were given by Dr. Sandra K. Erickson (University of California–San Francisco, San Francisco, CA), Dr. Mark P. Sowden (University of Rochester Medical Center, Rochester, NY), and Dr. John Y.L. Chiang (Northeastern Ohio Universities, Rootstown, Ohio), respectively.

Isolation and incubation of hepatocytes. Isolation of the secreted VLDL and biliary lipids. Parenchymal hepatocytes were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor as detailed in Reference 30. Cells were suspended at a density of 5×10^6 cells/mL in Krebs–Henseleit solution (pH 7.4), containing 2.5 mM $CaCl_2$, 10 mM glucose, and 2.5% FA-free BSA, and incubated for 2 or 4 h with 20 mU/mL SMase in 0.04% glycerol or 10 μM C_2-ceramide or C_6-ceramide in 0.05% DMSO. An equivalent amount of the corresponding solvent was added to control incubates. Cell viability, as determined by Trypan Blue exclusion, remained higher than 90% during incubation. At the end of the incubation, the cells were pelleted and VLDL was isolated by centrifugation (18 h at 10°C at 105,000 × *g*) after adjusting the density of the medium to 1.006 g/mL (31). The remaining lipoproteins were discarded by a second centrifugation in identical conditions after adjusting the density of the VLDL infranatant to 1.23 g/mL. The infranatant of the second centrifugation was desalted in SepPak C18 cartridges; bile acids were then recovered in methanol, and cholesterol and phospholipid were recovered in chloroform. The methanolic phase contained 95 ± 2% of secreted cholate (data not shown) and was considered to represent the biliary bile acid secretion.

Determination of VLDL lipids and apoB content and size. Aliquots of VLDL were used for lipid and apoB analysis, which were performed exactly as detailed previously (32), and for size analysis (23). In brief, lipids were extracted and separated by TLC, and the lipid classes were quantified by densitometry using an image analysis system from Bio Image Corporation (Ann Arbor, MI) (32). ApoB-100 and apoB-48 were separated by SDS-PAGE after concentration of VLDL particles with Cab-O-Sil (31). The protein bands were stained with Coomassie Brilliant Blue R-250, and apoB-100 and apoB-48 were quantified by densitometry using phosphorylase *b* as a standard (32). The mean diameter of the secreted VLDL particle population was estimated by quasi-elastic light scattering, using a Malvern Instruments, Inc. Zeta-Sizer instrument, as described in Reference 23.

Quantification of biliary bile acid and lipids. Bile acids were quantified using 3α-hydroxysteroid dehydrogenase as described previously (33). The mass of cholesterol and phospholipids was quantified as described above for VLDL lipids.

Determination of ACAT, cCEH, erCEH, and CYP7A activities. The hepatocyte pellets were homogenized in 20 mM Tris-HCl buffer (pH 7.4), containing 250 mM sucrose, 0.5 mM DTT, 2 mM EDTA, 0.01 mM leupeptin, and 1 mM benzamidin. Subcellular fractions were obtained by differential centrifugation (1000 × *g*, 10 min; 22,000 × *g*, 10 min; 105,000 × *g*, 60 min, twice). ACAT activity was assayed both in nontreated microsomes (105,000 × *g* pellet) and in microsomes

loaded with cholesterol (250 μM) to ACAT saturation by measuring the transfer of labeling from [1-^{14}C]oleoyl-CoA to cholesterol as detailed in Reference 23. CEH activity was estimated in the cytosol (105,000 × *g* supernatant) and microsomes by measuring the release of labeled oleic acid from a cholesteryl [1-^{14}C]oleate micellar substrate as described earlier (23). CYP7A was assayed both in nontreated microsomes and in microsomes loaded with cholesterol (25 μM) to CYP7A saturation by measuring the formation of 7α-hydroxycholesterol from [1-^{14}C]cholesterol as detailed in Reference 33.

RNA analysis. Northern blot analysis was performed according to Sambrook *et al.* (34). Total RNA was isolated from 10^7 cells with TRIzol (Invitrogen Life Technologies, Barcelona, Spain) and ~20 μg was separated on 1% (wt/vol) agarose gels containing 1 M formaldehyde using 20 mM MOPS, 5 mM sodium acetate (pH 7.0), and 1 mM EDTA as the running buffer. After transfer to nylon membranes and UV cross-linking, RNA blots were prehybridized for 2 h at 65°C in a solution of 500 mM disodium hydrogen phosphate, 1% BSA (pH 7.2), 7% SDS, 1 μM EDTA, and then hybridized overnight at 65°C with the corresponding probe labeled with [α-^{32}P]dCTP by random priming (Amersham Biosciences, Bucks, United Kingdom). The blots were exposed to an Imaging Screen-K™ (Kodak, Rochester, NY). The intensity of the bands was quantified after laser densitometric scanning (Molecular Imager FX and software Quantity One; Bio-Rad, Hercules, CA) and normalized to the hybridization signal of the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (pTRI-GAPDH-Rat; Ambion, Austin, TX). The membrane was stripped with boiled Tris-EDTA buffer in the presence of 1% SDS for 10 min and then rehybridized with the next probe.

Other analytical methods and statistical analysis. The amount of protein was measured by Bradford assay (35). Lipids from the hepatocyte homogenate, cytosol, and microsome were extracted with chloroform/methanol (2:1, vol/vol) (32), separated by TLC, and quantified as above for VLDL lipids. *De novo* synthesis of CE and TG in isolated hepatocytes was estimated as the amount of radioactivity associated with CE and TG after the incubation of cells with [^{3}H]oleate for up to 4 h followed by lipid analysis (23). After preincubation of cells with 0–20 mU/mL SMase for 30 min, 20 μM potassium [^{3}H]oleate (2 μCi/mL) was added and incubation was continued for a further 1–4 h in the continuous presence of 0–20 mU/mL SMase. The cells and incubation medium were harvested, lipids were extracted and separated, and the bands corresponding to CE and TG were counted for radioactivity. Cholesteryl [1-^{14}C]oleate (10,000 dpm) or glycerol tri[1-^{14}C]oleate (10,000 dpm) was added to each tube to assess recovery. Comparisons among groups were evaluated statistically by the unpaired Student's *t*-test. The level of significance was set at $P < 0.05$.

RESULTS

Effect of SMase treatment on the cellular balance and metabolism of CE in isolated rat hepatocytes. Variations in the content of cholesterol and CE in cell homogenate, cytosol, and microsome preparations, and the biosynthesis and hydrolysis of CE were analyzed in rat hepatocyte suspensions after treatment with 20 mU/mL of neutral SMase for 2 or 4 h. A preliminary analysis revealed that these treatments did not lead to morphological and viability changes in hepatocytes or to cholesterol efflux (data not shown). There was no change in the total cholesterol content of hepatocytes (data not shown) but, confirming previous data (4), profound alterations were registered in the distribution of cholesterol between its free and esterified forms and between the cytosolic and the microsomal cell compartments (Fig. 1). At the two time periods studied, SMase doubled the CE content in the cytosol and microsomes, and the amount of FC in the cytosol was ~20% above the control value, whereas in microsomes it diminished by ~20%. Biosynthesis of CE was assessed by measuring the incorporation of [^{3}H]oleate into CE in intact hepatocytes, and the activity of ACAT both in untreated microsomes and in a saturating level of cholesterol-loaded microsomes. Figure 2A (lower panel) shows that treatment of hepatocytes with SMase increased *de novo* formation of CE in an acute and phase fashion. At first, the increase in labeling was much more pronounced in that associated with cells (× 5 at 1 h) (Fig. 2A, upper panel), and then progressively equalized with that released to the medium (Fig. 2A, middle panel). ACAT

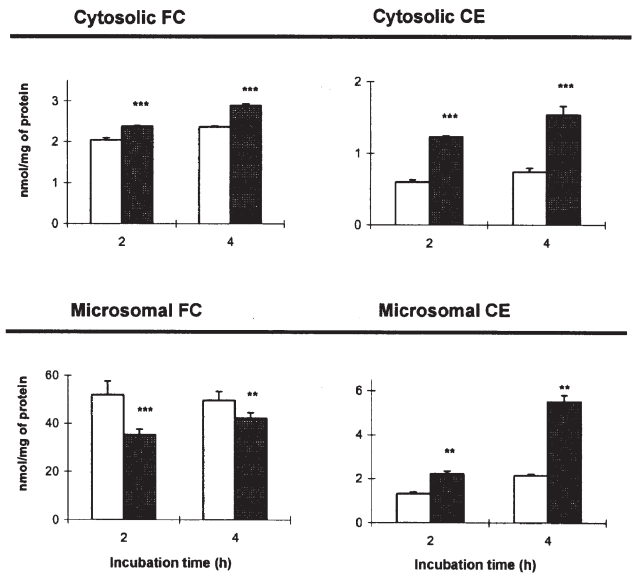

FIG. 1. Effect of sphingomyelinase (SMase) treatment on the content of free and esterified cholesterol in the cytosol and microsomes in isolated rat hepatocytes. Hepatocyte suspensions were incubated with 20 mU/mL SMase in 0.04% glycerol (solid bars) or the solvent alone (open bars) for 2 or 4 h. The cells were then homogenized and submitted to sequential fractionation. Lipids from the cytosol and microsomal preparations were extracted and separated, and the amounts of free cholesterol (FC) and cholesteryl ester (CE) were determined and expressed relative to the protein concentration in the corresponding preparation. The data are the mean ± SEM from five independent experiments. Statistical differences are denoted by $^{**}P < 0.01$ and $^{***}P < 0.001$ relative to control cells.

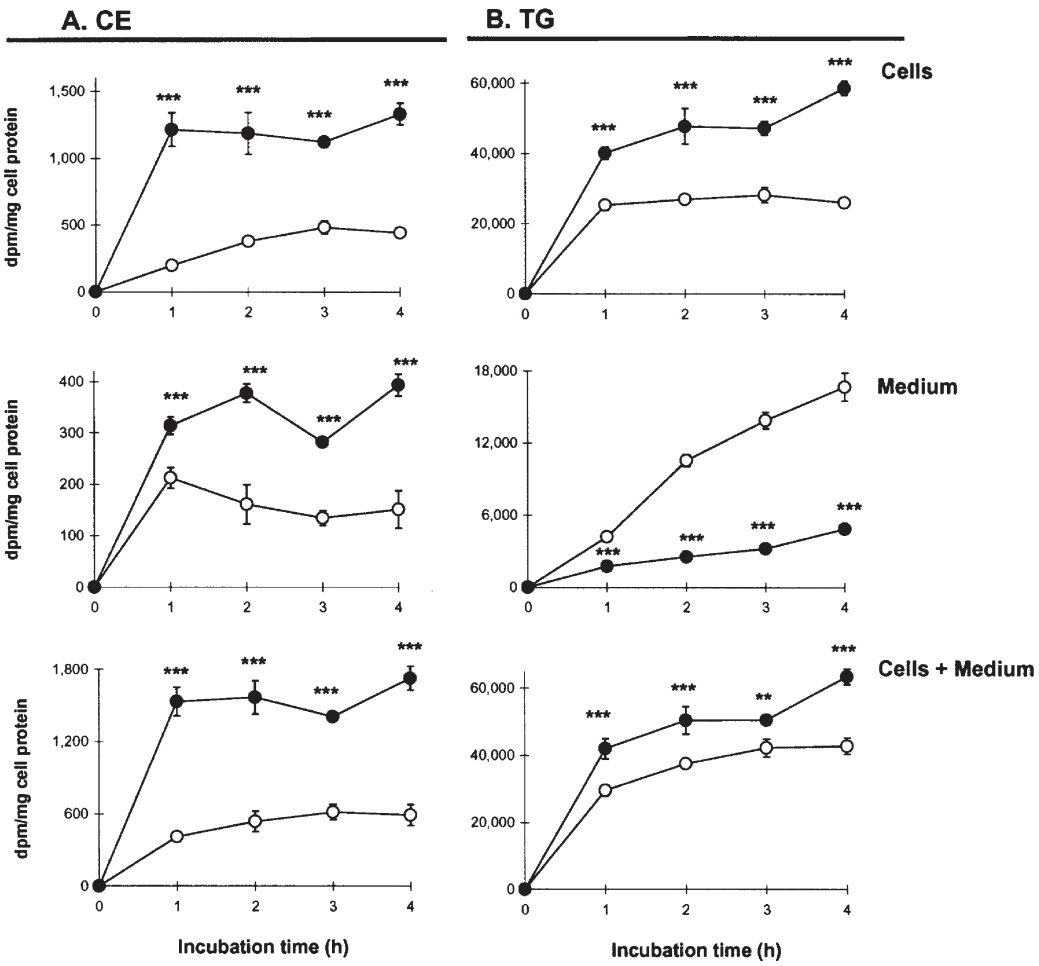

FIG. 2. Effect of SMase treatment on the incorporation of [^{3}H]oleate into CE and TG in isolated rat hepatocytes. After preincubation of hepatocyte suspensions with 20 mU/mL SMase in 0.04% glycerol (●) or the solvent alone (○) for 30 min, 20 μM potassium [^{3}H]oleate (2 μCi/mL incubation) was added, and the radioactivity incorporated into CE (A) and TG (B) of cells and media was measured and expressed relative to the protein concentration in cell homogenates. The data are the mean ± SEM from four independent experiments. Statistical differences are denoted by $^{**}P < 0.01$ and $^{***}P < 0.001$ relative to control cells. For abbreviations see Figure 1.

activity in untreated microsomes increased substantially by SMase (× 1.5 at 2 h and × 2.1 at 4 h, mean of three preparations). In addition, ACAT activity in microsomes that had been supplemented *in vitro* with a saturating level of exogenous cholesterol (36) increased 60 and 200% after 2 and 4 h of treatment, respectively (Fig. 3, left plot), indicating further nonsubstrate activation. Shown in Figure 4 is the lack of effect on the mRNA level for ACAT2, apparently the main gene product responsible for cholesterol esterification in rat hepatocytes (10,11). The hydrolytic side of the cholesterol–CE cycle was explored by measuring CEH activity in cytosol and microsomes, both supplemented with saturating cholesteryl oleate substrate (36). Figure 3 shows that cCEH (middle plot) and erCEH (right plot) exhibited profiles of activity consistently superior in SMase-treated cells than in control hepatocytes. Neutral SMase therefore largely forces the esterification and storage of cholesterol, but it also moderately forces the hydrolysis of CE in rat hepatocytes.

Because availability of cellular TG is a major determinant of how much apoB enters the VLDL assembly/secretion pathway (19), we also analyzed whether TG synthesis, measured by the incorporation of [^{3}H]oleate into TG, was affected by SMase treatment of hepatocytes. Figure 2B (lower panel) shows that labeling increased about 25% by SMase. However, while the nonsecreted newly formed TG doubled in 4 h (Fig. 2B, upper panel), the labeled TG released to the medium declined dramatically throughout the time period studied (Fig. 2B, middle panel), suggesting that SMase treatment of hepatocytes modestly increased the biosynthesis of TG but that its partitioning toward secretion was markedly reduced. This is supported by the observation that the intracellular accumulation of TG was greater in SMase-treated than in control hepatocytes [controls: 38.7 ± 1.5 and 42.7 ± 2.3; treated cells: 59.9 ± 3.8 and 61.2 ± 2.7 nmol/mg of cell protein (mean ± SEM, $n = 4$ preparations) at 2 and 4 h].

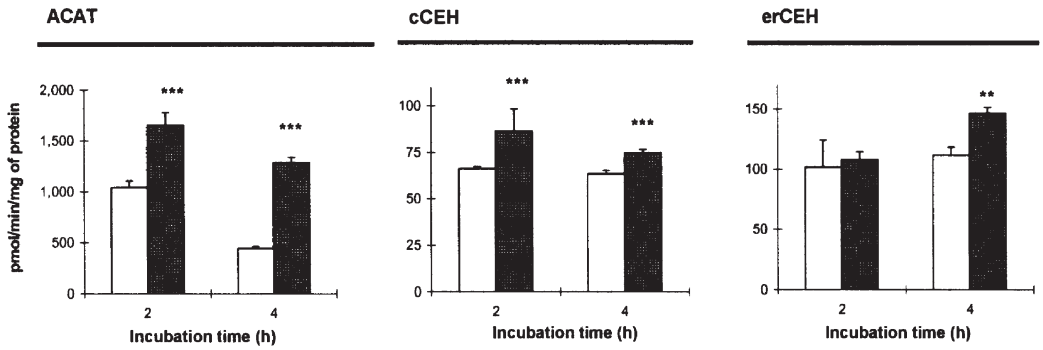

FIG. 3. Effect of SMase treatment on the activity of ACAT, cytosolic cholesteryl ester hydrolase (cCEH), and endoplasmic reticulum cholesteryl ester hydrolase (erCEH) in isolated rat hepatocytes. Hepatocyte suspensions were incubated with 20 mU/mL SMase in 0.04% glycerol (solid bars) or the solvent alone (open bars) for 2 or 4 h. The cytosol and microsomes were then prepared, and activity of ACAT in microsomes and CEH in the cytosol (cCEH) and microsomes (erCEH) was assayed and expressed relative to the protein concentration in the corresponding preparation. The data are the mean ± SEM from three (cCEH and erCEH) or four (ACAT) independent experiments. Statistical differences are denoted by $^{**}P < 0.01$ and $^{***}P < 0.001$ relative to control cells. For other abbreviation see Figure 1.

A

	2 h incubation			4 h incubation		
mRNA	Control	SMase	% of the content	Control	SMase	% of the content
ApoB	25.9 ± 0.1	19.3 ± 0.4*	74.5	34.5 ± 0.1	23.2 ± 2.5*	67.2
CYP7A 4 kb	6.08 ± 0.17	8.04 ± 0.36*	132.2	1.14 ± 0.18	1.05 ± 0.13	92.1
ACAT2	1.86 ± 0.09	1.91 ± 0.10	102.7	1.57 ± 0.07	1.73 ± 0.08	110.2

B

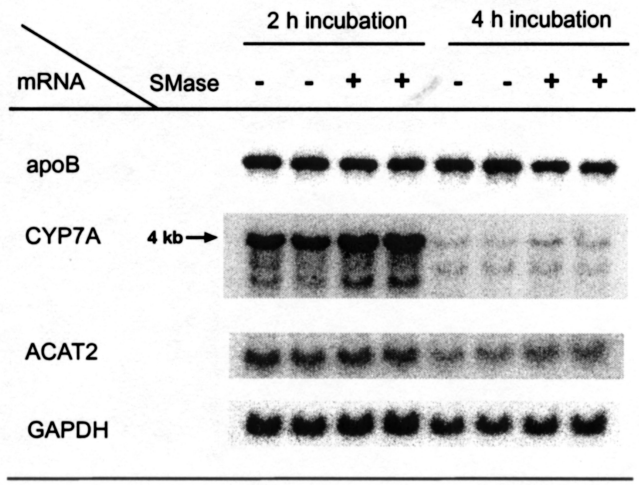

FIG. 4. Effect of SMase treatment on the mRNA levels for ACAT2, apolipoprotein B (apoB), and cholesterol 7α-hydroxylase (CYP7A) in isolated rat hepatocytes. Hepatocyte suspensions were incubated with 20 mU/mL SMase in 0.04% glycerol or the solvent alone for 2 or 4 h. Total RNA was then extracted, and ACAT2, apoB, and CYP7A mRNA levels were analyzed by Northern blotting. RNA was subjected to electrophoresis (20 μg/lane) and blot hybridization with the corresponding ^{32}P-labeled cDNA probe and a control ^{32}P-labeled probe directed against the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The membranes were exposed to an Imaging Screen-K^{TM} (Kodak, Rochester, NY) and analyzed by laser densitometric scanning. (A) The amount of each mRNA was calculated after correction for loading differences with GAPDH (OD unit ratio). Data are also given as a percentage of the control OD unit ratio values. The data shown are the mean ± SEM from four independent experiments. $^{*}P < 0.05$, at least, relative to control cells. (B) A typical blot of the changes in mRNA levels of apoB, CYP7A, ACAT2, and GAPDH. For other abbreviations see Figures 1 and 3.

TABLE 1
Effect of Sphingomyelinase (SMase) Treatment on Apolipoprotein B (apoB) and Lipids Secreted in VLDL and on VLDL Size in Isolated Rat Hepatocytes[a]

	Incubation with SMase for 2 h		Incubation with SMase for 4 h	
Parameter	Control	SMase	Control	SMase
ApoB (pmol/mg cell protein)	0.339 ± 0.007	0.188 ± 0.009***	0.550 ± 0.021	0.305 ± 0.029***
ApoB-48	0.288 ± 0.003	0.155 ± 0.009***	0.464 ± 0.022	0.252 ± 0.021***
Apo B-100	0.051 ± 0.004	0.033 ± 0.001**	0.086 ± 0.010	0.053 ± 0.007***
Lipid (nmol/mg cell protein)	25.54 ± 2.47	10.47 ± 1.31***	48.96 ± 4.56	23.74 ± 3.79***
TG	14.11 ± 1.30	4.67 ± 0.70**	29.81 ± 2.54	12.45 ± 2.22***
Cholesteryl esters	0.208 ± 0.017	0.205 ± 0.006	0.367 ± 0.024	0.381 ± 0.037
Phospholipid	10.19 ± 0.87	4.84 ± 0.56***	15.94 ± 1.48	9.81 ± 1.40***
Free cholesterol	1.031 ± 0.077	0.460 ± 0.012*	2.845 ± 0.212	0.830 ± 0.092***
Diameter (nm)	81 ± 3	107 ± 3**	86 ± 2	109 ± 4**

[a]Hepatocyte suspensions were incubated with 20 mU/mL SMase in 0.04% glycerol or the solvent alone for 2 or 4 h, VLDL were isolated from the $d < 1.006$ g/mL fraction after ultracentrifugation of medium samples, and the size and the content of apoB-48, apoB-100, and each lipid class in VLDL were determined. The data are the mean ± SEM from five independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ relative to control cells.

Effect of SMase treatment on VLDL secretion in isolated rat hepatocytes. The experiment shown in Table 1 was conducted to determine whether SMase treatment of hepatocytes affects the amount, composition, and size of the VLDL particles secreted from cells. ApoB secretion is indicative of the amount of VLDL that is secreted, as there is only one apoB per VLDL particle. Control hepatocytes secreted substantially more VLDL particles containing apoB-48 than apoB-100, resulting in a mass ratio of apoB-48 to apoB-100 of 5.6 ± 0.2 after 2 h, and of 5.4 ± 0.5 (mean ± SEM, $n = 5$ preparations) after 4 h of incubation. SMase treatment for 2 or 4 h caused a 45% reduction in the secretion of VLDL apoB; the decrease was slightly more pronounced in the secretion of apoB-48 (–45 and –47%) than of apoB-100 (–32 and –31%, respectively). The effect seems to be selective for these apoproteins, as there was no change in the overall synthesis and secretion of proteins, as determined by incorporation of [^{35}S]methionine into TCA-precipitable proteins of cells and medium (data not shown). Hepatocyte levels of mRNA encoding apoB were suppressed 25–30% by treatment with SMase for 2 or 4 h (Fig. 4).

Secretion of VLDL lipid was reduced drastically (–59% in 2 h and –51% in 4 h) by exogenous SMase (Table 1). TG, FC, and PL output decreased in a marked and reasonably constant way, whereas secretion of CE in VLDL was unaffected. Since VLDL particles contain much more cholesterol in its free than in its esterified form, the total amount of cholesterol exported from hepatocytes in VLDL decreased 46 and 62% after 2 and 4 h of treatment. From the values in Figure 2 and Table 1, we estimated the specific activities of the secreted CE, which increased (× 2.2 and × 2.8), and TG, which decreased (× 0.9 and × 0.6; 2 and 4 h, respectively). Figure 5 shows that SMase caused important changes in the VLDL composition affecting both the VLDL hydrophobic core lipids, with a notable decrease in TG that was substituted partly by CE, and the lipoprotein surface lipids, where FC tended to decline. The prominent detriment of VLDL TG in comparison with CE and PL, and the 34% rise in the molar ratio of the surface to core lipids supports the idea that the translocation of lipid components and/or the assembly process of the lipoprotein particle itself has been impaired. Indeed, physical changes in the VLDL particles were detected, as the mean particle diameter was largely augmented by SMase (Table 1).

Effect of SMase treatment on the synthesis and secretion of bile acids and secreted biliary lipids in isolated rat hepatocytes. We next investigated the effect of SMase on the

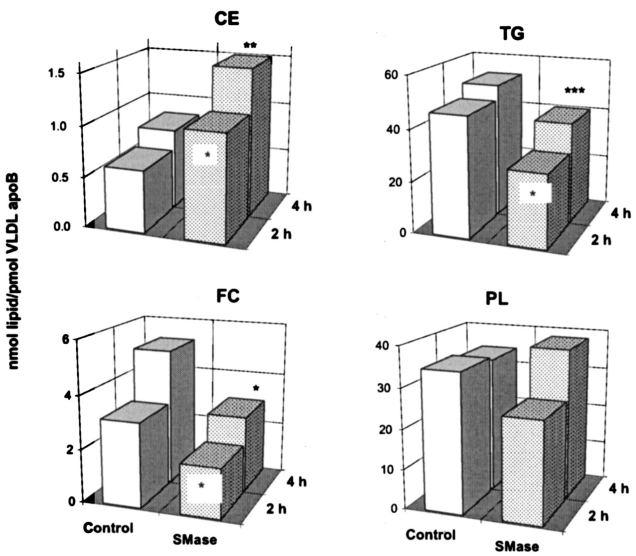

FIG. 5. Effect of SMase treatment on the lipid composition of VLDL particles secreted in isolated rat hepatocytes. Hepatocyte suspensions were incubated, VLDL isolated, and apoB and lipid composition analyzed as described in Table 1. The data are expressed as nmol of lipid/pmol of VLDL apoB and are the mean ± SEM from five independent experiments. Statistical differences are denoted by *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ relative to control cells. PL, phospholipid; for other abbreviations see Figures 1 and 4.

TABLE 2
Effect of SMase Treatment on Cholesterol 7α-Hydroxylase (CYP7A) Activity and on the Secreted Biliary Bile Acid, Cholesterol, and Phospholipid Amount in Isolated Rat Hepatocytes[a]

	Incubation with SMase for 2 h		Incubation with SMase for 4 h	
Parameter	Control	SMase	Control	SMase
CYP7A activity	57 ± 4	73 ± 5**	18 ± 1	35 ± 4***
Biliary bile acids	179 ± 12	312 ± 18***	372 ± 22	518 ± 35***
Biliary cholesterol	140 ± 2	168 ± 3**	162 ± 4	195 ± 3***
Biliary phospholipid	970 ± 33	1177 ± 40***	1260 ± 90	1449 ± 70*
Cholesterol/bile acids	0.782 ± 0.031	0.538 ± 0.020**	0.435 ± 0.018	0.376 ± 0.015**

[a]Hepatocyte suspensions were incubated with 20 mU/mL SMase in 0.04% glycerol or the solvent alone for 2 or 4 h. The cells were then washed and homogenized, the microsomes isolated, and the activity of CYP7A assayed. The biliary secretion was isolated from the $d > 1.23$ g/mL fraction, and the contents of bile acids, cholesterol, and phospholipid were analyzed. CYP7A activity is expressed as pmol 7α-hydroxycholesterol formed/min and mg of microsomal protein, and lipids are expressed as pmol/mg of cellular protein. The data are the mean ± SEM from four independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ relative to control cells. For other abbreviation see Table 1.

mass production of bile acids, cholesterol, and phospholipid in biliary secretion as well as on CYP7A activity, both in untreated microsomes and in saturating-level cholesterol-loaded microsomes, and mRNA level. After 2 h of treatment, SMase enhanced biliary bile acid (+75%), cholesterol (+20%), and phospholipid (+20%) secretion (Table 2) in parallel with the induction of CYP7A activity in untreated microsomes (+28%; Table 2) and enhanced the level of mRNA (+32%, Fig. 4A). The two major CYP7A mRNA species of 4 and 2.1 kb reported originally (27) were detected in control hepatocytes incubated for 2 h (Fig. 4B). The values shown in Figure 4A correspond to the major 4 kb CYP7A mRNA, and the extent of increase in the other species of CYP7A mRNA (Fig. 4B) was nearly identical (data not shown). A more prolonged (4 h) diversion of membrane cholesterol into intracellular compartments doubled the CYP7A activity in untreated microsomes and increased the secretion of biliary bile acids (+35%), cholesterol (+20%), and phospholipid (+15%). CYP7A mRNA species dropped to extremely low levels of expression after 4 h of incubation, although differences between control and SMase-treated hepatocytes were not significant (Fig. 4). When CYP7A activity was estimated in microsomes supplemented with cholesterol substrate at the saturation level (37), the extent of stimulation was slightly superior to that observed for CYP7A activity in untreated microsomes (data not shown). Collectively, these findings indicate that SMase treatment of hepatocytes enhanced cholesterol output *via* the bile by providing cholesterol substrate for bile acid synthesis by CYP7A and regulatory cholesterol, which upregulates the biliary secretion of bile acid, phospholipid, and cholesterol. Table 2 shows the decrease in the ratio of biliary cholesterol secreted to bile acids, suggesting that SMase treatment may result in a less lithogenic bile.

Effect of C_2- and C_6-ceramide on the cellular distribution and metabolism of CE and VLDL and on bile acid secretion in isolated rat hepatocytes. A series of experiments was conducted to analyze the possibility that the increased ceramide was responsible for the changes attributed to SMase. Short-chain N-acyl ceramides such as C_2 and C_6 have been used extensively to mimic the biological activity of endogenous ceramides because they enter cells rapidly when added exogenously and are nonmetabolizable compounds. Addition of 10 μM C_2- or C_6-ceramide for 2 or 4 h failed to mimic the effects of SMase reported in this study (data not shown).

DISCUSSION

We report that the treatment of intact rat hepatocytes with neutral SMase accelerates the metabolism of CE and modifies the partitioning of cholesterol toward the secretory routes by demonstrating that SMase not only promotes the formation, storage (Figs. 1–3), and hydrolysis of CE (Fig. 3) but also provides a pool of regulatory cholesterol that reduces apoB mRNA levels in hepatocytes (Fig. 4) and the secretion of VLDL apoB, TG, and cholesterol (Table 1). Conversely, it increases CYP7A activity (Table 2) and mRNA levels in hepatocytes (Fig. 4) as well as the secretion of biliary cholesterol, phospholipid, and bile acids into the medium (Table 2). We measured changes after treatment of hepatocytes in suspension with SMase for 2 or 4 h and ruled out the possibility that the increased ceramide was responsible for the observed changes. These findings suggest that plasma membrane neutral SMase may have a specific function in cholesterol homeostasis in hepatocytes, independent of the generation of ceramide.

Our experiments were based on extensive studies by others demonstrating that the treatment of cells with neutral SMase moves a substantial amount of cholesterol from the plasma membrane to the ER and other intracellular membranes (4), and that this can affect the function of membrane-bound proteins and protein complexes (38). Among them, ER membrane-bound proteins seem to be highly sensitive to increases in ER cholesterol levels. This is the case of the sterol sensing proteins SREBP (28) and SREBP cleavage-activating protein (SCAP) (29), which are retained in the membranes of the ER if sterols are abundant, thereby declining the transcription of the responsive genes (28). We rationalized that if proteins essential for the hepatocyte-specific output of cholesterol *via* VLDL (16,17) and bile (27) are located in the ER, then they might be particularly sensitive to neutral SMase

activation. The present study does not address the fact that different pools of sphingomyelin exist in the cell membrane and could participate in distinct pathways of cholesterol metabolism. The liver parenchymal hepatocyte is a polarized cell whose canalicular membrane contains more cholesterol and sphingomyelin than the sinusoidal membrane (39). Once isolated, there is a loss of polarity. Hence, we assumed that treatment of hepatocyte suspensions with SMase results in a regular centripetal trafficking of cholesterol from the plasma membrane to the nucleus.

The simultaneous intracellular buildup of CE, increased formation of new CE, and increased ACAT activity are the expected rational compensatory responses of cells to SMase treatment. Regardless of whether the sterol-sensing SCAP-SREBP system is functioning or whether cholesterol synthesis is (6) or is not (7) suppressed, most, if not all, of the new CE probably derives from the cholesterol found in the plasma membrane in origin and in the cytosol in transit. This is consistent with the increased mass of cytosolic FC seen in treated cells. Direct analysis revealed that treatment of intact cells with SMase increases the pool of ER cholesterol that is accessible to ACAT (40). In this study, the level of cholesterol in the ER-derived microsomes is not really known, as it cannot be assessed by measuring overall cholesterol in the membrane fraction. The cholesterol concentration in the ER is very low (3), and routine microsomal preparations also contain plasma membranes, endosomal membranes, and possibly some Golgi membranes. Given this situation, the decrease in microsomal FC in treated cells may be interpreted to mean that an important fraction of FC in the plasma membranes isolated in the microsomal fraction has moved to and been rapidly metabolized in the ER. SMase enhanced ACAT not only when the activity was estimated using the endogenous microsomal cholesterol as substrate but also when the assay was performed with an excess of substrate. Mechanisms controlling ACAT activity include altered protein mass, activation/deactivation, and viscotropic effects. The first mechanism is unlikely to be involved in SMase action because the abundance of mRNA for ACAT2 was not altered. Two ACAT genes exist in mammals (8–11), and although little is known with certainty, the ACAT2 seems to be the predominant ACAT expressed in the murine liver (11). Plausible explanations for the SMase action are that activation of ACAT operates through allosteric mechanisms involving cholesterol [at present these are poorly understood but are known to occur (9)], and/or through changes in the local lipid environment of the ER membrane where the protein resides (8). We conclude that ACAT is posttranslationally regulated by neutral SMase through a greater supply of the substrate cholesterol and an additional, as yet unknown, nonsubstrate activating effect on ACAT.

This study addresses the effect of hepatocyte plasma membrane sphingomyelin degradation on the neutral hydrolysis of CE for the first time. The two cCEH and erCEH enzyme activities were moderately activated by SMase, although the change in erCEH activity only reached statistical significance after 4 h of treatment. The enzymes were assayed in conditions of substrate saturation (36), whereby activation cannot be ascribed to the increased CE mass in microsomes and the cytosol. The mechanisms underlying cCEH and erCEH activation remain to be elucidated. Compensatory changes in hepatic cCEH at the activity, protein, and mRNA levels occur in response to hormones and treatments that perturb the cholesterol flux through the liver (41). Furthermore, the cCEH gene promoter seems to contain functional response elements that interact with sterols (42). If so, one might predict that cCEH activity would decrease with SMase treatment. The protein erCEH was recently purified from rat liver in this laboratory (43). Tissue distribution and subcellular localization analysis revealed that erCEH is exclusively localized in the ER of human and murine hepatocytes (14). *In vivo* studies showed that CEH activity in microsomes is modulated by altering the flux of cholesterol through the liver in hamsters (44) and rats (45), but the relationship between sterol availability and erCEH activity has not yet been clarified. The increase in cCEH and erCEH activities after treating hepatocytes with SMase may be interpreted to mean there is no limit to the proportion of excess cholesterol that could be converted to the ester but that it was further constrained by a higher CEH activity that would convert the accumulated CE back to FC. Directed back to the cell surface, this cholesterol would then result in the eventual stabilization of the defective plasma membrane. It is tempting to postulate that the increase in CEH activity is a protective response of the hepatocyte to preserve cholesterol in membranes if it goes below a threshold level that might compromise critical cellular functions.

Treatment of cells with SMase is expected to induce a persistent accumulation of ceramides (1,2). Hence, it was essential in this study to analyze the possibility that the increased ceramide was responsible for the changes attributed to SMase. This was achieved by treating hepatocytes with a 10-μM concentration of C_2- or C_6-ceramide for 2 or 4 h and monitoring its effects. With these treatments, the amount of ceramide that accumulates within cells can reach up to 100 pmol/nmol of phospholipid, which is comparable to those concentrations achieved after the prolonged response of hepatocytes to SMase or TNF-α (46). Neither ceramide treatment was found to replicate any of the observed effects of SMase. Both exogenous ceramide and the ceramide that is generated intracellularly accumulate within the Golgi apparatus (46); hence, protein secretion might be disrupted. The possible involvement of ceramide in the medium-term secretion of apoB and TG by cultured intestinal Caco-2 cells was postulated (47). However, we observed that in rat hepatocytes, the decrease in VLDL secretion (discussed below) is unrelated to the release of ceramide after sphingomyelin hydrolysis since neither C_2- nor C_6-ceramide altered the overall synthesis and secretion of proteins or the secretion of VLDL apoB and lipid classes.

VLDL biogenesis is known to require adequate levels of apoB, lipid, and MTP, and reductions in any one of these can limit the output of lipoproteins from hepatocytes (48). SMase

may decrease VLDL secretion *via* multiple mechanisms, including quantitative and qualitative changes in the apoprotein content and/or deficient lipidation and translocation of apoB across the ER membrane. Regulation of apoB secretion is primarily a posttranslational event brought about by proteolytic cleavage of the unlipidated or underlipidated forms of apoB (48). Transcriptional regulation of apoB mRNA has been reported to be caused by agents such as insulin (49), this mechanism being regarded as a secondary level of regulation in apoB secretion. It is known that the liver of rats secretes apoB-100 and apoB-48 in proportions that are subject to modification through apoB mRNA editing (48,50) and that susceptibility to proteasomal degradation of apoB is directly proportional to the length of the nascent polypeptide (51). Treatment of hepatocytes with SMase reduced apoB mRNA levels by ~25% and VLDL apoB secretion by ~45%, with the decrease of VLDL apoB-48 secretion being slightly more accentuated than that of VLDL apoB-100. This observation might be interpreted to mean that SMase, with its inherent changes in cellular cholesterol distribution, simultaneously downregulates apoB mRNA and impairs the translocation and/or intracellular degradation of apoB-48 more markedly than that of apoB-100. Whether the stability of apoB mRNA or transcriptional regulatory mechanisms are affected cannot be answered at present. In an elegant study, Nilsson and colleagues (52) demonstrated that protein translocation across the microsomal membranes is inhibited by enhanced ER cholesterol. It is therefore possible, although speculative at present, that the MTP translocation activity of hepatocytes exposed to SMase is inhibited by enhanced ER cholesterol and that the effect is more pronounced on the translocation apoB-48 than apoB-100 across the ER membrane. The possibility that apoB mRNA editing is affected by SMase treatment of hepatocytes cannot be excluded either.

Changes in the lipid levels of hepatocytes may be considered in general terms as more and less lipid being available for incorporation into the nascent or maturing VLDL particles, and specifically that they might alter the ability of apoB for translocation. Multiple experimental approaches suggest a role for cholesterol and CE (17–24) and TG (15–17) availability in regulating the rate of apoB production by hepatocytes. Even though CE and TG accumulated in SMase-treated hepatocytes, the cells secreted the same amount of CE and less FC and TG in fewer and larger VLDL particles. This indicates that regulatory events other than the availability of these lipids dominate the action of SMase and that serious alterations occur in the ER that impair cholesterol and TG recruitment and apoB translocation. Relatively few studies have been conducted on the involvement of phospholipid in VLDL production. SMase reduced the secretion of VLDL phospholipid by decreasing the number of particles secreted but not their phospholipid content. Sphingomyelin represents the second-most abundant phospholipid in plasma lipoproteins. It is known that provision of the most abundant PC is required for the recruitment of TG and the secretion of apoB, in addition to VLDL assembly (25,53,54). Of particular interest is the similarity of findings in choline-deficient hepatocytes, in which VLDL secretion was depressed by 60–70% and where the VLDL particles in the Golgi were larger than controls (53), and in the sphingomyelin-deficient hepatocytes analyzed here, in which VLDL secretion was inhibited by ~45% and the secreted particles were also larger than controls. Moreover, in both choline- (53) and sphingomyelin-deficient hepatocytes, the increase in the cellular levels of TG was accompanied by undersecretion of the newly formed lipid, indicating that a deficiency of sphingomyelin, or choline, triggers a program of lipid synthesis and sequestration, rather than one of lipoprotein lipid secretion. Such data, together with the finding that the size and lipid composition of secreted VLDL was modified by SMase, suggest the occurrence of abnormalities in the particle assembly or maturing processes and support a role for sphingomyelin in VLDL secretion. Evidence against such a hypothesis has been provided by Merrill and colleagues (55), who found that inhibition of sphingolipid synthesis in cultured rat hepatocytes reduced the amount of sphingolipid secreted but did not affect the secretion of VLDL apoB or cholesterol. One interesting possibility is that the SMase-mediated degradation of plasma membrane sphingomyelin may alter a sphingomyelin pool in the ER with consequences for VLDL biogenesis.

Bile cholesterol is entirely free and derives from preformed hepatic stores and from new hepatic synthesis. Changes in sterol balance across the hepatocyte can alter the rate of conversion of cholesterol to bile acids and, conversely, bile acids are potential regulators of hepatocyte cholesterol balance (26). In rodents, CYP7A gene expression is controlled by a set of orphan nuclear receptors (56), whose intricate interactions support the principles (i) that the CYP7A promoter contains a positive sterol response element that activates the gene in response to dietary cholesterol, and (ii) that a negative bile acid response element represses transcription in response to bile acids. The parallel increases in CYP7A activity and mRNA, as well as the biliary secretion of bile acids, cholesterol, and phospholipid seen in SMase-treated hepatocytes, indicate that bile acid synthesis and bile secretion are activated in a feed-forward manner, also by membrane-derived cholesterol. Broadly speaking, bile secretion and lipoprotein production are believed to be regulated interdependently. Thus, inhibition of ACAT in cultured rat hepatocytes was reported to supply substrate for bile acid synthesis and induce CYP7A gene expression (57). SMase treatment of isolated rat hepatocytes partly mimicked this situation, as it enhanced the pool of FC available for elimination *via* the bile either directly or after conversion to bile acids, as well as that available for esterification and storage. Induction of CYP7A by SMase seems to occur by providing cholesterol both as substrate for CYP7A and as an inducer of CYP7A mRNA levels, the latter indicating that the effect of positive regulators (i.e., cholesterol) dominates over that of suppresors (i.e., bile acids) of the CYP7A gene transcription in treated cells. Other posttranscriptional mechanisms involving cholesterol do not seem operative, as similar increases in CYP7A activity

were seen when the assays were performed in a saturating level of cholesterol-loaded microsomes or using the endogenous cholesterol as substrate.

Regardless of the precise mechanisms involved, the collective data in the current paper indicate that exogenous neutral SMase, and not the ceramides generated, regulates the intracellular turnover of CE and the partitioning of cholesterol toward the secretory routes in primary rat hepatocytes. Additional studies are required to establish the relevance of these findings. The mammalian liver expresses several neutral SMase with different subcellular localizations, including the plasma membrane (1,2,58,59), endoplasmic reticulum (60), and nuclei (61). Neutral SMase activity is believed to be responsible for the regulation of cellular growth, differentiation, and apoptosis, but the functions of each isoform *in vivo* are still under intense study. The findings reported here point to a ceramide-independent role for the cell membrane neutral SMase in the regulation of cholesterol output pathways in hepatocytes and provide a basis for better understanding the metabolic effects of plasma membrane neutral SMase-related abnormalities from injury.

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Distributions of Hydroperoxide Positional Isomers Generated by Oxidation of 1-Palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine in Liposomes and in Methanol Solution

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ABSTRACT: The differences in distribution of geometric isomers of unsaturated PC hydroperoxides generated by free radical oxidation were compared, as corresponding hydroxy analogs, in heterogeneous liposomes and in a homogeneous methanol solution by using HPLC with UV detection due to the presence of conjugated dienes. Identification of fractionated peak components was carried out by GC–MS. When the oxidation of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine, PC(16:0/18:2), was initiated in liposomes by a hydrophilic azo radical initiator, and in a methanol solution by a hydrophobic azo radical initiator, there was no significant difference in the relative percentages of 1-palmitoyl-2-(9-hydroxy-*trans*-10,*trans*-12-octadecadienoyl)-*sn*-glycero-3-phosphocholine (9-*t,t*-OH PC) and 1-palmitoyl-2-(13-hydroxy-*trans*-9,*trans*-11-octadecadienoyl)-*sn*-glycero-3-phosphocholine (13-*t,t*-OH PC) between the PC oxidized in liposomes and in the methanol solution. For the oxidation of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, PC(16:0/20:4), the relative percentage of 1-palmitoyl-2-(5-hydroxy-*trans*-6,*cis*-8,11,14-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine (5-OH PC) was significantly higher ($P < 0.01$) than that of 1-palmitoyl-2-(15-hydroxy-*cis*-5,8,11,*trans*-13-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine (15-OH PC) in liposomes. For the homogeneous methanol solution of PC(16:0/20:4), the relative percentage of 5-OH PC was close to that of 15-OH PC. For the PC(16:0/20:4) oxidized in bulk with added pentamethylchromanol, the individual amount of 15-OH PC, 1-palmitoyl-2-(11-hydroxy-*cis*-5,8,*trans*-12,*cis*-14-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine (11-OH PC), 1-palmitoyl-2-(12-hydroxy-*cis*-5,8,*trans*-10,*cis*-14-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine (12-OH PC), 1-palmitoyl-2-(8-hydroxy-*cis*-5,*trans*-9,*cis*-11,14-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine (8-OH PC), 1-palmitoyl-2-(9-hydroxy-*cis*-5,*trans*-7,*cis*-11,14-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine (9-OH PC), and 5-OH PC were close to each other compared to the corresponding values in liposomes and in methanol solution. The results obtained by gel permeation chromatography of the PC liposomes containing hydrophilic 2,2′-azobis-2-amidinopropane) dihydrochloride (AAPH) suggest that the AAPH added to the liposomes of PC(16:0/20:4) was partitioned into the water phase and out of the hydrophobic region of the fatty acyl moieties of the PC. These results confirm that the distance that exists in the bis-allylic carbons of the unsaturated fatty acyl moieties of PC from the interface between the hydrophilic region of PC and the water phases played an important role in influencing hydrogen abstraction to form a symmetrical distribution of hydroperoxide isomers in both the heterogeneous liposomes and the homogeneous methanol solution.

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The oxidizability of PUFA depends on the number of bis-allylic hydrogens in the FA. In homogeneous solutions, the oxidizability of PUFA increases with an increasing number of bis-allylic carbons (1,2). In contrast, the oxidizability of PUFA in aqueous micelles is known to decrease with an increasing number of bis-allylic carbons (3–5). One cause of this trend may be that the polar peroxyl radicals derived from the core migrate to the surface of the micelles. The accumulation of radicals would lower the oxidizability of PUFA by enhancing the termination reaction rate for polar peroxyl radicals and by reducing the rate of propagation since there is less oxidizable PUFA at the surface than in the micelle core (6,7). Previous studies suggested that, in the aerobic oxidation of PUFA having more than three double bonds, the mechanism of the oxidation is more complicated, as it includes the intramolecular cyclization of peroxyl radicals (8–10).

Numerous studies have looked at the distribution of positional isomers of hydroperoxides generated from unsaturated FA oxidized in the air (11–14). The composition of geometric isomers of hydroperoxides could differ between a bulk oil system and an emulsion system (15). Thus, the mechanism of hydroperoxide isomer formation could be influenced by certain reaction systems.

PC is an amphipathic molecule, consisting of both saturated and polyunsaturated acyl chains. The PUFA is usually susceptible to free radical oxidation and hence results in undesirable flavors and odors in food systems. These decomposition products of lipid oxidation usually cause on undesirable effect on biological membranes as well as a deterioration in the flavor of food (16–18). Therefore, understanding the mechanism of PC hydroperoxide formation is necessary to control lipid oxidation caused by free radical oxidation in complex systems.

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Abbreviations: AAPH, 2,2′-azobis-(2-amidinopropane) dihydrochloride; AMVN, 2,2′-azobis-(2,4-dimethylvaleronitrile); 9-OOH PC, 1,2-di(9-hydroperoxy-octadecadienoyl)-*sn*-glycero-3-phosphocholine; 13-OOH PC, 1,2-di(13-hydroperoxy-octadecadienoyl)-*sn*-glycero-3-phosphocholine; PC(16:0/18:2), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; PC(16:0/20:4), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PC-OH, hydroxy PC; PC-OOH, PC hydroperoxides; PMC, pentamethylchromanol; TMS, trimethylsilyl; Trolox®, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

The goal of this study was to evaluate the effect of radical initiators with different polarities on the distribution of hydroperoxide positional isomers during the oxidation of PC in heterogeneous liposomes and in a homogeneous methanol solution.

MATERALS AND METHODS

Lipids. 1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine [PC(16:0/18:2)] and 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine [PC(16:0/20:4)], both of over 99% purity, were purchased from Avanti Polar Lipids Inc. (Alabaster, AL) and used without further purification.

Free radical oxidation of lipids. For free radical oxidation of PC in a homogeneous solution, 12.5 mg of PC(16:0/20:4) was dissolved in 3 mL of methanol. Free radical oxidation was initiated by the addition of 2 mM 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN; Wako Pure Chemicals, Osaka, Japan) at 30°C in the dark. For the free radical oxidation in liposomes, 12.5 mg of PC(16:0/20:4) was dried in a glass beaker under a stream of nitrogen to form a thin film of PC. Subsequently, 1.5 mL of 50 mM Tris-HCl buffer (pH 7.4) was added and subjected to ultra-sonication with an ultrasonic disruptor (model UD-200; Tomy Co., Tokyo, Japan) in an ice bath for 5 min at an output power of 20 W to obtain liposomes. A quantity of 1.5 mL of 4 mM 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH; Wako Pure Chemicals) was then added to the prepared liposomes and further ultrasonication was carried out for 3 min to obtain a complete dispersion of AAPH in liposomes. Oxidation was initiated and carried out for up to 3 h in the dark.

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®; Aldrich, Milwaukee, WI) or α-tocopherol (Sigma, St. Louis, MO) was dissolved in 50 mM Tris-HCl buffer (pH 7.4) or methanol solution, respectively, of different concentrations then added to the dispersed PC film before sonication.

A dichloromethane solution consisting of 5 mM PC(16:0/20:4) and 2 mM AMVN as a radical initiator with or without 3.5 mM pentamethylchromanol (PMC) as a hydrogen donor was flash evaporated in a glass test tube under a stream of nitrogen to obtain a thin film of PC in bulk. The oxidation was initiated and carried out at 30°C in the dark.

Derivatization of lipids. For HPLC of PC hydroperoxides (PC-OOH), PC-OOH were extracted by chloroform and reduced to their corresponding hydroxy derivatives by triphenylphosphine (over 99% purity; Aldrich) in diethyl ether (5 mg/10 mL).

To identify the peak components, PC-OOH were reduced by sodium borohydride (Aldrich) to the corresponding hydroxy PC (PC-OH) and were subsequently hydrogenated by platinum (IV) oxide (Wako Pure Chemicals) to obtain saturated PC-OH. Following saponification with 0.5 M sodium hydroxide in methanol, acidified FFA were extracted by diethyl ether. The FFA obtained were methylated with ethereal diazomethane. Trimethylsilyl (TMS) ether derivatives of FAME were subjected to GC–MS to determine the position of the hydroxy group.

HPLC. Distribution of isomeric PC-OH was also analyzed by reversed-phase HPLC according to Milne and Porter (19). Briefly, hydroxy PC-OH, which was obtained by reducing PC-OOH, was separated on a reversed-phase column (Lichrosorb® RP-18, 250 × 4 mm i.d., 5 μm film thickness; Kanto Kagaku, Tokyo, Japan) using a mixture of water and methanol (5:95, vol/vol) as a mobile phase at a flow rate of 1.0 mL/min and a model LC-9A pump (Shimadzu). The conjugated PC-OH eluted from the column was monitored with a photodiode array detector (model 1050; Hewlett-Packard, Boise, ID).

GC–MS. The TMS ethers of FAME derived from the fractionated PC-OH were separated and analyzed by GC–MS to identify peak components as described previously (15). Briefly, the TMS ether derivatives of the hydroxy methyl octadecanoates were separated on a SUPELCOWAX 10 fused-silica open tubular column (0.25 mm i.d. × 30 m, 0.25 μm in film thickness; Supelco, Bellfonte, PA) attached to a model GC17A gas chromatograph (Shimadzu, Kyoto, Japan). The outlet of the column was connected directly to the electron impact source of a Shimadzu model QP5000 quadrupole mass spectrometer. The column oven temperature was programmed from 150 to 240°C at 3°C/min, and the injection port temperature was set at 250°C. Helium was used as carrier gas at a flow rate of 50 mL/min. Mass spectra were obtained in electron ionization mode (3 kV, 70 eV), with an interface temperature of 280°C, and set to the 40- to 650-amu range at 2 s per scan. Mass spectra data were acquired with a computer system equipped with Shimadzu Class-5000 software.

Gel permeation chromatography of liposomes. To elucidate the distribution of AAPH in the liposomes, the prepared liposomes were subjected to gel permeation column chromatography on a Sephadex G-15 column (2 cm i.d. × 40 cm; Pharmacia Fine Chemicals, Uppsala, Sweden). A 1.5-mL portion of PC liposomes was loaded onto the column and then eluted by 50 mM potassium chloride at a flow rate of 1.2 mL/min. The eluent was fractionated in 1.5-mL portions. Eluted liposomes and AAPH were monitored at 300 nm, owing to the slight opacity of the liposomes, and at 370 nm, owing to maximum absorption of AAPH. The fractionated liposome particles were dissolved in aqueous ethanol, and the amount of AAPH was determined spectrophotometrically. The recovery of AAPH was calculated from the amounts of AAPH loaded onto and eluted from the column. Preliminary experiments on the recovery of AAPH from the column revealed that the percentages of AAPH recovered from the outer hydrophilic phase of liposomes were 96.9, 96.7, and 99.4% when 40, 50, and 60 mM AAPH was added to the PC liposomes. These results confirmed that the added AAPH located preferentially in the hydrophilic phase of the liposomes.

Determination of AAPH. The liposome fraction recovered in gel permeation chromatography was dissolved in a mixture of equal volumes of ethanol and water (1:1, vol/vol) and made up to 50 mL. The AAPH fractions were collected and made up to 50 mL by water. Absorbance of the solutions was measured at 370 nm with a Shimadzu UV-160A spectrophotome-

ter. A calibration curve was obtained from standard solutions of AAPH at different concentrations in ethanol/water (1:2, vol/vol).

Determination of PV. PV of the oxidized PC was determined photometrically with the ferric thiocyanate method (20). The PV of oxidized PC in liposomes was determined in a chloroform solution after lipids were extracted.

Statistical analysis. The Microsoft Excel 5.0 (Microsoft Co., Redmond, WA) program was used for all statistical analyses. The data were analyzed with one-way ANOVA, and means were compared using Student's *t*-test (21). A difference at $P < 0.01$ was considered to be statistically significant.

RESULTS

Separation of the hydrophilic radical initiator in liposomes. The AAPH was separated from the liposomes by gel permeation chromatography as shown in Figure 1. Liposomes were eluted as a first peak between 27 and 40 mL of eluent under the present conditions. AAPH was eluted as a later peak between 46 and 62 mL of eluent. The amount of AAPH initially found in the 1.5 mL of liposomes was 7.8 mg, and the amount of AAPH recovered from the latter fraction was determined as 8.1 mg. Thus, the recovery of AAPH by gel permeation chromatography was calculated to be 103%, suggesting that AAPH was not included in the liposome fraction. These results confirmed that the added AAPH located preferentially in the hydrophilic phase of the liposome system.

Distribution of isomeric hydroxy methyl linoleate in oxidized PC(16:0/18:2). The PC-OH isomers derived from the hydroperoxy PC(16:0/18:2) that oxidized in liposomes were partially separated into three peaks by reversed-phase HPLC as shown in Figure 2. The bathochromic shift in the UV spectra with λ_{max} at 231 nm for peaks 1 and 3, and with λ_{max} at 235 nm for peak 2 (data not shown) reflected the *trans,trans* and *cis,trans* isomers of conjugated linoleoyl PC-OH, respectively. MS analyses of the corresponding TMS ether derivatives of hydroxy FAME derived from each peak compound (dominant fragment ion pairs of *m/z* 173 and 315 for both peak 1 and peak 2, and of *m/z* 229 and 259 for both peak 2 and peak 3) clearly showed that peaks 1, 2, and 3 were due to 1-palmitoyl-2-(13-hydroxy-*trans*-9,*trans*-11-octadecadienoyl)-*sn*-glycero-3-phosphocholine (13-*t,t*-OH PC), a mixture of 1-palmitoyl-2-(13-hydroxy-*cis*-9,*trans*-11-octadecadienoyl)-*sn*-glycero-3-phosphocholine (13-*c,t*-OH PC)

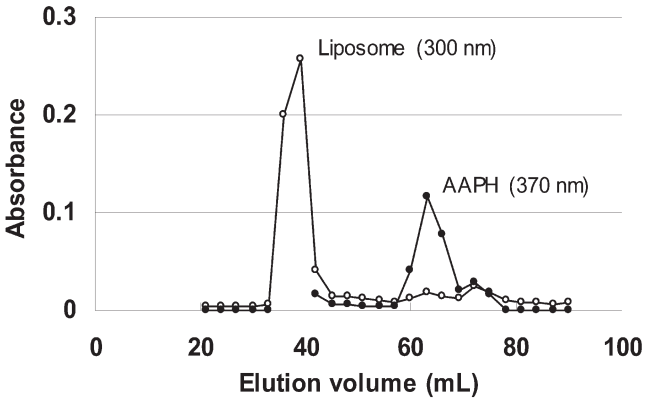


FIG. 1. Gel permeation column chromatography of PC liposomes containing 20 mM 2,2′-azobis-(2-amidinopropane)-dihydrochloride (AAPH) on a Sephadex G-15 column (2 cm i.d. × 40 cm). Liposomes were prepared with 50 mM Tris-HCl buffer (pH 7.4). A quantity of 50 mM KCl was eluted at a flow rate of 1.2 mL/min. Eluted liposomes and AAPH were monitored at 300 nm (owing to the slight opacity of the liposomes) and 370 nm (maximum absorption of AAPH), respectively, for the fractionated solution. The absorbance at 370 nm of the eluting fractions of the liposome was not determined owing to interference from slight opacity of the liposomes.

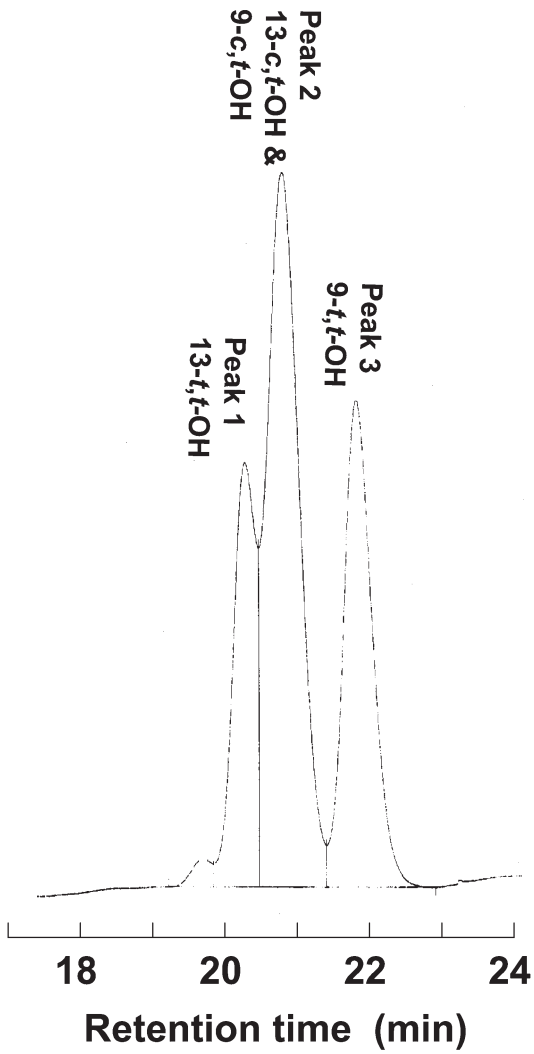


FIG. 2. Typical HPLC chromatogram of hydroxy 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine derived from reduction of the corresponding hydroperoxide by triphenylphosphine. Peak identifications were carried out by a combination of GC–MS and photospectrometry. 13-*t,t*-OH, 1-palmitoyl-2-(13-hydroxy-*trans*-9,*trans*-11-octadecadienoyl)-*sn*-glycero-3-phosphocholine; 13-*c,t*-OH, 1-palmitoyl-2-(13-hydroxy-*cis*-9,*trans*-11-octadecadienoyl)-*sn*-glycero-3-phosphocholine; 9-*t,t*-OH, 1-palmitoyl-2-(9-hydroxy-*trans*-10,*trans*-12-octadecadienoyl)-*sn*-glycero-3-phosphocholine; 9-*c,t*-OH, 1-palmitoyl-2-(9-hydroxy-*cis*-12,*trans*-10-octadecadienoyl)-*sn*-glycero-3-phosphocholine.

TABLE 1
Distribution of Isomeric Hydroxides Formed from 1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine [PC(16:0/18:2)] Initiated by Radical Initiators in Liposomes and in a Homogeneous Solution by GC–MS[a]

Hydroxide isomers	Liposomes (n = 8)	Solution (n = 8)
9-OH PC	45.70 ± 6.92[a]	45.20 ± 2.37[a]
13-OH PC	54.30 ± 6.92[b]	54.80 ± 2.37[b]

[a]Values are expressed as mean percentage ± SD. Values with different roman superscripts (a or b) are significantly different ($P < 0.01$). 9-OH PC, 1-palmitoyl-2-(9-hydroxy-octadecadienoyl)-*sn*-glycero-3-phosphocholine; 13-OH PC, 1-palmitoyl-2-(13-hydroxy-octadecadienoyl)-*sn*-glycero-3-phosphocholine.

and 1-palmitoyl-2-(9-hydroxy-*cis*-12,*trans*-10-octadecadienoyl)-*sn*-glycero-3-phosphocholine (9-*c,t*-OH PC), and 1-palmitoyl-2-(9-hydroxy-*trans*-10,*trans*-12-octadecadienoyl)-*sn*-glycero-3-phosphocholine (9-*t,t*-OH PC), respectively. As a consequence of the above observations, the compounds of peaks 1, 2, and 3 were identified as 13-*t,t*-OH PC, 13-*c,t*-OH PC and 9-*c,t*-OH PC, and 9-*t,t*-OH PC, respectively. PC-OH isomers derived from hydroperoxy PC(16:0/18:2) oxidized in the methanol solution showed a similar profile on the HPLC chromatogram. The relative distributions of the PC-OH isomers determined by GC–MS are summarized in Table 1. MS of the isomeric hydroxy FAME derived from the oxidized PC showed that the relative percentage of methyl 13-hydroxy-octadecadienoate (13-OH) was close to that of methyl 9-hydroxy-octadecadienoate (9-OH) when the oxidation of PC(16:0/18:2) was carried out in the methanol solution at different PV between 62 and 130 meq/kg. The relative percentages of 13-OH PC compared to those of 9-OH PC were similar when the oxidation was carried out in liposomes at different PV between 44 and 254 meq/kg. Thus, there was no significant difference in the relative percentages of the outer hydroxide isomers, 9-OH PC and 13-OH PC, between the homogeneous methanol solution and the heterogeneous liposomes.

The relative distribution of PC-OH isomers was calculated from the HPLC peak areas and is summarized in Table 2. Since the *cis,trans* isomers of PC-OH did not separate from each other, it was difficult to determine the ratio between the two isomers with a *cis,trans* configuration. However, the ratio between 13-*t,t*-OH PC and 9-*t,t*-OH PC was approximately 1:1 in liposomes as well as in the methanol solution, and there was no significant difference ($P < 0.01$) in the ratio of *trans,trans* isomers between the liposome system and the methanol solution.

These results showed that a symmetrical distribution of PC(16:0/18:2) hydroperoxide isomers was obtained even though the oxidation was performed in different reaction sys-

TABLE 2
Distribution by HPLC of Geometric Hydroxide Isomers Generated from PC(16:0/18:2) Initiated by Radical Initiators in Liposomes and in a Homogeneous Solution[a]

Hydroxide isomers	Liposomes (n = 4)	Solution (n = 4)
9-*t,t*-OH PC	23.85 ± 1.26[a]	28.23 ± 2.00[b]
13-*t,t*-OH PC	23.91 ± 2.58[a]	27.65 ± 2.90[b]
9-*c,t*-OH PC + 13-*c,t*-OH PC	52.24 ± 3.63	44.12 ± 4.46

[a]Values are expressed as mean percentage ± SD. Values with different roman superscripts (a or b) are significantly different ($P < 0.01$). 9-*t,t*-OH PC, 1-palmitoyl-2-(9-hydroxy-*trans*-10,*trans*-12-octadecadienoyl)-*sn*-glycero-3-phosphocholine; 13-*t,t*-OH PC, 1-palmitoyl-2-(13-hydroxy-*trans*-9,*trans*-11-octadecadienoyl)-*sn*-glycero-3-phosphocholine; 9-*c,t*-OH PC, 1-palmitoyl-2-(9-hydroxy-*cis*-12,*trans*-10-octadecadienoyl)-*sn*-glycero-3-phosphocholine; 13-*c,t*-OH PC, 1-palmitoyl-2-(13-hydroxy-*cis*-9,*trans*-11-octadecadienoyl)-*sn*-glycero-3-phosphocholine; for other abbreviation see Table 1.

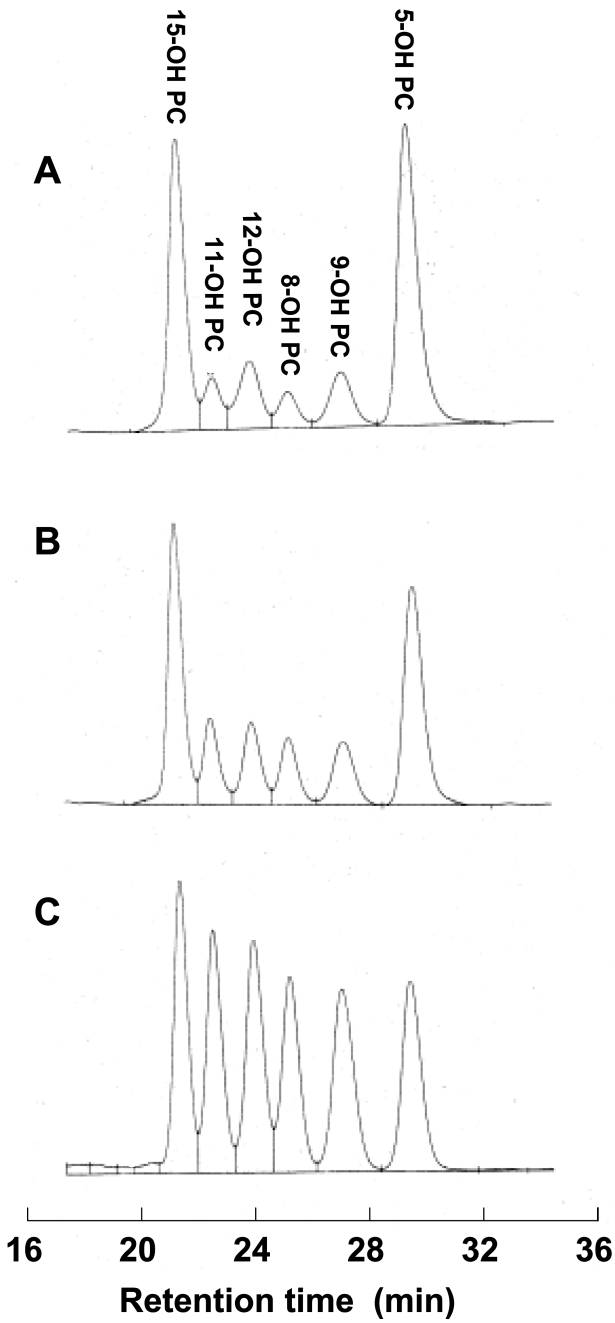


FIG. 3. Typical HPLC chromatograms of hydroxy 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine derived from reduction of the corresponding hydroperoxide by triphenylphosphine. The oxidation of phospholipid was initiated in heterogeneous liposomes (A), in a homogeneous methanol solution (B), and in bulk with added pentamethylchromanol (C). *n*-OH PC, 1-palmitoyl-2-(*n*-hydroxy-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine.

tems that included both heterogeneous liposomes and a homogeneous solution.

Distribution of isomeric hydroxy methyl arachidonate in oxidized PC(16:0/20:4). Typical HPLC chromatograms of hydroxy PC(16:0/20:4) derived from PC-OOH generated by oxidation in bulk, in methanol, and in liposomes are illustrated in Figure 3. The hydroxy isomers of PC obtained by the oxidation in bulk gave six well-separated peaks that eluted between 21 and 30 min of retention time as shown in Figure 3C. These peak components were identified by comparison with the published data (19) as 1-palmitoyl-2-(15-hydroxy-*cis*-5,8,11,*trans*-13-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine (15-OH PC), 1-palmitoyl-2-(11-hydroxy-*cis*-5,8,*trans*-12,*cis*-14-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine (11-OH PC), 1-palmitoyl-2-(12-hydroxy-*cis*-5,8,*trans*-10,*cis*-14-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine (12-OH PC), 1-palmitoyl-2-(8-hydroxy-*cis*-5,*trans*-9,*cis*-11,14-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine (8-OH PC), 1-palmitoyl-2-(0-hydroxy-*cis*-5,*trans*-7,*cis*-11,14-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine (9-OH PC), and 1-palmitoyl-2-(5-hydroxy-*trans*-6,*cis*-8,11,14-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine (5-OH PC) in order of elution from the shortest retention times. For oxidation of PC(16:0/20:4) without PMC in bulk, the same peaks appeared on the chromatogram (data not shown). In this case, however, the peaks correspondingto the inner hydroxy PC (11-OH PC, 12-OH PC, 8-OH PC, and 9-OH PC) were smaller compared to the outer hydroxy PC, 15-OH PC and 5-OH PC. A typical HPLC chromatogram of hydroxyl PC isomers obtained by oxidation in methanol is shown in Figure 3B. Six peaks with the same retention times were separated similarly to those of the PC-OH described above; however, the peaks for 15-OH PC and 5-OH PC were dominant in this particular case. The peaks for 15-OH PC and 5-OH PC were also dominant in the case of the oxidation of PC in liposomes as shown in Figure 3A. However, the peak for 5-OH PC was larger than that for 15-OH PC in this particular case. The relative percentages of hydroxy PC(16:0/20:4) obtained from the peak areas are shown in Table 3. For the oxidation of PC(16:0/20:4) in liposomes, the amount of 5-OH PC was 1.2 times higher than that of 15-OH PC ($P < 0.01$). In contrast, the amount of 5-OH PC was almost equal to that of 15-OH PC in the methanol solution; the ratio of 5-OH PC to 15-OH PC was 0.95. The relative percentage of 5-OH PC plus 9-OH PC was also predominant compared to 11-OH PC plus 15-OH PC ($P < 0.01$); the ratio of 5-OH PC plus 9-OH PC to 11-OH PC plus 15-OH PC was 1:1.2. In the methanol solution, the percentages of 5-OH PC plus 9-OH PC and of 11-OH PC plus 15-OH PC were close to each other. For the PC(16:0/20:4) oxidized in bulk, the individual amounts of 15-OH PC, 11-OH PC, 12-OH PC, 8-OH PC, 9-OH PC, and 5-OH PC were close to each other compared to the corresponding values in liposomes and in the methanol solution. These results demonstrate that the abstraction of the bis-allylic hydrogen from the 7-carbon of the arachidonoyl moiety of PC occurred predominantly in liposomes compared with its abstraction at the 13-carbon. In contrast, the hydrogen abstraction at the 7-carbon occurred at a frequency almost equal to that at the 13-carbon in the homogeneous methanol solution. For the oxidation of PC in bulk, the hydrogen abstraction occurred randomly in all bis-allylic carbons.

The addition of α-tocopherol to the PC liposomes resulted in an even distribution of the 5-OH PC and 15-OH PC as well as a tendency to increase the compositions of the isomers because of the internal hydroperoxides. The addition of Trolox to the PC liposomes resulted in an even distribution of 5-OH PC and 15-OH PC, differing from the PC liposomes with no antioxidative additives; however, the isomers from the internal hydroperoxides remained at lower concentrations as shown in Table 4.

TABLE 3
Distribution of Isomeric Hydroxides Generated from 1-Palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine [PC(16:0/20:4)] Initiated by Radical Initiators in Liposomes, in a Homogeneous Solution, and in Bulk[a]

Hydroxy isomers	Liposomes ($n = 5$)	Solution ($n = 5$)	Bulk with PMC ($n = 6$)
5-OH PC	39.6 ± 1.77[a]	31.3 ± 2.27[a]	16.0 ± 0.79[a]
8-OH PC	4.3 ± 0.82	7.3 ± 1.01	16.6 ± 0.37[a]
9-OH PC	7.8 ± 0.95	8.6 ± 0.73	14.8 ± 0.32[a]
11-OH PC	5.9 ± 0.87	8.9 ± 1.29	17.4 ± 0.25[a]
12-OH PC	9.6 ± 0.56	10.4 ± 0.69	16.5 ± 0.51[a]
15-OH PC	32.6 ± 1.43[b]	33.0 ± 2.30[a]	18.4 ± 0.66[a]
5-OH PC + 9-OH PC	47.4 ± 1.48[c]	40.2 ± 2.22[b]	32.7 ± 0.46[b]
11-OH PC +15-OH PC	38.5 ± 1.00[d]	41.9 ± 1.84[b]	34.9 ± 0.34[b]

[a]Values are expressed as mean percentage ± SD. Values with different roman superscripts (a, b, or c) in the same column are significantly different ($P < 0.01$). 5-OH PC, 1-palmitoyl-2-(5-hydroxy-*trans*-6,*cis*-8,11,14-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine; 8-OH PC, 1-palmitoyl-2-(8-hydroxy-*cis*-5,*trans*-9,*cis*-11,14-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine; 9-OH PC, 1-palmitoyl-2-(9-hydroxy-*cis*-5,*trans*-7,*cis*-11,14-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine; 11-OH PC, 1-palmitoyl-2-(11-hydroxy-*cis*-5,8-*trans*-12,*cis*-14-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine; 12-OH PC, 1-palmitoyl-2-(12-hydroxy-*cis*-5,8,*trans*-10,*cis*-14-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine; 15-OH PC, 1-palmitoyl-2-(15-hydroxy-*cis*-5,8,11,*trans*-13-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine; PMC, pentamethylchromanol.

TABLE 4
Distribution of Isomeric Hydroxides Generated from PC(16:0/20:4) Initiated by a Hydrophilic Radical Initiator in Liposomes With or Without Antioxidants[a]

Hydroxy isomers	PC[b] ($n = 5$)	PC + α-tocopherol ($n = 17$)	PC + Trolox® ($n = 7$)
5-OH PC	39.6 ± 1.77[a]	23.1 ± 1.92[a]	37.1 ± 2.86[a]
8-OH PC	4.3 ± 0.82	11.1 ± 0.67	5.1 ± 1.69
9-OH PC	7.8 ± 0.95	16.6 ± 0.70	7.1 ± 1.75
11-OH PC	5.9 ± 0.87	12.0 ± 0.62	6.2 ± 1.11
12-OH PC	9.6 ± 0.56	14.8 ± 0.66	8.2 ± 1.14
15-OH PC	32.6 ± 1.43[b]	22.4 ± 1.25[a]	36.3 ± 2.86[a]
5-OH PC + 9-OH PC	47.4 ± 1.48[c]	35.7 ± 2.40[b]	44.2 ± 2.18[b]
11-OH PC +15-OH PC	38.5 ± 1.00[d]	38.5 ± 3.09[b]	42.5 ± 2.19[b]

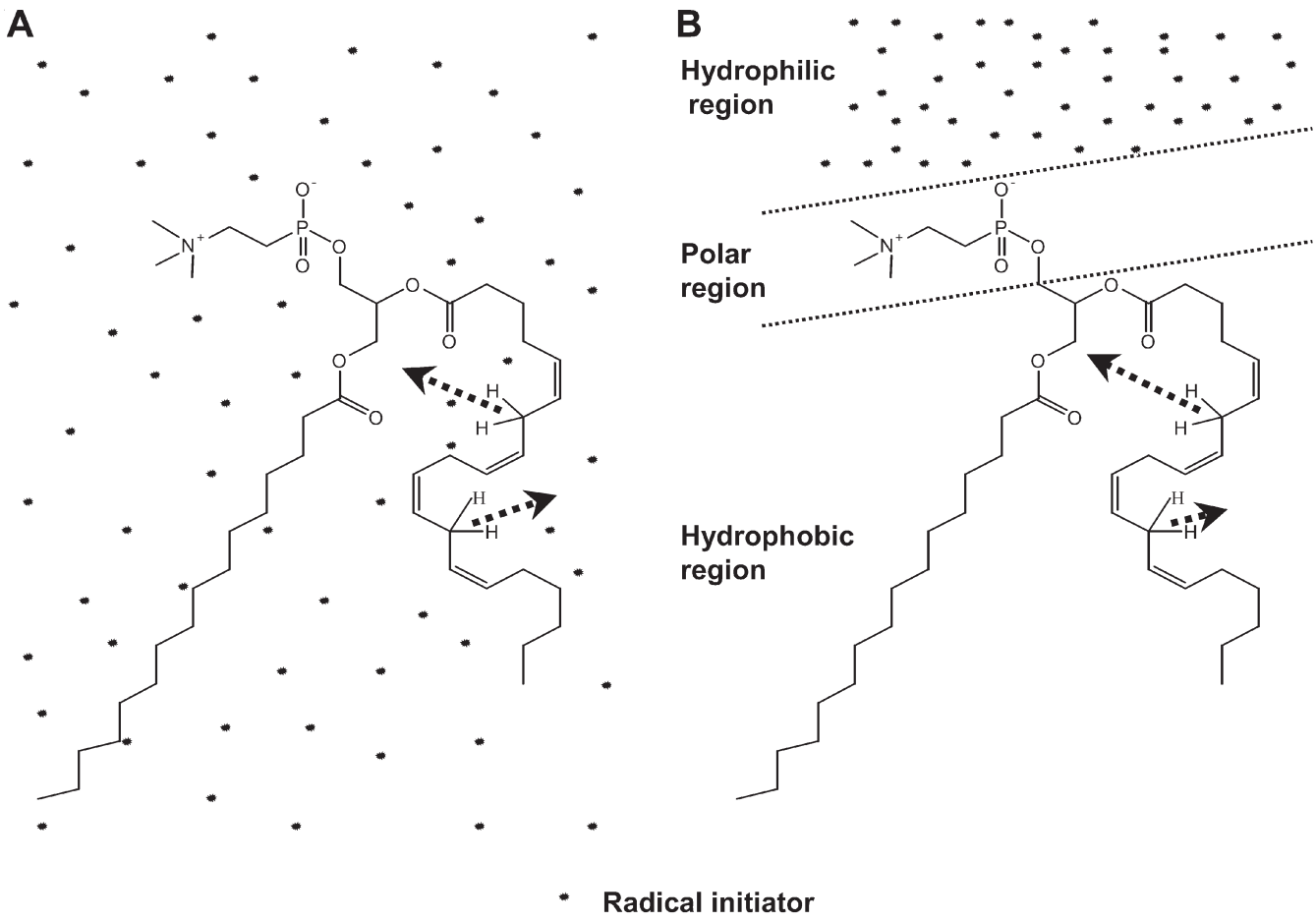
[a]Values are expressed as mean percentage ± SD. Values with different roman superscripts (a, b, or c) in the same column are significantly different ($P < 0.01$). Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; for other abbreviations see Table 3.
[b]Same data presented in Table 3.

DISCUSSION

The present study clearly demonstrates that the abstraction of bis-allylic hydrogen occurs at a high frequency at the carbon located closer to the interface between the hydrophobic phase and the hydrophilic water phase in liposomes when the oxidation of PC(16:0/20:4) is initiated by a hydrophilic initiator. Gel permeation chromatography of the liposomes strongly indicated that the hydrophobic initiator, AAPH, was partitioned out of the hydrophobic region of the PC, particularly in the water phase.

In the free radical oxidation of 1,2-dilinoleoyl PC inhibited by a hydrophilic antioxidant, Trolox, in liposomes, the ratios of 9-hydroperoxy PC (9-OOH PC) to 13-hydroperoxy PC (13-OOH PC) were constant between 0.9 and 1.3 (22). Similar symmetrical distributions of hydroperoxide isomers were obtained when α-tocopherol was added to the liposomes (23–27). The mechanism of this phenomenon has been explained as an easy diffusion behavior of polar peroxyl radicals from the nonpolar lipid phase to the polar interface where peroxyl radicals are trapped or terminated by a water-soluble antioxidant (24). Thus, the addition of a hydrogen donor is an important factor for peroxy radicals to alter the ratio of hydroperoxide isomers (15). This explanation seems quite logical since the hydrogen abstraction from the 11-carbon of the linoleoyl moiety of PC(16:0/18:2) led to the generation of an equal amount of outer hydroperoxides, including 9-OOH and 13-OOH (13,15). In the case of PC(16:0/20:4) oxidation, however, there are three bis-allylic carbons (7-, 10-, and 13-carbons) in the arachidonoyl moiety. Previous studies on packing characteristics of highly unsaturated bilayers of lipids suggest that the 7-carbon in the arachidonoyl moiety of the PC is located in a position closer to the interface of the liposomes compared to the 13-carbon (28,29). The mechanism producing the asymmetric distribution of isomeric hydroperoxides obtained in the present study is suggested to involve the bis-allylic hydrogen at the 7-carbon of arachidonate in the *sn*-2 position. This could move to a reasonable distance that would allow the hydrogen abstraction to be initiated more frequently because of the radicals' existing in the hydrophilic phase. In the homogeneous solution, however, the hydrophobic radical initiators locate a similar distance from all the bis-allylic carbons of the arachidonic moiety of PC(16:0/20:4). Thus, the hydrogen abstraction proceeds at an equal frequency at the 7- and 13-carbons, leading to the generation of equal amounts of outer hydroperoxide isomers in the homogeneous solution (Scheme 1).

Milne and Porter (19) recently reported that the oxidation of PC(16:0/20:4) with PMC in a thin film generated a rela-



SCHEME 1

tively even distribution of PC-OH isomers. On the other hand, Khaselev and Murphy (30) suggested a cooperation between the *sn*-1 vinyl ether substituent and the arachidonoyl substituent in the *sn*-2 of the arachidonate-containing plasmenyl glycerophosphocholine to the oxidation of the arachidonate ester at the 5-carbon. The mechanism of this selective oxidation at the 5-carbon of the arachidonate in the *sn*-2 position was considered to be due to the initial formation of the plasmalogen hemiacetal hydroperoxy radical in the *sn*-1 position where it could come within a reasonable proximity to permit the radical propagation reaction to proceed (31). It is very interesting that in the present study the hydroperoxy PC generated by the oxidation of PC(16:0/20:4) in the heterogeneous liposomes demonstrated an asymmetrical distribution of the outer hydroperoxide isomers, although the vinyl ether peroxides could not become an initiator of peroxidation at the bisallylic carbons of the arachidonoyl moiety of the PC. It has been reported that ferrous-catalyzed oxidation of methyl arachidonate in emulsion yields higher percentages of the outer hydroperoxides, 5-OOH and 15-OOH, accounting for approximately 35 and 41%, respectively (30). However, the molecular conformation of methyl arachidonate in emulsion particles is expected to be different from that of PC(16:0/20:4) in a liposome system.

The α-tocopherol added to methyl arachidonate in bulk inhibits the cyclization of the internal 8-, 9-, 11-, and 12-hydroperoxy radicals and results in an even distribution of the corresponding six isomers of hydroperoxides (32,33). Therefore, the tendency toward an even distribution of the isomers in the oxidized bulk PC(16:0/20:4) with added α-tocopherol obtained in the present study can be attributed to a similar mechanism. The tocopherol locating in the hydrophobic phase of the liposome seems to prohibit cyclization of the internal hydroperoxy radicals. A better hydrogen donor, PMC, performed more effectively in bulk. On the other hand, the addition of the hydrophilic antioxidant Trolox, which located in the hydrophilic region of the liposome, seemed to be less effective in preventing cyclization of the internal hydroperoxy radicals, leading to an asymmetrical distribution of the hydroperoxides.

The present study and the results of previous work (19) with added hydrogen donors such as α-tocopherol and PMC clearly showed that the oxidation of PC(16:0/20:4) in bulk yielded an even distribution of either the internal or the outer hydroperoxide isomers. The mechanism by which this phenomenon occurs is still obscure. However, we reported in an earlier work (15) that the higher concentration of hydrogen donors existing in the methyl linoleate particles of an oil-in-water emulsion favored the formation of the less stable *cis,trans* isomers. In this particular case, the lower concentration of methyl linoleate as hydrogen donor in the dichloromethane solution resulted in restricting hydrogen donation. As a result, more stable *trans,trans* isomers of methyl linoleate were generated under the condition of a relatively higher concentration of hydrogen donors, such as in the oil-in-water emulsion (4,15). Phospholipid molecules, α-tocopherol, and PMC in a relatively high concentration in the bulk system may serve as hydrogen donors to suppress the cyclization of internal hydroperoxy radicals.

The results obtained in the present study illustrate important aspects of the oxidizability of polyunsaturated glycerophospholipids in different reaction systems, such as complicated food and cell membrane matrices.

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Very Long Chain PUFA in Murine Testicular Triglycerides and Cholesterol Esters

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ABSTRACT: Very long chain (VLC) PUFA of the n-6 and n-3 series are known to occur in mammalian testis. The aim of this work was to characterize further two testicular lipid classes with VLCPUFA, cholesterol esters (CE) and total triglycerides (TG) in rat and mouse testis. The VLCPUFA predominating in these lipids were a series of n-6 pentaenes and tetraenes with 24 to 32 carbons, including small amounts of odd-chain PUFA, 28:5n-6 and 24:5n-6 prevailing in CE and TG, respectively. Most of the VLCPUFA of TG were concentrated in a small fraction of TG, made up by 1-*O*-alkyl-2,3-DAG. This TG subclass was absent altogether from the TG of sexually immature testis. The TG and the CE with VLCPUFA only occurred in testis of adult fertile animals. The proportion of VLCPUFA in total TG and CE was higher in rodents than in other mammals. In the n-6 PUFA-rich adult mouse testis, the amounts of testicular triacylglycerols decreased significantly after consumption of fish oil for 2 wk. Whereas 18:2n-6 was significantly reduced, the amounts of 22:5n-6 and longer n-6 PUFA were less affected in all major testicular lipids including PC and PE, where they were unchanged. The 1-*O*-alkyl-2,3-DAG and their n-6 VLCPUFA were virtually unaffected by the diet. The VLCPUFA-containing molecular species of CE and TG may represent a form of storage of cholesterol and polyenoic FA required to sustain spermatogenesis. *Via* chain-shortening, VLCPUFA stored in the neutral lipids may serve as precursors of the major C_{22} PUFA typical of cell membrane glycerophospholipids, protecting testicular cells against shifts in FA composition induced by dietary changes.

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A group of very long chain (VLC) PUFA of the n-6 and n-3 series was characterized several years ago in the mammalian genital tract. In rat testes (1) and in mouse spermatocytes and spermatids in culture (2,3), 24:4n-6 and 24:5n-6 were shown to be actively formed from labeled 20:4n-6. In rat testis, Grogan (4) showed that 20:4n-6 is the precursor of these and of even longer PUFA, including tetraenes and pentaenes with up to 30 carbon atoms. In testis and spermatozoa of a number of mammalian species, Poulos *et al.* (5) reported the occurrence of n-6 and n-3 VLC tetraenoic, pentaenoic, and hexaenoic FA. Complete homologous series of n-6 tetraenes and pentaenes up to C_{34}, including VLCPUFA with even and odd numbers of carbons, were characterized in the total lipid of rat seminiferous tubules (6). In the same article, such VLCPUFA were shown to be synthesized *in situ* by successive elongation of 24:4n-6 and 24:5n-6, two FA connected by an active 6-desaturation.

VLCPUFA were characterized in the retina of several vertebrates (7) and in the brain of rats (8) and humans (9,10). The VLCPUFA of retina are important components of the dipolyunsaturated molecular species of PC of retinal rod outer segments (11). In the PC of both retina (12) and brain (10), VLCPUFA are specifically esterified to the *sn*-1 position of the glycerol backbone. The proportion of VLCPUFA-containing molecular species of PC in human brain increases with development (9) and in the rat retina decreases with aging (13). As with the n-6 VLCPUFA of testis (4,6), the n-3 VLCPUFA of retina (14,15) are actively synthesized *in situ* by successive elongation of ordinary PUFA such as 20:5n-3 and 22:6n-3, with an important role being played by the 6-desaturation of 24:5n-3 to 24:6n-3.

The VLCPUFA of testis and spermatozoa occur in specific lipids. They were first reported to be located in unique molecular species of sphingomyelin (SM) (16). Robinson *et al.* (17) confirmed this finding in a variety of species and extended it by showing the occurrence, in rat and boar SM, of 2-hydroxylated derivatives of VLCPUFA. Further work in rat seminiferous tubules (6) detected the presence of VLCPUFA not only in SM but also in two neutral lipids, cholesterol esters (CE) and triglycerides (TG). The aim of this work was to study further the FA of these two lipid classes. It is shown that the testicular tissue of rodents contains important proportions of VLCPUFA of the n-6 series in these two lipids. Two subclasses of TG are described, the major one being the triacylglycerols (TAG), and the minor one containing TG with *O*-alkyl bonds. The latter is shown to concentrate most of the VLCPUFA present in testicular TG.

Since the levels of n-6 PUFA in tissue lipids may be affected by the consumption of diets enriched in n-3 FA, and since recently (18) we observed that the amount and proportion of 18:2n-6 and other n-6 PUFA in mouse plasma decreased after 2 wk on a fish oil-rich diet concomitantly with a reduction of TG, CE, and PC, we were interested in seeing how this condition would affect the FA of the same lipid classes from testis. In contrast to the liver, the testis did not incorporate much n-3 PUFA. Instead, it preserved the n-6 PUFA, especially the major 22:5n-6 of phospholipids (PL). The n-3-rich diet induced changes in the proportions of the TG and of their n-6 PUFA (18:2, 22:5, and VLCPUFA),

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Abbreviations: CE, cholesterol esters; LH, luteinizing hormone; PL, phospholipid; SM, sphingomyelin; TAG, triacylglycerols; TG, total triglycerides; VLC, very long chain. Fatty acids are named by the convention of: number of carbon atoms:number of double bonds.

which suggest that the levels of 22:5n-6 were sustained at the expense of the acyl groups stored in these lipids.

MATERIALS AND METHODS

The rat and mouse testicular tissues used in this study were obtained rapidly after the animals' death, whereas those from domestic animals were collected after standard surgical procedures. In the case of mice, testes from two groups of animals were analyzed: controls, having eaten the standard rodent lab chow diet, and fish oil-fed mice, receiving an n-3 FA-enriched diet for 2 wk. This diet consisted of 23% protein, 63% carbohydrates, 5% of a vitamin mixture, and 9% fish oil (w/w). The FA compositions of control and fish oil diets were as follows: saturates, 19.3 and 25.8%; monounsaturates, 22.4 and 29.6%; n-6 PUFA, 57.9 and 13.7%; and n-3 PUFA, 0.4 and 30.8%, respectively (18). The protocol for the study conformed to accepted norms of animal care and experimental procedures. The testes were decapsulated, and lipid extracts were prepared from the entire tissue or from portions of it according to the procedure of Bligh and Dyer (19). The extracts were dried under a stream of N_2, and the neutral lipids were separated on silica gel G TLC plates by using hexane/diethyl ether/acetic acid (80:20:1, by vol) up to the middle of the plates, to separate the TG from the CE, followed by *n*-hexane/diethyl ether (95:5, vol/vol) up to the top, in order to ensure separation of CE from the solvent front. All lipid bands were located under UV light after spraying the plates with 2′,7′-dichlorofluorescein in methanol.

The VLCPUFA of TG and CE were identified using the criteria described in previous work (6,7,11). The FA composition of lipids was determined by GC of their FAME derivatives. These were prepared by placing the lipid samples with 14% BF_3 in methanol overnight at 45°C under N_2 in Teflon® sealed tubes. Before GC, the methyl esters were purified by TLC using hexane/ether (95:5, vol/vol) on silica gel G plates that had been prewashed with methanol/ether (75:25, vol/vol). The methyl esters were recovered from the silica after thorough mixing and partitioning between water/methanol/hexane (1:1:1, by vol; three hexane extractions). Under the present GC conditions, the minor n-6 pentaenes with odd-numbered chains tended to coelute with (not resolve from) the corresponding hexaenes of the n-3 series with even-numbered chains having one carbon less (minor in mice and negligible in rats) (e.g., 27:5n-6 with 26:6n-3, 29:5n-6 with 28:6n-3, and so on). Something similar occurred between the n-6 tetraenes with odd chains and the n-3 pentaenes with even chains having one carbon less (e.g., 21:4n-6 with 20:5n-3, and so on) (Tables 1 and 2). To estimate the sums of n-6 PUFA and n-3 PUFA given in Figure 3, the methyl esters were resolved according to unsaturation by argentation TLC. Silica gel G plates, precleaned with methanol/ether (75:5, vol/vol) and dried, were impregnated by spraying with aqueous $AgNO_3$ (2 g per 10 g of silica), dried, and activated. The previously purified PUFA fractions were spotted and resolved into fractions by means of chloroform/methanol (90:10, vol/vol). HPLC-grade solvents were used throughout. A Varian 3700 gas chromatograph equipped with two (2 m × 2 mm) glass columns packed with 10% SP 2330 on Chromosorb WAW 100/120 (Supelco, Inc., Bellefonte, PA) was used. The column oven temperature was programmed from 155 to 230°C at a rate of 5°C/min and then kept at the upper temperature for about 30 min to allow VLCPUFA to elute from the column. Injector and detector temperatures were set at 220 and 230°C, respectively, and N_2 (30 mL/min) was the carrier gas. The FA peaks were detected with FID, operated in the dual-differential mode, and quantified by electronic integration (Varian Workstation). Statistical analyses of the results were performed using the two-tailed Student's *t*-test.

RESULTS

FA of TG and CE of murine testis. The FA compositions of CE and TG from (adult, fertile) rat and mouse testis are compared in Table 1. Both species contained VLCPUFA in TG and CE, most of them belonging to the n-6 series. These were virtually the only VLCPUFA type in the rat, the mouse containing somewhat more components of the n-3 series than the rat. In both animals, the single lipid class most highly enriched in molecular species with VLCPUFA was CE. The sum of VLCPUFA accounted for as much as nearly 40 and 20% of the FA of rat and mouse CE, respectively. The mouse had a significantly larger proportion of VLCPUFA of n-3 series (22:6n-3 and longer) than the rat in this lipid. In both animals, the most abundant VLCPUFA of testicular CE was 28:5n-6 (Table 1, Fig. 1).

In total testicular TG (Table 1), docosapentaenoic (22:5n-6) and linoleic (18:2n-6) acids were the major PUFA, the former predominating in the rat and the latter in the mouse. The sum of VLCPUFA (24 to 32 carbons) represented between 5 and 20% of the FA of total TG. Most of this sum was accounted for by 24:4n-6 and 24:5n-6, with a series of longer tetraenes and pentaenes up to 32 carbons long being responsible for the rest.

The solvents described in Materials and Methods for the separation of the neutral lipids resolved the TG into two subfractions, the minor upper one accounting in rodents for about 10–20% of the FA of total testicular TG. This subfraction of TG did not release glycerol after treatment with lipoprotein lipase, as did the major band of TG, containing the TAG. On TLC, the minor band of TG had an R_f corresponding to a standard of 1-*O*-palmityl-2,3-distearoyl-glycerol. After mild alkaline treatment, it produced FAME and a product having the same TLC migration, in two different solvents, as standards of two well-known 1-alkyl-diols, batyl and chimyl alcohol. It did not release fatty aldehydes after treatment with HCl fumes. It was concluded that the small subfraction of TG was a TG with an *O*-alkyl bond, 1-*O*-alkyl-2,3-DAG. This subclass concentrated most of the VLCPUFA that is present in the testicular TG as a class. Figure 1 (upper panel) shows the FA profiles of this lipid from rat and mouse testis. Pentaenoic FA of the n-6 series, from 22:5 to 30:5, were its major constituents, followed by tetraenoic FA from 20:4n-6 to 28:4n-6. The major acyl

TABLE 1
FA Composition of Major Neutral Lipid Classes from Murine Testis[a]

	Total triglycerides (TG)		Cholesterol esters		
	Rat	Mouse (C)	Rat	Mouse (C)	Mouse (FO)
14:0	0.8 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	1.2 ± 0.1	2.0 ± 0.2
15:0	0.1 ± 0.03	0.2 ± 0.04	0.4 ± 0.1	—	—
16:0	23.9 ± 0.4	13.4 ± 0.9	8.2 ± 1.2	7.0 ± 1.0	5.8 ± 0.8
17:0	0.2 ± 0.03	0.2 ± 0.04	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.4
18:0	2.6 ± 0.5	2.9 ± 0.3	2.0 ± 0.6	3.4 ± 0.6	2.6 ± 0.1
14:1	0.5 ± 0.1	0.04 ± 0.02	—	0.3 ± 0.1	0.4 ± 0.4
15:1	0.02 ± 0.01	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.3 ± 0.3
16:1	1.6 ± 0.1	4.2 ± 0.1	1.8 ± 0.4	2.9 ± 0.3	2.9 ± 0.1
17:1	0.1 ± 0.01	0.2 ± 0.02	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.4
18:1	11.3 ± 0.8	25.3 ± 0.5	5.4 ± 0.3	16.8 ± 3.3	14.3 ± 2.4
20:1	0.6 ± 0.1	0.1 ± 0.02	0.5 ± 0.1	0.3 ± 0.02	0.3 ± 0.1
18:2n-6	3.9 ± 0.6	28.1 ± 1.9	4.4 ± 0.2	2.9 ± 0.5	1.6 ± 0.3*
18:3n-6	0.1 ± 0.1	1.6 ± 0.5	0.2 ± 0.1	0.3 ± 0.2	0.5 ± 0.4
18:3n-3	0.5 ± 0.04	0.7 ± 0.1	0.2 ± 0.04	3.1 ± 0.5	2.8 ± 0.2
18:4n-3	—	0.3 ± 0.1	—	1.0 ± 0.2	0.7 ± 0.3
20:2n-6	—	0.3 ± 0.1	—	0.6 ± 0.1	0.9 ± 0.1
20:3n-9	—	0.1 ± 0.01	0.2 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
20:3n-6	1.6 ± 0.2	0.3 ± 0.04	0.7 ± 0.1	2.0 ± 0.2	0.5 ± 0.2
20:4n-6	3.8 ± 0.2	0.9 ± 0.2	11.8 ± 0.4	4.2 ± 0.5	1.2 ± 0.1*
20:4n-3	—	0.2 ± 0.03	—	1.1 ± 0.2	1.1 ± 0.1
20:5n-3, 21:4n-6, 22:3n-6	0.5 ± 0.03	0.5 ± 0.04	1.1 ± 0.3	1.1 ± 0.1	2.1 ± 0.2
22:4n-9, 22:3n-6	0.2 ± 0.02	0.2 ± 0.03	0.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.3
22:4n-6	4.8 ± 0.3	2.1 ± 0.4	2.2 ± 0.1	7.3 ± 1.1	1.8 ± 0.1*
22:5n-6, 23:3n-6	28.4 ± 0.7	5.9 ± 0.7	15.8 ± 0.2	7.5 ± 0.2	5.1 ± 0.7
22:5n-3, 23:4n-6	0.4 ± 0.01	0.4 ± 0.1	0.4 ± 0.05	2.7 ± 0.6	6.0 ± 0.9
22:6n-3, 23:5n-6, 24:3n-6	0.7 ± 0.25	2.0 ± 0.6	2.1 ± 0.2	10.6 ± 1.5	15.1 ± 2.7*
24:4n-6	3.5 ± 0.4	1.4 ± 0.1	3.4 ± 0.1	1.1 ± 0.3	0.9 ± 0.3
24:5n-6	6.3 ± 0.4	4.4 ± 0.4	5.7 ± 0.2	3.4 ± 0.9	1.8 ± 0.2*
25:4n-6, 24:5n-3	—	0.1 ± 0.03	—	0.3 ± 0.1	0.4 ± 0.4
25:5n-6, 24:6n-3	0.1 ± 0.08	0.4 ± 0.03	0.1 ± 0.1	0.7 ± 0.2	1.1 ± 0.4
26:4n-6	0.5 ± 0.1	0.1 ± 0.02	0.7 ± 0.1	0.7 ± 0.05	0.3 ± 0.3
26:5n-6	0.9 ± 0.1	1.1 ± 0.1	5.4 ± 0.2	3.2 ± 1.0	2.1 ± 0.4
27:4n-6, 26:5n-3	0.2 ± 0.03	0.03 ± 0.01	0.4 ± 0.04	0.4 ± 0.1	0.9 ± 0.1
27:5n-6, 26:6n-3	0.1 ± 0.04	0.3 ± 0.1	1.7 ± 0.1	0.5 ± 0.1	1.4 ± 0.3
28:4n-6	0.2 ± 0.1	0.05 ± 0.02	0.2 ± 0.1	0.7 ± 0.3	0.5 ± 0.4
28:5n-6	1.2 ± 0.2	0.7 ± 0.03	17.9 ± 2.5	8.9 ± 2.4	7.2 ± 0.3
29:5n-6, 28:6n-3	0.1 ± 0.04	0.1 ± 0.02	0.7 ± 0.2	1.9 ± 0.7	2.4 ± 0.6
30:4n-6	—	—	0.04 ± 0.03	0.7 ± 0.2	0.3 ± 0.4
30:5n-6	0.4 ± 0.1	0.2 ± 0.04	3.5 ± 0.6	0.8 ± 0.5	0.9 ± 0.6
31:5n-6	0.02 ± 0.01	0.01 ± 0.002	0.6 ± 0.1	0.1 ± 0.02	0.4 ± 0.4
32:5n-6	—	—	0.2 ± 0.1	0.1 ± 0.1	—
33:5n-6	—	—	0.1 ± 0.04	—	—
LC-PUFA	44.7 ± 0.8	42.4 ± 1.5	39.0 ± 1.3	42.8 ± 3.2	37.7 ± 3.7
VLCPUFA	13.6 ± 1.3	9.5 ± 0.6	40.9 ± 2.5	24.6 ± 6.6	22.4 ± 4.6

[a]Results are given as percentages of the total FA in each lipid (mean values ± SD from at least four animals). In the case of mice, (C) refers to controls and (FO) to animals consuming fish oil. Abbreviations: LC-PUFA, sum of PUFA with 18–22 carbons; VLCPUFA, sum of PUFA with >22 carbons. The asterisks point to significant differences due to FO consumption. Except for small amounts of 20:5, 22:5, and 22:6n-3, PUFA of the n-3 series were undetectable in rats and detectable though minor components in mice.

chains had an even number of carbon atoms, but odd-chain PUFA were found in both series. Separation of the pentaenoic and tetraenoic FA from both CE and TG by argentation TLC, followed by catalytic hydrogenation, gave rise in both cases to a complete homologous series of saturated FA (20:0 to 28:0 and 22:0 to 32:0), including the odd-chain saturates.

Most of the CE and TG with VLCPUFA observed in this work were contributed by cells located within the seminiferous tubules, as ascertained in rats by comparing the FA profile of lipids from whole testis, from seminiferous tubules, and from the cells that are discarded after the preparation of seminiferous tubules.

In rats, the FA composition of CE and TG, with the typical abundance of 22:5n-6 as well as VLCPUFA, was maintained

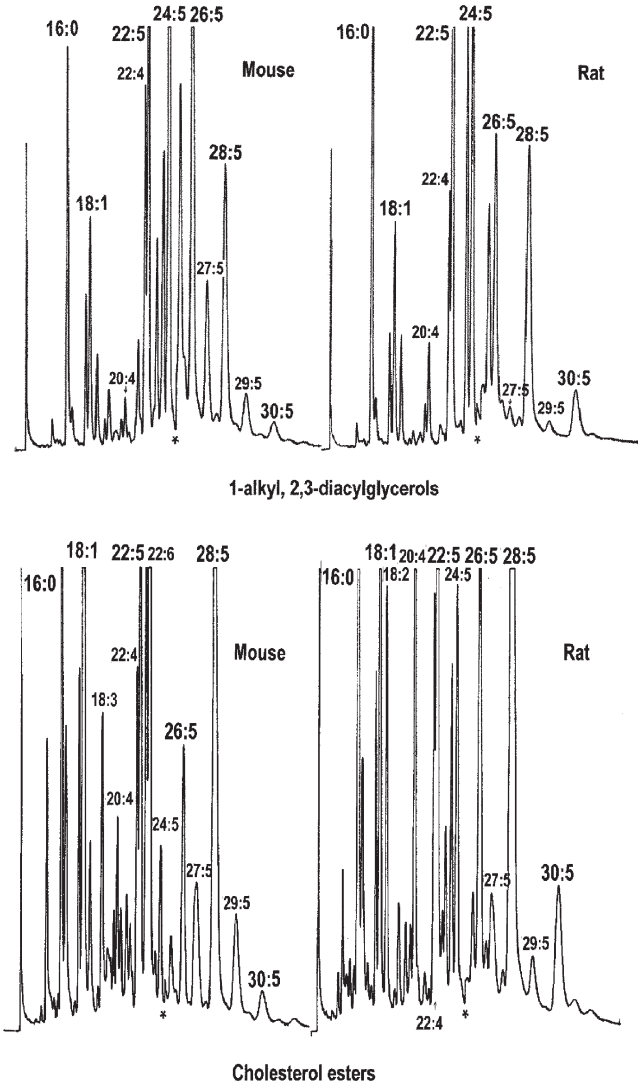

FIG. 1. FA from 1-*O*-alkyl-2,3-DAG (upper panel) and from cholesterol esters (lower panel) from mouse and rat testis. The FA, as methyl esters, were run on a set of conventional GC columns packed with a polar stationary phase as described in the Materials and Methods section. Total run time: 45 min. The asterisks denote a twofold increase in sensitivity.

with little or no change during aging, with no significant differences between young adults and rats aged 2.5 to 3 yr (against our expectations). Only in a group of rats that, incidentally, had been discarded due to reduced fertility (18 mon), were the VLCPUFA of these lipids significantly lower than in the normal fertile individuals. By contrast, all of the VLCPUFA shown in Tables 1 and 2 were absent altogether from the testicular CE and TG of 14-d-old rats. Moreover, both of these lipids had low levels of 22:5n-6 and other long-chain polyenes (data not shown) at that early age. Coincidentally, sexually immature rats and mice also lacked the 1-*O*-alkyl-2,3-DAG that carry most of the VLCPUFA associated with the TG class.

In the testis of other mammals, the proportion of VLCPUFA in CE and in total TG was smaller than in the two rodents of this study (Fig. 2). The total content of CE and TG per gram of testicular tissue (not shown) was lowest in bovine testis, intermediate in the two rodents studied here, and highest in cats and dogs. The epididymal tissue, examined in the same animals, contained vast amounts of TG, much more so at the caput than at the cauda regions, and negligible proportions of CE. Neither of these lipids contained VLCPUFA.

Changes induced by an n-3 PUFA-rich diet to the FA of mouse testicular lipids. After 2 wk of consuming a fish oil (FO)-rich diet, the major effect observed on the content of mouse testicular lipid classes was a significant decrease in TAG (6.6 ± 0.8 to 3.2 ± 0.2 mg/g of tissue), concomitant with a slight increase in the content of PL and CE, just as was the case with liver (18). The content of alkyl-DAG did not change significantly, as indicated by the amount of FA recovered from this lipid per gram of tissue. The FA modifications accompanying these lipid changes are shown in Tables 1 and 2, which disclose some differential effects of the n-3 diet on individual FA, and in Figure 3, which summarizes the effects on different groups of FA.

In the major membrane glycerophospholipids, PC and PE (Table 2), saturated and monounsaturated FA proportions remained practically unaltered, except for a slight increase of 18:1 in PC. In both lipids the proportion of the minor testicular n-3 PUFA increased with a concomitant reduction of the n-6 PUFA (changes in n-6/n-3 ratio: 5.8 ± 0.5 to 3.1 ± 0.1 for PC and 3.0 ± 0.2 to 1.7 ± 0.1 for PE, from controls to FO-fed mice, respectively). These changes were consistent with those seen previously in plasma and liver (18). However, in the testicular PL (Table 2), not all the n-6 PUFA were affected to the same extent. Within the n-6 PUFA group, the reduced FA were 18:2, 18:3, 20:2, 20:3, and 20:4n-6, whereas 24:4n-6,

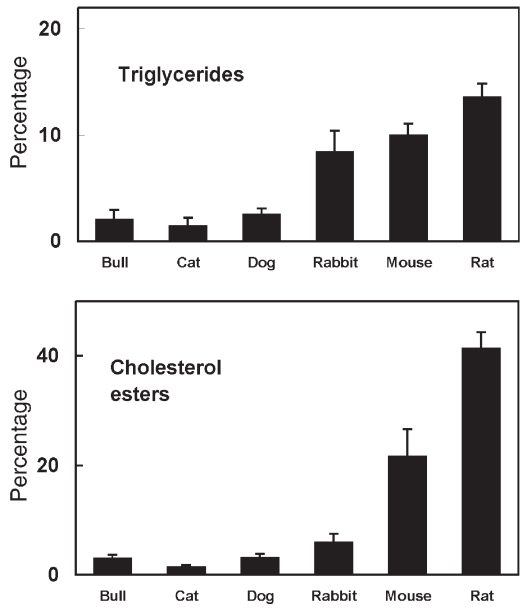

FIG. 2. Percentage of PUFA with very long chains in triglycerides and cholesterol esters in the testis from various mammals. The bars represent the sum of all FA with 24 to 32 carbon atoms. At least three animals per group were analyzed.

TABLE 2
FA of Testicular Glycerophospholipids and TG in Controls (C) and Fish Oil-Fed Mice[a] (FO)

	Choline glycerophospholipids		Ethanolamine glycerophospholipids		TAG		1-Alkyl-2,3-DAG	
	C	FO	C	FO	C	FO	C	FO
14:0	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.04	0.1 ± 0.03	0.8 ± 0.2	1.3 ± 0.6	0.8 ± 0.1	0.4 ± 0.2
15:0	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.02	0.2 ± 0.04	0.2 ± 0.1	0.7 ± 0.1	0.1 ± 0.1
16:0	29.7 ± 1.0	30.6 ± 0.8	21.5 ± 3.2	20.4 ± 1.8	17.3 ± 1.5	20.0 ± 1.7	7.2 ± 2.2	7.2 ± 0.5
17:0	0.2 ± 0.03	0.3 ± 0.04	0.3 ± 0.02	0.3 ± 0.03	0.4 ± 0.1	0.4 ± 0.1	0.1 ± 0.1	0.1 ± 0.04
18:0	9.3 ± 1.2	8.4 ± 0.2	8.3 ± 0.4	8.0 ± 0.5	1.9 ± 0.4	3.2 ± 0.6	1.6 ± 0.4	2.2 ± 0.2
14:1	0.1 ± 0.01	0.03 ± 0.02	—	—	0.2 ± 0.03	0.03 ± 0.03	0.5 ± 0.02	0.03 ± 0.02
15:1	0.1 ± 0.1	0.1 ± 0.02	0.1 ± 0.1	0.1	0.1 ± 0.04	0.1 ± 0.02	0.2	0.02 ± 0.02
16:1	0.8 ± 0.1	1.2 ± 0.1	0.6 ± 0.04	0.6 ± 0.1	7.8 ± 0.6	5.2 ± 0.4	1.2 ± 0.3	0.7 ± 0.1
17:1	0.1 ± 0.02	0.1 ± 0.01	0.2 ± 0.1	0.1 ± 0.03	0.6 ± 0.03	0.3 ± 0.3	0.02 ± 0.1	0.02 ± 0.02
18:1	14.2 ± 0.3	17.5 ± 0.5	9.8 ± 0.5	10.9 ± 0.7	32.6 ± 1.7	25.6 ± 3.5	4.5 ± 1.4	5.2 ± 0.5
20:1	—	—	—	—	0.1 ± 0.01	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.2
18:2n-6	3.0 ± 0.2	1.7 ± 0.1*	1.9 ± 0.5	1.0 ± 0.1*	27.6 ± 1.3	19.5 ± 2.9*	0.9 ± 1.2	0.7 ± 0.2
18:3n-6	0.2 ± 0.01	0.1 ± 0.01	0.3 ± 0.1	0.1 ± 0.02	1.3 ± 0.5	1.1 ± 0.3	0.6 ± 0.1	0.6 ± 0.1
18:3n-3	0.3 ± 0.02	0.4 ± 0.02	0.3 ± 0.3	0.3 ± 0.03	0.9 ± 0.3	1.3 ± 0.2	1.3 ± 0.1	1.5 ± 0.2
20:2n-6	0.5 ± 0.02	0.4 ± 0.01	0.2 ± 0.1	0.2 ± 0.02	0.3 ± 0.1	0.2 ± 0.02	0.5 ± 0.1	0.4 ± 0.2
20:3n-9	0.2 ± 0.1	0.5 ± 0.01	0.3 ± 0.2	0.4 ± 0.01	0.1 ± 0.01	0.2 ± 0.05	0.2 ± 0.03	0.1 ± 0.03
20:3n-6	2.3 ± 0.1	1.4 ± 0.1*	0.9 ± 0.2	0.7 ± 0.03	0.3 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	0.4 ± 0.1
20:4n-6	15.0 ± 0.7	10.3 ± 0.4*	19.0 ± 1.0	16.2 ± 0.5	0.7 ± 0.2	1.3 ± 0.3	2.0 ± 0.1	0.9 ± 0.2
20:4n-3	—	—	—	—	0.2 ± 0.03	0.5 ± 0.04	0.4 ± 0.1	0.5 ± 0.2
20:5n-3, 21:4n-6	0.4 ± 0.05	1.2 ± 0.04*	0.4 ± 0.1	1.7 ± 0.1*	0.3 ± 0.1	0.2 ± 0.1	2.5 ± 0.3	2.5 ± 0.1
22:4n-9	0.1 ± 0.02	0.1 ± 0.01	0.3 ± 0.2	0.3 ± 0.01	0.1 ± 0.02	0.1 ± 0.02	0.5 ± 0.2	0.5 ± 0.05
22:4n-6	0.9 ± 0.04	0.6 ± 0.02*	2.4 ± 0.1	1.8 ± 0.1	0.6 ± 0.3	1.1 ± 0.2	8.1 ± 1.0	5.9 ± 0.3
22:5n-6	13.8 ± 1.1	13.9 ± 0.7	14.5 ± 1.6	13.6 ± 0.7	3.3 ± 0.5	8.1 ± 1.3*	14.5 ± 0.3	12.6 ± 0.8
22:5n-3, 23:4n-6	0.4 ± 0.05	0.6 ± 0.03	0.6 ± 0.1	1.0 ± 0.05	0.2 ± 0.2	1.3 ± 0.3	0.3 ± 0.03	0.7 ± 0.04
22:6n-3	5.4 ± 0.3	7.6 ± 0.4*	12.9 ± 0.3	18.2 ± 1.6*	1.3 ± 0.3	4.5 ± 0.6*	5.0 ± 0.7	5.9 ± 1.0
24:4n-6	0.8 ± 0.1	0.7 ± 0.02	1.1 ± 0.1	0.9 ± 0.1	0.2 ± 0.2	0.5 ± 0.1	7.9 ± 0.2	7.0 ± 0.5
24:5n-6	1.4 ± 0.1	1.2 ± 0.1	2.2 ± 0.3	1.8 ± 0.2	0.7 ± 0.3	1.7 ± 0.4*	25.0 ± 0.5	25.2 ± 0.4
25:4n-6	—	—	—	—	0.1 ± 0.02	0.1 ± 0.04	—	—
24:6n-3, 25:5n-6	—	—	—	—	0.2 ± 0.1	0.6 ± 0.2*	1.9 ± 0.51	3.6 ± 0.3*
26:4n-6	—	—	—	—	0.1 ± 0.04	0.1 ± 0.04	0.5 ± 0.03	0.7 ± 0.1
26:5n-6	—	—	—	—	0.3 ± 0.2	0.3 ± 0.1	5.6 ± 0.2	5.8 ± 0.4*
27:4n-6	—	—	—	—	0.01 ± 0.01	0.02 ± 0.02	0.1 ± 0.03	0.1 ± 0.02
27:5n-6, 26:6n-3	—	—	—	—	0.1 ± 0.1	0.2 ± 0.1	1.1 ± 0.3	2.2 ± 0.05*
28:4n-6	—	—	—	—	0.01 ± 0.01	0.01 ± 0.01	0.1 ± 0.03	0.2 ± 0.1
28:5n-6	—	—	—	—	0.2 ± 0.1	0.20 ± 0.05	3.5 ± 0.5	3.9 ± 0.6
29:4n-6	—	—	—	—	0.01 ± 0.01	0.01 ± 0.01	0.1 ± 0.01	0.05 ± 0.02
29:5n-6, 28:6n-3	—	—	—	—	0.03 ± 0.02	0.1 ± 0.1	0.5 ± 0.1	0.7 ± 0.2
30:4n-6	—	—	—	—	—	—	0.1 ± 0.02	0.03 ± 0.01
30:5n-6	—	—	—	—	0.2 ± 0.03	0.1 ± 0.01	0.4 ± 0.1	0.4 ± 0.02
31:4n-6	—	—	—	—	—	—	0.04 ± 0.05	0.03 ± 0.01
31:5n-6	—	—	—	—	—	0.03 ± 0.02	0.1 ± 0.04	0.04 ± 0.01
LC-PUFA	42.3 ± 0.6	38.9 ± 1.0	53.9 ± 1.0	55.6 ± 2.9	37.2 ± 0.6	39.7 ± 0.3*	37.2 ± 2.5	33.3 ± 1.4
VLCPUFA	2.2 ± 0.1	1.8 ± 0.1	3.3 ± 0.4	2.7 ± 0.2	2.1 ± 0.9	3.9 ± 1.1	46.9 ± 1.4	50.0 ± 1.5*

[a]Results are presented as in Table 1.

24:5n-6, and the major 22:5n-6 were not significantly altered by the dietary modification.

The FA profiles of testicular TG and CE (Tables 1 and 2), in general, were affected by 2 wk of FO intake in the same way as the glycerophospholipids. In the presence of unchanged proportions of saturates and monoenes, except for a small decrease of 18:1 in TG, the n-6/n-3 ratio decreased in both lipids.

In CE, among the FA that were increased by the diet were 20:5n-3, 22:5n-3, 22:6n-3, and some n-3 VLCPUFA; and among those reduced were 18:2n-6, 20:3n-6, 20:4n-6, and 22:4n-6 (Table 1). Interestingly, in CE, as in the other lipids, the proportion of the major PUFA, 22:5n-6, was the less affected. The sum of VLCPUFA remained high and quantitatively unaffected by the FO diet, the total VLCPUFA accounting for about 20% of the CE FA in both dietary conditions (Fig. 3).

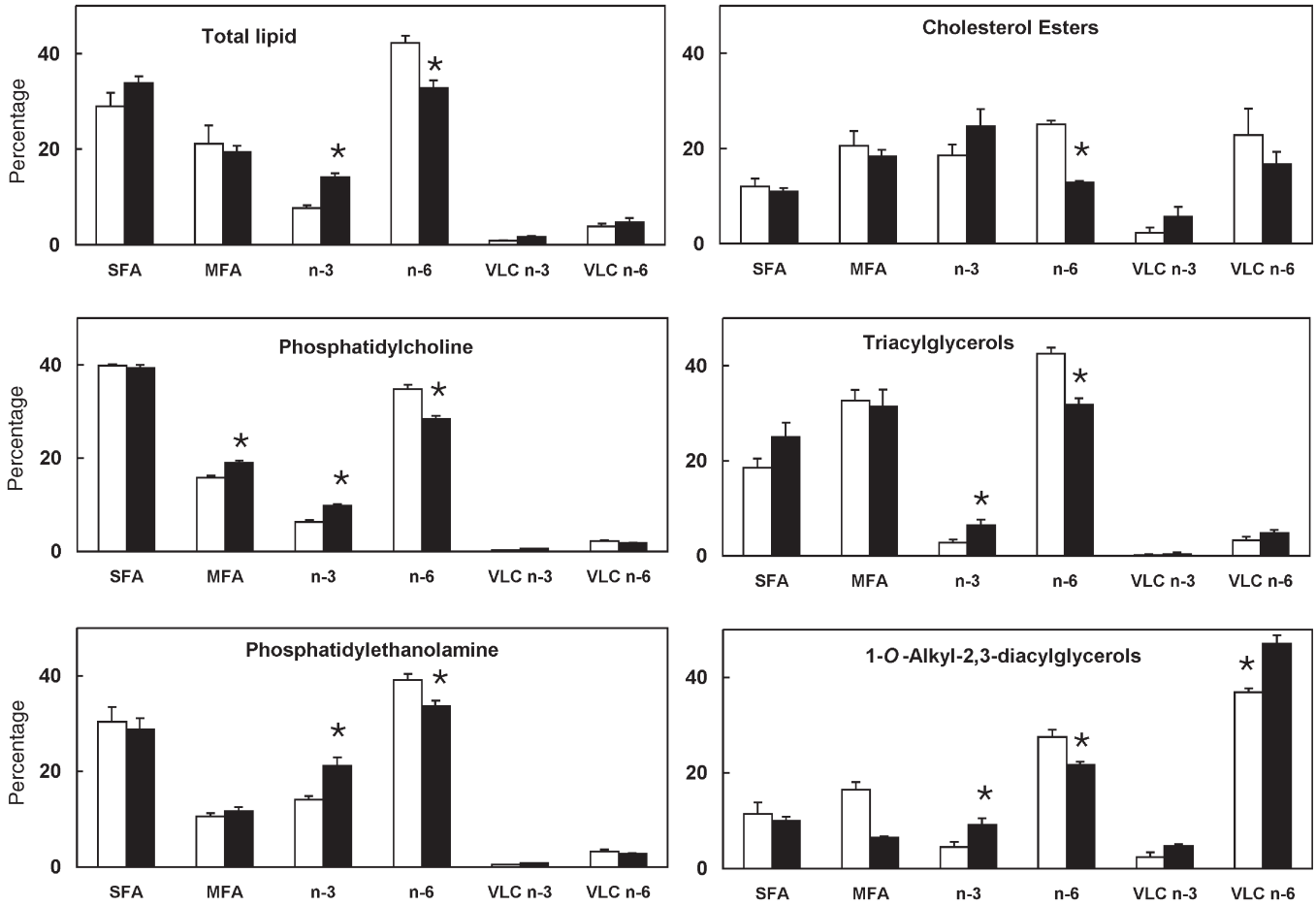

FIG. 3. Changes in the percentages of different groups of FA in mouse testicular lipids after 2 wk of consumption of an n-3 FA-rich diet. Open bars: controls, i.e., animals eating lab chow; solid bars: animals consuming 9% fish oil. The asterisks denote significant differences between both groups. The bars represent sums of FA as follows: SFA, saturated FA; MFA, monounsaturated FA; n-3 and n-6, PUFA with 18 to 22 carbon atoms; VLC n-3 and VLC n-6: PUFA with 24 to 32 carbon atoms. The sums of n-3 or n-6 PUFA and VLCPUFA were estimated after separating the FA by argentation TLC.

In focusing on the n-6 PUFA of TG, and especially considering that the amounts of testicular TAG were depleted after 2 wk on the FO diet, it was apparent that the 18:2n-6 available in this lipid was exhausted to a much larger extent than the longer and more highly unsaturated constituents (22:5n-6 and longer n-6 PUFA), whose proportions remained unchanged or even tended to increase in the abundance of n-3 dietary PUFA (Table 2). The depletion of 18:2 in TG was thus mostly responsible for the significant reduction in total n-6 PUFA seen in this lipid (Fig. 3). The 1-*O*-alkyl-2,3-DAG were the lipids whose FA composition changed less with the introduction of n-3 PUFA in the diet (Table 2). Instead of increasing the n-3 VLCPUFA, the diet resulted in a significant increase of total n-6 VLCPUFA in this lipid (Fig. 3).

DISCUSSION

The active and continuous formation and remodeling of cells that go along with spermatogenesis within the seminiferous tubule requires PL and free cholesterol for their membranes. The TG could play a role as intermediaries in which to temporarily store the polyenoic FA necessary as precursors (e.g., 18:2n-6 to be elongated and desaturated, 24:5n-6 to be chain-shortened) to give rise to PUFA with the "correct" length and unsaturation destined to be membrane PL (like 22:5n-6). The CE, after the action of CE hydrolases, could provide, besides PUFA, the necessary free cholesterol. This function, however, could be fulfilled by CE molecular species with much simpler acyl chains, not answering the question of why CE have PUFA with both high unsaturation and exceedingly long chains, such as 28:5n-6.

There is metabolic support to the interpretation that one possible role of the TG molecular species with PUFA and VLCPUFA could be to store preformed acyl chains, potential precursors of the major C_{22} PUFA that eventually constitute the membrane PL of tissues like testis. Thus, rat seminiferous tubules concentrate the highest levels of [^{14}C]-labeled PUFA into TG, whether exogenously provided (e.g., [^{14}C]24:4n-6) or endogenously produced from its desaturation ([^{14}C]24:5n-6) and elongation ([^{14}C]26:5n-6), suggesting that, of all seminiferous tubule lipid classes, it is TG that have a special role to play in the traffic and metabolism of testicular PUFA (6).

Something similar occurs in cell preparations from young rats testis, where most of the n-6 [^{14}C]22:4, 24:4, 24:5, and 22:5 formed after 24 h incubation with [^{14}C]20:4n-6 is concentrated in TG (24). Earlier work by Beckman and Coniglio (22) showed that when labeled 18:2n-6 and 20:4n-6 are injected *in vivo* in testis, they are taken up and metabolized primarily by Sertoli cells, PUFA like 22:5n-6 being synthesized and actively incorporated in TG, suggesting to these authors that TG may serve as a vehicle for transport of PUFA from Sertoli cells to germ cells, where they are incorporated into membrane PL.

The effects of the n-3-rich diet on testicular lipid FA support the interpretation that the acyl chains of the testicular neutral lipids may be used to sustain the PL with a minimum of change in their FA. Looking just as the components within the n-6 PUFA fraction of testicular TG as a class, it was evident that, whereas 18:2n-6 was considerably depleted, n-6 FA components with increasingly longer chains were much less affected by the diet. These TG may have acted not only as acceptors of PUFA (exogenously added, as in this case 22:6n-3, and endogenously synthesized, like 22:5n-6 from 18:2n-6) but also as donors of such acyl groups to the cellular PL, as suggested by the decreased TG concentrations. Moreover, the n-6 PUFA of TG not only decreased but also changed their quality, the synthesis of the longer n-6 PUFA of TG apparently being maintained at the expense of the shorter ones. Concurrently, the major testicular PL conserved the typical predominance of 22:5n-6, despite the drastic reduction in the availability of n-6 PUFA (18) caused by the dietary modification.

Although the structure, metabolism, and regulation of ether-linked neutral glycerolipids have been known for years (25), their cellular functions are still poorly understood. In rats and mice, 1-*O*-alkyl-2,3-DAG have been characterized as components of the lipid secreted by the Harderian gland of the eye (26) and of the lipid of skin (27). Alkyldiacylglycerols produced by acyl-CoA:alkylglycerol acyltransferase are characteristic markers in most tumors from animals and humans (25). They have also been described in the plasma membrane of goat epididymal sperm (28), but we have been unable to find previous references to the occurrence of these neutral lipids and their FA in testis. The present results show that testicular TAG differ from 1-alkyl-2,3-DAG not only in FA composition but also in the way they respond to a relative deficiency of n-6 FA in the diet. Whereas TAG were depleted of their PUFA moieties, 1-alkyl-2,3-diacyl-*sn*-glycerols retained their 22:5n-6 and longer n-6 PUFA, even increasing the latter. The ether-linked group may protect the acyl moieties at the 2- and 3-positions of the glycerol backbone from being hydrolyzed by the lipases that attack the TAG. The 1-*O*-alkyl, 2,3-DAG could function as a repository, temporarily storing the VLCPUFA that are potential precursors of 22:5n-6 (e.g., 24:5n-6).

Concerning CE, as is the case with the ether-linked neutral lipids, much more is known about the enzymes involved in their metabolism than about the CE themselves and their functions in most tissues, especially in relation to their FA. In rat testis, CE synthesis and hydrolysis are decreased as a result of hypophysectomy, the hydrolysis being reduced to a greater extent than the synthesis (29). There are two forms of testicular cytosolic CE hydrolase: The activity of one is inhibited with the elevation of testicular temperature above 37°C, and that of the other shows no temperature sensitivity (30,31). The temperature-stable form is present in Sertoli and Leydig cells and is induced by luteinizing hormone (LH), whereas the temperature-labile form is exclusive to Sertoli cells and is induced by follicle-stimulating hormone but not by LH. These enzymes have different M.W. and substrate specificities, with the temperature-stable form being more active toward CE with 18:1 and 20:4n-6, and the temperature-labile Sertoli cell form being more active toward CE with 18–24 carbons, especially with 24:1. The latter is not a component of testicular CE, but some of its properties probably resemble those of one the VLCPUFA described here (e.g., 28:5n-6). The Sertoli cell-specific, temperature-labile form has been purified and has been shown to be induced concomitantly with testicular maturation, in marked coincidence with the onset of testosterone synthesis and the beginning of spermatogenesis (32,33). A possible correlation between this enzyme and the CE with VLCPUFA described here is worth investigating, since these unusual lipids occur in cells located within the seminiferous tubules of fertile adult animals.

The lack of aging-related changes in the FA composition of testicular lipids in rats under a constant diet and the conservative changes shown by mouse testicular PL FA after consumption of fish oil suggest that one of the possible roles of the neutral lipids with VLCPUFA could be to protect testicular cells from eventual deficiencies, excesses, or inadequacies in the specific type of FA that is required for function, thus contributing to support spermatogenesis and to defend the genetic material.

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New Cerebrosides from the Basidiomycete *Cortinarius tenuipes*

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ABSTRACT: Five cerebrosides (**1–5**), including three new ones named cortenuamide A (**1**), cortenuamide B (**2**), and cortenuamide C (**3**), were isolated from the fruiting bodies of the basidiomycete *Cortinarius tenuipes*. The structures of those compounds were elucidated as (4*E*,8*E*)-*N*-D-2′-hydroxytetracosanoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine (**1**), (4*E*, 8*E*)-*N*-D-2′-hydroxytricosanoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8 sphingadienine (**2**), (4*E*, 8*E*)-*N*-D-2′-hydroxydocosanoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine (**3**), (4*E*, 8*E*)-*N*-D-2′-hydroxyoctadecanoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine (**4**), and (4*E*,8*E*)-*N*-D-2′-hydroxypalmitoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine (**5**) by spectral and chemical methods.

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Cortinarius (Cortinariaceae) is one of the largest genera in the subdivision Basidiomycotina in kingdom of fungi, comprising hundreds of species and widely distribute in the world (1). From the fruiting bodies of *Cortinarius* spp., a large number of toxins and/or pigments, including cyclic polypeptides (2), bipyridyl compounds (3), several types of anthraquinone derivatives, and chromogenic triterpenoids (4,5), have been isolated and characterized. Most investigations into the chemical constituents of *Cortinarius* have focused on toadstools in Europe and Australia. In continuing our studies on basidiomycete-derived bioactive secondary metabolites, we investigated chemical constituents of the mushroom *C. tenuipes*. This report describes the structural elucidation of five cerebrosides, including three new ones: (4*E*,8*E*)-*N*-D-2′-hydroxytetracosanoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine (**1**), (4*E*,8*E*)-*N*-D-2′-hydroxytricosanoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine (**2**) and (4*E*, 8*E*)-*N*-D-2′-hydroxydocosanoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine (**3**).

EXPERIMENTAL PROCEDURES

Instrumentation. Melting points were obtained on an XRC-1 apparatus (Sichuan, People's Republic of China) and are uncorrected. Optical rotations were measured on a Horiba Sepa-300 polarimeter (Horiba, Tokyo, Japan). ^{1}H, and ^{13}C NMR and 2-D NMR spectra were recorded on Bruker AM-400 and DRX-500 spectrometers (Karlsruhe, Germany), with chemical shifts (δ) in ppm relative to trimethylsilane (TMS) as internal standard and coupling constants in hertz (Hz). MS spectra were measured with a VG Autospec3000 mass spectrometer (VG, Manchester, England). IR spectra were obtained on a Bio-Rad FTS-135 IR spectrophotometer (Bio-Rad, Richmond, CA) with KBr pellets. GC–MS was performed on a Finnigan 4510 GC–MS spectrometer (San Jose, CA) employing the EI mode (ionizing potential 70 eV) and a capillary column (30 m × 0.25 mm) packed with 5% phenyl and 95% methylsilicone on 5% phenyl–dimethylsilicone (HP-5) (Hewlett-Packard, Palo Alto, CA). Helium was used as carrier gas, and the column temperature was increased from 160 to 240°C at a rate of 5°C/min.

Materials. Column chromatography was carried out on silica gel (200–300 mesh). TLC was carried out on plates precoated with silica gel F_{254} (Qingdao Marine Chemical Ltd., Qingdao, People's Republic of China). Reversed-phase chromatography was with RP-8 (LiChroprep, 40–63 μm; Merck, Darmstadt, Germany).

Fungal material. Fresh fruiting bodies of *C. tenuipes* were collected at Tenchong, Yunnan Province, People's Republic of China, in July 1999 and identified by Prof. P.G. Liu, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, People's Republic of China. A voucher specimen is deposited at the Herbarium of Kunming Institute of Botany, the Chinese Academy of Sciences, People's Republic of China.

Extraction and isolation. Fresh fruiting bodies of *C. tenuipes* (340 g) were extracted with 95% ethanol (5 L × 3), followed by extraction with chloroform/methanol (1:1, vol/vol) at 20°C. The combined extracts were concentrated *in vacuo* to give a crude extract, which was partitioned between water and chloroform to provide a chloroform-soluble extract (6.5 g) and a water-soluble fraction. The chloroform-soluble fraction was subjected to column chromatography by eluting with a solvent mixture of chloroform/methanol from 100:0 (vol/vol) to 70:30 (vol/vol) to give several fractions. The fraction eluted by chloroform/methanol (82:18, vol/vol) was concentrated to a small volume, and then a residue (0.32 g) was precipitated from the solution (one spot in normal TLC test). The components of the mixture were purified by reversed-phase chromatography (RP-8) by eluting with methanol/water from 50:50 (vol/vol) to 100% (vol/vol). Five cerebrosides—**1** (18.2 mg), **2** (5.5 mg), **3** (6.4 mg), **4** (13 mg), and **5** (32 mg)—were obtained as white powders.

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Abbreviations: DEPT, distortionless enhancement by polarization transfer; HR, high-resolution; LCB, long-chain base; TMS, trimethylsilane.

Acidic methanolysis. Compound **1** (12.1 mg) was refluxed with 1.5 mL of 0.9 M HCl in 82% (vol/vol) aqueous methanol at 80°C for 16 h. The reaction mixture was extracted with petroleum ether, and the petroleum ether layer was concentrated and chromatographed on TLC using silica gel (petroleum/ethyl acetate 9:1–7:3, vol/vol) to yield a FAME (**1b**) as a white solid, which was subjected to GC–MS. The result showed that **1b** was a methyl 2-hydroxytetracosanoate, which displayed major ion peaks at *m/z* 398 $[M]^+$, and 339 $[M - 59]^+$; its retention time was 35 min. $[\alpha]_D = -4.5°$ (c = 0.83, $CHCl_3$); EI–MS (70 eV) *m/z* 398 $[M]^+$; ^1H NMR (400 MHz, $CDCl_3$, in ppm) δ 4.19 (1H, *dd*, J = 4.2, 7.4 Hz, H-2), 3.79 (3H, *s*, OCH_3), 2.74 (1H, *bs*, OH), 1.76 (1H, *m*), 1.63 (1H, *m*), 1.10–1.25 (40H, *m*), and 0.88 (3H, *t*, *J* = 7.0 Hz, CH_3).

*Cortenuamide A (**1**).* High-resolution (HR) FABMS *m/z* 838.6772 $[(M - 1)^-$, calcd. for $C_{49}H_{92}NO_9$ 838.6775]; IR (KBr) ν 3441 (OH), 2925, 2857, 2840, 1646, 1536, 1465, 1079, 1036, 962, 720 cm^{-1}; EI–MS (70 eV) *m/z* (relative intensity, %) 822 $[M - OH]^+$ (1), 660 $[M - Glu - OH]^+$ (3), 481 (4), 427 (8), 410 (20), 385 (11), 321 (7), 292 (4), 276 (8), 262 (7), 222 (12), 180 (4); ^{13}C NMR (CD_3OD), see Table 1.

*Cortenuamide B (**2**).* HR FABMS *m/z* 824.6619 $[(M - 1)^-$, calcd. for $C_{48}H_{90}NO_9$ 824.6612]; IR (KBr) ν 3439 (OH), 2924, 2859, 2844, 1645, 1537, 1468, 1080, 1033, 960, 723 cm^{-1}; EI–MS (70 eV) *m/z* (relative intensity, %) 808 $[M - OH]^+$ (1), 646 $[M - Glu - OH]^+$ (3), 466 (3), 412 (6), 395 (17), 370 (11), 292 (6), 276 (8), 262 (6), 222 (13), 180 (7); ^1H NMR (500 MHz, CD_3OD): δ 5.75 (1H, *dt*, *J* = 15.4, 5.8 Hz, H-5), 5.51 (1H, *dd*, J = 15.4, 6.8 Hz, H-4), 5.11 (1H, *bt*, *J* = 6.8 Hz, H-8), 4.28 (1H, *d*, *J* = 7.8 Hz, H-1″), 4.14 (1H, *t*, *J* = 6.8 Hz, H-3), 4.11 (1H, *dd*, *J* = 10.4, 5.6 Hz, H-1a), 4.00 (1H, *m*, H-2′), 3.75 (1H, *dd*, *J* = 10.4, 3.6 Hz, H-1b), 3.34 (2H, *m*, H-6″), 3.30–3.27 (5H, *m*, H-2″, 3″, 4″, 5″), 2.10 (4H, *m*, H_2-10, H_2-7), 1.98 (2H, *m*, H_2-6), 1.61 (3H, *s*, H_3-19), 1.33 (2H, *m*, H-3′), 1.31–1.41 (aliphatic–CH_2–), 0.92 (6H, *t*, *J* = 7.0 Hz, Me-18, Me-24′). ^{13}C NMR (CD_3OD), see Table 1.

TABLE 1
^{13}C NMR (CD_3OD) Data of Cerebrosides 1–5[a]

C (DEPT)[b]	δ_C (**1**)	δ_C (**2**)	δ_C (**3**)	δ_C (**4**)	δ_C (**5**)
1(CH_2)	69.8	69.8	69.7	69.8	69.7
2(CH)	54.7	54.6	54.6	54.7	54.6
3(CH)	73.0	72.9	72.9	72.9	72.9
4(CH)	134.6	134.7	134.6	134.6	134.6
5(CH)	131.1	131.1	131.1	131.1	131.1
6(CH_2)	33.1	33.1	33.1	33.1	33.0
7(CH_2)	33.8	33.8	33.8	33.8	33.8
8(CH)	124.8	124.8	124.8	124.8	124.8
9(CH)	136.8	136.8	136.8	136.8	136.8
10(CH_2)	40.8	40.8	40.8	40.8	40.7
18(CH_3)	14.4	14.5	14.5	14.4	14.4
19(CH_3)	16.2	16.2	16.2	16.2	16.2
1′(C)	177.2	177.2	177.2	177.2	177.1
2′(CH)	73.2	73.1	73.1	73.2	73.1
3′(CH)	35.9	35.9	35.9	35.9	35.9
16′(CH_3)	—	—	—	—	14.4
18′(CH_3)	—	—	—	14.4	—
22′(CH_3)	—	—	14.5	—	—
23′(CH_3)	—	14.5	—	—	—
24′(CH_3)	14.4	—	—	—	—
1″(CH)	104.8	104.7	104.7	104.7	104.6
2″(CH)	75.0	75.0	75.0	75.0	75.0
3″(CH)	78.0	78.0	78.0	78.0	77.9
4″(CH)	71.7	71.6	71.6	71.6	71.6
5″(CH)	78.0	78.0	78.0	78.0	77.9
6″(CH_2)	62.8	62.7	62.7	62.7	62.7
Aliphatic –CH_2–	33.8	33.8	33.8	33.8	33.8
	33.1	33.1	33.1	33.1	33.0
	30.7	30.8	30.8	30.8	30.8
	30.4	30.5	30.5	30.5	30.4
	29.1	29.1	29.1	29.1	29.1
	28.7	28.7	28.7	28.7	28.7
	26.1	26.2	26.2	26.1	26.1
	23.7	23.8	23.7	23.7	23.7

[a]Compound **1**, (4*E*,8*E*)-*N*-D-2′-hydroxytetracosanoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine; **2**, (4*E*,8*E*)-*N*-D-2′-hydroxytricosanoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine; **3**, (4*E*,8*E*)-*N*-D-2′-hydroxydocosanoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine; **4**, (4*E*,8*E*)-*N*-D-2′-hydroxyoctadecanoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine; **5**, (4*E*,8*E*)-*N*-D-2′-hydroxypalmitoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine.
[b]DEPT, distortionless enhancement by polarization transfer.

*Cortenuamide C (**3**).* HR FABMS *m/z* 810.6459 [(M – 1)$^-$, calcd. for $C_{47}H_{88}NO_9$ 810.6449]; IR (KBr) ν 3444 (OH), 2927, 2863, 2843, 1647, 1537, 1466, 1081, 1035, 962, 721 cm^{-1}; EI–MS (70 eV) *m/z* (relative intensity, %) 794 [M – OH]$^+$ (1), 632 [M – Glu – OH]$^+$ (3), 452 (3), 398 (20), 381 (15), 356 (24), 292 (6), 276 (8), 262 (6), 222 (13), 180 (7); ^{1}H NMR (500 MHz, CD_3OD): δ 5.76 (1H, *dt*, *J* = 15.4, 5.8 Hz, H-5), 5.52 (1H, *dd*, *J* = 15.6, 6.6 Hz, H-4), 5.16 (1H, *bt*, *J* = 7.0 Hz, H-8), 4.29 (1H, *d*, *J* = 8.0 Hz, H-1″), 4.15 (1H, *t*, *J* = 7.0 Hz, H-3), 4.14 (1H, *dd*, *J* = 10.2, 5.6 Hz, H-1a), 4.02 (1H, *m*, H-2′), 3.77 (1H, *dd*, *J* = 10.2, 3.6 Hz, H-1b), 3.36 (2H, *m*, H-6″), 3.34–3.33 (5H, *m*, H-2″, 3″, 4″, 5″), 2.12 (4H, *m*, H_2-10, H_2-7), 2.02 (2H, *m*, H_2-6), 1.63 (3H, *s*, H_3-19), 1.35 (2H, *m*, H-3′), 1.32–1.42 (aliphatic–CH_2–), 0.94 (6H, *t*, *J* = 6.4 Hz, Me-18, Me-24′). ^{13}C NMR (CD_3OD), see Table 1.

*(4**E**,8**E**)-N-D-2′-Hydroxyoctadecanoyl-1-O-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine (**4**).* FABMS (negative) *m/z* 755 [M]$^+$; EI–MS (70 eV) *m/z* (relative intensity, %) 755 [M]$^+$ (1), 593 [M – Glu – OH]$^+$ (2), 397 (11), 356 (9), 292 (8), 276 (5), 262 (6), 222 (10), 180 (12); ^{1}H NMR (400 MHz, CD_3OD): δ 5.72 (1H, *dt*, *J* = 15.2, 5.4 Hz, H-5), 5.48 (1H, *dd*, *J* = 15.2, 7.0 Hz, H-4), 5.07 (1H, *bt*, *J* = 7.0 Hz, H-8), 4.26 (1H, *d*, *J* = 7.6 Hz, H-1″), 4.10 (1H, *t*, *J* = 7.0 Hz, H-3), 4.06 (1H, *dd*, *J* = 10.2, 5.4 Hz, H-1a), 3.98 (1H, *m*, H-2′), 3.72 (1H, *dd*, *J* = 10.2, 3.6 Hz, H-1b), 3.31 (2H, *m*, H-6″), 3.17–3.25 (5H, *m*, H-2″, 3″, 4″, 5″), 2.06 (4H, *m*, H_2-10, H_2-7), 1.95 (2H, *m*, H_2-6), 1.55 (3H, *s*, H_3-19), 1.31 (2H, *m*, H-3′), 1.28–1.40 (aliphatic –CH_2–), 0.89 (6H, *t*, *J* = 6.6 Hz, Me-18, Me-24′). ^{13}C NMR (CD_3OD) see Table 1.

*(4**E**, 8**E**)-N-D-2′-Hydroxypalmitoyl-1-O-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine (**5**).* FABMS (negative) *m/z* 727 [M]$^+$; EI–MS (70 eV) *m/z* (relative intensity, %) 727 [M]$^+$ (1), 709 [M – OH]$^+$ (3), 548 [M – Glu – OH]$^+$ (5), 508 (10), 397 (20), 372 (18), 262 (12), 222 (23), 180 (5); ^{1}H NMR (400 MHz, CD_3OD): δ 5.74 (1H, *dt*, *J* = 15.4, 5.4 Hz, H-5), 5.51 (1H, *dd*, *J* = 15.4, 6.8 Hz, H-4), 5.09 (1H, *bt*, *J* = 7.0 Hz, H-8), 4.29 (1H, *d*, *J* = 7.8 Hz, H-1″), 4.12 (1H, *t*, *J* = 6.8 Hz, H-3), 4.07 (1H, *dd*, *J* = 10.2, 5.6 Hz, H-1a), 4.01 (1H, *m*, H-2′), 3.73 (1H, *dd*, *J* = 10.2, 3.6 Hz, H-1b), 3.33 (2H, *m*, H-6″), 3.19–3.25 (5H, *m*, H-2″, 3″, 4″, 5″), 2.08 (4H, *m*, H_2-10, H_2-7), 1.98 (2H, *m*, H_2-6), 1.57 (3H, *s*, H_3-19), 1.35 (2H, *m*, H-3′), 1.31–1.43 (aliphatic –CH_2–), 0.93 (6H, *t*, *J* = 6.4 Hz, Me-18, Me-24′). ^{13}C NMR (CD_3OD), see Table 1.

2-Amino-1,3-dihydroxy-9-methyl-4,8-octadecanediene (**1c**). HR FAB. MS (positive) *m/z*: 438.3219 [(M + H), calcd. for $C_{47}H_{88}NO_9$ 438.3235]; EI–MS *m/z*: 438 [M + 1]$^+$ (5), 378 [M + 1 – HOAc]$^+$ (45), 318 [M + 1 – 2×HOAc]$^+$ (33), 257 [M – 3×HOAc]$^+$ (3). ^{1}H NMR (400M Hz, $CDCl_3$, in ppm): δ 5.38 (1H, *dt*, *J* = 15.4, 6.6 Hz, H-5), 5.45 (1H, *dd*, J = 15.4, 6.8 Hz, H-4), 4.25 (1H, *t*, *J* = 6.8 Hz, H-3), 4.07 (1H, *m*, H-2), 2.16 (3H, *s*, $COCH_3$), 2.08 (3H, *s*, $COCH_3$), 2.01 (3H, *s*, $COCH_3$), 1.98 (3H, *s*, Me-19), 1.85–1.97 (6H, *m*, CH_2-6, CH_2-7, CH_2-10), 1.22 (aliphatic –CH_2–), and 0.96 (3H, *t*, *J* = 7.0 Hz, Me-18).

GC–MS data of **1b**–**4b**. **1b**: (48.25 min), EI–MS *m/z*: 398 (100), 366 (5), 339 (85), 320 (17); **2b**: (40.89 min), EI–MS *m/z*: 384 (65), 352 (4), 325 (52), 306 (12); **3b**: (37.12 min), EI–MS *m/z*: 370 (75), 338 (3), 311 (62), 292 (18); **4b**: (24.14 min), EI–MS *m/z*: 314 (80), 282 (7), 255 (71), 236 (15).

RESULTS AND DISCUSSION

Cortenuamide A (**1**), $C_{49}H_{93}O_9N$ (HR FABMS showed [M – 1]$^-$ *m/z* 838.6772, calcd. for $C_{49}H_{92}NO_9$ 838.6775) was obtained as a white powder. Its IR spectrum exhibited strong hydroxyl absorption bands at 3440 cm^{-1} and bands at 1646 and 1537 cm^{-1} due to the amide group. EI–MS showed characteristic fragments ions at *m/z* 660 [M – Glu – OH]$^+$, 367 [$C_{24}H_{47}O_2$]$^+$, 339 [$C_{24}H_{47}O_2$ – CO]$^+$, and 293 [M – Glu – OH – $C_{24}H_{47}O_2$]$^+$. In its ^{13}C NMR spectrum, derived through distortionless enhancement by polarization transfer (DEPT), a D-glucopyranose moiety was indicated by the signals at δ 104.8 (CH), 75.0 (CH), 78.0 (CH), 71.7 (CH), 78.0 (CH), and 62.8 (CH_2). The signal at δ 177.2 was assigned to an amide carbon, and the signals at δ 136.8 (C), 134.6 (CH), 131.1 (CH), and 124.8 (CH) showed the presence of two double bond groups, which were also revealed by the signals at δ 5.78 (1H, *m*), 5.52 (1H, *dd*, *J* = 15.5, 7.0 Hz), and 5.18 (1H, *m*) in its ^{1}H NMR spectrum. The signals at δ_C 16.2 (CH_3), δ_H 1.62 (3H, *s*) were contributed by the methyl group vicinal to the quaternary double bond carbon, and the signals at δ_C 14.4 ($CH_3 \times 2$), δ_H 0.92 (6H, *br*, *J* = 6.8 Hz) were assigned to the two normal long-chain terminal methyl groups. Those data and literature precedents (6–8) led us to establish the structure of compound **1** as (4*E*, 8*E*)-*N*-D-2′-hydroxytetracosanoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine. This conclusion could be further confirmed as follows. The coupling constant between C-4 and C-5 olefenic protons ($J_{4,5}$ = 15.5 Hz), and the chemical shift of the methyl carbon at δ_C 14.3 ppm (CH_3-19) showed that the double bonds at positions 4 and 8 were all *E* geometry (7). The β-D-glucopyranoside linkage was assigned based on the coupling constant of the anomeric proton (4.30, *J* = 7.8 Hz, H-1″) (7). Assembly of NMR data revealed that the absolute configuration of **1** should be the same as those of literature precedents (6), and the length of the two side chains was determined by means of acidic methanolysis (9) in which **1** gave a characteristic long-chain FAME (**1a**), a characteristic long-chain base (LCB), and a methyl glucoside. The structure of **1a**, (2*R*)-2-hydroxytetracosanoyl, was confirmed by GC–MS analysis, which showed the molecular ion at *m/z* 398 [$C_{25}H_{50}O_3$]$^+$ and the characteristic fragment ion at 339 [M – $COOCH_3$]$^+$, and by its optical rotation value, $[\alpha]^{15}_D = -3.8$ (*c* = 0.23, $CHCl_3$); these values were consistent with literature data (10–12). The LCB was determined as 2-amido-1,3-dihydroxyl-9-methyl-4,8-octadecanediene (**b**) whose peracetylated derivative, 2-acetoamido-1,3-diacetoxy-9-methyl-4,8-octadecanediene (**c**), gave characteristic ion peaks at *m/z* 438 [M + 1]$^+$, 378 [M + 1 – HOAc]$^+$, 318 [M + 1 – 2 × HAc]$^+$, and 257 [M – 3 × HAc]$^+$. That evidence as well as the 2-D NMR data (Fig. 1) further supported the structure of **1** as shown in Figure 2.

Cortenuamide B (**2**), $C_{48}H_{91}O_9N$ (HR FABMS showed [M – 1]$^-$, *m/z* 824.6619, calcd. for $C_{49}H_{92}NO_9$ 824.6612) and

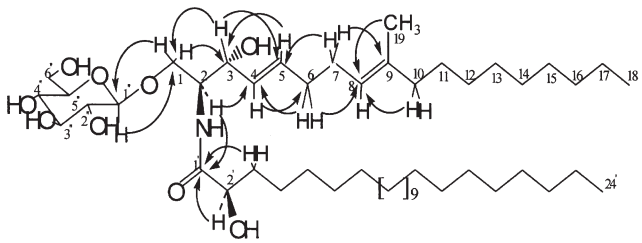

FIG. 1. Key heteronuclear multiple bond correlations of compound **1**.

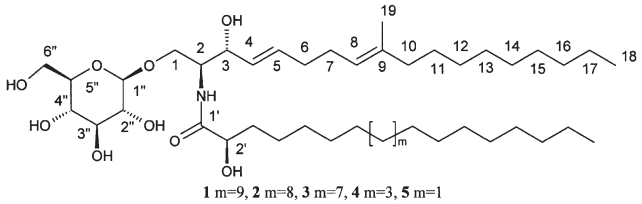

FIG. 2. Structures of five cerebrosides (**1–5**)

cortenuamide C (**3**), $C_{47}H_{89}O_9N$ (HR FABMS showed $[M - 1]^-$, *m/z* 810.6459, calcd. for $C_{49}H_{88}NO_9$ 810.6449) were obtained as white powders. By comprehensive comparison, we found that the ^1H and ^{13}C NMR (DEPT) data for **2** and **3** were almost the same as those of cortenuamide A (**1**). This suggested that cortenuamide B and C were cerebrosides with the same basic skeleton and absolute chemical configuration of cortenuamide A. The difference between **2**, **3**, and **1** was that **2** contained (2*R*)-2-hydroxytricosanoyl (**2a**) and **3** contained (2*R*)-2-hydroxydocosanoyl (**3a**) instead of (2*R*)-2-hydroxytetracosanoyl (**1a**) in the FA moiety of compound **1**. This was indicated by GC–MS analysis of **2a** (major ion peaks: *m/z* 398 $[M]^+$, 339 $[M - 59]^+$), and **3a** (major ion peaks: *m/z* 384 $[M]^+$, 325 $[M - 59]^+$). Acidic methanolysis experiments with **2** and **3** yielded the same LCB as that of **1**, as confirmed by the EI–MS and ^1H NMR of its peracetylated derivatives. The overall structures of cortenuamide B and C could then be established as (4*E*,8*E*)-*N*-D-2′-hydroxytricosanoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine (**2**) and (4*E*, 8*E*)-*N*-D-2′-hydroxydocosanoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine (**3**).

Cerebrosides **4** and **5** were two known compounds whose structures were characterizied as (4*E*, 8*E*)-*N*-D-2′-hydroxyoctadecanoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine (**4**) and (4*E*,8*E*)-N-D-2′-hydroxypalmitoyl-1-*O*-β-D-glycopyranosyl-9-methyl-4,8-sphingadienine (**5**) based on comparison spectroscopic (MS, IR, ^1H, and ^{13}C NMR) and physical data with **1–3** and the literature (6,7).

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Phospholipid FA from Indian Ocean Tunicates *Eudistoma bituminis* and *Cystodytes violatinctus*

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ABSTRACT: Two tunicates (*Eudistoma bituminis* and *Cystodytes violatinctus*, family Polycitoridae) were investigated for the FA content of their phospholipids. GC–MS analysis of their methyl esters and *N*-acyl pyrrolidides revealed 40 FA in *E. bituminis*, and 26 in *C. violatinctus*. In both cases, the most abundant FA were the saturated ones (C_{10} to C_{18}). *Cystodytes violatinctus* contained considerable oleic acid (20%). Both *E. bituminis and C. violatinctus* contained phytanic acid and Δ^{10}-unsaturated FA, which had not previously been found in such organisms. The two tropical tunicates contained only trace amounts of PUFA, which are usually predominant in this phylum.

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Few works have been published on the lipid composition of tunicates. The most comprehensive study was reported by Kostetsky *et al.* (1), who studied the phospholipid composition of 13 tunicates. The edible ascidian *Halocynthia roretzi*, very popular in Japan and Korea, also has been studied (2–4). Several studies of phospholipids of pelagic tunicates (i.e., gelatinous zooplankton) also have been undertaken (5–7). All species studied were shown to contain 22 to 32% saturated FA in their phospholipid fraction. Linear even-chain FA are predominant, i.e., 16:0 (9 to 18% of total FA), 18:0 (3 to 7%), and 14:0 (1 to 8%). Monounsaturated FA represented 13 to 25% of total FA and contained mainly linear even-chain FA: 18:1 (8 to 18% of total FA), 16:1 (2 to 5%), and 14:1 (0.2 to 0.3%). PUFA were the most important type, representing 41 to 59%. The distribution of these acids is not homogeneous among tunicate species; the most abundant FA were EPA (5,8,11,14,17-20:5) (13 to 26% of total FA), DHA (4,7,10,13,16,19-22:6) (4 to 20%), and linoleic acid (9,12-18:2) (2 to 26%). Isoprenoid FA were not characterized in any of the species studied.

No correlation between FA composition and tunicate families has yet been established, although the analysis of lipids in marine organisms can often provide valuable insights into the trophic interactions between marine consumers and their food supply (8,9), and can be indicators of physiological and reproductive status (10).

To our knowledge, the phospholipid composition of a warm-water ascidian has not previously been studied. Accordingly, we looked at the phospholipid composition of two ascidians of Mayotte Island (Comoro Islands, Indian Ocean) that had already been studied for their secondary metabolites (11–13).

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EXPERIMENTAL PROCEDURES

Materials. The two species of tunicates, *Eudistoma bituminis* and *Cystodytes violatinctus*, were collected on the same site, near the Dzaoudzi Beach of Mayotte Island (northwest of Madagascar in the Indian Ocean) at depth of 15–20 m, in July 1997 and September 1998.

The ascidians were immediately frozen with dry ice and carried in an insulated box by air to Réunion (3 h by air). There they were kept for several hours in a freezer until solvent extraction. Species identifications were carried out by Dr. Françoise Monniot (Museum National d'Histoire Naturelle, Paris, France). Voucher specimens were deposited at the Museum National d'Histoire Naturelle.

Isolation of phospholipid FA. The frozen tunicates were extracted with $CHCl_3/CH_3OH$ (2:1, vol/vol) at room temperature with a Waring blender. The $CHCl_3$ (pure, stabilized with 1% ethanol) and CH_3OH (denatured), from Labosi (Elancourt, France), were distilled before use. This procedure yielded crude total lipids in the following forms: a dark-brown gum (451 mg) for the *C. violatinctus* residue (dry weight, 45 g after extraction) and a dark-purple gum (1.9 g) for the *E. bituminis* residue (dry weight, 45 g after extraction). The lipids obtained were applied to a silica gel chromatography column (25 g of 40–63 mesh/g of extract; 2.5 × 53 cm; Merck, Darmstadt, Germany). The column was eluted with *n*-hexane (technical, 0.4 L/g of extract), dichloromethane (pure, 1.4 L/g of extract), acetone (pure, 3.0 L/g of extract), and methanol (0.8 L/g of extract). The *n*-hexane, dichloromethane, and methanol, from Labosi, were distilled before use. The *n*-hexane, dichloromethane, and methanol were obtained from Labosi. The less polar fraction and the first to elute contained hydrocarbons. All other fractions collected were characterized by TLC. The second column fraction contained sterols and TG (eluent: hexane/diethyl ether/acetic acid, 70:30:1; standards: cholesterol and tripalmitin; visualization of spots: vanillin). The third fraction contained glycolipids (eluent: dichloromethane/methanol,

100:20; standard: galactocerebroside; revelator: as above). Phospholipids were collected in a methanolic fraction (eluent: chloroform/methanol/water, 65:25:4; standard: phospholipid; revelator: as above). The phospholipid FA were transesterified to FAME by reaction (30 min under reflux) with 2% methanolic hydrogen chloride (R.P. Normapur, Prolabo, Fontenaylous Bois, France) and then dissolved in *n*-hexane. The resulting methyl esters were analyzed by GC–MS. *N*-Acyl pyrrolidides were prepared by direct treatment of the methyl esters with pyrrolidine (Acros Organics, Fairlawn, NJ)/acetic acid (p.a.; Acros Organics), 10:1 (vol/vol), under reflux (2 h) and were analyzed by GC–MS.

GC–MS. GC–MS of methyl esters and pyrrolidide derivatives was performed with a Hewlett-Packard 5890 gas chromatograph linked to a Hewlett-Packard 5989A mass spectrometer at 70 eV, equipped with an HP-9000/345 integrator, and using a DB-1 column (0.25 μm phase thickness, 0.32 mm × 30 m; Hewlett-Packard, Palo Alto, CA) with the oven temperature programmed from 170 to 300°C (ramped at 3°C/min).

RESULTS AND DISCUSSION

Contents of the total lipid classes of the two tunicates were as follows: (i) for *C. violatinctus*, total lipids: 1.00% (dry matter); hydrocarbons: 11.7%; phospholipids, 14.3%; TG, 38.6%; sterols, 3.6%; and other polar lipids, 31.8%; (ii) for *E. bituminis*, total lipids: 4.22% (dry matter); hydrocarbons: 27.8%; phospholipids, 20.7%; TG, 25.6%; sterols: 2.6%; and other polar lipids, 23.3%. The sterol composition of *E. bituminis* and *C. violatinctus* was reported previously (14). We noted in our previous work that low levels of the polar lipids, 44% for *E. bituminis* and 46.1% for *C. violatinctus*, are found in the two tunicates. Analyses in the literature (2–4,6,7) indicate that ascidian lipids are composed mainly of polar lipids (40 to 90% of total lipids). The predominant class is phospholipids, which are known to be major constituents of cell membranes. Forty FA in phospholipids of *E. bituminis* and 26 in *C. violatinctus* were identified by GC–MS analysis of their FAME and *N*-acyl pyrrolidides. Most of the FA listed in Table 1 were identified as methyl esters by comparing their equivalent chain length (ECL) values with those of commercial standards and other known compounds. For many compounds, *N*-acyl pyrrolidide GC–MS data allowed us to confirm their structures and to determine the double bond and branching positions. Mass spectral data for phytanic acid and 7-methyl-6-16:1 acid ester and pyrrolidide derivatives are in agreement with data in the literature (15,16). Phospholipids of *E. bituminis* contain more than 99% saturated FA: 77.6% of C_{10} to C_{18} FA of saturated chain length and 22.4% of C_{19} to C_{23} saturated chain length FA. Trace amounts of monounsaturated FA (C_{16} to C_{21}, with the exception of C_{20}) were detected. Phospholipids of *C. violatinctus* contained 64.2% of C_{10} to C_{18} saturated chain length and 23.6% of monounsaturated FA. Several C_{18} and C_{20} PUFA were detected in trace amounts in the two species.

Saturated FA. C_{10} to C_{18} even-chain FA were predominant in the two species studied: 16:0 (40.0% of total FA), 18:0 (16.5%), and 14:0 (10.0%) for *E. bituminis* and 18:0 (24.8% of

TABLE 1
Phospholipid FA from *Eudistoma bituminis* and *Cystodytes violatinctus*

FA	ECL[a]	Abundance (wt%)	
		E. bituminis[b]	*C. violatinctus*[c]
Dodecanoic (12:0)	12.00	Trace	1.2
Tetradecanoic (14:0)	14.00	10.0	4.5
13-Methyltetradecanoic (i-15:0)	14.59	Trace	0.7
Pentadecanoic (15:0)	15.00	Trace	1.5
14-Methylpentadecanoic (i-16:0)	15.62	Trace	0.5
6-Hexadecenoic (16:1)	15.69	Trace	1.26
Hexadecanoic (16:0)	16.00	40.0	21.2
7-Methyl-6-hexadecenoic (br-17:1)	16.50	Trace	2.43
15-Methylhexadecanoic (i-17:0)	16.60	3.9	1.2
14- Methylhexadecanoic (ai-17:0)	16.67	—	0.63
Heptadecanoic (17:0)	17.00	7.3	4.8
3,7,11,15-Tetramethylhexadecanoic (br-20:0)	17.68	16.5	Trace
9-Octadecenoic (18:1)	17.74	Trace	20.0
Octadecanoic (18:0)	18.00	16.4	24.8
17-Methyloctadecanoic (i-19:0)	18.62	Trace	0.6
Nonadecanoic (19:0)	19.00	2.0	3.6
Eicosanoic (20:0)	20.00	1.5	3.1
Heneicosanoic (21:0)	21.00	1.4	2.3
Docosanoic (22:0)	22.00	1.0	0.4

[a]ECL were determined using a DB-1 column (Hewlett-Packard, Palo Alto, CA) i, iso; ai, anteiso; br, branched. Trace, <0.1%.
[b]Minor FA identified (<0.1%): 11:0, 11.00; i-13:0, 12.61; 13:0, 13.00; i-14:0, 13.57; ai-14:0, 13.66; ai-15:0, 14.72; 9-16:1, 15.75; 11-16:1, 15.87; 8-17:1, 16.75; 9,12-18:2, 17.56; 11-18:1, 17.83; 5,8,11,14-20:4, 19.34; 23:0, 23.00.
[c]Minor FA identified (<0.1%): 10,13-18:2, 17.59; 10-18:1, 17.78; 11-18:1, 17.83; 5,8,11,14-20:4, 19.34; 23:0, 23.00.

total FA) and 16:0 (21.2%) for *C. violatinctus*. These percentages are considerable for tunicates. Until now, the highest percentages observed were in *H. roretzi,* with 18.0% of 16:0 reported in one study (4) and 7.2% of 18:0 in another (2). Furthermore, in *C. violatinctus* stearic acid was more abundant than palmitic acid. Other C_{10} to C_{18} linear-chain FA were less abundant except for 17:0 acid, which represented 7.3% of phospholipid FA of *E. bituminis*. C_{19} to C_{23} straight-chain FA represented 9% of phospholipid FA in *C. violatinctus* and 6% in *E. bituminis.* Iso and anteiso branched C_{10} to C_{18} FA were detected in trace amounts, except for i-17:0 in *E. bituminis*, which constituted 3.9% of phospholipid FA. Phytanic acid (3,7,11,15-tetramethyl-16:0), an isoprenoid FA usually found in marine organisms but identified for the first time in tunicates, represented 16.5% of the phospholipid FA of *E. bituminis*. Phytanic acid has been found in trace amounts in *C. violatinctus*. The origin usually accepted for this acid is oxidation of dihydrophytol, contained in chlorophyll. Phytanic acid is probably exogenous owing to the ubiquity of photosynthetic organisms in oceans (15).

Monounsaturated FA. These acids are commonly reported as being abundant in tunicates (13.0 to 24.8% of phospholipid FA). However, only very small quantities of C_{16} to C_{21} monounsaturated FA with n-5, n-7, or n-10 unsaturations were detected in *E. bituminis*. On the other hand, oleic acid was very abundant in *C. violatinctus,* constituting 20.0% of phospholipid FA. Trace amounts of 10-18:1 and 11-18:1 were also found in this ascidian. The FA 7-methyl-6-16:1, which is frequently found in other marine organisms, was found for the first time in tunicates and was identified in both species studied. The FA 6-16:1 and 7-methyl-6-16:1 represented 3.7% of phospholipid FA in *C. violatinctus*. The branched Δ^6 unsaturated FA is probably of bacterial origin (16).

PUFA. PUFA were detected in trace amounts in *E. bituminis* and *C. violatinctus. Eudistoma bituminis* contained trace amounts of arachidonic acid (5,8,11,14-20:4) and linoleic acid (9,12-18:2). Linoleic acid is the major FA in *Botryllus tuberatus* and *Botrylloides eligulatum* (family Styelidae, order Stolidobranchiata) (17). Trace amounts of arachidonic acid and a rare diunsaturated FA 10,13-18:2, were detected in *C. violatinctus*. The percentage of arachidonic acid commonly found in tunicates ranges between 2 and 7%. The dienoic acid 10,13-18:2 was synthesized and used in a comparative study with GC of dienoic FA by Christie and Holman in 1967 (18), and was found for the first time in a marine sponge by Barnathan *et al.* in 1992 (15).

The most remarkable characteristic of the FA composition of phospholipids of *E. bituminis* and *C. violatinctus* is the presence of only trace amounts of PUFA. Such low levels of PUFA were also observed for Senegalese gorgonians collected in warm water. This result is in agreement with a previous report of warmer water specimens having twice the saturated FA content of colder-water specimens (16). The influence of environmental temperature seems to be the plausible explanation, especially since the phenomenon of El Niño caused abnormally warm water to arrive in the lagoon of Mayotte during 1997–1998. Temperature is known to be one of the major environmental factors that causes changes in marine organisms. It is also generally reported that temperature may affect saturation level in FA and that there is a positive correlation between temperature and degree of saturation in FA of cell membranes (19). For example, a recent study provides evidence of the influence of diet and temperature on seasonal changes in abalone lipid profiles (20). Dietary input or symbionts may also account for the variation in PUFA levels.

Dinoflagellate endosymbionts are widely distributed among marine invertebrates, and their lipids are characterized by a high PUFA concentration (21). It is well known that the bleaching observed on coral reefs affects symbiosis in hosts ranging from cnidarians, sponges, and mollusks to tunicates, and tunicate-associated symbionts are either dinoflagellates, diatoms, or cyanobacteria (22).

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Dietary Fat, Obesity, and Health—From Theory to Practice

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INTRODUCTION

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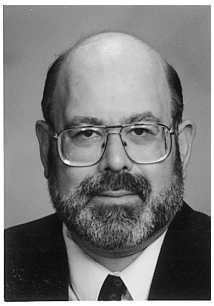

Mark Bieber

The Mark Bieber Symposium, "Dietary Fat, Obesity, and Health—From Theory to Practice," was organized to recognize Dr. Mark Bieber's contributions to the American Oil Chemists' Society (AOCS) and the fats and oils industry. The symposium was held at the AOCS Annual Meeting in Montréal on Tuesday, May 7, 2002.

Dr. Bieber was actively involved with the governance of the AOCS and with its programs; he was secretary of the Governing Board at the time of his death. Mark was a strong supporter of the Society's technical programs and the organizer of several very successful symposia. He was a strong advocate of the importance of science, but he was equally as fervent in his belief that science should have practical application to health and nutrition issues.

These papers were presented at the symposium organized by the Health and Nutrition Division of the AOCS in collaboration with the International Food Information Council (IFIC) and the International Life Sciences Institute of North America (ILSI-NA). In selecting speakers for the program, the organizing committee tried to address current theories and practical issues on the topic of obesity: the role of genetics vs. lifestyle; the question of dietary fat vs. carbohydrate and energy density; emerging areas such as DAG, CLA, and calcium in weight control; and finally, practical approaches to treating and preventing obesity.

Mark Bieber received his Bachelor of Science degree in chemistry, with honors, from the University of Pittsburgh in 1968. He received his Ph.D. in biochemistry from Michigan State University in 1973 and then spent four years as a postdoctoral fellow with the National Institutes of Health at Columbia University before joining the Bestfoods organization in 1977.

Dr. Bieber was a nutrition research associate with Unilever-Bestfoods in Somerset, New Jersey. In this company he worked as a senior nutritionist in research programs and in marketing. Dr. Bieber represented Unilever-Bestfoods in numerous professional and scientific societies.

Dr. Bieber joined the AOCS in 1977. He became active in the Northeast Section, serving in various section offices before being elected section president for 1987–1988. He was AOCS Division Council chairperson in 1995–1996 and was a past chairperson of the Health and Nutrition Division, 1993–1995. He was elected as a member-at-large of the AOCS Governing Board in 1997, then as AOCS secretary in 1999.

In addition to the AOCS, other organizations in which Dr. Bieber was active were the American Heart Association's Council on Arteriosclerosis, Thrombosis, and Vascular Biology (Fellow); American College of Nutrition; Society for Nutritional Education; Society for the Study of Ingestive Behavior; Institute of Food Technologists, IFIC; and numerous other scientific and honorary societies. He was a member of the FAO/WHO Expert Panel on Dietary Fats in Human Nutrition, a panel that met in Rome, Italy, in 1993. Dr. Bieber received a Movers and Shakers Award from the ILSI-NA in recognition of his work on ILSI committees.

We had the pleasure of working with Mark in the Health and Nutrition Division of AOCS for several years and the honor of assisting in the development of this program. Mark was an excellent scientist known for his probing questions and for his ability to synthesize complex issues, a person with a finely tuned sense of humor, and a great raconteur. In addition, he will be remembered for his fervent fostering of graduate students at AOCS meetings. His passing leaves us with a great sadness; however, he leaves a great legacy for those who were fortunate to work with him.

OBESITY—AN OVERVIEW

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In the United States, the body mass index (BMI) [BMI = $wt(kg)/ht(m)^2$] of men has increased by 3 to 4 units during the last 100 yr. The rise in the prevalence of overweight (BMI 25 to 30) and of obesity (BMI >30) during the last two decades is now recognized as a worldwide problem, with ominous implications for public health and health-related costs. The parameters generally considered to explain increases in BMI and in the prevalence of obesity include increased food avail-

ability, increased dietary fat content, greater energy density of foods, and decreased physical activity. Yet during the last 20 yr, these parameters appear to have remained fairly stable in the United States and in other affluent countries.

The predisposition toward obesity is also influenced by genetic factors, but the high prevalence of obesity in affluent societies makes it difficult to assess the relative importance of inherited vs. noninherited factors. Among the latter, it is helpful to distinguish between "circumstantial" factors that, together with genetic factors, are responsible for individual variability of BMI around the mean and "environmental" factors that cause mean BMI to rise, such as moving from a situation with occasional food scarcity into affluent conditions. This is a unidirectional effect, and its contribution to the development of obesity is underestimated when the relative importance of inherited vs. noninherited factors is assessed by the contribution of each to variance about the mean.

Because of their hydrophobic nature, lipids can be stored far more efficiently than glycogen (the animal equivalent of starch), which retains 3 g of water/g. In humans, the fat stored in adipose tissue provides an energy reserve that is typically two orders of magnitude greater than the body's limited glycogen stores. In evolution, the human body has therefore been compelled to develop regulatory mechanisms that gave higher priority to the control of its carbohydrate economy than to the control of fat metabolism. Thus, consumption of carbohydrate elicits a prompt increase in glucose oxidation (with a concomitant decrease in fat oxidation), whereas consumption of fat does not lead to increased fat oxidation. Even though progressive increases in the fat mass do promote fat oxidation, this is a long-term effect, and it is less powerful than the influence of the carbohydrate supply on the relative contributions made by fat and glucose to the fuel mix oxidized.

To understand body weight regulation, it is important to realize that the steady state of weight maintenance is only possible when the fuel mix oxidized matches the nutrient mixture consumed in amount and composition. This suggests that recent increases in the prevalence of obesity may be related to a trend of habitually maintaining glycogen levels within a slightly higher range, as this would require an expansion of the fat mass to maintain fat oxidation rates commensurate with fat intake. A further decline in physical activity could also be involved in causing increased fatness, in part by causing less glycogen depletion between meals. Although the impact of vigorous physical activity in preventing excess fat accumulation is readily recognized, the influence of modest levels of physical activity is less obvious. However, measurements of inactivity are more telling in this respect: They show that hours spent watching TV or sitting in front of a computer are correlated with fat accumulation in children.

Inherited traits are often thought to influence obesity by causing resting metabolic rates to be unusually low. When appropriately corrected for differences in body size and age, the observed differences are rather small, and, in any case, they are more than offset by the increase in energy expenditure associated with a larger body size. The size of the lean body mass is, in effect, the main predictor of resting energy expenditure. In the NHANES II data, height accounts for 10–16% of the variance in lean body mass, yet differences in stature explain less than 1% of the variance in body fat content. Thus, differences in resting metabolic rates appear to have a much smaller impact on obesity than is commonly believed. By contrast, inherited traits influencing energy expenditure by affecting individual predispositions toward physical activity can be expected to have a substantial impact on adiposity. Inherited traits also could exert major leverage on body weights by affecting the relative rates of carbohydrate and fat oxidation, particularly by altering the influence of the adipose tissue mass on the relative use of FA and glucose. Such effects have been difficult to prove, because their impact on the fuel ratio oxidized is much smaller than the effects thereon of the large daily variations in food intake and physical activity that occur in free-living individuals.

When considered over the long run, regulation of food intake leads to a remarkably good adjustment of energy intake to energy expenditure, as energy imbalances are generally less than 1 to 2% of energy turnover when considered over the period of a year. The fact that short-term variations in the energy balance are large shows not only that they are readily tolerated, but also that one cannot therefore expect physiological regulation of food intake to account for a large part of the variance in daily food consumption. This has made it very difficult to gain a realistic understanding of food intake regulation, and this constitutes a big obstacle in tackling the obesity problem.

The success of drugs in controlling body weight has been limited so far, perhaps because increasing adiposity seems to be a natural consequence of biological evolution, followed by economic evolution. Recommendations about lifestyle changes to control body weight are generally confined to altering circumstantial factors, whose leverage is weak compared to environmental and genetic influences. Creating opportunities to strive for weight control in group settings can be advantageous by providing a measure of change in the environment. Similarly, encouraging the acquisition of greater physical activity habits, notably in children and adolescents, offers the potential for improving the environment in population groups.

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Genetics of Obesity: More Complicated Than Initially Thought

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ABSTRACT: During the past several decades, there has been an explosion in the prevalence of obesity. Since our genes have not changed appreciably during that time, it stands to reason that the present epidemic is caused by our pervasive obesigenic environment, in which excess caloric intake and decreased physical activity conspire with one another. Despite an obesigenic environment, humans have great variability in their susceptibility to obesity, which is determined in large part by genetics. Current evidence suggests that genetic susceptibility to human obesity is the result of multiple genes, each with a modest effect, that interact with each other and with environmental provocations. Elucidation of obesity susceptibility genes through genome-wide and candidate gene approaches provides great promise in ultimately determining the genetic underpinnings of obesity. Further research will translate these new insights on the pathophysiological basis of obesity into new medications and diagnostic tests.

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Obesity is considered to be second-most important preventable cause of death, exceeded only by cigarette smoking (1). This is due to an explosion in the prevalence of obesity in the United States and other westernized countries over the last several decades (2). Obesity is a potent risk factor for type-2 diabetes, hypertension, and dyslipidemia (3–5), co-morbidities that markedly increase the risk of cardiovascular disease in the obese. Today, nearly two-thirds of the adult U.S. population is considered overweight or obese (4). The recent obesity epidemic is due in large part to our obesigenic lifestyle, specifically, excessive caloric intake accompanied by physical inactivity. This combination has led to a net positive energy balance and thus the increased prevalence of obesity.

GENETICS OF OBESITY

Twin, adoption, and family studies (6–10) strongly suggest an important genetic component to obesity susceptibility. Several such studies suggest that approximately 40–70% of the variation in body mass index can be accounted for by genetic influences. Current thinking is that genetic susceptibility interacts with environmental influences (i.e., our obesigenic environment) ultimately to render some individuals more susceptible than others. Some regard obesity susceptibility genes as "thrifty genes" (11), which may have provided a selective survival advantage throughout evolution when our environment was typically scarce in nutrients. It is likely that selective pressures for thrifty genes occurred very early in evolution, which would predict that several, or even many, obesity susceptibility genes have been selected over time.

Here we review what is currently known about the genetic influences on obesity. Although quite complex, the application of powerful genetic approaches to both rodent models and humans has provided a number of molecular and genetic insights into the biology of energy homeostasis and the pathophysiology of obesity. The identification of obesity susceptibility genes promises to provide new understandings of the molecular pathophysiology of obesity and, in so doing, opportunities for the development of new therapeutic agents with specific molecular targets that will be more effective and have fewer side effects. Furthermore, identification of these genes will allow us to offer genetic tests to determine an individual's genetic burden of obesity susceptibility genes for potential early intervention to mitigate future weight gain. Genetic testing also will allow us to subclassify obesity, which will be useful in directing therapy and may also have prognostic value with regard to obesity as well as its associated co-morbid complications.

MONOGENIC FORMS OF OBESITY IN RODENTS

There are several spontaneous monogenic mouse models of obesity in which genetic approaches have homed in on the specific genes that are responsible. Table 1 summarizes several obese mouse strains and the genes identified. These studies led to the identification of leptin, which is a hormone secreted by adipocytes in direct proportion to fat mass that acts in the hypothalamus to inhibit food intake (12). The *ob/ob* mouse has a mutation in leptin, rendering it leptin-deficient, whereas the *db/db* mouse strain has a mutation in its leptin receptor, thus rendering leptin inert and resulting in increased

TABLE 1
Spontaneous Monogenic Mouse Obesity Gene Mutations and Their (rare) Human Counterparts

Mouse	Mouse mutation	Human homolog mutation
ob/ob	Leptin	Leptin
db/db	Leptin receptor	Leptin receptor
fa/fa	Carboxypeptidase E	(Proconvertase 1)[a]
Agouti	Agouti-signaling protein	(Melanocortin-4 receptor)[a]
Tubby	Insulin-signaling protein	None known
Mahogony	Attractin	None known

[a]Human genes in parentheses are not strictly the mouse homologs, but rather are similar genes or genes involved in pathways similar to the corresponding mouse mutation.

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Abbreviation: PPAR-γ, peroxisome proliferator-activated receptor-γ; P/S, polyunsaturated fat to saturated fat ratio.

food intake and obesity (13). Other monogenic mouse mutations that cause obesity include mutations in carboxypeptidase E, which is responsible for the processing of a number of endocrine and neuroendocrine hormones involved in food intake (14). Mutations in the agouti-signaling protein, which acts through melanocortin receptors to modulate eating behavior, are the cause of obesity in the agouti mouse (15). The etiology of obesity in the tubby mouse (16) (caused by mutations in insulin signaling protein) and the mahogany mouse (17) (caused by mutations in the attractin gene) are less clear. The search for mutations in the human counterpart of these mouse obesity genes has led to the conclusion that they are quite rare forms of obesity in humans. However, a few families have been identified with mutations in their leptin or leptin receptor genes (18,19). Genetic variants in proconvertase 1 (20) (a processing enzyme similar to carboxypeptidase E) and the melanocortin-4 receptor (21) also have been reported to be associated with obesity.

GENETICS OF TYPICAL OBESITY IN HUMANS

Although there is a strong genetic component to typical obesity in humans, there is no simple Mendelian mode of inheritance. This suggests that multiple genes are likely to be involved in obesity and that these genes interact with each other and with environmental provocations to influence risk. Armed with essentially the complete sequence of the human genome, today's genetics researchers possess powerful approaches to identify disease-causing genes. The two broad categories of approaches include genome-wide analysis and candidate gene analysis. Both depend on the ascertainment of large numbers of obese individuals and their family members. With genome-wide approaches, hundreds of polymorphic markers distributed throughout the genome are assayed to determine which regions of the genome are shared more often in obese family members than would be expected by chance. Statistical evidence of excess sharing of a region of the genome in family members that also share the obese phenotype suggests that a gene influencing obesity exists in that region. Positional cloning of the obesity gene then can be performed using information from the human genome database as well as experimental methods. To date, several genome-wide scans for obesity susceptibility genes have been performed in diverse populations including Caucasians, African Americans, Mexican Americans and Pima Indians (reviewed in Ref. 22). These scans provide evidence for obesity susceptibility genes in several chromosomal regions including chromosome 10p, 11q24, 16p, 18q21, 20q13, and Xq24, among others. Positional cloning of the putative genes in these regions is under way in a number of laboratories.

Candidate gene approaches involve the investigation of genes whose products encode proteins that influence energy homeostasis and obesity. Comparison of the DNA sequences of these genes in obese and nonobese individuals may lead to the identification of specific gene variants that contribute to disease susceptibility. Over the years, many genes have been examined; however, the vast majority of candidate gene studies in humans so far have been negative, or alternatively the gene variant has been shown to play a relatively modest role in influencing the obesity phenotype. Two notable examples of candidate gene variants that are likely to play a role in obesity susceptibility, albeit a small one, include Trp64Arg β3 adrenoceptor (23–25) and Pro12Ala peroxisome proliferator-activated receptor-γ2 (PPAR-γ2) (26,27).

The β3 adrenoceptor is a seven-membrane-spanning receptor expressed predominantly in adipocytes. Stimulation *via* norepinephrine released by the sympathetic nervous system results in the activation of adenylyl cyclase, cAMP accumulation, protein kinase A activation, and lipolysis. In 1995, our group reported a common missense mutation in the β3 adrenocepter in which tryptophan was changed to arginine at codon position 64 (23). Several studies have shown that this nonconservative variant in the first intracellular loop results in a receptor of decreased function (28). The variant is quite common, with more than 50% of Pima Indians, 33% of Japanese, and 20–25% of Mexican Americans and Caucasians carrying at least one Arg64 allele. Although not all studies agree, subjects with the Arg64 allele tend to manifest signs of the insulin resistance syndrome including increased adiposity, particularly central/visceral fat (29–32). Although the influence of this variant by itself is modest, our recent studies suggest that the Trp64Arg β3 adrenoceptor variant acts as a modifier of other candidate gene variants, such that individuals with the Trp64Arg β3 adrenoceptor variant and a variant in another gene also involved in fat storage are significantly more obese than subjects with one or no copies of the variant genes. Examples of gene–gene interaction of the Trp64Arg variant include variants in the α2b receptor gene (33), PPAR-γ2 (34), type 2 deiodinase (35), and a co-activator of PPAR-γ, PPARGC1 (Shuldiner, A.R., unpublished data) (Fig. 1). Thus, as variants in candidate genes involved in converging pathways are identified and interactive effects examined, the pieces of the obesity genetics puzzle begin to fall into place. Additional candidate genes will undoubtedly uncover additional examples of gene–gene interactions.

PPAR-γ is a nuclear receptor important for adipogenesis and insulin signaling. In 1997, our group identified a common variant in PPAR-γ in which proline is changed to alanine at

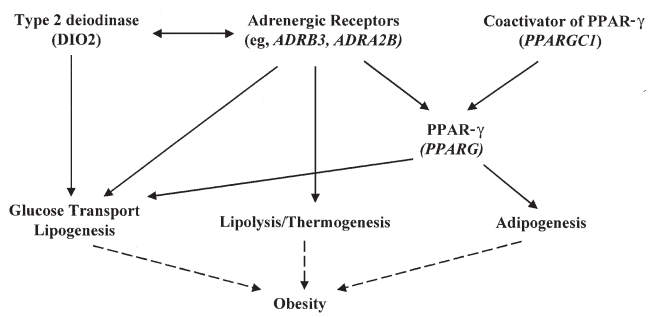

FIG. 1. The adipocyte, gene variants, and converging biochemical pathways. PPAR-γ, peroxisome proliferation-activated receptor-γ; ADRB3, β3 adrenoceptor; ADRA2B, α2b receptor.

codon 12 of the γ2 isoform (26). Studies (36) have shown that this single amino acid change results in a nuclear receptor of decreased activity. The alanine variant is quite common in Caucasian populations, with approximately 20–25% of individuals being either heterozygous or homozygous for the alanine allele (36). Studies indicate that individuals with the alanine allele are more sensitive to the effects of insulin to promote glucose uptake and are actually protected from the development of type-2 diabetes (36–39). In addition, they seem to be more efficient in their metabolism and have a greater propensity for weight gain and obesity (27,40,41). As mentioned earlier, individuals with the arginine allele of the β3 adrenoceptor and also the alanine allele of PPAR-γ are significantly more obese than individuals with only one or neither of these variants (34) (Fig. 2).

FA represent natural ligands for PPAR-γ. A recent study suggests a gene–nutrient interaction between the Pro12 ALA PPAR-γ2 variant and dietary FA (42). Subjects with the alanine allele of PPAR-γ who eat a diet with a low polyunsaturated fat to saturated fat (P/S) ratio are significantly more obese, whereas individuals with the alanine variant who eat a diet with a high P/S ratio are significantly leaner. These studies clearly point out the extraordinary complexity of energy homeostasis and the effects of common gene variants on susceptibility to obesity.

FUTURE PROSPECTS

Elucidation of obesity susceptibility genes through genome-wide approaches as well as candidate gene approaches provides great promise in ultimately determining the genetic underpinnings of obesity. New understandings of the pathophysiological basis of obesity will lead to new medications and diagnostic tests. It is envisioned that within the next 10 years, these discoveries will be translated to the clinical setting. With a blood test, physicians will be able to define their patient's genetic burden of obesity susceptibility genes as well as genetic constituents that define co-morbid risks and efficacy of various interventions for the treatment of obesity and its co-morbidities. Figure 3 depicts a hypothetical obesity gene diagnostic panel performed on a hypothetical patient in the year 2013. Such a genetic test will undoubtedly revolutionize the treatment and prevention of obesity.

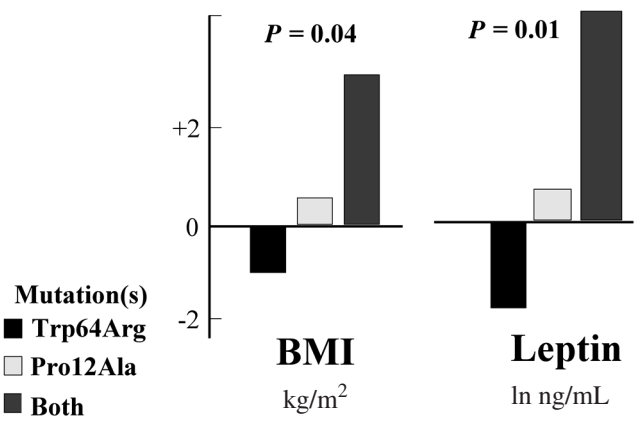

FIG. 2. Interaction between Trp64Arg ADRB3 and Pro12Ala PPAR-γ2. BMI, body mass index; for other abbreviations see Figure 1.

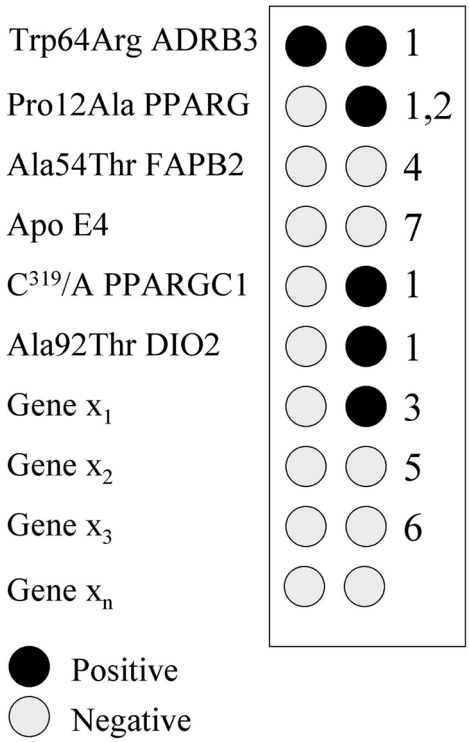

FIG. 3. A hypothetical obesity panel in the year 2013. *Interpretation:* Your patient, John Doe, is at 2.4-fold risk of becoming obese by age 45[1]. He is, however, at decreased risk of developing type-2 diabetes (RR: 0.77)[2] and hypertension (RR: 0.33)[3]. Effective preventive interventions include a diet with a low polyunsaturated/saturated fat ratio[2] and moderate exercise. Effective therapeutic agents include orlistat[4], Drug x[5], and Drug y[6]. β-3-agonist will not be effective[1]. Low HDL cholesterol will be relatively unresponsive to exercise[7] and should be treated with a PPAR-α agonist. Note: your patient is more prone to weight regain following weight loss and thus maintenance pharmacotherapy is recommended[2]. Superscripts indicate the predictive gene variants. RR: relative risk.

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Sedentary Lifestyle and Risk of Obesity and Type 2 Diabetes

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ABSTRACT: Obesity and type 2 diabetes have reached epidemic proportions in the United States. It is well-established that increasing physical activity plays an important role in reducing risk of obesity and diabetes. Few studies, however, have examined the association between sedentary behaviors such as prolonged television (TV) watching and obesity and diabetes. Using data from a large prospective cohort study, the Health Professionals' Follow-up Study, we have demonstrated that increasing TV watching is strongly associated with obesity and weight gain, independent of diet and exercise. Also, prolonged TV watching is associated with a significantly increased risk of type 2 diabetes. Men who watched TV more than 40 h per week had a nearly threefold increase in the risk of type 2 diabetes compared with those who spent less than 1 h per week watching TV. The increased risk was not entirely explained by the decreased physical activity and unhealthy eating patterns associated with TV watching. Thus, public health campaigns to reduce the risk of obesity and type 2 diabetes should promote not only increasing exercise levels but also decreasing sedentary behaviors, especially prolonged TV watching.

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Obesity and diabetes have reached epidemic proportions in the United States. According to the most recent data from the Behavioral Risk Factor Surveillance System (1), 20.2% of men (19.6 million) and 19.4% of women (19.2 million) are clinically obese [body mass index (BMI) ≥ 30 kg/m^2]; 6.5% of men (6.3 million) and 8.2% of women (8.7 million) also report having diagnosed diabetes. The adverse effects of obesity on health are indisputable. Overweight and obesity are central to the metabolic syndrome and are the single most important risk factors for type 2 diabetes (2) Overweight and obesity are associated with mortality from all causes, and the risk of death rises with increasing weight (3). In 2001 the U.S. Surgeon General issued a Call to Action, pointing out that "Overweight and obesity may soon cause as much preventable disease and death as cigarette smoking" in the United States (www.surgeongeneral.gov/topics/obesity/default.htm). Approximately 300,000 deaths a year in the United States currently are associated with obesity and overweight (compared to more than 400,000 deaths a year associated with cigarette smoking).

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Abbreviations: BMI, body mass index; CI, confidence interval; DPP, Diabetes Prevention Project; GI, glycemic index; HPFS, Health Professionals' Follow-up Study; IGT, impaired glucose tolerance; MET-h, metabolic equivalent-hours; RR, relative risk; TV, television; VCR, videocassette recordings.

Obesity is a complex problem resulting from a combination of genetic, behavioral, environmental, cultural, and socioeconomic influences. Although behavioral and environmental factors are considered primary determinants of obesity, specific lifestyle factors have not been clearly defined. In this paper, we review epidemiologic and clinical evidence regarding physical inactivity and a sedentary lifestyle in relation to obesity and type 2 diabetes.

PHYSICAL INACTIVITY, A SEDENTARY LIFESTYLE, AND OBESITY

Epidemiologic evidence strongly supports the role of exercise in preventing obesity and type 2 diabetes mellitus (4–11). However, less attention has focused on sedentary behaviors. Television (TV) watching represents a major sedentary behavior in the United States; on average, an adult male spends approximately 29 h per week watching TV, and an adult female spends 34 h per week (12). TV watching results in a lower metabolic rate compared with other sedentary activities such as sewing, playing board games, reading, writing, or driving a car (13). In several studies, time spent watching TV has been strongly associated with weight gain and obesity in both children (14,15) and adults (16–18). In a study of 51,529 male health professionals 40 to 75 years of age (18), the risk of being overweight was 50% lower [95% confidence interval (CI), 45–55%] for men in the highest quintile of physical activity level compared with men in the lowest quintile. Among men watching 41 h or more of TV or videocassette recordings (VCR) per week, the risk of being overweight was 4.06 times (95% CI, 2.67–6.17) greater than those for men watching no more than 1 h per week. In a prospective analysis, higher levels of physical activity and lower levels of TV or VCR viewing were independently associated with lower relative risks of becoming overweight between survey years. These results suggest that both physical activity levels and time spent watching TV or VCR contribute to the development of overweight in men, and that prolonged TV watching and physical activities represent separate aspects of lifestyle, each with independent risks for overweight (Fig. 1).

Coakley and colleagues (19) examined longitudinal predictors of weight change in the Health Professionals' Follow-up Study (HPFS) from 1988 to 1992. In this cohort of middle-aged men, vigorous activity was associated with weight reduction, and TV or VCR viewing and eating between meals was associated with weight gain. Quitting smoking and a history of voluntary weight loss prior to the study period were consistently related to a weight increase. Over the 4-yr

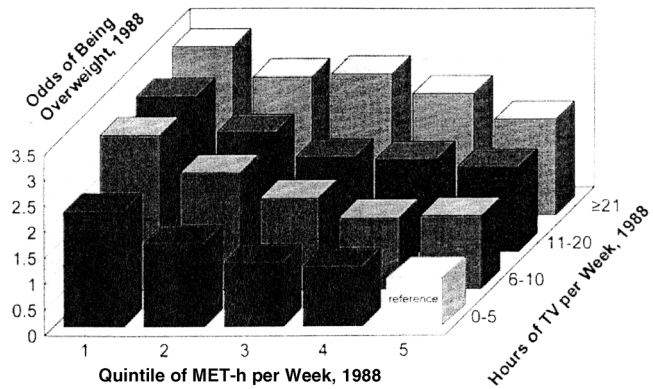

FIG. 1. Joint classification of quintile of metabolic equivalent-hours (MET-h), hours of television (TV) watching, and odds of being overweight among health professionals. Reproduced with permission from Reference 18.

follow-up period, men who increased their exercise, decreased TV viewing, and stopped eating between meals lost an average of −1.4 kg (95% CI, −1.1 to −1.6 kg), compared to a weight gain of 1.4 kg among the overall population. The prevalence of obesity among middle-aged men was lowest among those who maintained a relatively high level of vigorous physical activity over time, compared with those who were relatively sedentary (Fig. 2). These data suggest that increasing and maintaining vigorous activity, as well as decreasing TV use, are important for weight maintenance or a modest weight loss over 4 yr.

PHYSICAL ACTIVITY, TV WATCHING, AND PLASMA BIOMARKERS OF OBESITY AND CARDIOVASCULAR DISEASE RISK

Fung and colleagues (20) examined the relationship between physical activity and TV watching and plasma biomarkers of obesity and cardiovascular disease in a subsample of the HPFS participants. Multivariate linear regression analyses showed that increased levels of physical activity were significantly

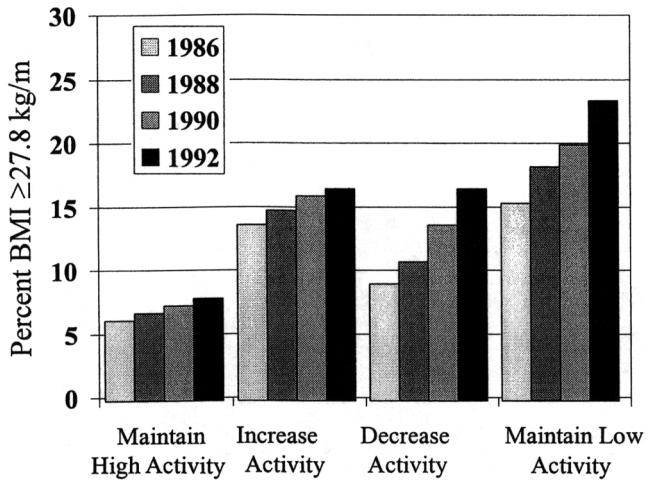

FIG. 2. Prevalence of obesity [body mass index (BMI) ≥27.8 kg/m^2] over time for different patterns of recreational vigorous physical activity. Reproduced with permission from Reference 19.

associated with HDL cholesterol (positively) and with leptin and C-peptide (inversely). The average number of hours of TV watching was significantly and positively associated with LDL cholesterol and significantly and inversely associated with HDL cholesterol and apolipoprotein A-1. Average hours of TV watching per week was positively associated with leptin levels ($P < 0.01$). The association of TV watching and vigorous activity with leptin and HDL cholesterol were independent of each other (Table 1). As expected, the most favorable leptin or HDL cholesterol profile was among men with the highest level of vigorous activity (tertile 3) and the lowest number of average hours of TV watching (tertile 1). In a study conducted between 1986 and 1994, men who had an average of 37.1 metabolic equivalent-hours (MET-h) of physical activity (tertile 3) and 3.4 h of TV watching (tertile 1) per week had 5.1 ng/mL (5% CI, −3.0 to −7.0) lower leptin levels and 12.8 mg/dL (95% CI, −2.2 to 27.8) higher HDL cholesterol levels compared with men with an average of 1.43 MET-h of physical activity (tertile 1) and 18.1 h of TV watching (tertile 3) per week. Across the same level of TV watching, HDL cholesterol generally increased and leptin tended to decrease with increasing levels of vigorous activity. Across similar levels of vigorous activity, more TV watching was associated with lower levels of HDL cholesterol and higher levels of leptin. An additional adjustment for BMI only slightly attenuated the results. Men at the highest level of physical activity and the lowest level of TV watching had a 9.7 mg/dL (95% CI, −4.4 to 23.9) higher HDL cholesterol level and a −3.6 ng/mL (95% CI, −2.1 to −5.2,) lower leptin level compared with those at the lowest level of physical activity and the highest level of TV watching.

SEDENTARY LIFESTYLE AND TYPE 2 DIABETES

In a subsequent analysis of the HPFS, Hu and colleagues (21) examined the relationship between physical activity and TV watching and the incidence of type 2 diabetes. A total of 1,058 cases of type 2 diabetes were diagnosed during 10 yr (347,040 person-yr) of follow-up. After adjusting for age, smoking, alcohol use, and other covariates, the relative risks (RR) of type 2 diabetes across increasing quintiles of MET-h)/wk were 1.0, 0.78, 0.65, 0.58, and 0.51 (P for trend < 0.0001). Time spent watching TV was significantly associated with a higher risk of diabetes. After adjusting for age, smoking, physical activity levels, and other covariates, the RR of diabetes across categories of average hours spent watching TV per week (0–1, 2–10, 11–20, 21–40, and >40) were 1.0, 1.66, 1.64, 2.16, and 2.87 (P for trend = 0.0005). The significant positive association persisted even after adjusting for BMI (RR comparing extreme categories = 2.31; 95% CI, 1.17–4.56; P for trend = 0.01). Further adjustment for intakes of saturated fat, monounsaturated fat, polyunsaturated fat, *trans* fat, and cereal fiber did not appreciably change the results.

In multivariate analyses, independent effects of TV watching and physical activity levels were observed (Fig. 3). Compared with men who were in the most active (>46 MET-h per week) and the lowest TV-watching (<3.5 h per week watch-

TABLE 1
Multivariate Linear Regression Coefficients for Average HDL Cholesterol and Leptin by Tertiles (range) of Average Weekly Hours of Television Watching and Average Vigorous Activities[a], Health Professionals Follow-up Study (HPFS), 1986–1994

Vigorous activities tertile (MET-h[b]/wk)	Television-watching tertiles (h/wk)					
	Leptin			HDL cholesterol		
	3 (12.9–32.9)	2 (6–12.8)	1 (0.1–5.8)	3 (12.9–32.9)	2 (6–12.8)	1 (0.1–5.8)
1 (0.0, 4.0)	0 (reference)	−2.7*	−2.5**	0 (reference)	−0.1	6.5
2 (4.1, 17.9)	−3.2**	−3.6**	−3.8**	−5.8*	−2.7	3.1
3 (18.0, 109)	−2.3*	−4.2**	−5.1***	7.6*	0.7	12.8

[a]Multivariate regression adjusted for age (continuous variable); alcohol (nondrinkers and 0.1–10, 10.1–20, and ≥20 g/d); smoking (never smokers, past smokers, current smokers of ≤14 cigarettes/d, current smokers of ≥14 cigarettes/d); fiber; saturated and polyunsaturated fats (quintiles). Number of subjects ranged from 43 to 60 in each stratum. *$P < 0.05$; **$P < 0.01$; ***$P \leq 0.001$. Reprinted with permission from Reference 20.
[b]MET-h, metabolic equivalent-hours.

ing TV) categories, those who were in the least active (<10 MET-h per week) and the most sedentary (>15 h per week watching TV) categories had a significantly increased risk of type 2 diabetes (RR = 2.92; 1.87–4.55). When a total physical activity score and time spent watching TV were simultaneously included in a multivariate model (without BMI), an increment of 2 h per day spent watching TV was associated with a 20% (95% CI, 8–32%) increase in risk of diabetes, whereas an increment of 18 MET-h per week (equivalent to very brisk walking for 40 min per day) was associated with a 19% (13–24%) reduction in risk. These data indicate that an increase in physical activity is associated with a significant reduction in risk of diabetes, whereas a sedentary lifestyle, indicated by prolonged TV watching, is directly related to risk. These findings suggest the importance of reducing sedentary behavior in preventing of type 2 diabetes.

TV WATCHING AND DIETARY AND OTHER LIFESTYLE FACTORS

In the HPFS, men who spent more time watching TV were more likely to smoke and drink alcohol and less likely to

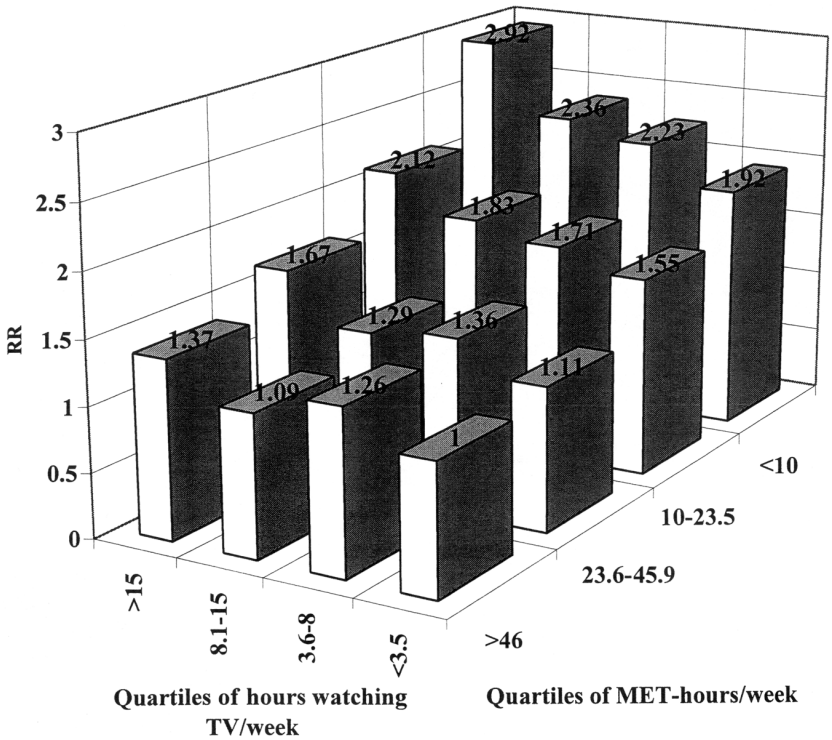

FIG. 3. Multivariate relative risks (RR) for type 2 diabetes mellitus according to categories of MET-h per week and average weekly time spent watching TV. For abbreviations see Figure 1. Reproduced with permission from *Arch. Intern. Med. 161,* 1542–1548 (2001). Copyrighted 2001, American Medical Association.

TABLE 2
Age-Standardized Characteristics According to Average Number of Hours Watching Television per Week in the HPFS at Baseline in 1988[a]

	No. of hours				
	0–1	2–10	11–20	21–40	>40
No. of men	1315	17434	9271	3173	186
Percentage of group					
Current smoking	5.6	7.3	9.5	12.4	20.4
Parental history of diabetes	18.7	19.8	20.9	20.6	19.2
Vitamin E supplement use	21.1	19.6	18.7	17.8	18.2
Hypertension	17.5	20.2	22.8	26.1	22.7
Hypercholesterolemia	15.8	17.9	19.7	22.3	21.5
Means					
Age (yr)	53.1	54.5	55.9	58.1	60.8
BMI	24.6	25.3	25.7	26.0	26.7
Alcohol use (g/d)	9.8	11.0	12.3	13.0	15.2
Physical activity (MET-h/wk)	29.8	29.1	27.4	24.7	19.8

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exercise (Table 2). They were substantially heavier and more likely to have hypertension and hypercholesterolemia. These men also had a higher intake of total energy, total and saturated fats, red meat, processed meat, french fries, refined grain products, snacks, sweets, and desserts and lower intakes of fish, vegetables, fruits, and whole grains (Table 3). Such an unhealthy eating pattern, which is directly related to commercial advertisements and food cues appearing on TV (22,23), has been directly associated with the risk of diabetes (24,25).

DIABETES PREVENTION THROUGH DIET AND LIFESTYLE MODIFICATION

Diet and lifestyle modification are widely considered the primary means to control weight. It is also the most important approach for diabetes prevention. Three clinical trials have demonstrated the beneficial effects of diet and lifestyle intervention on weight loss and diabetes prevention. Among 577 subjects with impaired glucose tolerance (IGT) in Da Qing, China (26), exercise and/or dietary interventions resulted in a 42–46% decrease in the progression from IGT to diabetes during 6 yr of follow-up. In the Finnish Diabetes Prevention Program, lifestyle modification reduced the incidence of type 2 diabetes by 58% in people with IGT (27). The intervention program included a modest weight loss of less than 10 lb (4.5 kg), combined with a healthy diet with less saturated fat and increased fiber intake and regular moderate exercise. In the Diabetes Prevention Project (DPP) (28), 3234 nondiabetic persons with IGT were randomly assigned a placebo, metformin (850 mg twice daily), or a lifestyle-modification program with the goals of at least a 7% weight loss and at least 150 min of physical activity per week. In this study, 50% of the participants in the lifestyle-intervention group had achieved the goal of a weight loss of 7% or more by the end of the curriculum (at 24 wk), and 38% had a weight loss of at least 7% at the time of the most recent visit; the proportion of participants who met the goal of at least 150 min of physical activity per week was 74% at 24 wk and 58% at the most recent visit. During the 2.8 yr of follow-up, the lifestyle intervention plan reduced participants' incidence of diabetes by 58% (95% CI, 48–66%) and metformin by 31% (95% CI, 17–43%) compared with the placebo. Lifestyle intervention was equally effective in both men and women and in different ethnic groups.

Despite the success of these trials, several important questions remain. During the follow-up in the DPP, many participants had regained their initial weight. Although this is a typ-

TABLE 3
Age-Standardized Characteristics According to Average Number of Hours Watching Television per Week in the HPFS at Baseline in 1988[a]

	No. of hours				
Nutrient intake (energy-adjusted)	0–1	2–10	11–20	21–40	>40
Total energy (kJ/d)	8192.3	8322.0	8501.9	8585.6	8669.2
Total fat (g/d)	69.9	71.0	72.5	73.7	73.9
Saturated fat (g/d)	24.0	24.4	25.5	25.6	26.1
Monounsaturated fat (g/d)	26.5	27.1	27.8	28.3	28.2
Polyunsaturated fat (g/d)	2.6	2.8	3.0	3.1	3.1
Trans FA (g/d)	2.6	2.8	3.0	3.1	3.1
Fiber (g/d)	23.6	22.5	21.4	20.6	18.7

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ical phenomenon in weight loss trials, it raises a question of long-term effectiveness of the intervention. A detailed analysis of predictors of weight regain would be helpful for improving long-term maintenance of weight loss. In addition, the dietary intervention in the DPP focused on low-fat, low-calorie diets. Although reducing the percentage of calories from dietary fat intake is commonly recommended for weight loss, long-term clinical trials have provided no convincing evidence that reducing dietary fat *per se* can lead to substantial weight loss (29,30). A hypocaloric moderate-fat diet, which allows greater variety in choosing foods and does not make people feel deprived, can have better long-term compliance and has led to better weight maintenance than a typical low-fat diet (31). In addition, recent evidence from experimental studies suggests that a diet high in carbohydrates, especially for those with a high glycemic index (GI), could play a role in the development of obesity (32,33). In animal studies (34), high-GI diets, compared with isocaloric low-GI diets, induced an increase in fat synthesis even when the total body weight remained constant. A recent review (33) suggested that consumption of low-GI foods and liquids was directly associated with a reduction in subsequent hunger and/or increased satiety in most of the short-term feeding studies in humans (lasting for a single meal or a single day). In addition, voluntary energy intake increased after consumption of high-GI meals compared with consumption of low-GI meals (33). These observations suggest that long-term consumption of high-GI diets may promote excess energy consumption and thus contribute to weight gain or to the maintenance of excess body weight, especially among susceptible individuals (e.g., sedentary or overweight subjects) (32). However, no long-term studies to date have tested the effects of GI on weight control.

SUMMARY AND CONCLUSIONS

Compelling evidence suggests that a sedentary lifestyle, indicated by prolonged TV watching, is an important risk factor for obesity and type 2 diabetes, whereas an increase in physical activity is associated with weight maintenance and a lower risk of obesity and type 2 diabetes. At least two explanations can be given for the observed positive association between TV watching and risk of diabetes. First, TV watching is directly related to obesity and weight gain, probably due to lower energy expenditure (i.e., less physical activity) and higher caloric intake. Second, participants who spent more time watching TV tended to eat more red meat, processed meat, snacks, refined grains, and sweets and fewer vegetables, fruits, and whole grains. Such an eating pattern, which is linked to commercial advertisements and food cues appearing on TV, may adversely affect the risk of diabetes.

Most adults in the United States do not engage in regular exercise, and a substantial proportion of the population is completely sedentary (1). Also, in the past several decades we have seen an increasing trend toward more sedentary behaviors, especially prolonged TV watching (12). The combination of a lack of exercise and an increase in sedentary behavior at least partially contributes to the increasing epidemic of obesity and type 2 diabetes in the United States and worldwide. A public health campaign is urgently needed not only to promote increasing physical activity but also to reduce sedentary behaviors, especially prolonged TV watching, in both adults and children.

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The Role of Energy Density

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ABSTRACT: Dietary energy density (ED) appears to have a major influence on the regulation of food intake and body weight. If people consume a fixed weight of food each day, then high-ED diets should be associated with high energy intakes and with overweight. In contrast, low-ED diets should result in lower daily energy intakes and therefore weight loss. For this approach to work, low-ED foods must be as palatable as high-ED foods and, calorie for calorie, have a greater satiating power. Each of those assumptions is debatable. Dietary ED depends chiefly on the water content of foods. As a rule, high-ED foods are more palatable but less satiating, whereas low-ED foods are more satiating but less palatable. Consumer preferences for high-ED foods can be explained in terms of good taste, low cost, and convenience. Low-ED foods, such as fresh produce, provide less energy per unit cost than do high-ED foods, which often contain added sugars and fats. Poverty and obesity may well be linked through the habitual consumption of a low-cost, high-ED diet.

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Dietary energy density (ED) is said to have a major effect on energy intakes (1–3) and on the long-term control of body weight (4–6). ED has replaced fat content as the principal dietary variable of interest in obesity research (5–8). The World Health Organization (WHO) recently identified high dietary ED as a principal contributor to the global obesity epidemic. High-ED foods and high-ED diets are increasingly held responsible for the rising prevalence of obesity and overweight (9).

Any connection between dietary ED and obesity rests on the premise that people consume a constant weight of food each day, as opposed to a constant amount of energy (3,7,10). Selecting high-ED foods would then increase daily energy intakes and lead to weight gain. In contrast, bulky low-ED foods provide fewer calories per unit volume and deliver less energy per eating occasion (3,12). In principle then, low-ED food choices should result in lower daily energy intakes and therefore weight loss. High-ED diets have been associated with obesity, whereas lowering dietary ED may be a promising approach to weight reduction (11–13).

For the ED approach to work, low-ED and high-ED foods should be equally palatable and have the same satiating power. The overall goal is to make the consumer feel full on fewer calories (10,13). Raw vegetables and fruit, salad greens, soups, and beverages provide between 0.1 and 0.5 kcal/g (5) and have long been available for human consumption. Yet consumers often prefer high-ED sweet and high-fat foods, including snacks, confectionery, sweets, and other desserts (14–16). The ED approach makes a secondary assumption that low-ED foods, those with fewer calories per unit weight, are as satiating as high-ED foods (10,13). In other words, energy density, palatability, and satiety must be independent variables (17).

Although ED and palatability have been separated under tightly controlled laboratory conditions (5,7,18,19), it is unclear whether they are separable in real life. High-ED foods tend to be more palatable than low-ED foods because they often contain fat, sugar, or both (15). Foods that are overconsumed are by definition less satiating than foods that are consumed in smaller amounts (5). As will be demonstrated below, palatability and satiety are inversely linked. As a rule, high-ED foods are palatable but not satiating, whereas low-ED foods are more satiating but less palatable (5,17). To complicate matters, high dietary ED may be linked to lower food costs. Generally, packaged high-ED foods are less expensive than perishable fresh produce, when expressed in terms of dietary energy per unit cost (kcal/$) (20). Reducing the ED of the total diet may therefore involve certain tradeoffs with respect to palatability, satiety, and the economics of food choice. Interactions among these variables are the focus of this review.

WHAT IS DIETARY ED?

The ED of individual foods is a function of their water content. Although early studies emphasized the links between ED and the fat content of foods (1,4), water was, in fact, the determining variable (5). Table 1 shows that the highest-ED foods are those that contain the least water. Dietary energy is provided by fat, carbohydrates, and in some cases sugar (5). The extremes of the ED spectrum are represented by vegetable oil (9 kcal/g) and diet soft drinks (0 kcal/g).

The relationships between ED and the nutrient composition of some common foods are summarized in Figures 1–5. These analyses are based on a 171-item food preference checklist (FPC) that listed a variety of meats, dairy products, grains, vegetables, and fruit as well as alcohol, sugars, oils, and fats (21–23). The food list was adapted from past analyses of National Health and Nutrition Examination Survey (NHANES) II data (24). Comparisons of the FPC with food

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Abbreviations: ED, energy density; FPC, food preference checklist; NHANES, National Health and Nutrition Examination Survey; SI, satiety index.

TABLE 1
Energy Density and Water Content of Selected Common Foods

Food item	Energy density (kJ/g)	Energy density (kcal/g)	Water content (g/100 g)
Safflower oil	36.8	8.9	0
Peanuts, dry	24.4	5.8	1
Candy, milk chocolate	22.6	5.4	1
Cereal, granola type	19.5	4.7	5
Doughnut, cake type	17.8	4.3	20
Potatoes, french-fried	12.9	3.1	40
Pizza, cheese	9.3	2.2	48
Ice cream sundae	7.5	1.8	60
Ham, lean	6.6	1.6	66
Spaghetti with tomato sauce	5.6	1.3	70
Potato, baked	4.6	1.1	71
Yogurt, fruit-flavored	4.4	1.1	85
Soup, split pea	3.1	0.8	86
Yogurt, low-fat plain	2.6	0.6	85
Orange, raw	2.0	0.5	87
Soda, cola type	1.7	0.4	89
Spinach, boiled	1.0	0.2	91
Tomato juice	0.7	0.2	94
Salad, green	0.7	0.2	96
Soda, diet type	0.0	0.0	100

frequency questionnaires have been published previously (21–23).

High-ED foods contain less water per unit weight (Fig. 1). Low-ED foods were vegetables and fruit, whereas high-ED foods included spreads, oils, and fats. The fat content was also related to the ED of foods (Fig. 2). Multiple regression analysis showed that water content alone accounted for 85% of the variance in ED, whereas water and fat together accounted for 99%. Carbohydrate (including sugar), protein, and fiber content of foods played a decidedly lesser role, as indicated in Figures 3–5.

Because of their high water content, raw vegetables and fruit rarely provide more energy than 0.5 kcal/g (2 kJ/g). High water content also keeps the ED of beverages, juices, and soups below 1.0 kcal/g (4.0 kJ/g). Yogurt, ice cream, and some meats are other foods with a relatively high water content. As an alternative to adding water, the ED of foods can be lowered by replacing caloric sugar and fat with low-energy equivalents. Intense sweeteners reduce the ED of soft drinks to zero while maintaining their sweetness (16). To some extent, the ED of foods can also be lowered by incorporating dietary fiber or partially digestible starches, gels, and gums that mimic the texture and mouthfeel of dietary fat (15). The challenge is to reduce dietary ED while keeping palatability constant.

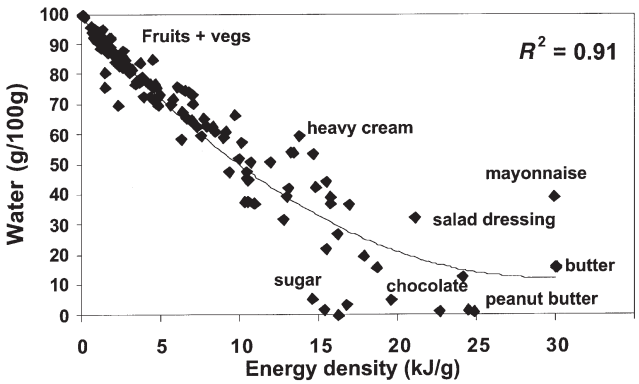

FIG. 1. The relationship between energy density (kJ/g) and water content of foods.

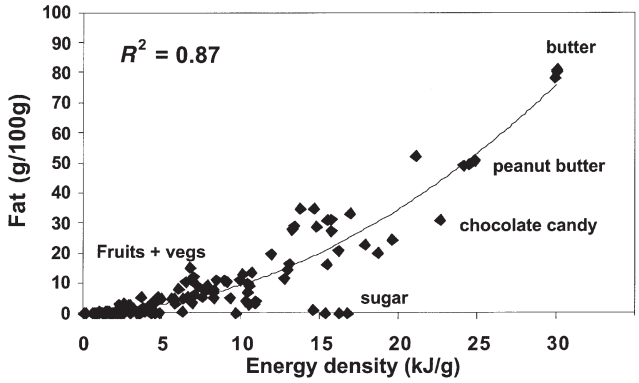

FIG. 2. The relationship between energy density (kJ/g) and fat content of foods.

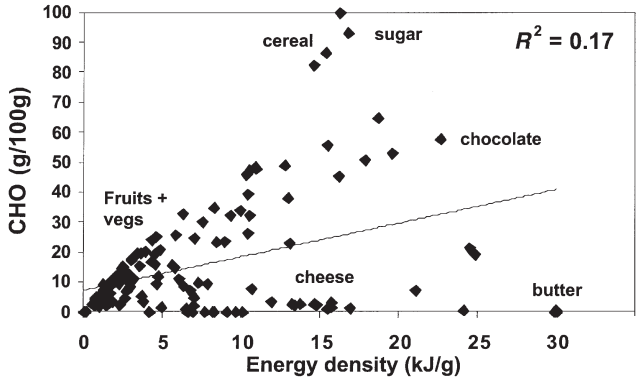

FIG. 3. The relationship between energy density (kJ/g) and carbohydrate (CHO) content of foods.

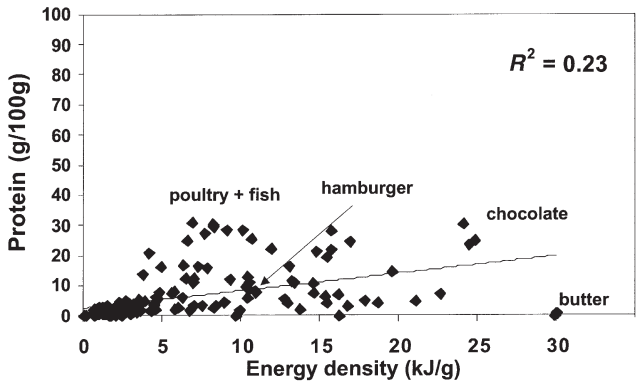

FIG. 4. The relationship between energy density (kJ/g) and protein content of foods.

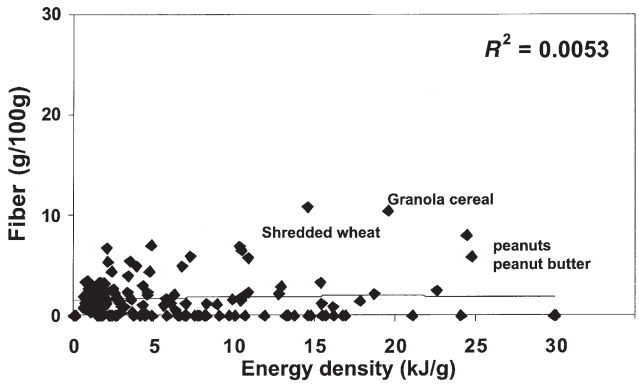

FIG. 5. The relationship between energy density (kJ/g) and fiber content of foods.

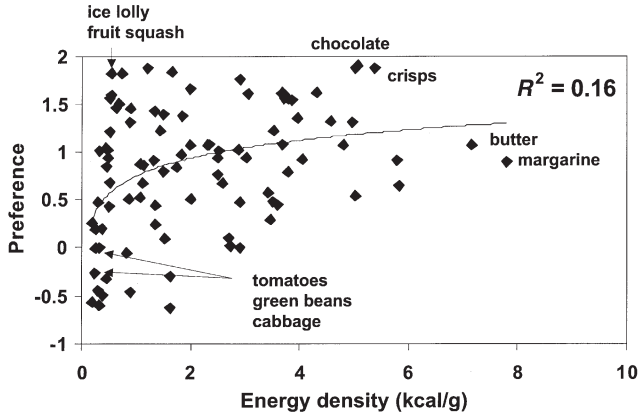

FIG. 6. The relationship between energy density and self-reported food preferences for 4-yr-old children in the United Kingdom [based on data from Wardle *et al.* (27)].

ED AND PALATABILITY

Arguably, foods are palatable *because* they are energy dense (5,17). Sensory enjoyment of sugar and fat is directly related to the fact that both represent concentrated and readily available sources of dietary energy. Although concentrated energy is rewarding *per se*, there seems to be no physiologic drive to select bulky low-ED foods that provide a fraction of a calorie per gram. High-ED chocolate tastes better to most people than low-ED spinach, and is consumed with more pleasure and satisfaction. Given a choice, young children prefer high-ED foods to foods that deliver less energy per unit weight (25,26).

The question is whether high-ED foods are indeed more palatable than low-ED foods. Chocolate cake, hamburgers, and french fries provide from 12 to 18 kJ/g. Chocolate candy and peanut butter provide 22–24 kJ/gram. In contrast, most salad greens are low-ED foods, providing <1 kJ/g. By definition, there are no high-ED beverages. ED values of regular cola, orange juice, and 1% milk are almost exactly the same at 2 kJ/g.

Apart from anecdotal reports, the relationship between ED and food palatability is not well documented. Food preference studies tend to rely on self-reported preferences for food names, not for actual foods. The best such data were obtained from persons least subject to social desirability concerns or dietary restraint, in other words, children. A reanalysis of data obtained with 4-yr-old children in the United Kingdom (27) showed that their food preferences were related to the sweetness and ED of foods (Fig. 6). The best-liked foods were either energy dense or sweet. A positive relationship between ED and food preferences had also been observed in young and mostly male Army personnel (5,28).

Food preference data obtained from college-age women are altogether different. Although children and young men prefer high-ED potato chips to yogurt and fruit, many young women report precisely the opposite. In a sample of 159 college women, raspberries were the most highly preferred food, outranking chocolate, french fries, and pizza. There was no significant correlation between ED and food preferences. In past studies, young women suffering from the eating disorder anorexia nervosa also reported higher preferences for salad greens than for ice cream or chocolate (29,30). Similarly, older women reported higher preferences for fresh vegetables and fruit than for higher-ED foods (21). The relationship between ED and food palatability, as measured by self-reported food preferences, is strongly influenced by age, gender, and diet-related attitudes and behaviors. Although observed among children and young men, it has not been observed consistently among women.

DIETARY ED AND SATIETY

Feeling full on fewer calories requires that satiety be influenced not only by dietary energy but also by the weight or volume of the foods consumed (10,13). Most laboratory studies on ED and satiety have addressed this issue using a study design known as the preload paradigm (17). In such studies, ingestion of a caloric preload was followed by repeated measures of hunger and satiety and by the eventual consumption of a test meal. Preload ED was manipulated by keeping preload energy constant while altering volume, or by keeping volume constant while altering energy. In most studies, preload volume was altered by using water. Preload energy was also altered by using fat, sugar, intense sweeteners, fat replacement products, and sometimes fiber (11,12,18,19). In fact, many studies in this area were focused not so much on ED but on the effect of intense sweeteners and fat replacements on hunger and satiety (31–34).

Studies that provided subjects with preloads of constant volume but different energy yielded inconsistent results. Some did demonstrate differences in hunger and satiety between low-ED and high-ED conditions, whereas others did not. In our studies (18,19), participants consumed a breakfast consisting of large volume (500 g) of fromage blanc (a soft creamy white cheese) that was sweetened with sucrose (700 kcal) or aspartame (400 kcal). Although preload volume was constant, the ED varied from 2.5 to 5.8 kJ/g. Participants reported being equally satiated by high-ED and low-ED preloads in the first 30 min postingestion. However, it would be

incorrect to assume that energy had no effect on satiety. Measurable differences in hunger, fullness, and satiety scores became apparent later, reaching a peak ~2 h postingestion. Studies in which the time interval between preload and the test meal is <20 min are liable to conclude, incorrectly, that preload energy has no effect on satiety. Although regular and diet soft drinks may have the same initial effect on hunger and satiety, the effect of calories does become apparent with time (35).

Studies that kept preload energy constant and varied preload volume showed more agreement. Isoenergetic preloads with higher volume promoted satiety more effectively, at least in the short term (2,3). Those data are consistent with the notion that satiety is influenced primarily by dietary energy and that preload volume may have some additional effects that are observed shortly postingestion.

Recently, studies of ED have gone beyond the standard manipulations of water, sugar, and fat. One study varied ED by altering the proportion of vegetables to pasta in a pasta salad (3). Study subjects given meals of varying ED consumed the same amount of food by weight so that energy intakes were a direct function of ED level (3). As a result, subjects in the low-ED condition consumed 30% less energy over a 2-d period without a major reported effect on hunger or satiety. Matching foods for palatability was a critical part of study design because reduced consumption of low-ED foods might be explained by their reduced appeal and lower palatability.

As semiliquid preloads are replaced by real foods, the palatability issue becomes more of a problem. The satiety index (SI) of foods was an early attempt to map the relationship between ED and satiety. In that study (36), satiety ratings were measured every 15 min for 2 h after the consumption of 240-kcal portions of 38 common foods. The SI was calculated by dividing the area under the curve for each food by that for white bread and multiplying by 100%. SI values were correlated most highly with food weight, i.e., with ED. A ration of 240 kcal was provided by 38 g of peanuts or by 625 g of oranges (36). As might be expected, the more bulky and low-ED foods had the highest SI values, with potatoes, porridge, and fish proving to be more satiating than chocolate, cookies, and cakes. However, in that study, as in many others, the most satiating foods were also rated the least palatable (36).

PALATABILITY AND SATIETY

Researchers have struggled to explain why people overeat high-ED foods, especially those that contain sugar and fat. Some have argued that the high palatability of sweet and high-fat foods overrides normal satiety signals, thereby leading to overeating and overweight (37–40). The question was whether such foods were overeaten because of their high ED or their high fat content. Some researchers have argued that satiety effects were macronutrient specific, and that fat was less satiating than an isocaloric amount of carbohydrate or protein. Indeed, the phrase "passive overconsumption" was launched to account for the overeating of fat-rich foods (40). Other researchers believe that satiety is volume driven, such that bulky low-ED foods are more satiating, independent of their fat content.

Palatability and satiety, or more properly satiation, have opposite effects on food intakes. Palatability increases appetite and therefore energy intakes, whereas satiation limits consumption by reducing meal size and satiety delays the time to the next meal (5,17). Palatability is the property of the food itself, coupled with the appetitive state of the consumer. Commonly used measures of palatability include the rated pleasantness of a given food, intent to eat, and the amount of food consumed (5).

Satiation, defined as an internal state of energy repletion after a meal, has been measured in terms of fullness, reduced intent to eat, and reduced amount of food consumed (5,17). Satiety has also been measured in terms of reduced palatability. For example, sensory-specific satiety was defined in terms of reduced palatability of the just-consumed food relative to other foods (41). In other words, our measures of palatability and satiation are inversely linked (5,17). The most palatable foods are, by definition, the least satiating and *vice versa*. As shown by Holt *et al*. (36), the more palatable cake, cookies, and chocolate were less satiating than the less palatable porridge, potatoes, and boiled fish. If palatability and satiety are inversely linked, then the creation of highly palatable and yet satiating foods is a contradiction in terms.

Laboratory studies on ED and satiety have tried to keep palatability constant. Their chief intent was to demonstrate that low ED foods could be just as palatable as high-ED foods and just as acceptable to the consumer. In some cases, reducing ED had no effect on palatability ratings. However, the ED of most test foods was already low (3-4 kJ/g) and was allowed to vary within only a narrow range, from 3 to 6 kJ/g (2,3,7). All test stimuli had a high water content and included yogurts, soups, and a semiliquid white cheese, fromage blanc (5). Maintaining good taste while varying ED over a broader range may present more of a challenge.

ED OF THE DIET

Most studies on ED have been based on individual foods, single dishes, or single meals (5,6), although a few have addressed the ED of the total diet (42–44). Dietary ED is difficult to establish because there is little consensus concerning whether water, noncaloric beverages, caloric beverages, soups, semiliquids, or semisolids ought to be included or excluded from analyses. Current research suggests that water consumed as a beverage and water incorporated into fruit, juices, or soups may have a differential effect on satiation and satiety (45). Diet structure may turn out to be the more important variable because different types of diets may have similar ED values.

Whether people consume a fixed weight or volume of food and beverages each day is not always clear. Although this may be the case under laboratory conditions (3,5), epidemiologic surveys suggest that the weight of foods and beverages

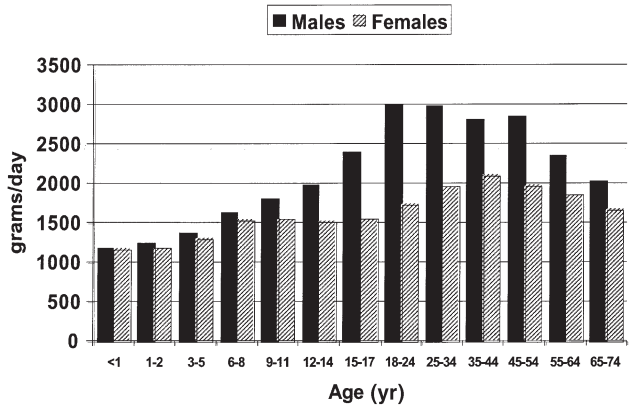

FIG. 7. Mean amounts of food (g) consumed by age group in the NHANES II data (1976–1980).

declines sharply with age. A reanalysis of 1976–1980 data from the NHANES II survey (46) showed that both energy intakes and the weight of foods and beverages declined with each advancing decade of life (47). Older adults in the NHANES II data set consumed progressively less food (Fig. 7). Energy intakes also declined as a function of age.

The mean dietary ED was influenced by both sex and age (47) (Fig. 8). Dietary ED dropped from a peak of 1.2 kcal/g in childhood and adolescence to a low of 0.7 kcal/g for adult women 45–54 yr old. Given that intakes of vitamin C (mg/1000 kcal) increase steeply after the age of 35 yr, particularly for women, it seems likely that the reduction in dietary ED was achieved through increased consumption of vegetables and fruit. Age-associated increases in vegetable and fruit intakes, observed in other studies, are wholly consistent with that hypothesis. In fact, an argument has been made that varying the energy density of foods is the body's prime mechanism for regulating energy intakes during the life cycle. Although children, adolescents, and young adults consume high-ED diets, older adults may reduce energy intake by reducing both food volume and dietary ED (48).

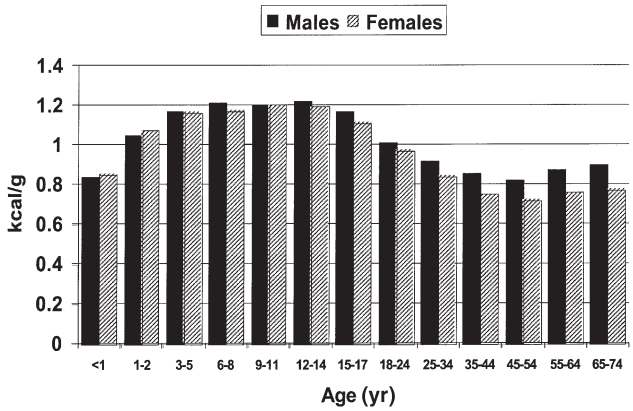

FIG. 8. Mean energy density of the diet (kcal/g) by age group in the NHANES II data (1976–1980).

Dietary ED is strongly affected by age. Aging is associated with lower energy intakes, lower intakes of fat and saturated fat, and higher intakes of dietary fiber, vegetables, and fruit (46,49). Older adults consume more whole grains, vegetables, more cruciferous vegetables, green leafy and other vegetables, and more fruit than do younger adults (49). Adults over the age of 59 yr are more likely to consume fruit, tomatoes, breakfast cereals, and whole-grain breads than those 18–59 yr old and less likely to consume sweets, snacks, and carbonated sugar-based beverages (50,51). Consumption of at least five servings of fruit and vegetables per day (50) also rises with age. Data obtained from 23,699 adults in 16 U.S. states, as part of the Behavioral Risk Factor Surveillance System, showed that twice as many women over the age of 65 yr (29%) complied with the five-a-day diet compared with women aged 18–24 yr (14%) (51).

DIETARY ED AND FOOD COSTS

The fact that dietary ED is primarily a function of the water content of foods has economic consequences. Packaged, dry, high-ED foods with a stable shelf life provide more energy per unit cost than perishable low-ED fresh produce with a high water content. As noted in Table 2, lower-ED foods typically provide fewer calories per unit cost than do high-ED foods. Consumers select foods on the basis of taste, cost, and convenience (52), with cost being of most concern to low-income families. Not surprisingly, higher-income groups in both Britain and the United States tend to have higher-quality diets (53–55). Obesity, in turn, is associated with limited economic resources, and low levels of education and income.

Compliance with dietary guidelines for health promotion is often poor, especially among low-income respondents. Studies on dietary compliance in weight loss have addressed a variety of metabolic and cognitive variables, from glycemic index to information about the fat content of foods. Yet concerns about the increased cost of low-ED diets have not become a part of mainstream obesity research. The possibility remains that food expenditures are a major barrier to dietary change. Simply put, palatable high-ED foods containing sugar and fat provide more energy per unit cost than any low-ED

TABLE 2
Energy Density and the Cost of Selected Foods (kcal/$)

Food item	Energy density (kcal)	(kcal/g)	(kcal/$)
Oil (1 tbs)	120	8.8	3852
Peanuts, roasted (1 oz)	180	5.9	1147
Chocolate bar, Hershey (1.5 oz)	230	5.3	460
Cheese nachos (7 oz)	810	4.0	967
Ice cream, Häagen Dazs (1 cup)	540	2.5	332
Sirloin steak (8 oz)	390	1.6	260
Fruit yogurt, low-fat (8 oz)	230	1.0	291
Apple (1 fruit)	90	0.6	270
Broccoli, cooked (1/2 cup)	20	0.3	170
Spinach, raw (1 cup)	10	0.2	90

lalternatives. A relationship between ED and food costs for selected foods is outlined in Table 2.

DIETARY ED AND BODY WEIGHT

Higher-ED diets are linked to increased consumption of fast foods, sweets, and desserts. Lower-ED diets are associated with increased consumption of vegetables and fruit. Studies have linked obesity to fat consumption and to the percentage of fat energy in the total diet. Although dietary ED and dietary fat content are linked, there is no population-based evidence at this point that high-ED diets are associated with obesity in either women or men. It remains to be demonstrated that ED alone, independent of fat content, is the causal factor in promoting body weight gain.

Reducing dietary ED can be accomplished by selecting more bulky and lower-ED foods that provide fewer calories in a larger volume. Laboratory studies suggest that such foods can be as palatable and as satisfying as high-ED foods. Real-life experience suggests otherwise. High-ED foods containing fat, sugar, and sometimes salt tend to be more palatable than lower-ED options. Packaged high-ED foods are arguably more convenient to use, and eating away from home provides a variety of high-ED options. Perhaps most important, high-ED is often associated with lower food costs, expressed as calories per dollar. Lowering dietary ED may be a potent option for weight reduction and it is currently being promoted as such. However, consumers' food choices are based largely on taste, cost, and convenience. For the purposes of obesity research, food costs and the economics of food choice merit a closer look.

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Dietary Glycemic Index and the Regulation of Body Weight

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ABSTRACT: Prevalence rates of overweight and obesity have risen precipitously in the United States and other developed countries since the 1960s, despite comprehensive public health efforts to combat this problem. Although considerable attention has been focused on decreasing dietary fat and increasing physical activity level, the potential relevance of the dietary glycemic index to obesity treatment has received comparatively little scientific notice. This review examines how the glycemic and insulinemic responses to diet may affect body weight regulation, and argues for the potential utility of low glycemic index diets in the prevention and treatment of obesity and related complications.

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All dietary carbohydrate can be digested or metabolically transformed into glucose. However, carbohydrate-containing foods can alter blood glucose and insulin levels in markedly different ways. For this reason, Jenkins and colleagues (1) proposed a systematic classification of carbohydrates according to glycemic response. The glycemic index (GI) is defined as the 2-h area under the glucose response curve after consumption of 50 g carbohydrate from a test food compared with the area under the curve after consumption of 50 g carbohydrate from a control food, either white bread or glucose (2). A related term, glycemic load (GL), was recently proposed to account for differences in macronutrient composition among foods, meals, or diets. GL of a food or meal is defined as the individual or average GI multiplied by the grams of available carbohydrate present (3). To date, >100 studies have been published describing the GI of most commonly consumed carbohydrate-containing foods, as compiled by Foster-Powell and colleagues (4).

The GI of a food is determined by carbohydrate type and by other dietary factors affecting food digestibility, gastrointestinal motility, or insulin secretion (2,5–10). Because the hydrolysis of glucose polysaccharides in the gut is not rate limiting (11), the GI of refined starchy foods approach that of glucose itself (12). By contrast, fructose and galactose require metabolic transformation in the liver, a slow process conferring these sugars with a relatively low GI (12). Food structure may slow digestion for mechanical reasons; thus, whole-kernel grains tend to have a lower GI than milled grain products (7). The method of food preparation may influence GI, leading to the difference, for example, between pasta *al dente* vs. fully cooked. Fat or fiber in a meal lowers the GI by delaying gastric emptying or by acting as a physical barrier to glucose diffusion in the small intestine, respectively (8–10, 13). Finally, protein tends to lower GI by augmenting insulin secretion (10,14). In general, nonstarchy vegetables, fruits, and legumes have a low GI, whereas processed grain products and potato have a high GI. The GI of individual food items may be used to predict that of a mixed meal or, by implication, a diet (12).

DISCUSSION

Studies of GI and short-term regulation of food intake. Satiety after a meal appears to be inversely related to glycemic and insulinemic responses. For example, Haber and colleagues compared the effects of various apple-based meals, each containing equal amounts of carbohydrate and eaten at the same rate (15). Apple juice induced high insulin levels, reactive hypoglycemia, and low satiety scores. By contrast, whole apples produced low insulin levels, no reactive hypoglycemia, and high satiety scores. Apple purée yielded intermediate values for these parameters. Among various breakfast cereals, GI was inversely related to cholecystokinin levels and satiety (16). Glycemic and insulinemic responses to rice preparations (17) or starch-based meals (18) were inversely related to satiety. Addition of a low- (bean) compared with a high- (potato) GI starch to a test meal delayed the return of hunger (19). Finally, fructose, a low-GI sugar, when administered 2.25 h before a test meal, significantly decreased food intake compared with water or glucose preloads (20–22). Of 16 published studies on GI and appetite regulation, all but one demonstrated increased satiety, delayed return of hunger, or decreased food intake after low- compared with high-GI foods (23), although some of these studies did not control for other potentially confounding dietary variables.

We examined the potential physiologic mechanisms relating GI to the short-term regulation of food intake in a study comparing three meals with identical energy content but differing in GL (24). The high- and medium-GL meals were oatmeal preparations differing in food structure but controlled for macronutrient composition, fiber content, energy density, and palatability. The low-GL meal (a vegetable omelet with fruit), differed in many of these variables from the oatmeals,

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Abbreviations: GI, glycemic index; GL, glycemic load.

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TABLE 1
Meal Composition Based on Sample Test Meals Containing 1.65 MJ[a]

	Low glycemic load	Medium glycemic load	High glycemic load
Food	55 g whole egg 45 g egg white 40 g low-fat cheese 200 g spinach 30 g tomato 185 g grapefruit 115 g apple slices	63.9 g steel-cut oats 160 g 2% milk 15 g Half and Half cream 16.0 g fructose 0.0 g saccharine 397 g water	60.9 g instant oatmeal 160 g 2% milk 15 g Half and Half cream 19.0 g dextrose 0.2 g saccharine 397 g water
% Energy			
From carbohydrate	40	64	64
From protein	30	16	16
From fat	30	20	20
Energy density (kJ/g)	2.46	2.52	2.52

[a]Meal size individualized to provide 18.5% of predicted resting metabolic rate. Food quantities are precooked weights. Reproduced by permission of *Pediatrics* (Ref. 24).

but was included to increase the possible magnitude of effect beyond what could be achieved by manipulating food structure alone (Table 1). The subjects, 12 obese teenage boys, were admitted to the General Clinical Research Center at Children's Hospital, Boston, the day before each study, given a standard dinner and bedtime snack, and put to bed at 2200 h. The next morning, the boys were awakened at 0645 h, an intravenous line was placed, and one of the three test meals was given. During the subsequent 5 h, blood samples were obtained every 30 min for measurement of hormones and metabolic fuels. At lunch, a second test meal (identical to the breakfast meal) was given and voluntary food intake (quantity of food consumed *ad libitum* from snack platters) was determined over 5 h. Each of the three admissions was separated by a 1- to 2-wk washout period.

The hormonal and metabolic responses to the three test meals are shown in Figure 1. Glycemic and insulinemic responses differed among the meals as expected (high GL > medium GL > low GL). The relatively high insulin and low glucagon concentrations observed after the high-GL meal would be expected to promote uptake of glucose in muscle, liver, and fat tissue; restrain hepatic release of glucose; and inhibit lipolysis. As a consequence, access to the two major metabolic fuels was diminished in the postabsorptive period, as demonstrated by the lower concentrations of blood glucose (nadir was –0.5 mmol/L lower, $P = 0.02$) and FA (mean concentration from 2.5 to 4.5 h, $P < 0.05$) after the high- compared with the low-GL meal. A surge in the counterregulatory hormones, epinephrine and growth hormone, underscored the significance of these metabolic changes.

During the 5 h after the test meal given at lunch, subjects consumed significantly more energy after the meal with the high GL (5.8 MJ) than after the meal with the medium GL (3.8 MJ, $P < 0.05$) or low GL (3.2 MJ, $P = 0.01$). Thus, high glycemic response meals appear to induce a sequence of metabolic changes that limit access to metabolic fuels in the postabsorptive period and cause overeating. This difference in voluntary energy intake, if maintained over several months, would be expected to produce substantial differences in body weight.

Effects of glycemic response on body weight "set-point." Studies suggest that body weight is regulated within a specific range (25), and this body weight "set-point" is believed to have a substantial genetic basis (26). However, the increasing prevalence of obesity among genetically stable populations (27) indicates that environment must also play an important role in body weight regulation. Glycemic response patterns, by affecting availability of stored metabolic fuels, may modify the physiologic adaptations to energy restriction.

We compared the effects of high- and low-GL diets on the physiologic adaptations to energy restriction in a randomized, crossover, in-patient study (28). The two diets differed in macronutrient composition (high GL: 67% carbohydrate, 15% protein, 18% fat; low GL: 43, 27, and 30%, respectively) but had similar total energy, energy density, and fiber content. The subjects, 10 moderately overweight young men, were admitted for 9 d on two separate occasions. On days 1 to 0, they consumed self-selected foods *ad libitum* and baseline measurements were obtained. On days 1–6, they received the high- or low-GL diet with energy intake limited to 50% of predicted energy expenditure (29). On days 7–8, they continued on the diets but were allowed to eat *ad libitum*.

Both high- and low-GL diets resulted in similar degrees of weight loss (3.6 ± 1.1 vs. 3.2 ± 0.9 kg, $P = 0.26$) as expected because the two treatments provided identical amounts of energy. The area under the glycemic response curve was twofold greater ($P = 0.001$), and the area under the insulinemic response curve was 1.5-fold greater ($P = 0.01$), on day 1 of the high-compared with the low-GL diet. Serum leptin decreased more rapidly and to a greater extent during energy restriction with consumption of the low-GL diet compared with the high-GL diet ($P = 0.03$). Resting energy expenditure

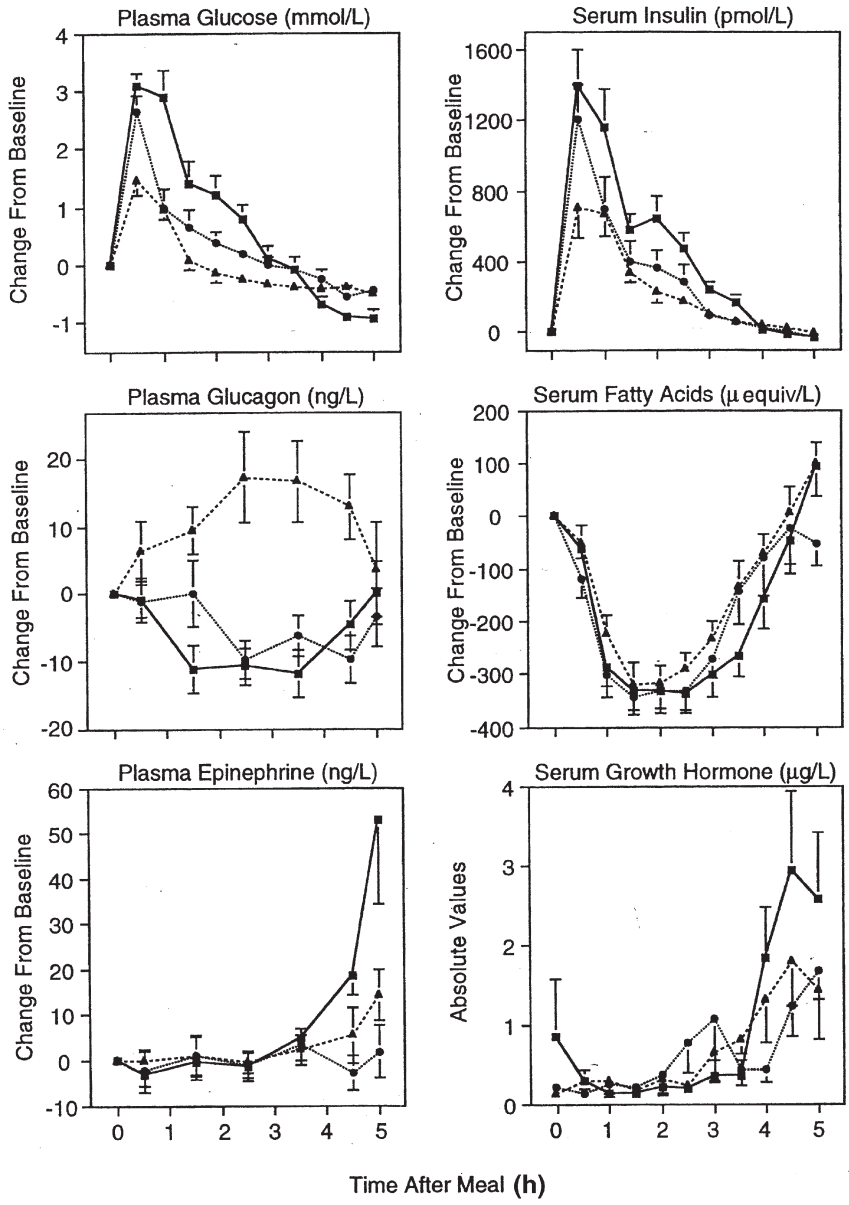

FIG. 1. Hormonal and metabolic changes after test breakfasts. High-glycemic load (GL) meal (■); medium-GL meal (●); low-GL meal (▲). Reproduced with permission of *Pediatrics* (Ref. 24).

declined by 10.5% during energy restriction with consumption of the high-GL diet, but by only 4.6% with the low-GL diet (resting energy expenditure on days 5 and 6, 7.38 ± 0.39 vs. 7.78 ± 0.36 MJ/d, $P = 0.04$) (Fig. 2). Energy intake from snacks consumed *ad libitum* on days 7 and 8 was 25% greater with the high- compared with the low-GL diet (17.0 ± 2.2 vs. 13.5 ± 1.9 MJ , $P = 0.009$). Nitrogen balance tended to be more negative with the high- than with the low-GL diet [–9.7 ± 5.5 vs. +25.7 ± 14.1 mg N/(kg·d), $P = 0.06$], despite the fact that both diets were protein sufficient (providing 56 or 100 g/d, respectively, for the typical subject). In summary, this study demonstrates that a diet designed to elicit a low glycemic response may have beneficial effects on energy expenditure, voluntary food intake, and nitrogen balance.

Effects of glycemic and insulinemic response on weight loss. Slabber and colleagues (30) conducted a 12-wk study of an energy-restricted diet designed to minimize postprandial insulin response compared with that of a standard diet. The subjects, 30 hyperinsulinemic but otherwise healthy obese women, consumed diets containing ~5 MJ/d as outpatients. Both the low-insulinemic and standard diets contained 50% carbohydrate, 20% protein, and 30% fat but differed in the GI of the carbohydrate-containing foods (e.g., lentils and pasta vs. white bread and potatoes) and the temporal pattern of macronutrient consumption. Fasting insulin concentrations decreased to a greater extent with consumption of the low-insulinemic diet than the standard diet (91.3 ± 61.8 vs. 21.0 ± 71.5 pmol/L, $P < 0.05$). Interestingly, subjects in the crossover phase of the study lost

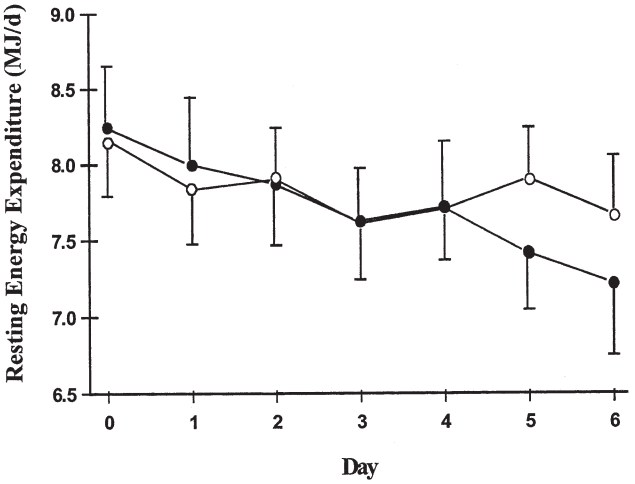

FIG. 2. Change in resting energy expenditure during energy restriction with the consumption of low- (○) and high- (●) GL diets. Reproduced with permission of the American Society for Clinical Nutrition. © *Am. J. Clin. Nutr.,* American Society for Clinical Nutrition (Ref. 28). For abbreviation see Figure 1.

2.94 kg (95% confidence interval 0.14–5.75) more with consumption of the low-insulinemic than the standard diet. Bouche and colleagues (31) recently reported results from a crossover study involving 11 overweight men treated with isoenergetic low- and high-GI diets for 5 wk. Body fat mass was 700 g lower among subjects consuming the low- compared with the high-GI diet. Our group conducted an outcomes assessment of 107 patients attending an academic obesity treatment clinic over a mean of 4 mon (32). We found that children instructed to follow a low-GI diet with *ad libitum* consumption lost significantly more weight than those instructed to follow an energy-restricted low-fat diet (−2.03 vs. +1.31 kg, $P < 0.001$).

SUMMARY

To date, there are no published long-term, randomized controlled trials of a low-GI diet in the treatment of obesity, perhaps because of inherent difficulties of experimental design. The glycemic and insulinemic responses to a meal, as discussed above, are affected by a variety of dietary factors. To obtain maximal differences in these responses for the purposes of a clinical trial, it is necessary to vary the macronutrient composition and fiber content of the experimental and control diets, but such a study cannot attribute any experimental outcomes to the specific effects of GI alone. Short-term feeding studies in a metabolic ward can achieve meaningful differences in these responses between experimental and control diets, while controlling for potentially confounding factors such as macronutrient composition, by manipulations of carbohydrate type, food form, and preparatory methods. However, such studies may have limited relevance to understanding long-term body weight regulation in individuals consuming food *ad libitum*. Conversely, it can be difficult to control for macronutrients and fiber content and also to maintain substantial differences in glycemic responses over the long term in an outpatient setting in which subjects prepare their own meals.

The challenges involved in examining the long-term effects of GI should not be taken to mean that this dietary principle is irrelevant. To the contrary, theoretical and empirical work suggests that the glycemic and insulinemic responses to diet may indeed influence energy metabolism and body weight regulation. Acutely, low-GI meals appear to improve access to stored metabolic fuels and promote satiety. Over more extended periods, the lower insulin levels associated with low-GI diets may facilitate weight loss through a variety of physiologic mechanisms (33). Innovative, well-designed prospective studies are warranted to determine the actual role of low-GI and low-GL diets in the prevention and treatment of obesity. The need for this work is clear in view of the poor long-term success of conventional dietary treatments options (34).

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Dietary Fat and Body Weight Control

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ABSTRACT: The global obesity epidemic has heightened the debate about dietary factors contributing to weight gain. Media stories have promulgated the notion that obesity has increased despite reductions in dietary fat intake. Some have even speculated that lower dietary fat levels may be driving the rapid rise in weight gain within the population. A close examination of the science reveals a different picture and supports the hypothesis that dietary fat, within the context of the total dietary composition consumed by many populations, promotes obesity. Hence, dietary fat control is still an important strategy as part of an overall approach to body weight management in our modern environment. Dietary fat increases the energy density of foods. Abundant evidence from preclinical and clinical studies indicates that fat promotes excess energy intake and positive energy balance. Dietary fat does not promote its own oxidation in the body and is stored efficiently, promoting a positive fat balance. Thus, both the behavioral and metabolic responses to dietary fat increase the probability of positive energy balance and body fat gain. Restoring fat balance when consuming diets rich in fat requires increasing the size of the body fat mass, increasing physical activity, or reducing dietary fat intake. Numerous epidemiologic, preclinical, and controlled clinical studies have shown that body fat is positively associated with dietary fat intake and that dietary fat manipulation leads to appropriate changes in body fat mass. Finally, data from the National Weight Control Registry, a database of >3000 individuals who have successfully maintained a substantial weight loss, indicate that moderating dietary fat intake is a key strategy for long-term management of body weight.

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There has been heightened debate in recent years about the optimal dietary composition to promote body weight control (weight loss and maintenance). Much of this discussion has been driven by the observation that the prevalence of obesity has risen rapidly during the last two decades, whereas nutrition surveys indicate that dietary fat intake has gone down as a percentage of total energy consumption (1). The decade of the nineties saw the introduction of thousands of new food products that were fat free or reduced in fat, yet the population continues to gain weight. Does this mean that fat reduction is an ineffective strategy for body weight control? Might reduced-fat foods and diets actually promote weight gain? What should we tell people about dietary fat in the face of the emerging obesity epidemic?

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A reasoned answer to any of these questions requires a deeper examination of the scientific evidence about dietary fat and its effects on total energy intake, energy expenditure, and fuel oxidation, and the interaction of these with body weight and body composition. This brief review will consider these issues as well as the importance of considering the total dietary and lifestyle context in which dietary recommendations about fat and weight control are made.

HAS FAT INTAKE REALLY GONE DOWN?

National nutrition surveys have reported that fat intake has gone down as a proportion of total energy intake from ~42% of total energy in the mid-1960s to ~35% of energy by 1990 (2). These same surveys indicate that absolute fat intake (g/d) also declined between the 1960s and through the 1980s (2). After that, absolute fat intake stabilized and actually began increasing in the mid-1990s (2). The stabilization and increase in daily grams of fat intake coincide with the period of most rapid increase in obesity prevalence (3). These recent increases in fat intake also coincide with an upward trend in energy intake within the population. Media stories have speculated that the increase in total energy intake concomitant with a drop in the percentage of dietary fat energy may suggest that consumers have taken license to overconsume fat-free and reduced-fat foods as if there were no consequences for total energy intake. There is, however, little evidence for this because use of reduced-fat foods remains very low in the population (2,4) and cannot explain either the population-based decline in relative fat intake or the rising prevalence of obesity affecting all population segments.

It is critical that we recognize these trends in absolute fat intake, given that the propensity of dietary fat to promote obesity is not a pure function of its relative level in the diet but also of the absolute level in the diet in concert with the prevailing levels of carbohydrate consumed from most typical diets.

DIETARY FAT AND ENERGY INTAKE

The influence of any dietary nutrient or composition on energy balance and body weight is a function of its composite effect on energy intake and energy expenditure, i.e., total energy balance. To promote weight gain, the diet must promote excess energy intake and/or reduced energy expenditure compared with the weight stable condition. Evidence from a variety of sources supports the hypothesis that dietary fat increases the probability of excess energy intake.

Fat increases the energy density of foods. Fat contains the greatest amount of metabolizable energy per gram (9 kcal/g) compared with the other major energy-yielding nutrients in the diet, carbohydrate and protein (4 kcal/g for each). The fat content of a food, therefore, has a disproportionate effect on its caloric density relative to the contents of other macronutrients (5). Because of this, for a given volume of food consumed, people will consume more energy if the food is high in fat compared with one lower in fat. At least in the short term, there is evidence that people tend to consume a constant volume or weight of a given food, independent of its energy density (6). Thus, short-term energy intake is generally directly related to the energy density and fat content of the food consumed.

High energy density is not an intrinsic property of high-fat foods. For example, a salad served with oil and vinegar dressing is high in fat calories as a percentage of total energy, but low in energy density owing to the high water and fiber content of the vegetables. However, from a practical point of view, high fat-energy foods that tend to be prevalent in the food supply and that are often hypothesized to contribute to weight gain are also high-calorie, high energy density foods. These foods are generally low in both moisture and fiber, such as popular fried foods and snacks. Under these typical conditions, fat content is a surrogate for the energy density of the food.

Fat promotes excess energy consumption. High levels of dietary fat are associated with so-called "passive overconsumption," denoting the propensity of people to spontaneously eat excess energy when offered high-fat food compared with when they are offered reduced-fat versions of the same food (7). This phenomenon is linked to both the high palatability and high energy content of high-fat food and to the relatively weak satiating effect of fat compared with carbohydrate or protein on a kcal/kcal basis (8).

Probably the most potent influence of dietary fat on energy balance is its effect on energy intake. Numerous animal studies have shown clearly that dietary fat promotes excess energy consumption and obesity relative to lower-fat diets (9). These studies generally control for the level of physical activity, allowing more precise isolation of the effect of the dietary manipulation. The inability to control for physical activity in human epidemiologic and other population-based studies often confounds the ability to observe the independent effect of the diet and its components on energy balance.

Controlled dietary manipulation studies in humans are consistent with the animal data and show that energy intake is positively related to dietary fat content. Clinical studies ranging from 1 to 11 wk showed that when the dietary fat level was raised from 15–20% of energy as fat to 30–60% of energy as fat, total energy intake increased from 114 to >800 kcal/d in the different studies (10–14). These findings were relatively consistent in both men and women and among lean and obese subjects.

A recent meta-analysis of *ad libitum* dietary intervention studies ranging in duration from 9 wk to 1 yr in humans found that when dietary fat was reduced by 3–18% from habitual levels, energy intake was spontaneously reduced by 200–750 kcal/d (15). Thus, both animal and human data consistently indicate that when the dietary fat level is increased, energy intake is elevated and when the fat level is reduced, energy intake is reduced.

Is the effect of dietary fat on energy intake due to fat per se *or to energy density?* Because energy density varies as a function of fat content in most foods, many studies examining dietary effects on energy intake are confounded by the inability to separate the independent effects of energy density vs. fat, *per se*. Recent research has shed some light on this and has shown that energy density has important effects on energy intake, independent of fat content (16,17). Although energy density plays an important role, other evidence suggests that fat has a further independent effect to promote positive energy balance (8). From a practical standpoint, given the close association of fat and energy density in foods popular in Western culture (e.g., fast foods), it may be less important to quantify the precise independent contribution of each obesity-promoting mechanism and more important to make sure dietary recommendations account for foods that have low energy density, but are high in fat-energy.

DIETARY FAT, ENERGY EXPENDITURE, AND FUEL METABOLISM

Dietary composition and total energy intake have relatively minor effects on energy expenditure in the short term. The body has only a limited capacity to adjust metabolic energy expenditure to offset changes in energy intake to maintain body energy stores (18). Changes in energy expenditure are generally seen as a secondary consequence of alterations in body weight, which is the major determinant of total energy expenditure in sedentary individuals (18). Dietary composition, however, can have a pronounced effect on the fuel mixture burned by the body. This in turn can have important effects on body composition and the regulation of appetite, whose interaction determines the steady-state body weight maintained by an individual over the long term (19).

Consumption of dietary fat does not promote its own oxidation. This has been observed under conditions of *ad libitum* dietary consumption and under conditions of overfeeding (12,20–22). By contrast, ingestion of either protein or carbohydrate is accompanied by a rapid increase in the oxidation of each, such that the balances of these macronutrients are maintained over the short term (12,20). The implications of the lower metabolic priority for fat oxidation is that fat balance is rarely achieved in the short term, and this increases the risk of a more permanent increase in body fat mass.

Whether the excess energy storage in the short term leads to a persistent increase in fat mass depends on whether there is physiologic or behavioral feedback to reduce subsequent food intake and/or increase energy expenditure. In both animals and humans, there appears to be a weak behavioral compensatory response to excess energy intake stemming from dietary fat compared with other nutrients (8). Fat has been described as being less satiating on a calorie-for-calorie basis

compared with carbohydrate and protein. This phenomenon is conceivably related to the sluggish metabolic response to fat consumption, which may provide weaker signals to the brain for adjustment of subsequent food intake. In addition to the weak metabolic and food intake compensation, there does not appear to be a robust mechanism that drives spontaneous physical activity to limit body energy gain.

Clearly, individuals are able to achieve stable body weight and body composition over the long term while consuming diets differing in macronutrient composition. The level of body fat at which steady state is reached may differ, however, as a function of dietary fat level, especially in sedentary individuals. Over the long term, there are two main mechanisms that can modulate fat oxidation in response to persistent changes in fat intake to maintain nutrient balance. The first is to increase the level of physical activity. Fat is a key metabolic fuel for aerobic exercise. At a given level of fat intake, the level of energy and fat balance is lower in active individuals than in sedentary individuals (23). The second mechanism for increasing fat oxidation is to increase the size of the fat mass (24). Fat oxidation in the body is driven by the availability of nonesterified FA. As fat mass increases, more FA are released into the blood to fuel oxidation in muscle. In a sedentary society consuming appreciable levels of dietary fat (and carbohydrate), it would appear that increased fat mass and obesity represent a necessary condition to achieve weight stability.

There is one additional means to increase fat oxidation in the absence of exercise or increased fat mass. If dietary carbohydrate is severely restricted such that blood insulin levels are substantially reduced, the body will deplete its glycogen stores and will dramatically increase its oxidation of fat. This condition is associated with high rates of gluconeogenesis and ketosis, and this metabolic response is the basis of the popular high-protein, high-fat, low-carbohydrate diets on the market today. Although this dietary composition may have utility for accelerating weight loss, such extreme limitation of carbohydrate may limit the ability to lead a physically active lifestyle, making it difficult to meet current physical activity guidelines for optimal health.

DIETARY FAT, ENERGY BALANCE, AND BODY WEIGHT: DOES FAT MAKE YOU FAT?

Weight gain and excess body fat are products of cumulative positive energy balance. Regardless of diet composition, excess body fat cannot accumulate unless total energy balance is positive. As stated previously, the main effect of fat on energy balance is its ability to promote excess energy intake, a phenomenon related to its weak effect on satiety. Furthermore, the limited capacity of the body to increase fat oxidation in response to increased fat intake promotes positive fat balance, which, if subsequently uncompensated, leads to an increase in body fat mass.

The answer to the question of whether dietary fat makes you fat is that it depends on whether the particular high-fat foods consumed result in excess energy intake. Dietary fat will promote obesity under dietary conditions favoring excess energy intake, and these conditions are made more probable by a sedentary lifestyle. The environment in the United States would seem ideal for dietary fat to promote obesity because high-fat, energy-dense foods are ubiquitous and available in huge portions at a reasonable cost. In addition, the sedentary lifestyle typical of Americans severely limits the body's main mechanism for burning fat, the muscular work of exercise.

Data from animal trials in which physical activity is controlled at a very low level and the diet is a single high-fat food clearly indicate that body fat is proportional to dietary fat over the range of dietary fat levels typical of human diets (25). These types of studies are valuable because they allow examination of the basic behavioral and metabolic responses to dietary fat uncomplicated by other environmental and behavioral factors such as physical activity. Under these circumstances, the data are remarkably consistent in showing that increasing dietary fat increases the probability of obesity.

Interestingly, most human dietary fat intervention trials have not modeled the animal studies looking at the effect of fat on weight gain, but rather have addressed the question of whether dietary fat reduction leads to spontaneous weight loss. There are as yet no large, randomized prospective intervention trials that have looked at the effect of reducing dietary fat on primary prevention of weight gain. One published study examined body weight over a 6-mon period in subjects provided access to "regular-fat" foods or "reduced-fat" foods (26). Subjects given access to regular-fat foods gained ~0.5 kg, whereas those with access to reduced-fat foods did not gain weight. These data provide preliminary evidence that fat reduction may aid in preventing primary weight gain, in agreement with the animal study literature. It would seem that this is a critical area for future research because health authorities (3) are now recommending primary prevention as the most promising approach to combating the rising prevalence of obesity.

Data from human intervention trials up to 1 yr in length that examined the effect of fat reduction on body weight support the conclusion that dietary fat reduction leads to modest, but consistent, reductions in body weight (15,27). In some of these trials, body weight reduction was greater in the early stages of the intervention than at the end. One might interpret this observation to suggest that subjects were metabolically adapting to the reduced-fat diet or compensating for reduced energy intake, thus reducing the effect on body weight. However, it is equally likely that, over time, dietary compliance was reduced and weight crept up as a function of increased fat and calorie intakes.

Support for this latter interpretation comes from studies of the fat substitute olestra, which has organoleptic properties identical to dietary fats but is not digested or absorbed, so it provides no energy. Using olestra as a tool, it is possible to maintain the sensory characteristics of a high-fat diet yet still reduce the level of digestible fat intake. Under these conditions, studies in both lean and obese men and women have shown that reduction in digestible fat intake leads to weight

loss (28,29). For example, Bray *et al.* (28) studied 45 overweight men randomized to consume *ad libitum* either a control diet (33% of energy as fat), a reduced-fat diet (25% of energy as fat), or a fat-substituted diet having 25% of digestible energy from fat but having the sensory properties of the diet with 33% of energy as fat. All three groups lost ~3 kg of body fat during the first 3 mon. Thereafter, the fat-reduced group gained back about half of the body fat that was lost by the end of the 9-mon trial. In contrast, the subjects receiving the fat-substituted diet lost an additional 3 kg of body fat during the remainder of the trial and were still losing weight when the trial ended. Thus, under conditions in which dietary compliance was maintained, a diet reduced in fat energy is associated with reduced energy intake and weight loss and no adaptation was evident.

Data from epidemiologic studies generally support the association of dietary fat with body weight (30,31), although there is much debate about the relevance of such studies owing to the confounding effects of lifestyle factors that cannot be assessed accurately in such studies. For example, there is a strong interaction between dietary fat level and exercise on energy balance. At any given level of dietary fat, energy balance is less positive (and even negative in some cases) when individuals are more physically active than when they are less active or sedentary (23). This observation makes sense, given that aerobic exercise is one of the best mechanisms for increasing fat oxidation. Under conditions of an active lifestyle, fat balance can be maintained at a lower body fat level, despite a high intake of fat, a circumstance that would confound the dietary fat–body weight association in epidemiologic studies.

Perhaps the most practically relevant evidence that reduction of dietary fat intake can improve weight control comes from data collected on >3000 subjects in the National Weight Control Registry. Subjects in the registry have lost an average of 30 kg and have maintained it for >5 yr (32–34). Among the most commonly reported characteristic behaviors of subjects in this group are adherence to a reduced-fat diet (~25% of energy) and regular physical activity representing an energy expenditure of ~400 kcal/d. Although these data are self-reported and the subjects do not represent a random population sample, this information indicates that dietary fat reduction can be a key strategy to prevent weight gain among susceptible individuals.

SUMMARY AND CONCLUSIONS

Collectively, the available evidence suggests that it is difficult to achieve fat balance at a normal body weight on typical fat-rich Western diets and in the context of prevailing sedentary lifestyles. Several different lines of evidence indicate that elevated dietary fat levels increase the probability of excess energy intake and positive fat balance. Because diet composition has such small effects on energy expenditure, the main effect of dietary fat in promoting body weight gain arises from its ability to promote excess energy intake. To resist a positive fat balance when consuming diets high in fat, the level of fat oxidation has to be increased to match the level of fat intake. The main mechanisms that can increase fat oxidation are to increase physical activity or to increase the size of the fat mass. In largely sedentary populations, such as the United States, achieving fat balance while consuming diets rich in fat generally requires increasing the size of the fat mass.

Substantial evidence exists from preclinical, clinical, and randomized intervention trials documenting that high levels of dietary fat promote increased body fatness, consistent with its effects on food intake and metabolism from shorter-term experimental studies. Although much of the discussion about the effect of dietary fat on weight has focused on whether fat reduction causes weight loss, the most promising utility of controlling fat intake may be in prevention of weight gain. Once obesity is established, the ability of dietary macronutrient manipulation alone to reduce weight is driven almost solely by how much it permanently reduces uncompensated energy intake. Current evidence suggests that fat reduction alone, in the absence of other behavioral changes, leads to only modest weight reduction. Data from the National Weight Control Registry suggest that, in combination with moderate physical activity, fat reduction is a strategy that can help prevent secondary weight gain among individuals susceptible to obesity.

The debate over what dietary composition is optimal for weight control in the population is likely to continue. Many alternative dietary schemes have been suggested as better alternatives to fat reduction for controlling body weight (1). However, there are few empirical data that these are effective for controlling body weight under either controlled or real-life conditions. Given the evidence discussed here and the absence of evidence-based alternatives, it would be a mistake to recommend consumption of higher levels of dietary fat to the population.

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Nutritional Characteristics of DAG Oil

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ABSTRACT: Excess calorie intake in industrialized countries has prompted development of fat substitutes and other lower-calorie dietary items to enhance health. DAG cooking oils, with a 1,3 configuration, taste and have the texture of commonly used TAG cooking oils. Because they are not hydrolyzed to 2-MAG in the gut, the absorption and metabolism of DAG oil differs from that of TAG. Among the physiological differences are lower postprandial lipemia and an increased proportion of FA being oxidized instead of stored. Preliminary studies suggest that these differences in energy partitioning between DAG and TAG may be usefully exploited to reduce the amount of fat stored from cooking oil and oil components of food items. Over 70 million bottles of DAG oil have been sold in Japan since its introduction in February 1999, and the product is being test-marketed in the United States. It is hoped that wider use of DAG oil may provide one additional means of preventing obesity.

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Over the past 40 yr, the role of dietary fat in promoting health or disease has come under scrutiny. Public health agencies and professional health organizations have issued guidelines and enacted programs that encourage diet and exercise modifications to improve overall health (1–3). Common recommendations from these groups include lowering total fat consumption, especially saturated fat, balancing the types of fat in the diet, and increasing the amount of physical activity.

Dietary fat and its metabolism have been the focus of considerable research to understand their link with lifestyle-related diseases, particularly obesity (4). As a result of this research, both nutritional and pharmaceutical products commonly have focused on limiting fat digestion and/or absorption (i.e., with structured lipids, nondigestible fats, lipase inhibitors, or fat absorbers) or enhancing fat catabolism (i.e., with caffeine or ephedra). Newer products have focused not on limiting fat digestion and absorption but on the ways edible oils influence fat metabolism and therefore affect body weight. Such products include DAG-rich oils and medium-chain TAG (MCT)-rich oils. (MCT have been used extensively for several decades in special clinical settings to treat fat malabsorption and burn patients.) DAG, the main component of DAG oil, have been used by the food industry as emulsifiers for some time. However, the Kao Corporation of Japan introduced a DAG cooking oil in 1999 that contains more than 80% DAG. DAG oil looks and tastes like conventional edible oils, being pale yellow with a light, bland flavor.

TABLE 1
Relative Contribution of Mono-, Di-, and Triacylglycerols in Selected Edible Oils

	MAG	DAG	TAG	Others
Soybean	—	1.0	97.9	1.1
Cottonseed	0.2	9.5	87.0	3.3
Palm	—	5.8	93.1	1.1
Corn	—	2.8	95.8	1.4
Safflower	—	2.1	96.0	1.9
Olive	0.2	5.5	93.3	2.3
Rapeseed	0.1	0.8	96.8	2.3
Lard	—	1.3	97.9	0.8

[a]1% weight of total glyceride content.

DAG occur naturally in edible oils to varying degrees (Table 1) (5,6). Cottonseed and olive oils contain greater amounts of DAG than other commonly used edible oils. Unlike conventional edible oils, DAG oil contains predominantly DAG while having a FA composition primarily made up of oleic (38% by weight), linoleic (54% by weight), and linolenic (5% by weight) acids. The majority of DAG in the DAG oil have FA in the 1,3 configuration as compared to the 1,2 configuration (Table 2). This enriched composition of DAG in DAG oil makes an important difference in their absorption and subsequent metabolism, particularly because of the 1,3-DAG.

The first observation of a metabolic difference between DAG oil and TAG was observed in animals with regard to plasma TAG metabolism. Consumption of DAG oil resulted in findings not commonly associated with conventional TAG oil consumption. When substituted for a conventional TAG oil with a closely matched FA composition, the DAG oil was absorbed in a manner that resulted in a lower secretion of lymph, lower serum TAG, and decreased levels of post-meal TAG-rich particles in the blood (7,8).

In human studies, similar differences in post-meal serum TAG levels were observed. DAG oil showed the ability to change the degree of increase in serum TAG with the strongest

TABLE 2
Typical Relative Glyceride Portion of DAG Oil

Glycerides	% by weight	Glycerides	% by weight
DAG[a]	82	TAG	17
sn-1,3	57	MAG	1
sn-1,2	25		

[a]Minimum content.

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Abbreviations: ACO, acyl-CoA oxidase; DAGT, DAG acyltransferase; MGAT, MAG acyltransferase; MCT, medium-chain triacylglycerol(s); UCP-2, uncoupling protein-2.

effects shown for a single dose of >20 g (9). The most recent study, which provided a single 55-g dose of DAG oil, found a significant reduction in serum TAG at 2 and 4 h after consumption compared to a conventional TAG oil with a matched FA profile (10). Levels of remnant lipoprotein-cholesterol were decreased significantly at 2 and 4 h postconsumption as well.

When Japanese type 2 diabetics with elevated fasting serum TAG (>150 mg/dL) substituted a quantity of 10 g/d of DAG oil for conventional oil for 12 wk as part of a low-fat diet, they showed a significant decrease in fasting TAG (11). Additionally, the diabetics consuming DAG oil showed a reduction in glycosylated hemoglobin over the course of the study, indicating improved blood sugar control compared to diabetics consuming only conventional oil.

Further animal studies were conducted to explore possible mechanisms underlying these observations. Activities of enzymes related to FA oxidation in the liver [carnitine palmitoyltransferase, acyl-CoA dehydrogenase, acyl-CoA oxidase (ACO), enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 2,4-dienoyl-CoA reductase and d3/d2-enoyl-CoA isomerase] were observed to increase following 14 d of DAG oil consumption (12). Correspondingly, activities of enzymes related to FA synthesis (FA synthase, glucose-6-phosphate dehydrogenase, and malic enzyme) were observed to decrease. Changes in liver enzyme activities were accompanied by a lower hepatic TAG content and lower serum cholesterol. In additional studies, animals were observed to have different fat digestion products in the gut as well as a tendency to use greater amounts of oxygen after DAG oil consumption (13). Mice (C57BL/6J) prone to diet-induced obesity were observed to maintain lower body weights during their lifespans when they consumed DAG oil *ad libitum* in place of conventional oil (14,15). Consistent with the increased ACO activity, ACO mRNA levels also were significantly increased in the livers of mice fed the DAG oil compared to a conventional TAG oil (15). Additionally, DAG oil increased β-oxidation in the small intestine in mice fed DAG oil compared to a TAG oil (12). The increased β-oxidation in the small intestine was accompanyied by an increased expression of genes associated with β-oxidation and lipid metabolism including ACO, medium-chain acyl-CoA dehydrogenase, liver-FA binding protein, FA transporter, and uncoupling protein-2 (UCP-2). These changes in β-oxidation and mRNA expression were not observed in the liver.

The maintenance of lower body weight and body fat following DAG oil consumption in animals also has been observed in humans. Two well-controlled studies have been conducted in humans to examine the impact of DAG oil on body weight and body fat. In subjects consuming approximately 5% of total calories from DAG oil for 16 wk, significantly greater reductions in body weight ($P < 0.01$) and body fat area ($P < 0.05$) were observed compared to subjects consuming conventional oil (16). In a larger study, overweight subjects consuming 15% of total energy from DAG oil for 6 mon as part of a mildly hypocaloric diet (~500 to 800 kcal/d) had a greater loss of body weight ($P < 0.025$) and body fat ($P < 0.037$) over the period of dietary intervention compared to subjects consuming a conventional TAG oil (17). These changes appear to be similar in overweight adult Americans and in Japanese individuals. In both studies, DAG oil was incorporated as the oil ingredient in food items such as mayonnaise, crackers, muffins, and instant soups. DAG oil appeared to confer a greater loss of body fat and body weight compared to conventional oil when used as a dietary aid as part of a healthy diet or caloric management plan.

With the apparent energy value of DAG oil being nearly identical to that of conventional oil (18), the process of fat digestion and absorption must be examined to understand how DAG oil affects fat and energy metabolism. Conventional TAG oils are digested into 2-MAG and FFA by the *sn*-1,3-specific pancreatic lipase. These digestion components are absorbed into cells that line the gut. They are reassembled into TAG by sequential addition of FFA by MAG acyltransferase (MGAT), a process that is most efficient in using 2-MAG (19), then by DAG acyltransferase (DAGT). TAG are packaged into chylomicra that are secreted into the lymph, which carries fat-rich particles away from the gut and to the bloodstream for circulation throughout the body. The level of fat-rich particles increases in the blood temporarily, then decreases as body tissues take up FA before eventually reaching the liver.

The body digests DAG oil as it would a conventional oil, yielding MAG and FFA. However, the 1-MAG produced during digestion of 1,3-DAG is different from the 2-MAG resulting from TAG hydrolysis (13). The gut normally reassembles TAG using 2-MAG, but starting with 1-MAG results in lower amounts of fat-rich particles appearing in serum following consumption of a single dose of DAG oil. As a result, fewer fat-rich particles appear in the blood following a meal containing DAG oil. This difference leaves a pool of FA that must be handled by the gut.

No significant difference in energy value has been observed; thus, the differences in weight and body fat loss observed as a result of DAG oil consumption must be due to either increased energy expenditure or decreased energy intake. Experimental evidence in animals shows increased oxygen consumption, indicating increased energy expenditure; increased FFA in the portal vein; increased β-oxidation in the liver and small intestine; and increased mRNA expression of ACO and UCP-2 in the small intestine following 1,3-DAG or DAG oil consumption (12–15). These results suggest that the fat used for energy is increased through greater energy expenditure rather than being stored as fat following absorption of DAG oil through the differential metabolism of 1,3-DAG. This shift in fat metabolism may explain, in part, animal and human results observed following DAG oil consumption. However, the possibility exists that increased fat oxidation may affect appetite. A growing body of scientific evidence indicates that enhanced β-oxidation or inhibition of FA synthase might enhance appetite suppression (20–23).

DAG cooking oil is approved by the Japanese government as a food for specific health use (FOSHU) to control post-

meal blood lipids and body fat (24). Additionally, a professional association of Japanese physicians (Japanese Society of Human Dry Dock, affiliated with the Japan Hospital Association) officially recommends DAG cooking oil as part of a healthy diet. This recognition indicates to the Japanese public that the use of DAG oil to regulate post-meal blood lipids, body weight, and body fat in a healthy manner has considerable support. In fact, DAG oil (marketed as Healthy Econa oil) is a leading cooking oil in Japan, with over 70 million bottles sold since it was launched commercially in February 1999.

In the United States, DAG oil has been self-affirmed as generally recognized as safe (GRAS) for numerous food categories based on an expert panel review (25). DAG oil can be used as an edible oil (for home use) as well as a component of spreads, salad dressings, baked goods, health bars, and numerous additional food product categories.

New products promoted as "altering fat digestion" or "altering fat absorption" are often carefully scrutinized. Commercial examples are such products as nondigestible fat substitutes (i.e., Olestra) and pancreatic lipase inhibitors (i.e., Orlistat). With these products, the consequences of undigested fat reaching the lower bowel and of causing decreased serum levels of certain fat-soluble nutrients remain a problem. Because DAG oil is digested and absorbed like a conventional oil, fatty stools are not observed and fat-soluble vitamins appear to be absorbed normally (18,26).

The Japanese marketplace is clearly an environment that provides consumers with a variety of unique dietary fats with healthful characteristics, including DAG oil and oils containing MCT. As we understand its impact on body weight and lipid metabolism, DAG oil offers the potential for incorporating dietary fat into the U.S. diet in a way that may have a favorable impact on obesity as well as on both fasting and postprandial serum TAG.

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CLA and Body Weight Regulation in Humans

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ABSTRACT: CLA comprises a group of unsaturated FA isomers with a variety of biological effects in experimental animals. CLA reduces body fat accumulation in animal models and has been suggested to have significant effects on lipid and glucose metabolism, e.g., antidiabetic effects in obese Zucker rats. It has been proposed that the *trans*10-*cis*12 isomer is the active isomer associated with the antiobesity and insulin-sensitizing properties of CLA. The metabolic effects in humans in general, and isomer-specific effects specifically, are not well characterized. In a series of controlled studies in humans, we investigated the effects of CLA (given as the commercially available mixture of isomers and as the purified *trans*10-*cis*12 CLA isomer) on anthropometry, lipid and glucose metabolism, and markers of lipid peroxidation. Preliminary results indicate that CLA may slightly decrease body fat in humans also, particularly abdominal fat, but there is no effect on body weight or body mass index. There is no simultaneous improvement in lipid or glucose metabolism. Rather, the *trans*10-*cis*12 CLA isomer unexpectedly caused significant impairment of the peripheral insulin sensitivity as well as of blood glucose and serum lipid levels. In addition, CLA markedly elevated lipid peroxidation. Thus, the metabolic effects of CLA in humans seem complex; further studies, especially of isomer-specific effects and for longer time periods, are warranted.

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CLA comprises a group of positional and geometric isomers of conjugated octadecadienoic acid, derived from linoleic acid (18:2n-6) and produced by bacterial biohydrogenation in the ruminant gut (1). In human food, CLA is derived mainly from dairy and ruminant meat sources (2). CLA is found in human serum lipids (3) as well as in other tissues including adipose tissue (4).

CLA has been shown to reduce body fat and increase lean body mass in growing animals by as yet unknown mechanisms (5–8). Recent data have shown beneficial effects of CLA on several components of the metabolic syndrome in Zucker diabetic fatty rats such as normalized glucose tolerance and reduced hyperinsulinemia; plasma levels of FFA (9) and a significantly improved blood lipid profile were reported in CLA-fed hamsters (10).

The metabolic syndrome represents a cluster of risk factors (insulin resistance, dyslipidemia, hypertension, and abdominal obesity) predisposing for coronary heart disease and type-2 diabetes (11). Abdominal visceral adiposity seems to be a key factor in the metabolic syndrome (12). The sagittal abdominal diameter (SAD) has been suggested to be the best simple anthropometric measurement of visceral fat (13) and is strongly associated with increased cardiovascular risk (14,15) and insulin resistance (16). Epidemiologic data from a population of elderly men showed an inverse correlation between the estimated dietary intake of milk fat and abdominal obesity (17), suggesting some indirect evidence for the postulated metabolic effects of CLA also in humans.

The antiobesity properties and metabolic effects of CLA have at present been studied mainly in animals and should therefore be tested also in humans. We performed a series of studies to investigate the effects of CLA, and of specific CLA isomers, on body composition and adiposity in humans. In addition, we investigated the effects of CLA on aspects of lipid and glucose metabolism as well as on markers for lipid peroxidation and inflammation. This paper reviews some of our studies and compares the results with those of other recent human studies with CLA.

Effects of CLA on body fat in humans. Although considerable data exist in growing animals indicating a reduced body fat accumulation after CLA, there have up to now been surprisingly few studies in humans. Nearly all of the studies have used commercially available mixtures of isomers, usually containing similar proportions of the *cis*9-*trans*11 and *trans*10-*cis*12 CLA isomers, or other preparations containing several CLA isomers. Table 1 summarizes present knowledge regarding effects of CLA on body mass index (BMI) and body fat in humans.

In a randomized, double-blind study, Atkinson (18) supplied obese people, who were instructed to follow a weight loss regimen with a reduction in calories and increase in exercise, with a mixture of CLA (2.7 g/d) or placebo for 6 mon. Both groups lost a similar amount of body weight and body fat and there was no significant difference between the groups, although a *post hoc* analysis suggested that there could have been a subpopulation in the CLA-fed group that gained lean body mass and lost body fat. In contrast, Blankson *et al.* (19) reported reductions in body fat in a randomized,

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Abbreviations: BMI, body mass index; PG, prostaglandin; SAD, sagittal abdominal diameter; UCP, uncoupling protein.

double-blind study of overweight or obese volunteers ($n = 47$). The subjects were divided into five groups receiving 1.7, 3.4, 5.1, or 6.8 g CLA or a control preparation (olive oil), respectively, for 12 wk. As in all other studies of supplementation with CLA in humans, there were no differences in body weight or changes in BMI, but there was a significantly higher reduction in body fat mass in the CLA groups as a whole compared with the placebo ($P < 0.03$). The reduction of body fat was significant for the 3.4- and 6.8-g CLA groups without any clear suggestion of a dose–response effect. The authors suggested that CLA may reduce body fat mass in humans and that no additional effect is achieved with doses >3.4 g CLA/d.

In a carefully performed study, Zambell *et al.* (20) investigated the effect of CLA supplementation on body composition and energy expenditure in healthy, normal-weight women. Women ($n = 17$) were confined to a metabolic suite in which diet and activity were controlled and held constant; they were fed either a CLA preparation or placebo for 64 d. Body composition was determined by total body electrical conductivity as well as by dual X-ray absorptiometry; energy expenditure, fat oxidation, and respiratory exchange ratio were measured. CLA had no significant effect on either body composition or energy expenditure. The amount of CLA given in this study was rather low, i.e., 2 g/d, with a mixture of different CLA isomers including 0.8 g/d of the two isomers *cis*9-*trans*11 and *trans*10-*cis*12 taken together.

Mougios and coworkers (21) examined the effect of CLA supplementation in healthy, normal-weight young women and men. In a placebo-controlled fashion, the study group consumed 0.7 g CLA/d for 4 wk followed by a second 4-wk period of consuming 1.4 g CLA/d. The proportion of body fat, estimated from the sum of 10 skinfold measurements, did not change significantly during the study compared with a control group.

We investigated the effects of supplementation with CLA, administered as the commercially available mixture of isomers or as a purified preparation of the *trans*10-*cis*12 CLA isomer, in humans. In a double-blind study, Smedman and Vessby (22) studied a group of healthy men and women ($n = 53$) given either a mixture of CLA containing 3.4 g CLA/d or a control preparation for 12 wk. There was a significant reduction in body fat by 3.8% ($P < 0.001$) in the CLA group. The body fat change, calculated from the three-compartment model based on caliper measurements of skinfold thickness and bioimpedance (23), was significantly different from that in the control group ($P = 0.050$). Body weight, BMI, and SAD were unchanged.

When giving the same amount and type of CLA preparation to a group of abdominally obese men during a 4-wk double-blind randomized study, Risérus *et al.* (24) demonstrated a significant mean decrease of the SAD by 0.6 cm ($P = 0.003$) in the CLA group compared with the control group ($P = 0.04$). There were also significant reductions of the waist/hip ratio and the waist circumference within the CLA group, but these changes were not significantly different from those of the control group.

The *trans*10-*cis*12 CLA isomer is suggested to have antiobesity and antidiabetic properties in animals. For the first time in humans, we were able to test a CLA preparation containing purified *trans*10-*cis*12 CLA and compare that with the conventional mixture of CLA isomers and a control preparation (25). This was again a trial in abdominally obese men with the metabolic syndrome; the goal was to determine whether CLA could improve insulin sensitivity, lipid metabolism, or body composition during a 3-mon treatment period. The amount of CLA ingested corresponded to 2 g/d, either as a mixture of the two main isomers or as purified *trans*10-*cis*12 CLA. At the end of the 3 mon, there were no significant differences between the groups associated with the changes in BMI, body fat, waist girth, or SAD. However, within the *trans*10-*cis*12 CLA group, there were significant reductions of BMI, waist girth, and body fat, whereas SAD and body fat decreased within the CLA group. SAD tended to decrease ($P = 0.07$) after both CLA treatments compared with the control group.

Effects of CLA on lipid and glucose metabolism in humans. In contrast to some of the animal studies, it has not been possible to show any improvement of the metabolic status in humans after supplementation with CLA. On the contrary, the *trans*10-*cis*12 CLA isomer induced insulin resistance in abdominally obese men with the metabolic syndrome (25), resulting in simultaneously increased glycemia and reduced concentrations of HDL cholesterol. The impairment of insulin sensitivity may be an isomer-specific effect. The CLA mixture did not significantly affect glucose metabolism but lowered HDL cholesterol. A significant reduction in HDL cholesterol after supplementation with CLA was noted also in other controlled studies (19,21), and Medina *et al.* (26) reported a tendency to increased insulin levels in plasma after CLA supplementation.

Effects of CLA on antioxidants and lipid peroxidation in humans. CLA is easily oxidized, and it has been suggested that increased lipid oxidation may contribute to the antitumorigenic effect demonstrated in experimental studies (27,28). When given to humans, CLA induces lipid peroxidation (29,30), as indicated by significantly increased levels of urinary 8-iso-prostaglandin (PG) $F_{2\alpha}$, a major F_2-isoprostane, and 15-keto-dihydro $PGF_{2\alpha}$, a major metabolite of $PGF_{2\alpha}$, which are direct measurements of nonenzymatic (31) and enzymatic (32) lipid peroxidation *in vivo*, respectively. F_2-isoprostanes are probably the most reliable and clinically relevant markers of oxidative stress available (33). Both inflammation (34) and oxidative stress (35) have been suggested to contribute to insulin resistance. The increased lipid peroxidation may also be an isomer-specific effect.

The lipid peroxidation after CLA ingestion disappears rapidly when CLA supplementation is interrupted (24). No reductions in antioxidant capacity or in the levels of plasma tocopherols have been demonstrated (29,30). The mechanisms behind the increased lipid peroxidation after CLA and their clinical importance are currently under investigation.

DISCUSSION

There is no evidence from any human studies that supplementation with CLA would reduce body weight or BMI. Although

TABLE 1
Effects on Body Weight and Body Fat of CLA: Human Studies[a]

Reference number	CLA[b] dose (g)	Participants			Effects on		
		n	Sex	BMI	BMI	% Body fat	SAD
18	2.7	2 × 40	M/W?	Obese	0	0	NM
19	1.7–6.8	5 × 12	M/W	28–30	0	Reduction	NM
20	0.8 (several isomers)	7/10	W	23	0	0	NM
21	0.7–1.4	10/12	M/W	23	0	0	NM
24	3.4	10/15	M	32	0	NM	Reduction
22	3.4	24/26	M/W	25	0	Reduction	0
25	2.0	3 × 20	M	30–31	0	0	0 (—)

[a]Abbreviations: BMI, body mass index; SAD, sagittal abdominal diameter; M/W, men/women; NM, not measured.
[b]Sum of CLA isomers *c*9-*t*11 and *t*10-*c*12.

not convincingly shown in all studies, there is a suggestion of an effect on body fat, and possibly especially on abdominal fat, by CLA in several studies (Table 1). A significant body fat reduction was not found in humans with CLA doses <3 g/d. However, in a study in overweight subjects, there was no clear evidence of a dose–response effect (19), and no further reduction of body fat was seen when the CLA dose was >3.4 g/d. A reduction in abdominal fat was demonstrated in obese men after only 4 wk of CLA supplementation (24), and it has not yet been shown that the putative effect of CLA on body fat increases with time. A certain reduction in body fat has been indicated in both normal-weight and obese subjects.

The lack of an effect in some of the studies might be due to the small numbers of participants and the rather large measurement errors in determining body composition in humans. Quite clearly, the effect on body fat, if any, is moderate and not accompanied by any metabolic improvement. It is too early to speculate whether there is an isomer-specific effect on body composition also in humans.

The mechanism behind a possible reduction in body fat by CLA is not known. CLA has been suggested to affect the rate of *de novo* lipogenesis and/or the rate of lipolysis. Increased lipolysis and decreased lipoprotein lipase activity have been observed *in vitro* in adipocytes when CLA was added to the medium (36). Reduced production of antilipolytic PG in adipose tissue has been suggested as a cause for increased lipolysis (37). One might postulate that dietary supplementation with CLA, as a possible inducer of catecholamine-related lipolysis, could cause a selective reduction in visceral fat, indirectly measured as a reduction in SAD. A possible thermogenic effect of CLA is suggested by tissue analyses of uncoupling protein (UCP) expression in prediabetic CLA-fed rats, which showed an increased expression of brown adipose tissue UCP-2 mRNA compared with control rats (38).

Supplementation with the *trans*10-*cis*12 CLA isomer led to a tendency toward reduced abdominal fat coinciding with significantly impaired insulin sensitivity, increased fasting glucose, and dyslipidemia—an apparently paradoxical result. This suggests that the metabolic effects of CLA in humans are different from those reported in diabetic Zucker rats (9) but are in agreement with the effects in female C57BL/6J mice, which became severely insulin resistant and lipodystrophic after CLA supplementation (39). Similarly, male AKR/J mice showed signs of insulin resistance after CLA (40). An intriguing possibility is that CLA, and possibly the *trans*10-*cis*12 isomer specifically, induces adipocyte apoptosis, with reduction of the fat mass and an induction of insulin resistance. The *trans*10-*cis*12 CLA isomer may also inhibit the formation of new, small, insulin-sensitive fat cells, possibly *via* downregulation of peroxisome proliferator-activated receptor γ (39). Reduced activity of lipoprotein lipase, as shown *in vitro* (36), might also contribute to reduced fat accumulation with a concomitant impairment of serum lipid levels owing to decreased clearance of VLDL TG.

Although the putative reduction of body fat, and possibly mainly of abdominal fat, would suggest a positive metabolic effect of CLA, the metabolic impairment after supplementation with the *trans*10-*cis*12 isomer, i.e., a significantly impaired insulin sensitivity, is cause for concern. Estimated from dose–response clamp studies in obese subjects and lean controls (41), the relative decrease of insulin sensitivity after *trans*10-*cis*12 CLA was equivalent to having an excess body weight of ~15 kg, at a current mean weight of ~100 kg, indicating a rather powerful effect of this isomer. The amounts of *trans*10-*cis*12 in the diet (in which the major isomer is *cis*9-*trans*11) are very low, but commercial CLA mixtures do contain 20–45% *trans*10-*cis*12, indicating that long-term use of these preparations may be of concern. However, more clinical trials over longer time periods are required to make firm conclusions regarding the effects of CLA and different CLA isomers on body composition and metabolism as well as on the clinical safety of (specific isomers of) this FA. Specifically, it would be interesting to determine whether other CLA isomers have other effects and whether CLA supplementation may prevent weight regain, in line with the reduced body fat accumulation in animals. This could be tested, e.g., after a period of body weight reduction or in connection with smoking cessation.

SUMMARY

Preliminary results indicate that CLA may slightly decrease body fat in humans, particularly abdominal fat, but that there is no simultaneous reduction of body weight or improvement

of lipid or glucose metabolism. Rather, the *trans*10-*cis*12 isomer unexpectedly caused significant impairment of peripheral insulin sensitivity as well as of blood glucose and serum lipid levels. In addition, CLA markedly elevated lipid peroxidation. The mechanism behind these changes is at present unknown. Importantly, the safety of CLA supplements should be evaluated more closely because a large number of people are currently using commercially available CLA preparations as antiobesity agents. Thus, the metabolic effects of CLA in humans seem complex, and further studies, especially of isomer-specific effects and for prolonged time periods, are warranted.

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Role of Dietary Calcium and Dairy Products in Modulating Adiposity

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ABSTRACT: Dietary calcium plays a pivotal role in the regulation of energy metabolism. High-calcium diets attenuate adipocyte lipid accretion and weight gain during overconsumption of an energy-dense diet and increase lipolysis and preserve thermogenesis during caloric restriction, thereby markedly accelerating weight loss. Our studies of the *agouti* gene demonstrate a key role for intracellular Ca^{2+} in regulating adipocyte lipid metabolism and TG storage. Increased intracellular Ca^{2+} resulting in stimulation of lipogenic gene expression, and lipogenesis and suppression of lipolysis resulting in adipocyte lipid filling and increased adiposity. Moreover, we recently demonstrated that the increased calcitriol produced in response to low-calcium diets stimulates adipocyte Ca^{2+} influx and, consequently, promotes adiposity. Accordingly, suppressing calcitriol levels by increasing dietary calcium is an attractive target for obesity intervention. In support of this concept, transgenic mice expressing the *agouti* gene specifically in adipocytes (a human-like pattern) respond to low-calcium diets with accelerated weight gain and fat accretion, whereas high-calcium diets markedly inhibit lipogenesis, accelerate lipolysis, increase thermogenesis, and suppress fat accretion and weight gain in animals maintained at identical caloric intakes. Further, low-calcium diets impede body fat loss, whereas high-calcium diets markedly accelerate fat loss in transgenic mice subjected to caloric restriction. Dairy sources of calcium exert markedly greater effects in attenuating weight and fat gain and accelerating fat loss. This augmented effect of dairy products is likely due to additional bioactive compounds in dairy that act synergistically with calcium to attenuate adiposity. These concepts are confirmed by both epidemiological and clinical data, which demonstrate that increasing dietary calcium results in significant reductions in adipose tissue mass in obese humans in the absence of caloric restriction and markedly accelerates the weight and body fat loss secondary to caloric restriction, whereas dairy products exert significantly greater effects. These data indicate an important role for dairy products in both the prevention and treatment of obesity.

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A substantial body of evidence has emerged over the last 3 yr to support what may appear to be a very unlikely concept: that dietary calcium may play a role in the regulation of energy metabolism and in modulating obesity risk. We first observed an "antiobesity" effect of dietary calcium accidentally, during the course of a study investigating the antihypertensive effect of dairy products in African Americans. We noted that adding calcium-rich dairy foods (yogurt) to the daily diet resulted in significant reductions in body fat and circulating insulin (1) as well as a sustained reduction in intracellular calcium and an antihypertensive effect (2,3). Twelve months of yogurt supplementation, sufficient to raise daily calcium intake from approximately 400 to approximately 1,000 mg/d, resulted in significant decreases in both serum insulin (from 22 ± 3 to 14 ± 4 μU/mL, $P < 0.03$) and body fat (from 32.3 ± 2.6 to 27.4 ± 3.1 kg fat, $P < 0.01$ by repeated measures comparison). Although these data were inexplicable to us at the time, our recent studies of the mechanism of action of the agouti gene in obesity and insulin resistance have provided a compelling mechanism that has now been confirmed in a series of studies described in this review. These data demonstrate a key role for intracellular Ca^{2+} in the regulation of both murine and human adipocyte metabolism, resulting in modulation of adipocyte TG stores, as described below. Since intracellular Ca^{2+} can clearly be modulated by calcitrophic hormones, including 1,25-dihydroxy-vitamin D [1,25-$(OH)_2$-D] and parathyroid hormone, these data provide a theoretical framework that may explain our earlier clinical trial observations (1). The next portion of this review is devoted to a portrayal of these findings.

AGOUTI, INTRACELLULAR CALCIUM, AND OBESITY

Most of the data describing the role of intracellular Ca^{2+} in human adipocyte metabolism derives from our recent studies of the mechanism of action of *agouti*, the first of the obesity genes to be cloned (4). The C-terminal region of agouti protein, which retains full functional activity relative to the intact protein in an *in vitro* assay system (5), exhibits a striking spatial homology in both number and spacing of cysteine residues to spider and snail venoms (ω-conotoxins, plectoxins), which target Ca^{2+} channels (6). Accordingly, the C-terminus may form a 3-D structure that is functionally similar to these venoms and may thereby serve to modulate Ca^{2+} transport. Indeed, we have reported that obese *agouti* mutant mice (viable yellow, A^{vy}) exhibit increases in both steady-state intracellular Ca^{2+} and Ca^{2+} influx in several tissues (7,8). This increase in intracellular Ca^{2+} was closely correlated with both

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Abbreviations: ACE, angiotensin-converting enzyme; FAS, fatty acid synthase; 1,25-(OH_2)-D, 1,25-dihydroxy-vitamin D; RAS, renin-angiotensin system; SUR, sulfonylurea receptor; UCP2, uncoupling protein 2.

the degree of ectopic *agouti* expression and body weight (8), suggesting the possibility of a causal mechanism between intracellular Ca^{2+} and obesity in these animals. Since A^{vy} mice exhibit elevated rates of adipocyte lipogenesis and increased adipocyte size relative to lean controls (9,10), we explored the links between *agouti*, intracellular Ca^{2+}, and regulatory enzymes in lipid metabolism.

INTRACELLULAR CALCIUM REGULATES ADIPOCYTE LIPID METABOLISM

Recombinant agouti protein directly increased Ca^{2+} influx and steady-state intracellular Ca^{2+} in a variety of cell types, including both murine and human adipocytes (7,8). This regulation occurs in response to physiologically meaningful concentrations of *agouti* (EC_{50} of 18–62 nM, depending on cell type), and, although studies in HEK-293 cells demonstrate the dependence of this effect on the presence of intact melanocortin receptors, it is not dependent on melanocortin receptor antagonism (7). The role of these increases in Ca^{2+} in lipogenesis has been explored using fatty acid synthase (FAS), as this multifunctional enzyme is highly regulated by nutrients and hormones and is a key enzyme in *de novo* lipogenesis. FAS expression and activity are markedly increased in A^{vy} relative to control mice (11), and nanomolar concentrations of agouti protein stimulate *ca.* twofold increases in FAS gene expression and activity and TG accumulation in 3T3-L1 adipocytes as well as in human adipocytes, similar to the maximal increases stimulated by insulin (11). These increases are mediated by a distinct *agouti*/Ca^{2+} response sequence in the FAS promoter (12). This sequence maps to the –435 to –415 region of the FAS promoter and is upstream of the insulin response element, which maps to –67 to –52, consistent with the observed additive effects of *agouti* and insulin on FAS gene transcription (12). Further, we recently reported that *agouti* exerts a regulatory effect on human FAS expression *in vivo*, and that there is a strong correlation between *agouti* expression and FAS expression in adipose tissue obtained from normal volunteers (13). This *agouti* modulation of FAS transcription appears to be mediated *via* intracellular Ca^{2+}, as it can be inhibited by Ca^{2+} antagonism (11,14) and can be mimicked in the absence of *agouti* by either receptor- or voltage-mediated Ca^{2+} channel activation (15).

In addition to activating lipogenesis, recent data also indicate that increasing intracellular Ca^{2+} may also contribute to increased TG stores by inhibiting lipolysis. Increasing Ca^{2+} influx with either arginine vasopressin or epidermal growth factor was reported to inhibit lipolysis in rat adipocytes in a Ca^{2+} dose-responsive fashion (16). Further, we have shown that the *agouti* gene product similarly inhibits lipolysis in human adipocytes *via* a Ca^{2+}-dependent mechanism (17). This inhibition can also be mimicked in the absence of *agouti* by either receptor- or voltage-mediated Ca^{2+} channel activation (17). The antilipolytic effect of intracellular Ca^{2+} is due to a direct activation of phosphodiesterase 3B, resulting in a decrease in cAMP and, consequently, reduced ability of agonists to stimulate phosphorylation and activation of hormone- sensitive lipase (13). Thus, *agouti* regulation of adipocyte intracellular Ca^{2+} appears to promote TG storage in human adipocytes by exerting a coordinated control of lipogenesis and lipolysis, serving to stimulate the former and inhibit the latter simultaneously.

However, it is important to note that *agouti* interaction with insulin is required for the full expression of *agouti*-induced obesity. *Agouti* and insulin exert independent, additive effects on FAS transcription and lipogenesis (12). Since increased intracellular Ca^{2+} is the proximate signal for insulin release, and *agouti* regulates Ca^{2+} in several cell types (7), it is reasonable to speculate that *agouti* may stimulate insulin release as well. Indeed, we recently found that *agouti* is expressed in human pancreas and stimulates Ca^{2+} signaling in rat, hamster, and human pancreatic β cells (18). Further, hyperplasia of β cells precedes the development of obesity in *agouti* mutant mice, suggesting that hyperinsulinemia may be a direct effect of *agouti* acting on the pancreas and that the combination of this hyperinsulinemia and *agouti*-stimulated adipocyte Ca^{2+} influx may lead to obesity. In support of this concept, transgenic mice expressing *agouti* at high levels in adipose tissue under the control of the aP2 promoter become obese if they are also hyperinsulinemic as a result of either exogenous insulin or a high-sucrose diet, whereas hyperinsulinemia was without effect in nontransgenic littermate controls (19–21). Since humans exhibit a similar pattern of adipocyte *agouti* expression (22), similar *agouti*/insulin/Ca^{2+} interactions may result in excessive adipocyte TG storage.

Taken together, these data indicate that regulation of adipocyte and pancreatic intracellular Ca^{2+} may be an important target for the development of therapeutic strategies for the prevention and treatment of obesity (14). This concept is summarized in Figure 1.

To further evaluate this hypothesis, *agouti*-expressing transgenic mice were treated with high doses of a Ca^{2+} channel antagonist, nifedipine. This treatment resulted in an 18% reduction in fat pad mass and completely normalized the *agouti*-induced hyperinsulinemia over a 4-wk treatment period in the transgenic mice, but was without effect in the nontransgenic littermate controls (23). Thus, adipocyte and/or pancreatic β-cell Ca^{2+} appears to be a reasonable therapeutic target for the treatment and/or prevention of obesity.

We recently extended this concept by demonstrating that human adipocytes express a sulfonylurea receptor (SUR) that exerts a regulatory effect on the Ca^{2+} channel and, consequently, modulates adipocyte lipid accumulation (15,24). Compounds acting on the pancreatic SUR to increase (e.g., glinbeclamide) or decrease (e.g., diazoxide) intracellular Ca^{2+} (indirectly, *via* a K^+-ATP channel) cause corresponding increases and decreases in weight gain, although these effects have previously been attributed to the effects of these compounds on circulating insulin. However, the identification of SUR expression in human adipocytes (15) suggests that it may modulate adipocyte Ca^{2+} flux and thereby regulate lipid metabolism. Indeed, the SUR agonist glinbeclamide increases human adipocyte intracellular Ca^{2+} and thereby causes

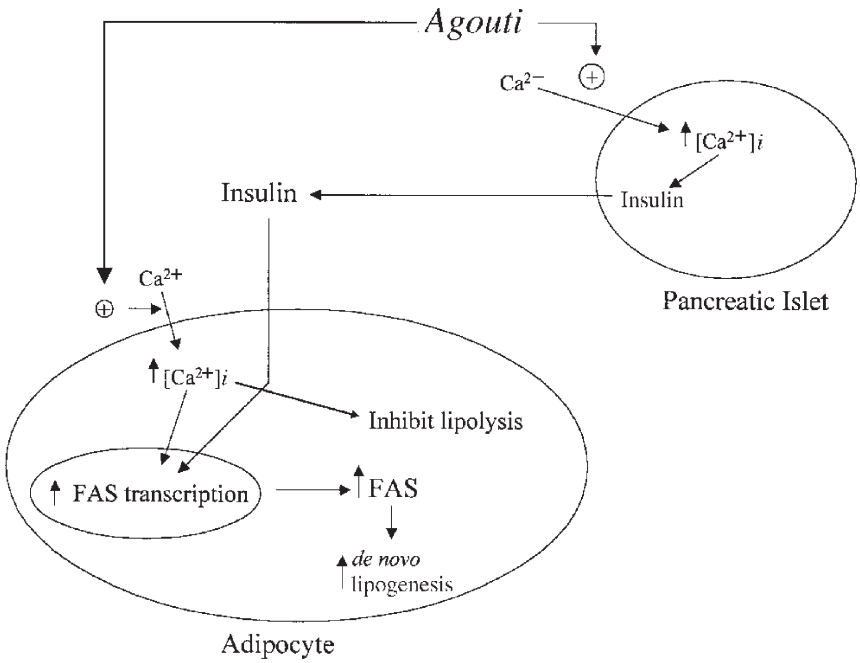

FIG. 1. Ca^{2+}-mediated mechanisms of *agouti* regulation of adiposity. FAS, fatty acid synthase.

marked increases in lipogenic enzyme activity and inhibition of lipolysis. Moreover, inhibition of the adipocyte SUR-regulated Ca^{2+} channel with diazoxide completely prevents each of these effects. Accordingly, the adipocyte SUR may represent a new target for the development of pharmacological interventions in obesity (15). In support of this concept, diazoxide has been demonstrated to exert significant antiobesity effects in both obese Zucker rats and hyperinsulinemic obese adults (25–27). Although this effect was attributed to actions on pancreatic β-cell insulin release, we subsequently found diazoxide treatment significantly suppresses adipose tissue FAS and lipoprotein lipase in obese Zucker rats (24).

ROLE OF 1,25-$(OH)_2$-D IN REGULATING ADIPOCYTE Ca^{2+} AND LIPID METABOLISM

Based on these findings and on our earlier observations of dietary calcium-induced reductions in adiposity, we proposed that the elevations in 1,25-(OH_2)-D that occur in response to low-calcium diets may stimulate adipocyte Ca^{2+} influx and thereby increase adiposity. Indeed, we recently reported that 1,25-(OH_2)-D stimulates significant Ca^{2+} influx and sustained dose-responsive increases in steady-state intracellular Ca^{2+} in primary cultures of human adipocytes (1). Moreover, treatment of human adipocytes with 1,25-(OH_2)-D resulted in a coordinated activation of FAS and inhibition of lipolysis, similar to the action of *agouti* on these cells (1,28). Consequently, suppression of 1,25-(OH_2)-D with high-calcium diets would be anticipated to reduce adipocyte intracellular Ca^{2+}, inhibit FAS, and activate lipolysis, thereby exerting an antiobesity effect. This concept is summarized in Figure 2.

DIETARY CALCIUM MODULATION OF ADIPOSITY

This concept was confirmed in transgenic mice expressing *agouti* in adipose tissue under the control of the aP2 promoter. Mice placed on low-calcium (0.4%)/high-fat/high-sucrose diets for 6 wk exhibited marked increases in adipocyte lipogenesis, inhibited lipolysis, and accelerated increases in body weight and adipose tissue mass. However, high-calcium (1.2%) diets reduced lipogenesis by 51% and stimulated lipolysis three- to fivefold, resulting in 26–39% reductions in body weight and adipose tissue mass (1). The magnitude of these effects depended on the source of dietary calcium, with dairy sources of calcium exerting significantly greater effects than calcium carbonate.

These data are consistent with our observation that 12 mon of yogurt supplementation, sufficient to raise daily calcium intake from approximately 400 to 1000 mg/d, resulted in a 4.9-kg reduction in body fat in obese African Americans without an accompanying reduction in caloric intake. The relevance of this finding at the population level was assessed *via* analysis of the National Health and Nutrition Examination Survey; odds ratios for percent body fat as a function of calcium intake were estimated by logistic regression, with age, race/ethnicity, activity level, and caloric intake as covariates. The odds of being in the highest quartile of body fat were reduced from 1.0 for the first quartile of calcium intake to 0.75, 0.40, and 0.16 for the second, third, and fourth quartiles of calcium intake, respectively, for women (1). The regression model for males similarly demonstrated a significant inverse relationship between dietary calcium and body fat, although the same simple dose–response relationship found in women was

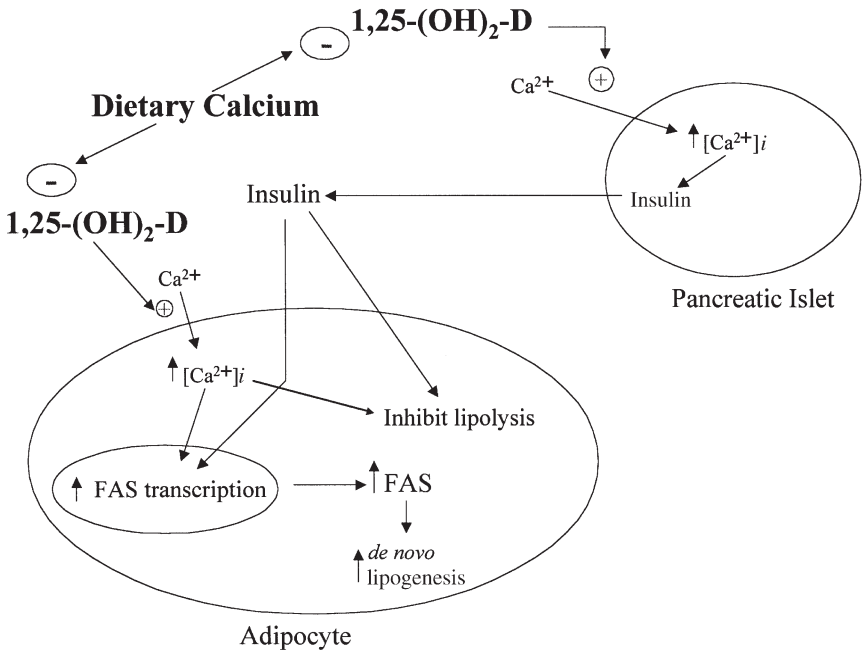

FIG. 2. Dietary calcium modulation of adiposity is mediated *via* 1,25-dihydroxy-vitamin D [1,25-(OH_2)-D] regulation of Ca^{2+} flux.

not evident (1). Notably, the strength of the relationship was improved for both genders when dairy product intake was included in the analysis compared to when total calcium intake was evaluated without consideration of calcium source.

These data have significant implications for the prevention or attenuation of diet-induced obesity but do not directly address the issue of whether high-calcium diets will exert any effect on established obesity. Accordingly, we next conducted a study to extend these findings by determining whether dietary calcium and calcium-rich dairy products reduce metabolic efficiency and accelerate fat loss secondary to caloric restriction in the same mouse model following dietary induction of obesity (29). Mice (aP2-*agouti* transgenic) similar to those in the studies described above were used. They were fed the same basal low (0.4%)-Ca/high-fat/high-sucrose diet for 6 wk as in the preceding study.

Administration of the low-calcium (0.4%), high-fat, high-sucrose diet to aP2-*a* mice for 6 wk resulted in a ~100% increase in adipocyte $[Ca^{2+}]_i$ (128 ± 18 vs. 267 ± 15 nM, $P < 0.001$), with a corresponding body weight gain of 29% ($P < 0.001$) and twofold increase in total fat pad mass ($P < 0.001$), demonstrating that diet-induced dysregulation of adipocyte $[Ca^{2+}]_i$ is associated with increased adiposity in aP2-*a* mice.

All three calcium diets, including the high-calcium diet (1.2% Ca derived from $CaCO_3$), the medium-dairy diet (1.2% Ca derived from nonfat dry milk replacing 25% of protein), and the high-dairy diet (2.4% Ca derived from nonfat dry milk replacing 50% of protein), caused a 50% decrease in adipocyte $[Ca^{2+}]_i$ ($P < 0.001$). In contrast, $[Ca^{2+}]_i$ in adipocytes from mice maintained on the energy-restricted basal low-calcium diet remained at the same elevated level as that of animals fed *ad libitum*.

Energy restriction resulted in a body weight loss of 11% ($P < 0.001$), compared to the *ad libitum* group. However, markedly greater weight reductions of 19, 25, and 29% were observed in the high-calcium, medium-dairy, and high-dairy groups, respectively ($P < 0.01$ vs. basal energy-restricted group). Consistent with this result, energy restriction caused only 8% lower fat pad mass (not significant), compared to the basal diet *ad libitum* group, whereas the high-calcium diet caused a 42% decrease ($P < 0.001$), which was further reduced by 60 and 69% by the medium- and high-dairy diets ($P < 0.001$ vs. basal energy-restricted group), respectively.

The high-calcium diet caused a 35% decrease in FAS activity ($P < 0.05$ vs. basal energy-restricted group), which was further reduced by 63 and 62% by the medium- and high-dairy diets ($P < 0.05$), respectively; FAS mRNA followed a similar trend. Increasing dietary calcium caused a corresponding increase in lipolysis. Although the basal energy-restricted diet did not affect adipocyte lipolysis, the high-calcium diet caused 77% stimulation in lipolysis ($P < 0.05$), which was further increased in the medium- and high-dairy diet groups ($P < 0.05$ vs. basal energy-restricted group). Increased lipolysis, coupled with decreased lipogenesis, may represent a metabolic state in which the efficiency of energy metabolism is shifted from energy storage to energy expenditure.

This shift in energy metabolism was further confirmed by a dietary calcium-induced increase in core temperature. All three high-calcium diets exerted stimulatory effects on core temperature, with 0.48, 0.57, and 0.67°C increases on the

high-calcium, medium-dairy, and high-dairy diets, respectively ($P < 0.05$), whereas the basal energy-restricted diet did not affect core temperature. A possible physiological basis underlying the increased core temperature is that the expression of uncoupling protein 2 (UCP2), which has been implicated in thermogenesis, was upregulated in white adipose tissue, with an 80% increase in all three high-calcium diets ($P < 0.05$). However, the role of UCP2 in thermogenesis is not clear, and further study is required to address the precise mechanism whereby dietary calcium regulates UCP2 expression. Although this upregulation of UCP2 expression may result directly from inhibition of $[Ca^{2+}]_i$, it is also possible that it is merely a result of increased substrate (FA) flux secondary to increased lipolysis. However, we recently found that 1,25-$(OH)_2$-D directly suppresses UCP2 expression in isolated human adipocytes and that this effect is independent of FA flux (30).

Collectively, these data demonstrate that high-calcium diets suppress adipocyte $[Ca^{2+}]_i$ and thereby reduce energy storage and increase thermogenesis during energy restriction, with greater effects exerted by dairy products than by elemental calcium. Recent findings from other laboratories support a beneficial role for calcium in weight control. In a 2-yr prospective study of 54 normal-weight women participating in an exercise intervention, the dietary calcium/energy ratio was a significant negative predictor of changes in both body weight and body fat (31); moreover, increased total calcium and dairy calcium intakes predicted fat mass reductions independently of caloric intake for women at lower energy intakes (below the mean of 1876 kcal/d) (31). A similar beneficial effect of dietary calcium on body fat mass accumulation has been demonstrated in growing children, as a significant inverse relationship between dietary calcium and body fat was recently reported in a 5-yr longitudinal study of preschool children ($R^2 = 0.51$) (32).

Davies *et al.* (33) conducted a series of calcium intervention studies designed with primary skeletal end points, and have recently re-evaluated these data with a body weight end point. The re-analysis involved 780 women who participated in five clinical trials (i.e., four observational and one double-blind, placebo-controlled, randomized trial). They noted significant negative associations between calcium intake and body weight for all age groups (third, fifth, and eighth decades of life), and an odds ratio for being overweight of 2.25 for young women in the lower half vs. the upper half of calcium intake (33). Data from the randomized controlled trial demonstrated a calcium treatment effect of 0.325 kg weight loss per year over 4 yr with no intentional change in caloric intake; overall, the relationships derived from this re-analysis indicate that a calcium intake increase of 1,000 mg/d is associated with an 8-kg reduction in body weight (33).

We recently studied the efficacy of a calcium-fortified breakfast cereal, alone or with a small amount of milk, in attenuation of weight and fat gain in the aP2-*a* transgenic mouse (34). Male mice placed on a basal low-calcium (0.4%)/high-fat (25 energy %)/high-sucrose diet for 6 wk exhibited *ca.* twofold increases in $[Ca^{2+}]_i$ and both visceral and subcutaneous fat mass. However, addition of a calcium-fortified breakfast cereal sufficient to increase dietary calcium to 1.2% with macronutrient adjustments to ensure identical carbohydrate, protein, and fat levels with the basal diet resulted in a 41% decrease in adipocyte $[Ca^{2+}]_i$ ($P < 0.001$) and 25–30% decreases in weight gain ($P < 0.03$) and total fat pad mass compared to the basal diet ($P < 0.001$), whereas food consumption was unaffected. Comparable decreases were found in both subcutaneous and visceral fat compartments. A second control group, which received the basal diet supplemented with the same amount cereal without calcium fortification (with macronutrient adjustment) was not significantly different from the basal control group.

We also found the calcium-fortified cereal to have similar effects in markedly accelerating weight and fat loss secondary to caloric restriction in these mice. Interestingly, addition of sufficient nonfat dried milk to bring the calcium content of the calcium-fortified cereal diet from 1.2 to 1.3% (with macronutrient adjustment) resulted in substantial amplification of these effects. Thus, a calcium-fortified breakfast cereal is effective in reducing adiposity and accelerating fat loss during caloric restriction in this model of obesity, whereas addition of a small amount of milk significantly amplifies this effect further.

We recently confirmed the utility of calcium-rich diets in accelerating fat loss during a 6-mon clinical trial in obese patients (35). Obese adults ($n = 32$) were maintained for 24 wk on balanced deficit diets (500 kcal/d deficit) and were randomized to control (0–1 serving/d and 400–500 mg Ca/d supplemented with placebo), high-calcium (control diet supplemented with 800 mg Ca/d), or high dairy (3–4 servings of low-fat dairy products/d, total calcium intake of 1200–1300 mg/d). Control patients lost 6.4 ± 2.5% of their body weight, which was increased by 26% on the high-calcium diet and 70% (to 10.9 ± 1.6%) on the high-dairy diet ($P < 0.01$). Fat loss (*via* dual X-ray absorptiometry) followed a similar trend, with the high-calcium and high-dairy diets augmenting the fat loss found on the low-calcium diet by 38 and 64%, respectively ($P < 0.01$).

An unexpected finding was a marked change in the distribution of body fat loss (35). Patients on the low-calcium diet lost 5.3 ± 2.3% of their trunk (abdominal region) fat on the low-calcium diet. This was increased to 12.9 ± 2.2% on the high-calcium diet and 14.0 ± 2.3% on the high-dairy diet ($P < 0.025$ vs. low-calcium and high-calcium diets). Consequently, fat loss from the abdominal region represented 19.0 ± 7.9% of the total fat lost on the low-calcium diet, and this was increased to 50.1 ± 6.4% of the fat lost on the high-calcium diet ($P < 0.001$) and 66.2 ± 3.0% on the high-dairy diet ($P < 0.001$). Thus, increasing dietary calcium not only accelerates weight and fat loss secondary to caloric restriction but also shifts the distribution of fat loss to a more favorable pattern, with more fat lost from the abdominal region on the high-calcium diet. Moreover, dairy products exert a substantially greater effect on both fat loss and fat distribution compared to an equivalent amount of supplemental calcium.

Consistent with this, Melanson *et al.* (36) recently reported that higher calcium intakes are associated with higher rates of whole-body fat oxidation measured in a whole-room calorimeter, with significant effects noted over a 24-h period, during sleep, and during light exercise.

ROLE OF ADDITIONAL DAIRY-DERIVED BIOACTIVE COMPOUNDS

Data accumulated from experimental animal and human studies clearly support a beneficial role for dietary calcium in weight management, but markedly greater effects are evident from dairy products vs. nondairy sources of calcium. Although the additional components of dairy products responsible for the differential effects between calcium and dairy products are not yet know, work is under way to determine their identity. At present, preliminary data suggest that this additional activity resides in the whey fraction of milk. Whey is recognized as a rich source of bioactive compounds (37) that may act independently or synergistically with the calcium to attenuate lipogenesis, accelerate lipolysis, and/or affect nutrient partitioning between adipose tissue and skeletal muscle. Notably, whey proteins have recently been reported to contain significant angiotensin-converting enzyme (ACE) activity (38,39). Although ACE inhibitory activity may appear to be more relevant to an antihypertensive effect of dairy than to an antiobesity effect, recent data demonstrate that adipocytes have an autocrine/paracrine renin–angiotensin system (RAS), and that adipocyte lipogenesis is regulated, in part, by angiotensin II (reviewed in Ref. 40). Thus, activation or suppression of the adipocyte RAS may exert corresponding effects on adipocyte lipid metabolism independently of the circulating RAS. Indeed, inhibition of the RAS mildly attenuates obesity in rodents, and limited clinical observations support this concept in hypertensive patients treated with ACE inhibitors (40). Thus, it is possible that whey-derived ACE-inhibitory activity may contribute to the antiobesity effect of dairy products (41). However, it is also possible that other whey bioactive compounds may contribute or, alternatively, that a synergistic effect of multiple factors, along with the aforementioned effects of the calcium, are responsible. For example, Layman (42) has recently proposed that the rich concentration of leucine in whey protein may play a significant anabolic role in skeletal muscle and thereby contribute to greater maintenance of skeletal muscle mass during weight loss. Accordingly, the high concentration of leucine and other branched-chain amino acids in dairy products may also be an important factor in the repartitioning of dietary energy from adipose tissue to skeletal muscle.

CONCLUSION

A growing body of evidence now clearly demonstrates a beneficial role for dietary calcium in the partitioning of dietary energy, resulting in reductions in body fat and an acceleration of weight and fat loss during energy restriction. Interestingly, dairy sources of calcium exert substantially greater effects than supplemental or fortified sources of calcium. There is a strong theoretical framework in place to explain the "anti-obesity" effects of dietary calcium; however, the mechanism whereby dairy products augment this anti-obesity effect is not yet clear, although it may be mediated by whey peptides. These data have important implications for the prevention of both pediatric and adult obesity, especially in light of the marginal calcium intakes exhibited by the majority of the population (Fig. 3) and the population-based data indicating protection from obesity and the insulin resistance syndrome in populations consuming greater amounts of calcium and dairy products (1,43,44).

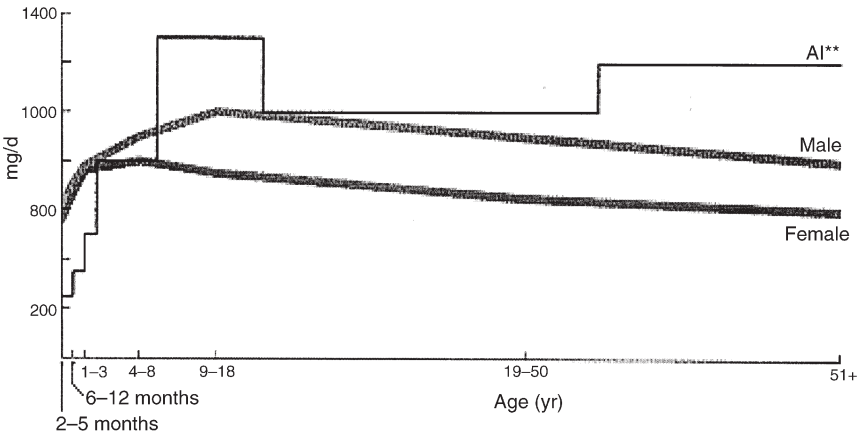

FIG. 3. Median calcium intake from food of the U.S. population, 1988–1994. **AI, adequate intake. 1997 Dietary Reference Intakes, Food and Nutrition Board, Institute of Medicine, National Academy of Sciences. *Source*: Centers for Disease Control/National Center for Health Statistics (CDC/NCHS), National Health and Nutrition Examination Survey (NHANES III) 1988–1994. Figure used by permission of the National Dairy Council.

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Treatment and Prevention of Obesity: What Works, What Doesn't Work, and What Might Work

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ABSTRACT: We provide a very broad conceptual overview of some of the issues involved in the treatment and prevention of obesity. Data suggest that clinicians have some ability to promote positive changes with obesity treatment. The environment, though important in influencing one's degree of adiposity, has largely transient effects that do not tend to carry over from one time to substantially later times. In contrast, the genetic influences on body mass index at any one time do tend to carry over to later times. This information influences the types of approaches that are and are not likely to be successful in terms of preventing obesity or reducing obesity on a population level. A second issue concerns the composition of weight lost. Conditional on fat loss, weight loss has been associated with an increased mortality rate (MR) whereas, conditional upon weight loss, fat loss has been associated with a decreased MR. This suggests that we should seek treatments that maximize the proportion of weight lost as fat. Third, the efficacy of current treatments is far below patients' expectations and desires. We need both to increase the efficacy of our treatments dramatically and help patients adjust their expectations so that they can take satisfaction in smaller weight losses. Perhaps, with continued efforts at enhancing treatments, we will see incremental advances in the treatment and prevention of obesity.

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FACTORS INFLUENCING OBESITY

Before discussing treatment and prevention, let us first consider what we know about the causes of obesity, as this may influence the choice of treatment or prevention strategies. Consider the two sets of data in Figure 1. The first set, plotted in a steadily downward-descending line, is the change in back fat thickness among Canadian Yorkshire pigs from 1980 to 1995 (1). In contrast, the upward-sloping line is the average body mass index (BMI; kg/m^2) of American adults over the last three decades (2). While pigs are getting leaner, Americans are getting fatter. This figure shows the importance of both genetic and environmental factors in influencing the degree of body fatness. The line for pigs is going downward because they are being subjected to selective breeding for leanness. Most geneticists believe that there is no stronger evidence for genetic influence on a trait than the ability to selectively breed to a higher or lower value of that trait. In contrast, the upward-sloping line for humans is almost certainly a result of environmental factors. In the relatively short time captured in this line (three decades), it is unlikely that sufficient changes have occurred to the human gene pool to be responsible for population changes in phenotypic expression of any important magnitude. Thus, these data show that both genes and environments play critical roles in determining degree of adiposity.

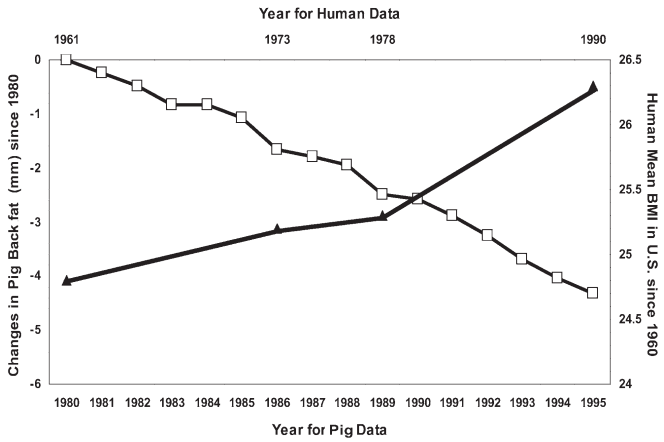

FIG. 1. Changes in pig back fat (□) and human mean body mass index (BMI) (▲) from 1961 to 1990. Pig (Candian Yorkshire) data from the Canadian Swine Breeders Association (1); human data from The National Health and Nutrition Examination Surveys (2).

That both environmental and genetic factors influence the degree of adiposity may seem obvious in the 21st century, but the belief that obesity could have important genetic determinants has not always been considered a reasonable hypothesis. Obesity is often thought to be a disorder of both behavior and "willpower," and many people believe that these factors are orthogonal to genetic factors. It had been pointed out previously that spouses, who are presumably genetically unrelated, are similar to each other with respect to BMI. It had even been noted that obese dog owners are more likely to have obese dogs than are nonobese dog owners (3). Although both of these observations are correct, they do not necessarily imply that family members are similar to one another with respect to degree of adiposity because of common environmental rather than genetic factors. People do not select spouses (and presumably dogs) at random. Although we have no evidence on how people come to choose particular dogs as pets, there is evidence that people tend

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Abbreviations: BMI, body mass index; DPP, Diabetes Prevention Program; MR, mortality rate; SES, socioeconomic status.

to choose mates who are similar to them. Indeed, Allison *et al.* (4) published evidence that couples who would eventually become spouses were correlated by BMI even before marriage and cohabitation. Similarly, Knuiman *et al.* (5) found that length of marriage was unrelated to degree of correlation for BMI between husbands and wives. Thus, husbands and wives apparently are similar to each other with respect to BMI not because they grow to become more alike due to common environmental influences, but rather because they tend to pick mates who resemble them with respect to BMI.

In Table 1 are descriptions of four different types of studies that have been used to estimate the heritability of BMI. Heritability can be defined as the ratio of phenotypic variance due to genetic variations within the population to total phenotypic variance within the population. Heritability is conceptually akin to the R^2 that would be obtained if one could correlate all genetic factors of influence with BMI in a multiple regression equation. That is, heritability is the proportion of within-population variance in BMI, which is explained by within-population genetic factors. All of the twin-based studies (Table 1) tend to obtain heritability estimates around 70%. In contrast, family and adoption studies tend to obtain estimates of 25 to 50% (6). The detailed mathematics of why these different studies may yield different answers is beyond the scope of this paper. In brief, family and adoption studies are effective at estimating heritability when all factors are added and environment is static. On the other hand, twin studies are effective at estimating heritability when there is a great deal of non-additivity, such as occurs with gene/gene and gene/environment interaction, as there is with respect to obesity (7). Therefore, we believe that the more valid estimates come from twin studies and that a very reasonable estimate of the broad or total heritability of BMI tendency is approximately 70% (8). For more detailed discussion of these issues, see References 7 and 9.

At least three genetic studies have shown that, although the environment does play a large role in influencing one's degree of adiposity, the effects of the environment are transient (10–12). That is, an environmental influence on BMI at one point in time does not tend to carry over to later points in time to any great extent. In contrast, the genetic influences on BMI at any one time do tend to carry over. This point is critical because it suggests what type of approaches are and are not likely to be successful in terms of preventing obesity or reducing obesity on a population level.

It is often stated that obese children grow up to be obese adolescents, and obese adolescents grow up to be obese adults—that is, BMI "tracks." These statements are undeniably correct.

TABLE 1
Studies of Heritability of Body Mass Index

Type of study	Heritability (%)	Reference
Family and adoption	~25–50	6
Ordinary twins	~50–85	6
Monozygotic twins reared apart	~70	8
Virtual twins[a]	~65	9

[a]Same-age unrelated siblings.

However, it is sometimes assumed from these simple observations that if we prevent obesity early in life, we will prevent obesity throughout life. This statement assumes that environmental factors that influence obesity early in life will have effects that are present throughout life. This contradicts observations from the genetic studies, which show us that people's BMI values track mainly because of the constant influence of the genotype. Therefore, any environmental influence that is put into place to reduce obesity levels on a population scale must be put in place throughout life. The idea of simply "inoculating" children against the environmental influences that will lead to obesity in adulthood seems out of step with everything we know about the ontogeny of obesity.

A second important point that we learn from genetic studies is what the likely impact of environmental changes might be. Although more than half of the variance of BMI within human society can be attributed to genetic variations, this fact in no way implies that the environment is "impotent" with respect to the ability to change population levels of BMI. Adapting an idea from Hewitt (13) and considering that the heritability of BMI is approximately 0.70 and the SD of BMI among adults in the United States is approximately 5.8, we then can write the following equation:

$$\text{BMI} \approx 5.8\left(\sqrt{0.7}\cdot G+\sqrt{1-0.7}\cdot E\right) \quad [1]$$

where E represents environmental factors and G represents genetic factors. This equation implies that a 1 SD decrease in the environmental factors that increase BMI would reduce BMI approximately 3.2 units. Across both men and women, the average American is approximately 68 in. or 173 cm tall. For such a person, a BMI reduction of 3.2 units would correspond to a loss of approximately 21 lb or 9.6 kg. The average obese person who comes for treatment in a clinical trial usually weighs approximately 100 kg. Thus, this reduction would represent a loss of approximately 10% of body weight. It has been shown that a loss of 10% of body weight is greater than is needed to achieve a clinically meaningful health benefit (14,15) and is equivalent to the same amount of weight loss that can be produced by the best available pharmaceuticals today (16). To put a 1-SD decrease in environmental factors in perspective, consider that if the environmental liability to obesity were roughly normally distributed, then a 1-SD decrease might entail, for example, moving someone from the 68th percentile of environmental liability to the 50th percentile. That is, one would not have to move down in environmental liability to be abnormally low in risk but would only have to move from the upper part of the distribution to more average levels. This seems to be a plausible achievement for our society.

WHY SHOULD WE CARE ABOUT TREATING AND PREVENTING OBESITY?

In *Henry IV Part II,* Act 5, Shakespeare wrote, "Leave gourmandizing. Know that the grave doth gape thrice wider for thee than for other men." Shakespeare used this language to

have Henry warn Falstaff of the dangers of his obesity. Several hundred years later we now have ample evidence that obesity is associated with an increased rate of mortality (17). Indeed, Allison *et al*. (17) estimated that, in the United States, obesity could have accounted for 300,000 deaths during year 1991. Given recent increases in the prevalence of obesity (18), this number may even be higher today. That extremes of relative body weight or BMI are associated with increased mortality rate (MR) is virtually incontrovertible. Nevertheless, many questions remain as well as some controversy.

One recent controversy concerns the relationships among cardiorespiratory fitness, fatness, and MR. Cardiorespiratory fitness is an important predictor of MR (19). It also is correlated with and may be a good marker for activity levels (19). Based on such observations, one occasionally hears the conjecture that one should not be concerned about how fat a person is but only about how fit. In our opinion, such a view is erroneous for several reasons. First, the activities we would advise people to undertake to increase their cardiorespiratory fitness may be the very same activities we would advise them to undertake to decrease their body weight. To the extent that this is true, then from a public health and clinical point of view, the question of whether we should advise people to maintain a low body weight or a high degree of cardiorespiratory fitness is of minimal relevance because the behaviors required will be the same to achieve either end. Second, cardiorespiratory fitness and BMI tend to be correlated. Therefore, statements about the independent causal effects of one vs. the other are difficult to make without assuming some scheme of causation. It is possible that becoming obese leads to lesser cardiorespiratory fitness. In this case, while it might be true that, independent of cardiorespiratory fitness, BMI would have no impact on MR, the point would be irrelevant because the way to achieve greater cardiorespiratory fitness would be to lower body weight. Finally, there are actually data to suggest that both cardiorespiratory fitness and relative body weight are important predictors of MR. Indeed, it seems odd to us that questions about the relations between cardiorespiratory fitness, obesity, and MR are ever cast in the framework of either/or. Given the possibility of independent effects, asking which is more important, "fitness or fatness," seems akin to asking, "Which is more important, not smoking cigarettes or wearing a helmet while riding a motorcycle?" If one wants to prolong one's life, it is good neither to smoke cigarettes nor to ride a motorcycle without wearing a helmet.

This issue of fitness vs. fatness is made clear by an analysis of data from the Cooper Institute. Wei and Wallance (20) published an abstract in which they analyzed the joint effects of fitness and fatness on over 11,000 men. The results are depicted in Figure 2. Wei and Wallance found that, in men, obesity was a predictor of cardiovascular disease as well as mortality, even after adjustment for cardiorespiratory fitness and conventional risk factors (20). Thus, these data reinforce the concept that maintaining a non-obese body weight is an important factor in prolonging life.

Another question about the nature of the relationship between body weight and MR concerns the question, "How much of the apparent effect of obesity on MR is an effect of obesity *per se* or caloric excess?" It has been demonstrated in many studies of laboratory animals that caloric restriction prolongs life. Indeed, caloric restriction is the only treatment known to prolong life in multiple species including mammals. This has led some caloric restriction investigators to speculate that body weight or obesity itself is irrelevant and only caloric intake matters. This empirical question can be addressed. Recently, data from the Biosure Study, which involved 1,200 Wistar rats given varying diets and followed until late adulthood, were analyzed (Wang, C., Weindruch, R., Fernandez, J., Patel, P., and Allison, D., unpublished data) to identify whether the effects of body weight and caloric intake on MR in rats were independent (21). Figure 3 shows MR among these rates as a function of their degree of body weight in quintile-defined categories, where the weight was taken at 141 d of life. The hazard ratios displayed on the *y*-axis have been adjusted for caloric

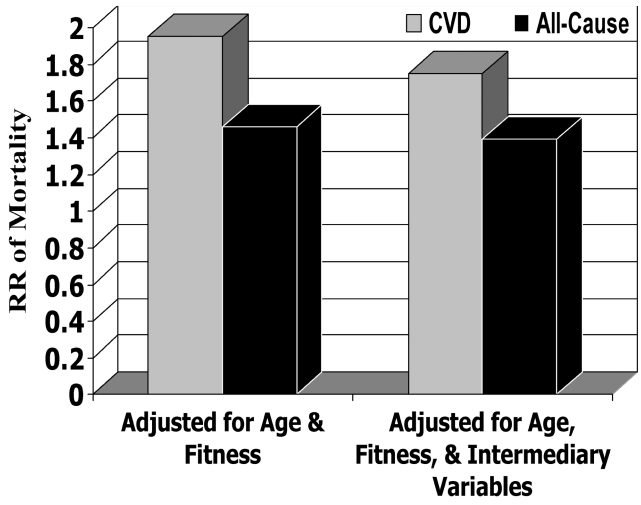

FIG. 2. Independent effects of obesity on relative risk (RR) of mortality. Obesity was a predictor of cardiovascular disease and all-cause mortality in the long run, even after adjustment for cardio-respiratory fitness and conventional risk factors in men. Subjects: 11,610 men ages 20–89 yr with 20–24 yr of follow-up. Obesity: body mass index (BMI) ≥ 30. Data from Reference 20.

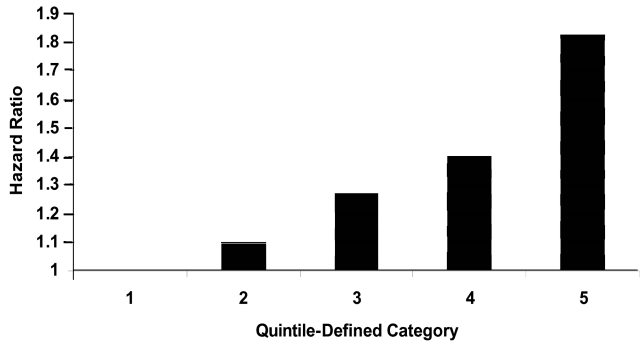

FIG. 3. Biosure Study of Wistar rats given different caloric diets. Higher weight is associated with increased mortality rate monotonically and independently of dietary (caloric) restriction (Wang, C., Weindruch, R., Fernandez, J., Patel, P., and Allison, D., unpublished data). Results represent mortality rate as a function of weight at day 141.

intake. As can be seen, higher body weight is monotonically positively related to MR. Thus, it appears that body weight and caloric intake have independent effects on longevity.

WHAT WILL WORK TO IMPROVE HEALTH?

Having reviewed evidence on the origins of variations in adiposity levels and on the deleterious effects of obesity on MR, which should provide an impetus in considering potential ways to reduce obesity levels, we can now ask, "What methods will work to improve health among obese persons?" For several years obesity researchers stated that small weight losses of only 5 to 10% of initial body weight are sufficient to produce important improvements in health. However, truly compelling data in support of such statements have only recently become available. One study in this regard is the Diabetes Prevention Program (DPP) (15). The DPP found that subjects who lost, on average, less than 8 kg of body weight and who, at an average of 2.8 yr follow-up, maintained less than 4 kg of body weight loss on average, experienced a 58% reduction in the incidence of new onset diabetes (15). This study shows that a weight loss that many have previously dismissed as trivial can in fact have profound health consequences.

Despite the health benefits of weight loss among obese people, many have questioned whether weight loss treatment is perhaps not ill-advised for other reasons. One question that has been raised is whether weight loss and the regain that so often accompanies weight loss attempts have long-term adverse psychological effects. This has now been investigated by a number of scientists, perhaps most notably Foster and colleagues. Foster *et al.* (21) prospectively assessed psychological effects of weight loss and regain in obese women. They found that even after a 21-kg cycle of weight loss and regain, women did not have deleterious changes in psychological function. In contrast, they reported significant improvements in both mood and eating behavior (21). These data and others collected by Foster and colleagues suggest that, whereas we should be vigilant about possible adverse effects of weight loss on individuals, adverse psychological effects are not common consequences of weight loss treatment. In fact, not only does weight loss treatment appear not to have deleterious psychological effects, but also it actually appears to have beneficial psychological effects. Fontaine *et al.* (22) studied quality of life in several domains before and after weight loss treatment. They found that weight loss treatment significantly improved quality of life in multiple domains.

With respect to MR, the evidence is still equivocal as to what the effects of weight loss are among obese persons. Although a number of studies have been reported on this topic, almost all are observational epidemiological studies in which the causes of weight changes are not well understood. Even among studies of so-called intentional weight loss, the subjects for whom weight changes are observed are merely subjects who at one point state that they intend to lose weight. It is not clear whether the weight loss that they achieve, if any, is due to their intention or to other factors beyond their intention

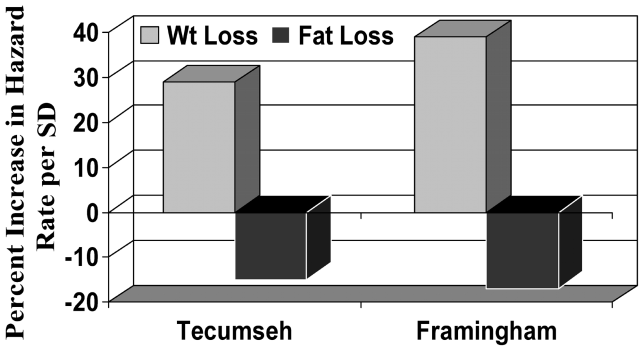

FIG. 4. Weight loss vs. fat loss from two independent cohort studies, the Tecumseh (26) and Framingham data sets (27). Weight loss increases and fat loss decreases all-cause mortality rate.

such as pre-existing subclinical disease. Moreover, whether an individual intends to lose weight may be less important than the methods by which they lose the weight. In observational epidemiological studies, these methods are uncontrolled. Thus, we really know very little about the effects of weight loss on MR among humans at this time. For a balanced review on this topic see articles by Yang *et al.* (23) and Sorenson (24).

Recently, there has been increased recognition that body composition may be a more important variable than body weight *per se*. This is not surprising given that obesity is, by definition, an excess of fatness. We only operationally define it as an excess of body weight. Thus, we might expect greater health benefits when individuals lose fat *per se* than when they simply lose undifferentiated weight. This conjecture is supported by results from two independent epidemiological studies. Allison and colleagues (25) demonstrated that, conditional on fat loss, weight loss was associated with an increased MR whereas, conditional on weight loss, fat loss was associated with a decreased MR (see Fig. 4). This suggests that if we are to help people improve their health we should seek treatments that maximize the proportion of weight that is lost as fat.

HARM REDUCTION AS A NOVEL APPROACH

Yet another approach to alleviating the health problems produced by obesity is harm reduction. The concept of harm reduction is that it may not be possible or desirable to stop people from engaging in a particular behavior that increases their risk of some adverse health event. However, one may be able to set up circumstances that, despite the behavior, minimize the risk or extent of harm that accrues to people. Here, creative environmental solutions may be useful. Consider the fact that some of the most deleterious effects of obesity are on the risk of cardiovascular disease. A major contributor to cardiovascular disease is believed to be saturated fat intake. One of the leading sources of saturated fat is the consumption of animal flesh. Although some might argue it would be appropriate to suggest to the public they abandon eating animal flesh and instead eat fresh vegetables, the majority of the public is unlikely to take this action the majority of the time. However, perhaps there are ways that people can have their

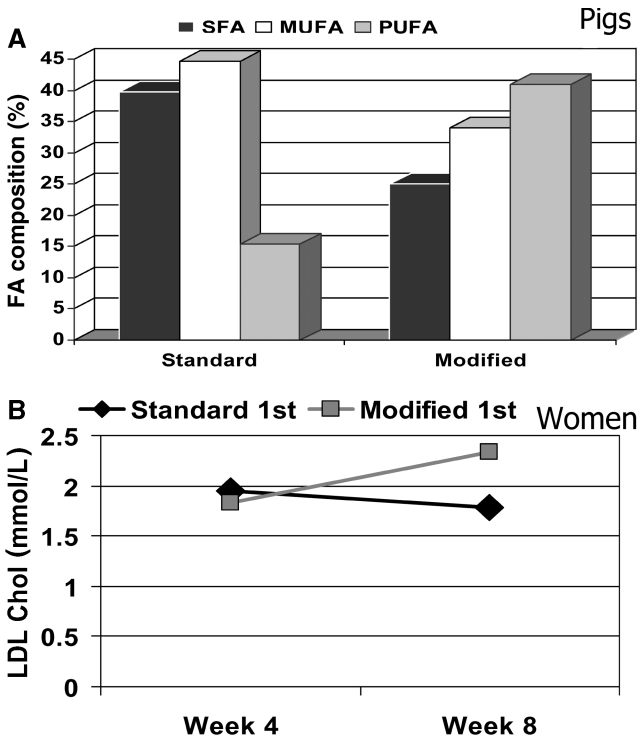

FIG. 5. Harm Reduction as an approach to nutritional excess. Data were analyzed by Stewart *et al.* (28) and reflect the results of a modified pork diet. In the two-stage study, pigs were randomized to be fed either a standard diet or a diet with added soy protein oil. Women were randomized to be fed pork from the two groups of pigs in a cross-over design. SFA, saturated FA; MUFA, monunsaturated FA; LDL Chol, LDL cholesterol.

cake and eat it too. Stewart *et al.* (28) conducted an interesting two-stage study (see Fig. 5) in which pigs were first randomized to be fed either a standard diet or a diet with added soy protein and soybean oil. The flesh of the pigs fed the modified diet subsequently contained substantially more PUFA and less saturated FA (i.e., you are what you eat). The pigs were slaughtered, and the pork from these animals was then fed to women who participated in phase two of the experiment, a cross-over study in which the women were first given pork from pigs fed the modified diet and then from pigs fed the standardized diet, or the reverse. Results showed that pork from pigs fed the modified diet substantially lowered the LDL cholesterol of the women to whom it was fed. Thus, if we allow ourselves to fantasize a bit, we can imagine a physician speaking with a patient whose LDL cholesterol is getting a bit high and, rather than saying, "You need to stop eating so much pork and begin eating more broccoli," instead saying, "You need to start eating more pork chops to get that LDL cholesterol down."

Another form of harm reduction involves preventive medical care. Several studies have shown that obese women are less likely to receive certain preventive medical examinations than are non-obese women (29,30). Reasons for this are not entirely clear but obesity is more common in lower socioeconomic groups, and there may be cost issues or social discomfort on the part of the patient or social prejudice on the part of the health-care providers. Regardless of the underlying cause of this less frequent utilization of preventive medical examinations, to the extent that such examinations are efficacious in preventing illness, some of the adverse health effects associated with obesity may result from lack of preventive medical care. One way to reduce the adverse health effects of obesity at least partially would be to alter environmental conditions such that obese individuals received preventive medical care with greater frequency.

"CONVENTIONAL" CLINICAL TREATMENT

Considering more "conventional" treatments of obesity, we can ask, "Do patients know what is best for them?" It is not uncommon to see presentations of data from surveys in which people comment on the causes of their obesity, what they perceive to be the most helpful ways to lose weight currently existing, or what they perceive to be the methods of weight loss that would be most likely to help. Although such studies have some merit, their results are often taken, inappropriately, at face value. Consider an admittedly small, old study by Murray (31). Murray investigated whether self-control or determination-raising training for obesity was more effective. Murray studied these treatments among people who preferred one treatment to another and found no evidence that the preferred treatment had any interaction effect with the treatment received. That is, treatments were equally efficacious, or non-efficacious, regardless of which treatment one preferred.

TREATMENT EFFICACY

How efficacious is treatment? Previous research has consistently shown that most subjects do not lose weight such that they move from being non-obese to being in the "normal" weight category and then maintain that weight loss for a period of, for example, more than 5 yr. Based on this observation and historical papers, it is not uncommon to read quotations such as "research has shown that approximately 95% of dieters failed to maintain a weight loss of at least 20 lb for 2 yr or more" (32). However, such statements are not based on careful reviews of modern data. Perhaps the most thorough review of modern evidence regarding the efficacy of nonsurgical treatment of obesity comes from Ayyad and Andersen (33). These authors conducted a meta-analysis and found that, across many studies, the median success rate for patients entering obesity treatment was 15%, where success was operationally defined as maintenance of all weight initially lost or maintenance of at least 9–11 kg of weight loss for at least 3 yr of followup (33). Nine to 11 kg corresponds roughly to the 20 lb mentioned previously by Connors and Melcher (32). Although a 15% success rate is hardly stellar, it is three times the often-quoted 5% success rate. These data suggest that clinicians do have some ability to promote positive changes with obesity treatment, that treatments have become better over the years, and, perhaps, that continued efforts at enhancing treatments will continue to produce slow, incremental advances in outcome.

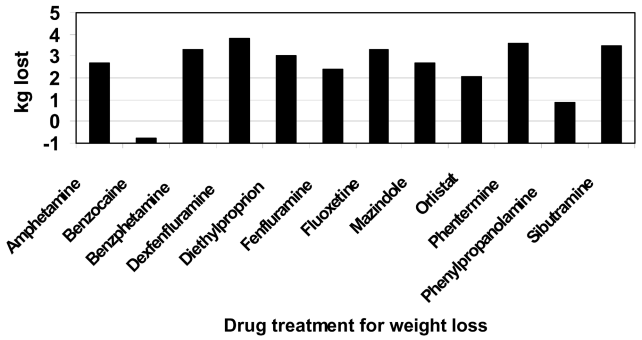

FIG. 6. Efficacy of pharmaceutical treatment of obesity.

What about pharmaceutical treatment? Recently, Haddock *et al.* (16) published a meta-analysis of pharmaceutical treatment for obesity. Their results, depicted in Figure 6, show that, with the exception of two over-the-counter drugs (benzocaine and phenylpropanolamine), all currently available pharmaceuticals for weight loss tend to produce 2–4 kg of additional weight loss above and beyond that produced by placebo control (16). Referring to the earlier results from the DPP (15), this seemingly small weight loss can be seen to be nontrivial. Nevertheless, despite the clinical importance of this weight loss, from the patient's perspective, such small incremental weight losses may be quite disappointing. Foster *et al*. (34) surveyed patients regarding their expectations for obesity treatment. They found that a 15% weight loss or less would be considered disappointing by most patients. Given that the average patient enters treatment weighing approximately 100 kg, a 15% weight reduction would be 15 kg. In contrast, the best available pharmaceuticals on the market produce approximately 10% reduction in body weight or a 10-kg weight loss, which is only 2 to 4 kg above and beyond that produced by placebo. Thus, although we currently have methods available that have important health benefits, we both need to increase the efficacy of our treatment modalities dramatically in order to make them acceptable to patients and to help patients adjust their expectations so that they can take satisfaction in the smaller weight losses that we have the ability to help them achieve.

What about over-the-counter supplements for weight loss? The use of herbal, botanical, and other over-the-counter supplements for weight loss is quite popular. A recent review of the evidence (or lack thereof) supporting the safety and efficacy of such supplements was provided by Allison *et al.* (35), who found, with the exception of supplements containing combinations of ephedrine and caffeine, no compelling evidence for the efficacy of any over-the-counter supplements marketed for weight loss. In addition, although the efficacy of some supplements containing ephedrine and caffeine has been documented, even for this class of supplements, questions about safety continue to be raised (36).

PUBLIC HEALTH APPROACHES, POLICY, AND RELATED APPROACHES

There is increasing recognition that the strategy of simply having individual patients come into clinics to have verbal interactions with treatment providers and/or fellow patients and then go back out into the same "obesigenic environment" in which we all live may not be the optimal approach to the environmental treatment of obesity. Instead, many investigators now recognize that if we have been successful in changing the environment to produce dramatic increases in obesity rates over the last several hundred years, then it may be possible to make changes to the environment to reduce obesity rates. The question is, what changes can and should we make? It is tempting to say glibly that we should simply enter the schools with better provision of food products in lunch programs, better provision of exercise, and proper education programs around the issues of nutrition, activity, and body weight. In addition to associating the more nutritious school lunches and higher activity levels of students in times past with leaner children, there is a belief that, because obese children grow up to be obese adults, if we can prevent obesity in childhood, we can prevent it in adulthood. However, as we discussed above, the foundation for such a belief may be wanting. Moreover, even if this belief were well-founded, one could still question whether any particular program offered to children, in schools or otherwise, would necessarily be a good thing.

Arguing with the merits of education, activity promotion, and reduction of the intake of junk food in schools seems like arguing against the value of "mom and apple pie." However, the scientific literature is rich with examples of prevention and intervention strategies that hardly anyone would have argued with *a priori* yet produced untoward effects. For example, who could disagree with trying to prevent sexual abuse, suicide, and eating disorders among children and adolescents? Yet work by Carter *et al.* (37), Callahan (38), and Taal and Edelaar (39) all are studies describing examples of prevention programs that actually had deleterious effects on youth. What these studies suggest is that we should not naively assume that all preventive efforts aimed at children in schools that are built around seemingly positive things like physical activity and nutrition education will necessarily have positive consequences. Well-controlled research is needed to evaluate the actual effects of treatment and prevention strategies we propose, however "obviously" beneficial they may seem on the surface.

Consider the following set of, perhaps superficially, unrelated facts. Gosler (40) studied fat storage in the birds called great tits (*Parus major*). These birds increase their fat reserves when food is scarce and variable. That is, when birds do not know where their next meal is coming from, they actually eat more and become fatter. Moreover, this effect is primarily seen among birds lower in the social hierarchy. Sobal and Stunkard considered fat storage (as indexed by BMI) in humans (41). Humans of lower socioeconomic status (SES) report more frequent food insecurity. Food insecurity is akin to "not knowing where your next meal is coming from." Such humans of lower SES also have been shown to have increased fat reserves. Is there a parallel? If so, what might this say about proposals to alter the environment to reduce obesity levels that restrict access to food during certain periods of

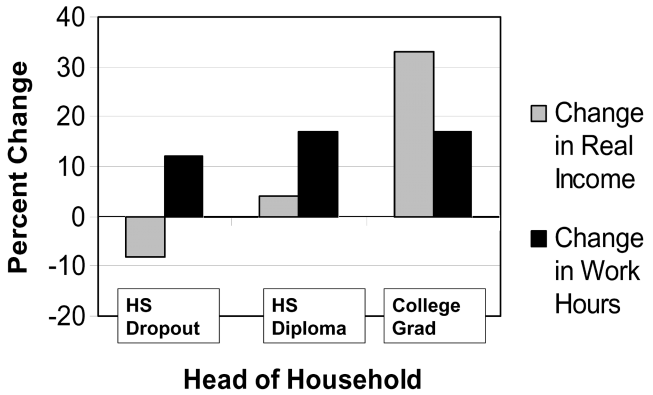

FIG. 7. Changes in real income and hours of work for two-earner families from 1970 to 1990 (47) according to head of household.

time or in certain circumstances? Is it possible that certain restrictions might actually lead to overconsumption in other environments where such food is available? Might this suggest, for example, that in the school or workplace, restricting access to foods or certain foods unequivocally might be less beneficial than simply making it inconvenient to get certain foods or all foods during certain times? We will only know the answers to these questions if we are prepared to conduct rigorous research evaluating them.

What have we learned from school-based obesity research programs? At least four large-scale, expensive, school-based obesity prevention programs have been conducted: CATCH (42), Pathways (43), Apples (44), and SPARK (45). Four out of four of these studies showed no effect on body weight. Why were these studies unsuccessful? The current authors make no pretense of knowing the answer to this question. However, it seems important that investigators who are proposing the next school-based obesity prevention program, if there are such investigators, carefully study these past failures at preventing obesity in childhood lest they serve as examples of Santayana's truism, "Those who do not know their history are doomed to repeat it."

What about financial incentives? Another common suggestion for altering the environment in such a way to reduce obesity levels is to tax high-fat, high-calorie, energy dense, or "nutritionally low value" foods and/or financially supplement their complements. There is evidence to suggest that some financial manipulation can produce valuable changes in food purchasing behavior (46). However, although the economic dimension may be one that is easy to focus on because it is so easily quantified, there may be at least one additional dimension that is perhaps more salient. Specifically, the dimension of time may be an under-studied influence on food choice. Consider Figure 7 containing data from Bluestone and Rose (47). These data show that from 1970 to 1990, across almost all households within the United States, real income increased substantially. In addition, across virtually every class of households in the United States, hours worked also increased substantially. Although the U.S. economy presently is not doing as well as it was a few years ago, the data in Figure 7 suggest the possibility that the average American adult is short on time but not on cash. To the extent that this is true, manipulating the price of different foods may be far less important than manipulating the ease, speed, and convenience with which they can be obtained. Clearly this is an area meriting considerably more investigation.

ACTIVITY PROMOTION AS AN INTERVENTION

What about activity promotion? It has been remarked that there seems to be an imbalance in the biomedical research community's attention to food intake modulation as a means of treating or preventing obesity relative to physical activity modulation (48). Moreover, not only does physical activity have potential as a means for treating or preventing obesity, but also it has a number of positive effects independent of any effects it may have on body weight (49,50). Therefore, many authors believe that we should focus considerably less on food intake and considerably more, perhaps even exclusively, on activity promotion. While the current authors would not dispute the idea that activity promotion is important and should be investigated, the extreme view that activity promotion is a panacea that can supplant all other approaches to treating or preventing obesity seems naïve. In a recent review, Bouchard and Blair (51) wrote, "The body of knowledge on physical activity as relevant to obesity outcomes is extremely limited. There are few randomized clinical trials that have lasted one year or more, with reasonable statistical power, adequate moderating of intervention protocols, high levels of compliance, and a proper measurement of outcome variables. The net result is a general lack of a solid research database regarding the role of physical activity in the prevention and treatment of overweight and obesity as well as their co-morbidities." Thus, as Gard and Wright (52) so forcefully point out, the prevailing view that activity promotion in general and activity promotion among children in schools in particular is necessarily going to have important benefits for obesity reduction is not well substantiated at this time. Moreover, there are several reasons to be skeptical about simple proposals for moderate activity as a singular method for reducing obesity levels. For example, clinical trials in women (but not men) show that moderate exercise in the absence of caloric restriction promotes little or no weight loss (53). Among rodents, greater activity promotes lower body weight among males, but greater activity has no apparent effect on body weight among females unless that activity level is truly extreme (54). As stated previously, intervention studies among children such as CATCH (42), which have increased activity, at least as measured by self-report, have not resulted in reduced body weight (55). Finally, "prospective studies with physical activity measured at baseline . . . gave inconsistent results regarding the effects of increased physical activity on weight change" (56). Collectively, these observations suggest that, though clearly important, physical activity promotion alone is unlikely to be the sole solution to our obesity problems.

Above and beyond directly promoting activity, trying to reduce rates of extreme inactivity may be at least equally

important. Two studies (57,58) have reported on effects of interventions aimed at reducing childhood television watching, which is known to be associated with a greater prevalence of obesity. Both studies by Faith *et al.* (59) and by Robinson *et al.* (60) showed that interventions can reduce television watching as well as indicators of adiposity.

SUMMARY

Based on the foregoing data and reasoning, we offer the following tentative conclusions. First, moderate environmental changes may make meaningful differences in population BMI levels. Second, we should be skeptical about proposals in which activity promotion is the sole method proposed to reduce obesity, especially for females. Third, seemingly innocuous environmental changes, including but not limited to restricting access to food in certain circumstances, may have untoward consequences. Fourth, immediacy and convenience may be more salient dimensions than cost in determining food choice. Finally, environmental changes, if they are to be effective in reducing obesity rates throughout the developmental lifespan, most likely need to be in place throughout the developmental lifespan.

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In vitro Comparison of Hepatic Metabolism of 9*cis*-11*trans* and 10*trans*-12*cis* Isomers of CLA in the Rat

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ABSTRACT: Hepatic metabolism of the two main isomers of CLA (9*cis*-11*trans,* 10*trans*-12*cis* $C_{18:2}$) was compared to that of oleic acid (representative of the main plasma FA) in 16 rats by using the *in vitro* method of incubated liver slices. Liver tissue samples were incubated at 37°C for 17 h under an atmosphere of 95% O_2/5% CO_2 in a medium supplemented with 0.75 mM of FA mixture (representative of circulating nonesterified FA) and with 55 μM [1-^{14}C]9*cis*-11*trans* $C_{18:2}$, [1-^{14}C]10*trans*-12*cis* $C_{18:2}$, or [1-^{14}C]oleate. The uptake of CLA by hepatocytes was similar for both isomers (9%) and was three times higher ($P < 0.01$) than for oleate (2.6%). The rate of CLA isomer oxidation was two times higher (49 and 40% of incorporated amounts of 9*cis*-11*trans* and 10*trans*-12*cis*, respectively) than that of oleate ($P < 0.01$). Total oxidation of oleate and CLA isomers into [$^{14}CO_2$] was low (2 to 7% of total oxidized FA) compared to the partial oxidation (93 to 98%) leading to the production of [^{14}C] acid-soluble products. CLA isomers escaping from catabolism were both highly desaturated (26.7 and 26.8%) into conjugated 18:3. Oleate and CLA isomers were mainly esterified into neutral lipids (70% of esterified FA) and, to a lesser extent, into polar lipids (30%). They were slowly secreted as parts of VLDL particles (<0.4% of FA incorporated into cells), the extent of secretion of oleate and of 10*trans*-12*cis* being 2.2-fold higher than that of 9*cis*-11*trans* ($P < 0.02$). In conclusion, this study clearly showed that both CLA isomers were highly catabolized by hepatocytes, reducing their availability for peripheral tissues. Moreover, more than 25% of CLA escaping from catabolism was converted into conjugated 18:3, the biological properties of which remain to be elucidated.

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CLA is a collective name used for a mixture of positional and geometric isomers of linoleic acid that contain two conjugated double bonds. CLA exhibits several important beneficial properties for human health with regard to cancers, heart disease, diabetes, bone formation, growth modulation, and immunity (1). However, factors such as genetics, species studied, sex, and current state of health may influence the impact of CLA supplementation (2). Indeed, a few studies demonstrated negative effects of CLA in some experimental animal models such as insulin-resistant states, hepatomegaly, FA deposition in aortic tissues, and acceleration of autoimmune processes (2), suggesting that the long-term effect of CLA supplementation requires further investigations.

CLA is found mainly in ruminant products such as meat and milk, pasteurized dairy products, and processed cheeses (3). They are also present to a lesser extent in partially hydrogenated or frying oils (4) and as trace amounts in fish meat and vegetable oils (3). The major source of CLA in human tissues is the diet (5). The daily consumption of CLA was estimated to be 212 and 151 mg for men and women, respectively (6).

The presence of CLA in ruminant products is a consequence of bacterial biohydrogenation and *trans* isomerization in the rumen of linoleic and linolenic acids derived from plant matter (7), leading to production of different isomers dominated by the 9*cis*-11*trans* isomer (80–90% of total CLA). CLA are intermediates in the biohydrogenation of PUFA, and a portion escapes the rumen and is incorporated into milk and body fats. In addition, the animal itself may synthesize 9*cis*-11*trans* CLA from 11*trans*-octadecenoic acid, another intermediate in ruminal biohydrogenation that is similary absorbed. This synthesis involves the activity of the Δ9-desaturase, which is present in mammary (lactation) and adipose (growth) tissues (8). Under certain dietary conditions (high-concentrate, low-fiber diets), distribution of CLA isomers can be altered to favor incorporation of the 10*trans*-12*cis* isomer into milk fat (7). Synthetic CLA also can be produced by industry, resulting in approximately equal amounts of 9*cis*-11*trans* and 10*trans*-12*cis* isomers and other minor isomers with double bonds mainly in the 8,10 or 11,13 positions (9).

In the liver of different species such as rats (10–12), lambs (13), or mice (14) fed CLA-enriched diets, CLA was converted into conjugated 18:3 probably *via* the action of the Δ6-desaturase (11,15). These CLA derivatives were subsequently elongated and desaturated into conjugated 20:3 and conjugated 20:4 (12). However, the site of bioconversion and the proportion of CLA isomers converted into conjugated 18:3 were poorly documented except in a report of Belury and Kempa-Stec (14). These authors showed, by *in vitro* assay using liver microsomes of mice, that CLA was desaturated into a nonchemically identified 18:3 product to the extent of around 10% in 30 min. Moreover, although these studies reported the presence of CLA derivatives in the liver of animals, they did not demonstrate clearly that this CLA conversion took place in this organ; different tissues or organs

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Abbreviation: ASP, acid-soluble products; CEA, Commissariat à l'Energie Atomique.

(adipose tissue, mammary gland) might have metabolized FA, the liver being simply an organ of storage.

However, among the different tissues or organs that might metabolize FA, including CLA, the liver appears to play a central role in lipid metabolism (16) because of its large capacity for lipogenesis, desaturation, and bioconversion of FA. Moreover, in hepatocytes, FA may be totally oxidized to CO_2, partially oxidized to acetate and ketone bodies, or esterified to form phospholipids, cholesteryl esters, and especially TAG, which might be exported as part of VLDL.

Although the recent explosion of interest in CLA was ignited by the spectrum of biological activities attributed to this minor FA from ruminant products, the metabolism of different isomers of CLA once absorbed, and especially how they are channeled toward oxidation, esterification, or bioconversion pathways, is poorly documented. This information is important since these pathways determine the availability of CLA to elicit their biological effects. This study is the first to compare, by using the *in vitro* method of liver slices in incubation, the metabolism of highly purified CLA isomers (9*cis*-11*trans* vs. 10*trans*-12*cis* isomers) with that of oleate (representative of the main plasma FA) in the liver of rats. For this purpose, we studied the successive steps of hepatic metabolism of FA, i.e., uptake, oxidation, desaturation, and esterification of FA, and finally their secretion as part of VLDL particles.

EXPERIMENTAL PROCEDURES

Reagents. The medium used for liver slice incubations (RPMI-1640), BSA free of FA, FA (8:0, 10:0, 14:0, 16:0, 18:0, 18:1n-9, 18:2n-6), and antibiotic-antimycotic cocktail were purchased from Sigma Chemical Corp. (St. Louis, MO). [1-^{14}C]Oleic acid, [^{3}H]trioleylglycerol, and [^{3}H]PC were purchased from Amersham International (Bucks, United Kingdom), [1-^{14}C]9*cis*-11*trans* and [1-^{14}C]10*trans*-12*cis* CLA were graciously supplied by CEA (Saclay, France).

Liver slice preparation and incubation. Sixteen male Wistar rats (16 ± 1 wk) were fed a standard chow diet *ad libitum* and allowed free access to water. Liver tissue samples were taken up under general anesthesia by diethyl ether, quickly rinsed in an ice-cold saline solution (KCl 0.4 g/L, NaCl 6 g/L, and Na_2HPO_4 52 mg/L, NaH_2PO_4 13.5 mg/L, pH 7.4) containing D-glucose (2 g/L), trimmed of blood and connective tissue, and cut into slices of 0.5 mm thickness, as previously detailed (17). Approximately 150 mg of fresh liver was cut into four or five slices of equivalent size and placed on stainless steel grids positioned either on a plastic organ culture Petri dish (for measurement of FA bioconversion, esterification, and secretion) or in a 25-mL flask equipped with suspended plastic center wells (for measurements of CO_2 production) in the presence of RPMI-1640 medium free of FA. They were incubated for 2 h at 37°C in a 95% O_2/5% CO_2 atmosphere saturated with water to deplete hepatocytes of intracellular FA and to create a requirement for substrate by the cells. Medium was then replaced by the fresh RPMI-1640 medium (0.9 mL for a dish and 1.4 mL for a flask) supplemented with an antibiotic-antimycotic mixture (100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 µg/mL amphotericin B). To approximate physiological conditions, a mixture of FA (18) representative of plasma nonesterified FA (0.75 mM final concentration) was added into the medium. This FA mixture was composed of 16 µM 8:0, 16 µM 10:0, 58 µM 14:0, 200 µM 16:0, 220 µM 18:0, 213 µM 18:1n-9, and 26 µM 18:2n-6 in the presence of 55 µM [1-^{14}C]9*cis*-11*trans* (1.97 GBq/mmol) or [1-^{14}C]10*trans*-12*cis* (2.0 GBq/mmol) complexed to BSA (FA/albumin molar ratio of 4:1) to test CLA metabolism. In the same way, to compare oleate (representative of the main plasma FA) and CLA metabolism, the same mixture of purified FA (except for 18:1n-9) with 55 µM [1-^{14}C]oleic acid (2.06 GBq/mmol) was complexed to BSA and added to the medium of liver slices. Liver slice incubations corresponding to a dish and a flask for each FA tested were stopped after 17 h of labeling. Briefly, after media had been removed, liver slices were washed with 2 mL of saline solution and homogenized in 2 mL of 25 mM Tris-HCl (pH 8.0)/50 mM NaCl buffer with a Dounce homogenizer. Viability of liver cells was verified by determining kinetics of albumin synthesis and secretion, as previously described (19).

Animal experiments were conducted in a manner compatible with national legislation on animal care (Certificate of Authorisation to Experiment on Living Animals no. 7740, French Ministry of Agriculture).

[^{14}C]FA oxidation. CO_2 excreted by liver cells to the atmosphere was complexed by hyamine hydroxide (500 µL) placed in the suspended plastic center wells inside flasks at the beginning of the incubation. After 17 h of incubation, wells were introduced into scintillation vials containing 4 mL of Ready Safe scintillation cocktail for radioactivity quantification (17). Ketone bodies were prepared as acid-soluble products (ASP) by treating cell homogenates (250 µL) and media (500 µL) with ice-cold perchloric acid (0.2 mM final) (17).

[^{14}C]FA bioconversion. Total liver lipids were extracted according to the method of Folch *et al*. (20), and their corresponding FA were liberated and methylated by transmethylation at room temperature with sodium methylate (1 M) for 30 min followed by boron trifluoride in methanol (14%, vol/vol) for 30 min according to Christie *et al*. (21). FAME containing [^{14}C]FAME were analyzed by GLC by using an HP 5890 series II gas chromatograph (Hewlett-Packard, Palo Alto, CA), equipped with a splitless injector and a fused Stabilwax wide-bore silica column (60 m × 0.53 mm i.d.; film thickness, 0.50 µm; Restek, Evry, France). The effluent from the column was split between an FID (10%) and a copper oxide oven heated at 700°C to transform the [^{14}C]FA into $^{14}CO_2$ (90%). Radioactivity was quantified with a radiodetector (GC-RAM) (LabLogic, Sheffield, United Kingdom) by counting $^{14}CO_2$ after it had been mixed with a 9:1 ratio of argon/methane, as previously described (22). Data were computed using Laura software (LabLogic).

[^{14}C]FA esterification into neutral and polar lipids. Total liver lipids were extracted according to the method of Folch *et al*. (20) after addition of standard (nonradioactive) liver homogenate (850 µL containing approximately 10 mg of lipids)

as a lipid carrier and of [^{3}H]trioleylglycerol (67 Bq) and [^{3}H]PC (100 Bq) as internal TAG and phospholipid standards (17). Neutral and polar lipids were separated by affinity-LC on an aminopropyl-activated silica Sep-Pak® cartridge according to Kaluzny *et al*. (23). They were directly collected into scintillation vials, evaporated to dryness under an air stream, and counted for radioactivity.

Hepatic secretion of [^{14}C]VLDL. Three milliliters of medium containing [^{14}C]VLDL newly secreted by liver cells was purified by flotation ultracentrifugation according to Graulet *et al*. (17). Thus, medium was supplemented with purified plasma bovine VLDL (0.3 mg of VLDL-TAG per 12 mL tube) as a carrier for VLDL during ultracentrifugation, adjusted to the density of 1.063 g/L with potassium bromide, and overlaid with 9 mL of saline solution. VLDL particles were purified by ultracentrifugal flotation at 100,000 × *g* for 16 h at 15°C in a Kontron Centrikon T-2060 ultracentrifuge equipped with a TST 41-14 rotor (Kontron, Zürich, Switzerland). Two milliliters was collected at the top of each tube and recentrifuged in the same conditions, except that pure albumin (50 mg/tube) was added to remove traces of free [^{14}C] FA adsorbed onto VLDL particles. Finally, purified VLDL (2.5 mL) were counted for radioactivity in scintillation vials.

Statistical analysis. Values are expressed as the means ± SE for the six (CLA isomer metabolism) or four (oleic acid metabolism) independent assays. Comparison of means between the different FA was tested by using Student's *t*-test.

RESULTS

FA uptake. Uptake of FA by liver slices (expressed in nmol/g fresh liver) was calculated individually for 9*cis*-11*trans*, 10*trans*-12*cis* isomers, and oleic acid. It corresponded to the sum of FA incorporated into neutral and polar lipids in the homogenate, of FA oxidized into ASP, and of FA excreted as CO_2. After 17 h of incubation, hepatic uptake of both CLA isomers was similar (Fig. 1A) and amounted to 40.2 and 45.9 nmol/g fresh liver for 9*cis*-11*trans* and 10*trans*-12*cis* isomers, respectively, but oleic acid uptake was significantly lower (16.4 nmol/g fresh liver, $P < 0.01$) than that of either CLA isomer. In the same way, when expressed as the percentage of radioactivity introduced into the incubation medium (Fig. 1B), uptake of the two CLA isomers by liver cells was not significantly different but was higher than that of oleic acid (9.0 vs. 2.6%, $P < 0.01$).

FA oxidation. The extent of oxidation of FA (expressed as the percentage of FA converted into CO_2 and ASP out of the total FA incorporated into cells) was nearly 50% for both CLA isomers (Table 1). It was 20.3% higher for 9*cis*-11*trans* than for the 10*trans*-12*cis* isomer ($P < 0.03$). In contrast, the extent of oxidation of oleic acid was significantly ($P < 0.01$) lower than that of the CLA isomer; the former was only *ca.* 23%. Partial oxidation of FA was quantified by determining the amounts of [^{14}C]ASP that were produced in cell homogenates and secreted into the medium. After 17 h of incubation, the amount of total [^{14}C]ASP was similar for the two

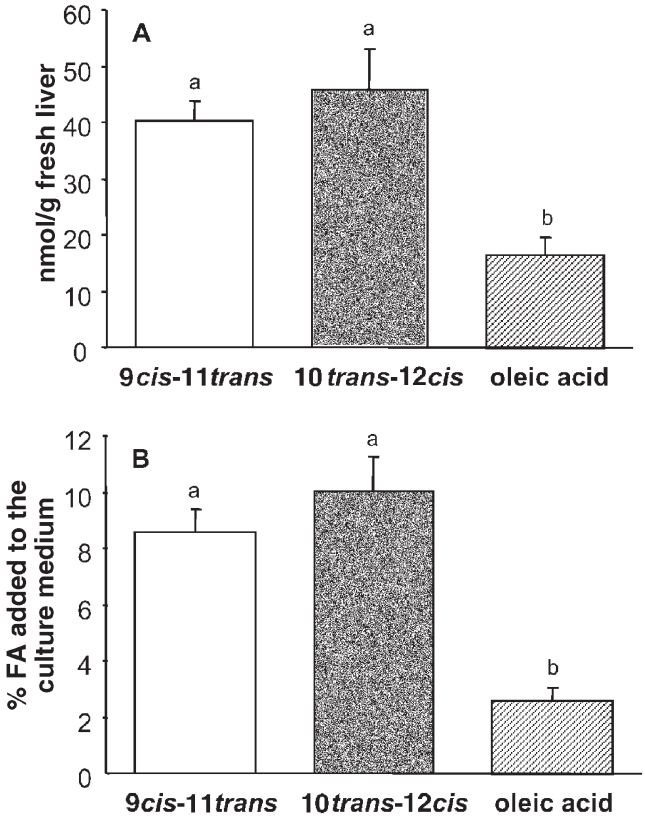

FIG. 1. Uptake of CLA isomers (9*cis*-11*trans* and 10*trans*-12*cis*) and of oleic acid by rat liver slices. Liver slices from rats were incubated in medium containing a mixture of FA representative of physiological plasma nonesterified FA (0.75 mM) and 55 μM [1-^{14}C]9*cis*-11*trans*, [1-^{14}C]10*trans*-12*cis* or [1-^{14}C]oleic acid for 17 h. Uptake of FA by liver slices was calculated as the sum of FA converted into CO_2 (complexed with hyamine hydroxide) and into acid-soluble products (ASP) (purified by perchloric acid treatment), and incorporated into total cellular lipids (neutral plus polar lipids) after 17 h of incubation; measurement of the radioactivity was carried out with a scintillation counter. Values were expressed per g of fresh liver (A) or as the percentage of FA introduced in culture medium (B). Data are means ± SE of six animals per group for CLA and four animals for oleic acid uptake. Mean values with unlike superscript letters were significantly different using Student's *t*-test ($P < 0.01$).

CLA isomers and equal to about 97% of total oxidized CLA, whereas for oleic acid it was slightly but significantly lower (93%, $P < 0.05$). The total amount of $^{14}CO_2$ produced by total oxidation of FA after 17 h of incubation (Table 1) was not different for the CLA isomers (2.4 and 3.3% of oxidized 9*cis*-11*trans* and 10*trans*-12*cis* isomers, respectively) but was significantly higher for oleic acid (6.6%, $P < 0.05$).

CLA conversion into conjugated metabolites. The FA composition of total lipids extracted from liver homogenates, as determined by radio-GC, showed a partial conversion of 9*cis*-11*trans* (Fig. 2A) and 10*trans*-12*cis* (Fig. 2B) isomers into conjugated metabolites. These conjugated metabolites were identified in both experiments using 9*cis*-11*trans* and 10*trans*-12*cis* isomers as conjugated 18:3 (presumably 6*cis*-9*cis*-11*trans* and 6*cis*-10*trans*-12*cis* 18:3) according to the similarity of their respective retention times to those of the corresponding pure standards of conjugated 18:3. The extent

TABLE 1
Extent of Oxidation and Catabolism of CLA Isomers (9*cis*-11*trans* and 10*trans*-12*cis*) and of Oleic Acid into Acid-Soluble Products and CO_2 by Rat Liver Slices After 17 h of Incubation[a]

	9*cis*-11*trans* (n = 6)	10*trans*-12*cis* (n = 6)	Oleic acid (n = 4)
Extent of oxidation			
(% of FA incorporated by cells)	49.2 ± 2.7[a,c]	40.9 ± 1.7[a,d]	22.6 ± 1.9[b]
Acid-soluble products			
(nmol of FA incorporated by cells)	19.2 ± 1.7[a]	17.9 ± 2.7[a]	3.6 ± 1.1[b]
(% of oxidized FA)	97.6 ± 0.2[c]	96.7 ± 0.5[c]	93.4 ± 1.6[d]
CO_2			
(nmol of FA incorporated by cells)	0.49 ± 0.07[c]	0.55 ± 0.05[c]	0.24 ± 0.06[d]
(% of oxidized FA)	2.4 ± 0.22[c]	3.3 ± 0.51[c]	6.6 ± 1.6[d]

[a]Values are expressed as mean ± SEM. [a,b]$P < 0.01$; [c,d]$P < 0.05$ (Student's t-test).

of the bioconversion of 9*cis*-11*trans* and 10*trans*-12*cis* isomers into conjugated 18:3 was similar and represented 26.7 and 26.8% of total [^{14}C]FA in liver homogenates following 17 h of labeling for 9*cis*-11*trans* and 10*trans*-12*cis* isomers, respectively (Figs. 2A,B).

FA esterification into cellular lipids. The extent of esterification (expressed as the percentage of FA converted into neutral and polar lipids out of the total FA incorporated into cells) of 9*cis*-11*trans* 18:2 was slightly lower (−16.3%, $P < 0.03$) than that of 10*trans*-12*cis* 18:2 (Table 2), but for these isomers, the extent of esterification was 1.5 and 1.3 times lower, respectively, than that of oleic acid ($P < 0.01$). FA were primarily esterified (>66%) into neutral lipids (mainly TAG) and secondarily into phospholipids (<33%, Table 2).

FA secretion into VLDL lipids. Incorporation of CLA into lipids secreted as part of VLDL particles was 2.2-fold lower ($P < 0.02$) for 9*cis*-11*trans* 18:2 than for 10*trans*-12*cis* 18:2 or oleic acid (Fig. 3A). When expressed as the percentage of FA taken up by hepatocytes (Fig. 3B), incorporation of 9*cis*-11*trans* 18:2 into VLDL lipids was two and seven times lower than that of 10*trans*-12*cis* 18:2 and of oleic acid, respectively ($P < 0.02$).

DISCUSSION

Numerous studies have been published in the past decade on the beneficial physiological effects of CLA for humans as determined by use of animal or cell models (24). However, the specific metabolism of different isomers of dietary CLA, once they have been absorbed by the small intestine, and especially how they are channeled toward the esterification, oxidation, or bioconversion pathways in tissues or organs, is poorly documented in spite of the importance of CLA availability to elicit their biological effects. This paper reports for the first time the comparative metabolism of highly purified CLA isomers (9*cis*-11*trans* vs. 10*trans*-12*cis* isomers) in the liver of the rat by using the *in vitro* method of incubated liver slices.

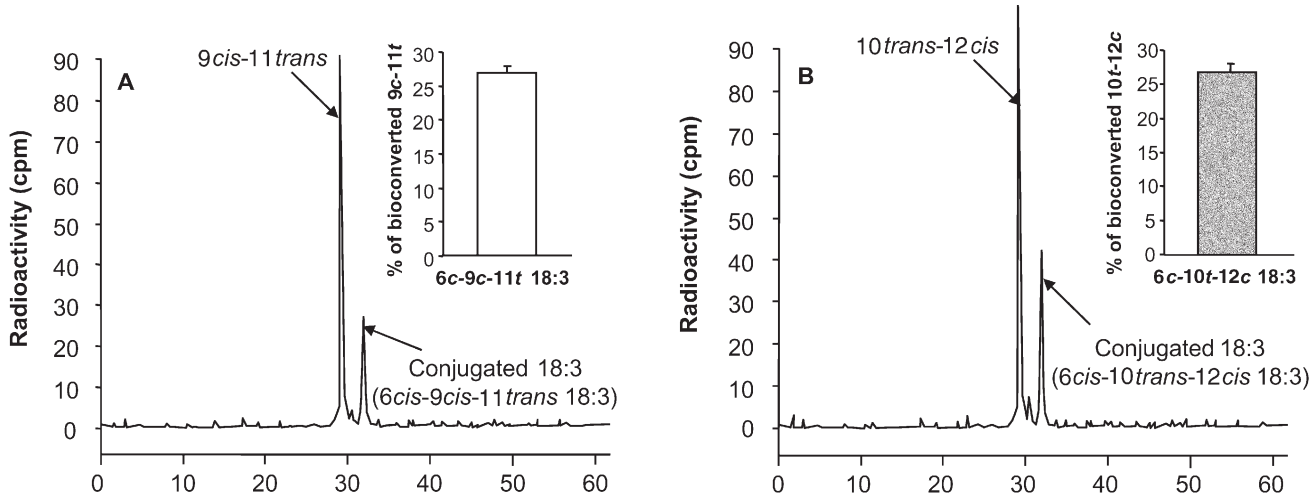

FIG. 2. Representative radiochromatograms of [^{14}C]FA and proportion of the two CLA isomers bioconverted into conjugated 18:3 in rat liver slices. Liver slices from rats were incubated in medium containing a mixture of FA representative of physiological plasma nonesterified FA (0.75 mM) and 55 μM [1-^{14}C]9*cis*-11*trans* (A) or [1-^{14}C]10*trans*-12*cis* (B) for 17 h. Cellular lipids were extracted, transformed into methyl esters, and analyzed by GLC. The outflow from the column was split between an FID (10%) and a copper oxide oven heated at 700°C to transform the labeled FA into $^{14}CO_2$ (90%). The radioactivity was determined with a radiodetector by counting $^{14}CO_2$ after mixing it with a 9:1 ratio of argon/methane. The proportion of each CLA isomer converted into conjugated 18:3 was calculated as the ratio between the radioactivity corresponding to the conjugated 18:3 and the sum of the radioactivity present in peaks of CLA isomer plus their corresponding conjugated 18:3. Data are means ± SE for six animals per group.

TABLE 2
Extent of Esterification and Incorporation of CLA Isomers (9*cis*-11*trans* and 10*trans*-12*cis*) and of Oleic Acid into Neutral Lipids and Phospholipids by Rat Liver Slices After 17 h of Incubation[a]

	9*cis*-11*trans* ($n = 6$)	10*trans*-12*cis* ($n = 6$)	Oleic acid ($n = 4$)
Extent of esterification			
(% of FA incorporated by cells)	50.8 ± 2.7[a,c]	59.1 ± 1.7[a,d]	77.4 ± 1.9[b]
Phospholipids			
(nmol of FA incorporated by cells)	5.8 ± 1.1[c]	6.8 ± 0.6[c]	3.9 ± 1.0[d]
(% of esterified FA)	28.3 ± 4.5	27.0 ± 3.0	33.2 ± 9.5
Neutral lipids			
(nmol of FA incorporated by cells)	14.8 ± 2.0[c]	20.6 ± 4.0[c]	8.6 ± 2.4[d]
(% of esterified FA)	71.7 ± 4.5	73.0 ± 3.0	66.8 ± 9.5

[a]Values are expressed as mean ± SEM. [a,b]$P < 0.01$; [c,d]$P < 0.05$ (Student's *t*-test).

These two CLA isomers were studied because of their abundance in the human diet [9*cis*-11*trans* 18:2 (7)] and in commercial supplements [43% 9*cis*-11*trans* 18:2 and 45% 10*trans*-12*cis* 18:2 (9)]. Moreover, although 9*cis*-11*trans* 18:2 is by far the main isomer synthesized in ruminant animals given conventional diets, a significant amount of 10*trans*-12*cis* 18:2 has been reported in the milk of animals given low-fiber diets (7). These two CLA isomers exhibit different biological activities independently as well as in concert (1).

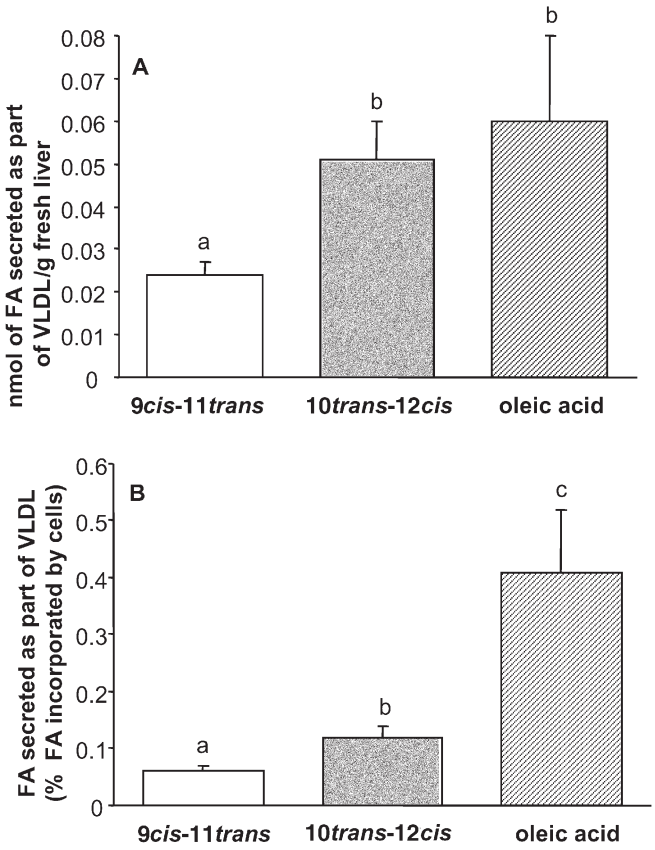

FIG. 3. Secretion of CLA isomers (9*cis*-11*trans* and 10*trans*-12*cis*) and of oleic acid as part of lipids in VLDL particles by rat liver slices. Liver slices from rats were incubated in medium containing a mixture of FA representative of physiological plasma nonesterified FA (0.75 mM) and 55 μM [1-^{14}C]9*cis*-11*trans,* [1-^{14}C]10*trans*-12*cis* or [1-^{14}C]oleic acid for 17 h. VLDL were purified by ultracentrifugal flotation, and the radioactivity was measured in a scintillation counter. Values were expressed per g fresh liver (A) or as percentage of FA incorporated into liver slices (B). Data are means ± SE of six animals per group for CLA and four animals for oleic acid secretion measurements. Mean values with unlike superscript letters were significantly different using Student's *t*-test ($P < 0.02$).

From the point of view of the experimental procedure, levels of CLA isomers added to medium in the present study (55 μM) were similar to those used in different cell systems, such as human cancer cells (25) and isolated rat hepatocytes (26), which had no cytotoxic effects as assessed by cell viability measurement (trypan blue exclusion and lactate dehydrogenase leakage) (26). Moreover, when compared with the previous *in vivo* study carried out in mice by Belury and Kempa-Steczko (14), amounts of CLA added to the medium in our study were close to those present in the portal vein (available for liver) when 0.5–1.5% of CLA was added in the diet of mice.

In our study, the particular conformation of CLA isomers (*trans/cis* conjugated double bonds) might explain the higher efficiency of their uptake and incorporation into hepatocytes compared to those of oleate, which is taken as representative of the usual FA. However, the rate of incorporation of oleic acid by hepatocytes in our experimental conditions was lower than that in perfused rat liver (about 90% of the perfused oleate extracted by the liver after 2 h) (27) or in isolated rat hepatocytes (50% of oleate incorporated after 6 h) (28). Among possible reasons for this discrepancy, closer contact between cells and medium in the perfused liver model or in isolated hepatocytes might explain why those values were higher than those obtained in this study with the incubated liver slice model.

Products of oleic acid oxidation by rat liver slices were dominated in our study by ASP (products of partial oxidation), which represented more than 93% of total oxidation products; the remainder was composed of CO_2 (the product of total oxidation), as previously demonstrated on rat liver slices (17) and on cultured rat hepatocytes (29). In the same way, the oxidation of both CLA isomers led mainly to the production of ASP (nearly 97% of total oxidation products). However, the higher extent of oxidation of both isomers of

CLA compared to that of oleate was in agreement with previous data (30,31), which showed that liver from CLA-fed rats produced significantly more ketone bodies than that from linoleic acid-fed rats. CLA itself did not exert a direct influence on FA oxidation but increased mitochondrial activity of carnitine palmitoyltransferase (32). Stimulated ketogenesis was inversely related to the reduced secretion of TAG by the liver of rats given CLA supplements, mechanisms by which beneficial hypolipemic effects of dietary CLA might be partly explained (30).

CLA isomers (9*cis*-11*trans* and 10*trans*-12*cis*) that escaped from a β-oxidation pathway were converted to a similar extent (>25% after 17 h of incubation) into conjugated 18:3. This conversion has been described previously in the liver of rats (10–12), lambs (13), and mice (14), probably *via* the action of the Δ6-desaturase (11,15). Moreover, these CLA derivatives can subsequently be elongated and desaturated into conjugated 20:3 and conjugated 20:4 (12). 9*cis*-11*trans* 18:2 is converted into conjugated 18:3 and 20:3, the latter being identified as Δ8,11,13-20:3 (33). 10*trans*-12*cis* 18:2 appeared to be converted mainly into an 18:3 having double bonds in Δ6,10,12, whereas smaller quantities of conjugated 16:2 (having the ethylenic position in Δ8 and Δ10) and of conjugated 20:3 have been detected, probably because the biosynthesis of these compounds is very low (12). In our experimental conditions, 16:2, 20:3, and 20:4 conjugated metabolites were not detected, probably because the incubation time for rat liver slices was too short to allow subsequent elongation, desaturation, or retroconversion. The extent of conversion of CLA into conjugated 18:3 is poorly documented except in the report of Belury and Kempa-Steczko (14), which showed, by *in vitro* assay on liver microsomes of mice, that desaturation of a mixture of CLA into an unidentified 18:3 occurred to the extent of 10% in 30 min. These authors speculated that CLA would compete with linoleate for Δ6-desaturase activity (a rate-limiting step for the conversion of linoleate into arachidonate), leading to an overall decrease in arachidonate-derived eicosanoids. Since eicosanoids are involved in the stimulation of cancers, immune function, and cardiovascular diseases, such a reduction of arachidonate by CLA might explain, in part, the beneficial role of CLA against carcinogenesis and other diseases in humans (14). However, the biological properties of conjugated derivatives of CLA are still unknown, and preparation of labeled metabolites by organic synthesis would be necessary to study the impact of these particular FA on lipid metabolism.

Oleic acid, both CLA isomers, and derivatives that escaped the β-oxidation pathway were esterified in our rat liver slices. The major product of esterification was neutral lipids (close to 70% of total esterified FA) dominated by TAG, confirming previous experiments showing that TAG represented more than 90% of neutral lipids in the rat liver (34). The notable esterification of CLA into neutral lipids observed in our study is consistent with previous studies using rat hepatocyte suspension cultures incubated with a synthetic mixture of CLA (26) or using liver of rats fed butter-enriched CLA (35). From these results, we can speculate that CLA isomers and their derivatives escaping from catabolism by β-oxidation were esterified in a form more easily exportable by the liver (TAG) rather than in phospholipids, which are preferential structural lipids for hepatocyte membranes.

The extent of secretion of oleic acid, CLA isomers, and of their derivatives incorporated into TAG as part of VLDL particles by rat liver slices was very low under our experimental conditions (0.06 to 0.4% of FA incorporated into cells) possibly because of the short time of incubation of liver slices. Indeed, it was previously demonstrated that newly synthesized TAG represented only a small part of the lipids secreted as part of VLDL (36). Neosynthesized TAG are stored primary in cytosolic droplets from which they enter into a hydrolysis/esterification cycle to return to the endoplasmic reticulum for VLDL assembly (28). In our experimental conditions, the time of incubation of liver slices appeared too short to allow the return of a significant amount of TAG to the secretion pool. However, the extent of secretion of the 10*trans*-12*cis* CLA isomer appeared comparable to that of oleic acid but twofold higher than that of the 9*cis*-11*trans* CLA isomer when expressed in nanomoles of FA secreted per gram of fresh liver. When expressed as the percentage of total FA incorporated into cells, the extent of oleic acid secretion appeared significantly more important than that of CLA isomers, but this difference is probably due to the lower incorporation of oleic acid compared to CLA isomers. The higher extent of secretion of the 10*trans*-12*cis* compared to the 9*cis*-11*trans* CLA isomer might be linked to a preferential utilization of this isomer by peripheral key tissues probably in relation to its specific biological properties (12,22,37).

In conclusion, the liver slice model allows us to compare the metabolism of two main isomers of CLA (9*cis*-11*trans* and 10*trans*-12*cis*) that are commercially available. We have clearly demonstrated that their respective metabolisms were not significantly different except that the secretion extent as part of the VLDL particles is higher for 10*trans*-12*cis*. However, compared with oleic acid metabolism, a great part (50%) of these CLA isomers is catabolized *via* the β-oxidation pathway, which implies their reduced availability for peripheric tissues. Moreover, more than a quarter of the CLA isomers that escaped from catabolism were converted into conjugated 18:3, of which the biological properties are still to be determined.

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Effects of Policosanols and Phytosterols on Lipid Levels and Cholesterol Biosynthesis in Hamsters

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ABSTRACT: The current study was carried out to examine the effects of policosanols and phytosterols, alone and in combination, on lipid profiles, cholesterol biosynthesis, and tissue histopathological changes in hamsters. Fifty male Golden Syrian hamsters, weighing 100 to 120 g, were fed a regular rodent chow for 2 wk before being randomly assigned into 5 groups of 10 animals each fed semisynthetic diets for 4 wk. Group 1 was given a control diet that contained 0.25% cholesterol and 5% fat with a PUFA to saturated FA ratio of 0.4. Groups 2 to 5 were fed the control diet and given Octa-6 [a policosanol mixture from sugar cane wax, 25 mg/kg body weight (BW)], Ricewax (a policosanol mixture from rice wax with 50% being converted to the corresponding acids, 50 mg/kg BW), phytosterols (Cholestatin™; 1,000 mg/kg BW), and Ricewax (50 mg/kg BW) plus phytosterols (1,000 mg/kg BW), respectively. The results showed that there was no difference between Octa-6 and Ricewax treatments in any of the lipid parameters measured, and both had similar levels of triglyceride (TG), total cholesterol (T-C), and HDL cholesterol (HDL-C) as the control. Octa-6 but not Ricewax increased ($P = 0.03$) non-HDL-C as compared with the control. Phytosterols reduced T-C ($P < 0.0003$) and HDL-C ($P < 0.004$) without a significant effect on TG and non-HDL-C as compared to the control. Ricewax plus phytosterols had effects similar to those with phytosterols alone. Free cholesterol synthetic rates were not different among the treatments. Policosanols or phytosterols did not show any toxic effects in liver, heart, brain, or kidney. Results suggest that, although phytosterols reduce T-C and HDL-C levels, policosanols have no significant favorable effect in changing lipid levels in hamsters.

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Policosanols represent a mixture of aliphatic primary alcohols isolated and purified from sugar cane wax or other plant waxes. Major components of policosanols isolated from sugar cane wax include octacosanol, triacontanol, and hexacosanol; other alcohols including tetracosanol, heptacosanol, nonacosanol, dotriacontanol, and tetratriacontanol exist as minor components (1,2). Over the last decade policosanols have been studied for their cholesterol-lowering effect (3–7). After oral administration, policosanols are oxidized to FA through β-oxidation and esterified with sterols and phospholipids (8,9). Meanwhile, β-oxidation shortens the chain length of policosanols (10–12). Therefore, very long chain FA are structurally and metabolically related to their corresponding alcohols. It has been reported that very long chain saturated FA lower the plasma cholesterol levels in rats (13). A similar or even greater effect has been observed in rabbits given very long chain FA as compared with the corresponding alcohols isolated from the same source, sugar cane wax (14).

Mechanisms through which policosanols alter lipid profiles have not yet been clearly identified. It has been reported that policosanol mixtures isolated from sugar cane wax inhibit cholesterol biosynthesis after addition to cultured fibroblasts (15). *In vitro* studies indicate that policosanols may inhibit cholesterol synthesis at a step before mevalonate formation (15,16). This finding has been supported by *in vivo* studies in rabbits (5) as well as by data showing that policosanols do not inhibit the activity of HMG-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis (15,17). Increased LDL binding, uptake, and degradation have been observed, suggesting another mechanism through which policosanols reduce cholesterol levels (5,16). Although inhibition of *de novo* biosynthesis of cholesterol by policosanols has been suggested, direct evidence is lacking.

The efficacy of phytosterols in lowering plasma cholesterol has now been unquestionably established through many earlier (18) and recent (19–24) studies. As well, developments in our understanding of the extent to which phytosterols suppress cholesterol absorption in humans have also been made in the recent past. Several studies have quantified the efficacy by which sterols (21) and stanols (21–23,25) inhibit intestinal cholesterol absorption *in vivo* using both direct and indirect techniques. This inhibition of absorption is, however, partially compensated by a reciprocal increase in the cholesterol biosynthesis rate (21,22).

In light of the reciprocal increase in cholesterol biosynthesis observed with consumption of phytosterols, the goal of this study was to determine whether policosanols and phytosterols act synergistically on circulating cholesterol levels by suppression of both absorption and biosynthesis. The efficacy and mechanism of action of policosanols from two different plant sources on plasma cholesterol and triglyceride (TG) levels were investigated.

MATERIALS AND METHODS

Animals and diets. The Golden Syrian hamster has been considered to be one of the most suitable choices of animal model for studying dietary effects on cholesterol metabolism (26). In the current study, 50 male Golden Syrian hamsters weighing 100 to 120 g were housed in individual steel cages and subjected to a 12-h light/dark cycle. Animals were fed *ad libitum* for 2 wk with regular rodent chow and had free access to water. Then the hamsters were weighed and randomly

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Abbreviations: BW, body weight; FC-FSR, free cholesterol fractional synthetic rate; HDL-C, HDL-cholesterol; non-HDL-C, VLDL + intermediate density lipoprotein (IDL) + LDL-cholesterol; T-C, total cholesterol.

assigned to 5 groups of 10 animals each prior to commencing the study. The experimental diets were in accordance with AIN-76 formulation. (i) Group 1 was given a control diet containing, as wt%, casein, 20; cornstarch, 28; sucrose, 36.08; beef tallow/safflower oil, 5; cellulose, 4.95; DL-methionine, 0.5; mineral mix, 4; vitamin mix, 1; choline bitartrate, 0.2; cholesterol, 0.25; and BHT, 0.02. The PUFA to saturated FA ratio of the control was 0.4. Groups 2 to 5 were fed the control diet and given (ii) Octa-6 [25 mg/kg body weight (BW)/d], (iii) Ricewax (50 mg/kg BW/d), (iv) phytosterols (Cholestatin™; 1,000 mg/kg BW/d), or (v) Ricewax (50 mg/kg BW/d) plus phytosterols (1,000 mg/kg BW/d). All the test materials were provided by Degussa Bioactives (Champaign, IL). Octa-6 is a policosanol mixture extracted from sugar cane by using ethanol. It comprises approximately 60% octacosanol and 40% other very long chain fatty alcohols, including triacontanol, hexacosanol, tetratriacontanol, dotriacontanol, nonacosanol, tetracosanol, and heptacosanol. Ricewax is a mixture of very long chain fatty alcohols extracted from rice wax with 50% converted to the corresponding very long chain FA. The purity of Octa-6 and Ricewax is over 90%. The combination of Ricewax with phytosterols permits an examination of whether a synergistic action exists between phytosterols and either the alcohols or the acids. Octa-6 and Ricewax were mixed into the oil component of the diets by heating at 55°C and microwaved for 30 s. Phytosterols in finely milled form were mixed into the diets directly. Diets were prepared weekly and stored at –20°C.

Hamsters were fed experimental diets for 28 d. Food intake was recorded daily and animals were weighed weekly. Two hours prior to sacrifice on day 28, animals were given 0.5 mL of deuterium oxide (CDN Isotopes, Montréal, Canada) by intraperitoneal injection. Two hours after injection, animals were anesthetized by carbon dioxide inhalation, and blood samples were collected by decapitation. Plasma and red blood cells were separated and stored at –80°C. Brain, heart, liver, and kidney were collected and fixed in formalin. The experiment was reviewed and approved by the Animal Care and Research Ethics Committee, McGill University, and was conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Plasma lipid analysis. Plasma total cholesterol (T-C), HDL cholesterol (HDL-C), and TG levels were measured in duplicate using a VP Autoanalyzer (Abbott Laboratories, North Chicago, IL) in conjunction with commercial enzymatic kits, standardized reagents, and appropriate standards (Abbott Diagnostics, Montréal, Quebec). Measurement of HDL-C in plasma was done after the precipitation of apolipoprotein B containing lipoproteins with dextran sulfate and magnesium chloride. Because the Friedewald equation may not have been applicable to hamsters (27), non-HDL-C [VLDL + intermediate density lipoprotein (IDL) + LDL cholesterol] instead LDL-C was used. The non-HDL-C was calculated by subtracting HDL-C from T-C.

Histopathology assessment. Brain, liver, heart, and kidney were assessed for histopathological changes using microscopic procedures.

Cholesterol biosynthesis measurement. Approximately 1.5 g red blood cells was placed into a 50-mL Pyrex (Corning Glass Works, Corning, NY) tube with addition of 8 mL methanol and heated for 15 min at 55°C. Lipids were extracted with 24 mL hexane/chloroform (4:1, vol/vol) and 2 mL water. The mixture was shaken mechanically for 15 min and then centrifuged at 500 × *g* for 10 min. The upper solvent phase was removed, and the remaining aqueous phase was re-extracted twice. Solvent phases were combined and dried under nitrogen. Extracts were redissolved in 200 µL chloroform and spotted onto thin-layer silica plates (Whatman Inc., Clifton, NJ). Plates were developed using petroleum ether/diethyl ether/acetic acid (135:15:1.5, by vol). After drying, individual lipid bands were identified by developing in iodine vapor followed by comparison with co-chromatographed standards. Free cholesterol bands were scraped from the plates and saponified with freshly prepared methanolic KOH solution. Water was added followed by 5 mL of petroleum ether; samples were vortexed and then centrifuged for 15 min at 500 × *g*, before solvent was removed. The extraction was repeated twice. Solvent layers were combined and thoroughly dried under nitrogen. Free cholesterol was redissolved in chloroform and transferred into a pre-annealed 20 cm × 6 mm Pyrex combustion tube containing 0.6 g cupric oxide and a 2 cm × 1 mm length of sterling silver wire. After drying under nitrogen, tubes were evacuated to <10 mtorr and then flame-sealed. Samples were then combusted at 520°C for 4 h and cooled to room temperature. The combustion water was vacuum-distilled into pre-annealed 15 cm × 6 mm Pyrex tubes containing 60 mg zinc reagent (Biogeochemical Laboratories, Indiana University, Bloomington, IN). Tubes were flame-sealed under vacuum and heated at 520°C for 30 min to reduce the combustion water to hydrogen gas. Analysis of plasma water deuterium enrichment was carried out after dilution of 2-h post-dose plasma samples (1:60 with deionized distilled water of known isotope abundance) to obtain deuterium enrichments within analytical range of the mass spectrometer. Triplicate 2-µL samples were vacuum-distilled into 15 cm × 6 mm Pyrex tubes containing 60 mg zinc reagent. These tubes were frozen with liquid nitrogen, evacuated, and flame-sealed before heating at 520°C for 30 min to generate hydrogen gas.

Deuterium enrichment of the hydrogen was analyzed by isotope ratio mass spectrometer (VG Isomass, 903D, Cheshire, United Kingdom) with an internal analytical error of 0.17 per mil (‰). The H^+ contribution was checked daily, and appropriate correction factors were applied (28). The mass spectrometer was calibrated daily using three standards.

The proportion of central-pool free sterols derived from synthesis, expressed as the free cholesterol fractional synthetic rate (FC-FSR), was calculated by using Equation 1,

$$\text{FC-FSR (pools/d)} = \frac{[\delta \text{ cholesterol (‰)} \cdot 24 \text{ h}/2 \text{ h}]}{[\delta \text{ plasma water (‰)} \cdot 0.478]} \quad [1]$$

where δ cholesterol is the change in cholesterol deuterium enrichment over the interval period between injection and sacrifice and δ plasma water is the mean deuterium enrichment. The multiplication factor of 0.478 accounts for the fraction of deuterium atoms obtained from body water during cholesterogenesis.

Statistical analysis. Data across treatment groups were

analyzed by one-way ANOVA using the general linear model procedure of SAS software (29). Where a significance level of less than 0.05 was achieved, differences between group means were evaluated using the least squares means test. Data were expressed as means ± SD.

RESULTS

Body weight and food intake. After the 4-wk feeding period, feed intake was not affected by the dietary treatments (Table 1). Body weight also was not different among the groups (Table 2).

Lipid profiles. There was no difference between Octa-6 and Ricewax groups in either of the lipid parameters measured (Table 3). Compared with control, Octa-6 and Ricewax did not alter the levels of T-C and HDL-C; the level of non-HDL-C was, however, increased ($P = 0.03$) by Octa-6. Feeding of phytosterols lowered ($P < 0.0001$) T-C and HDL-C levels but not non-HDL-C levels as compared with control. Hamsters given phytosterols had lower T-C ($P < 0.0001$), HDL-C ($P < 0.002$), and non-HDL-C ($P < 0.007$) levels than those given Octa-6 or Ricewax. Ricewax plus phytosterols had effects similar to those of phytosterols alone on the lipid profiles. Hamsters after Ricewax plus phytosterols had lower T-C ($P = 0.0003$) and HDL-C ($P < 0.0002$) but not non-HDL-C than control. TG levels were not affected by the dietary treatments.

FC-FSR. The FC-FSR values were not different among the treatments (Fig. 1). No significant correlations were detected between FC-FSR and any of the lipid parameters measured within each treatment.

Histopathological changes. In liver, compared with the control, the incidence of inflammatory foci was not increased by feeding hamsters with any of the experimental diets (Table 4). Animals after consumption of phytosterols or Ricewax plus phytosterols showed a reduced incidence of inflammatory foci. In those animals receiving phytosterols, a reduction in the degree of cytoplasmic rarefaction was observed in a proportion of the animals examined. In the livers of a majority of animals, a generalized hepatocellular rarefaction observed was probably indicative of glycogen deposition. The other histopathological changes seen were only occasional tubular basophilia in the kidneys of a small number of animals (1 of 10 in Ricewax and Ricewax plus phytosterols, respectively) (data not shown). This was considered to be incidental and not associated with treatment. No lesions were found in sections of brain and heart (data not shown).

TABLE 1
Effects of Policosanols and Phytosterols on the Feed Intake of Hamsters[a]

	Feeding period (wk)			
Diet[b]	1	2	3	4
Control	8.82 ± 0.78	8.28 ± 0.76	8.04 ± 0.53	7.80 ± 0.84
Octa-6	9.23 ± 0.85	8.26 ± 0.89	8.23 ± 0.66	8.20 ± 0.44
Ricewax	8.95 ± 1.33	8.10 ± 0.89	8.17 ± 1.00	7.95 ± 1.08
Phytosterol	8.82 ± 1.02	8.20 ± 0.79	8.43 ± 0.73	8.15 ± 0.67
Ricewax/ phytosterol	8.94 ± 1.04	8.12 ± 0.99	8.25 ± 0.87	8.06 ± 0.80

[a]Values are means ± SD (g/animal/d). In each week, no difference was detected among the treatments ($P \geq 0.05$).
[b]Octa-6, a mixture of very long chain fatty alcohols isolated from sugar cane wax (60% octacosanol and 40% other alcohols); Ricewax, a mixture of very long chain fatty alcohols isolated from rice wax with 50% being converted to the corresponding very long chain FA.

DISCUSSION

The results of the current study demonstrate that the oral administration of policosanol mixture isolated from sugar cane wax, Octa-6 (25 mg/kg BW/d), had no influence on TG, T-C, and HDL-C levels but elevated non-HDL-C levels in the diet-induced hyperlipidemic hamsters. These results contrast with those of previous studies in animals (3–5), where oral administration of policosanol purified from sugar cane wax reduced serum cholesterol levels in normocholesterolemic and hypercholesterolemic rabbits (3) and monkeys (4). In rabbits, feeding a cholesterol-raising diet to which policosanol isolated from sugar cane wax was added lowered T-C and LDL-C levels compared with feeding a cholesterol-raising control diet alone (5). Human studies have demonstrated similar effects of policosanols in reducing blood cholesterol concentrations (2,30).

Very long chain fatty alcohols can be oxidized to very long chain FA through β-oxidation in the body (8–12). It has been suggested that very long chain FA are the major active metabolites of policosanols (31). The experiments carried out with a mixture of very long chain FA isolated from sugar cane wax have demonstrated similar or even greater pharmacological effects than the mixture of the corresponding alcohols (14,15,32). Ricewax, a mixture of 50% very long chain fatty alcohols and 50% of their corresponding acids isolated from sugar cane, administered at a dose of 50 mg/kg BW/d showed 4, 3, and 7% lower T-C, HDL-C, and non-HDL-C levels, respectively, than when feeding Octa-6, but these effects were not significantly different

TABLE 2
Effects of Policosanols and Phytosterols on the Body Weight of Hamsters[a]

	Feeding period (wk)				
Diet[b]	0	1	2	3	4
Control	111.50 ± 9.13	117.34 ± 9.50	125.99 ± 11.87	129.93 ± 10.97	134.73 ± 9.65
Octa-6	111.67 ± 8.27	117.50 ± 8.63	124.21 ± 11.30	131.96 ± 11.56	137.68 ± 13.55
Ricewax	111.55 ± 8.18	116.27 ± 8.13	124.67 ± 9.44	130.09 ± 9.03	136.15 ± 8.91
Phytosterol	112.27 ± 5.89	116.84 ± 5.94	125.80 ± 9.05	132.42 ± 10.77	139.14 ± 11.97
Ricewax/phytosterol	112.23 ± 9.29	115.72 ± 7.63	123.48 ± 11.01	131.71 ± 9.85	136.32 ± 11.90

[a]Values are means ± SD (g). In each week, no difference was detected among the treatments ($P \geq 0.05$).
[b]For description of diets see Table 1.

TABLE 3
Effects of Policosanols and Phytosterols on the Lipid Profiles of Hamsters[a]

Diet[b]	TG[c]	T-C	HDL-C	Non-HDL-C
Control	2.88 ± 0.71	7.20 ± 0.93[a]	5.64 ± 0.95[a]	1.56 ± 0.62[b,c]
Octa-6	3.52 ± 0.64	7.46 ± 0.95[a]	5.49 ± 0.78[a]	1.97 ± 0.81[a]
Ricewax	3.53 ± 1.08	7.18 ± 1.16[a]	5.34 ± 0.69[a]	1.84 ± 0.89[a,b]
Phytosterol	3.65 ± 0.62	5.60 ± 0.80[b]	4.29 ± 0.45[b]	1.31 ± 0.54[c]
Ricewax/phytosterol	3.12 ± 0.67	5.92 ± 0.75[b]	4.40 ± 0.71[b]	1.52 ± 0.63[b,c]

[a]Values are means ± SD (mmol/L). For each lipid parameter, values with different superscripts (a–c) are significantly different ($P < 0.05$).
[b]For description of diets see Table 1.
[c]HDL-C, HDL cholesterol; non-HDL-C, VLDL + intermediate density lipoprotein (IDL) + LDL cholesterol; T-C, total cholesterol; TG, triglyceride.

TABLE 4
Incidence of Histopathological Changes in the Liver of Hamsters Fed Policosanols and Phytosterols

	Diet[a]				
	Control	Octa-6	Ricewax	Phytosterol	Ricewax/phytosterol
Number of animals	10	10	10	10	10
Inflammation	4	3	2	1	0
Reduced rarefaction	0	0	0	3	5

[a]For description of diets see Table 1.

from those of feeding Octa-6. Compared to control, Ricewax did not affect any lipid parameters measured in the current study.

The inconsistency between the current study and previous investigations may be a result of multiple factors that affect lipid metabolism, including research model, lipid baseline, and the formula/delivery form of policosanols. Results of previous studies in other species indicate that reductions of cholesterol levels by policosanols occur mainly in the LDL-C fraction, with no change or increased HDL-C levels observed (3–5). However, hamsters carry most cholesterol in HDL particles, explaining why policosanols tested in the current study did not show a significant effect on lipid levels. The dietary formulation of policosanols could be another important factor influencing the effectiveness of policosanols on cholesterol metabolism. In the current study, Octa-6 and Ricewax were mixed into the diet after being mixed into the oil at 55°C. In contrast, those experiments showing a significant effect of policosanols provided these materials within an acacia gum-water vehicle through gastric gavage (3–5,14,32).

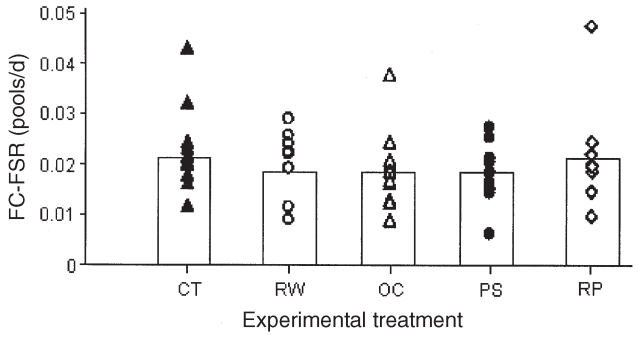

FIG. 1. Effects of policosanols and phytosterols on free cholesterol fractional synthetic rate (FC-FSR) in hamsters fed cornstarch/cheese/sucrose-based moderately atherogenic diet for 28 d. Data were analyzed using one-way ANOVA. No significant dietary effect was detected ($P \geq 0.05$). Values are means ± SD. CT, control; OC, Octa-6, a policosanol mixture isolated from sugar cane wax with 60% octacosanol and 40% other alcohols [25 mg/kg body weight (BW)/d]; RW, Ricewax, a policosanol mixture isolated from rice wax with 50% being converted to the corresponding very long chain FA (50 mg/kg BW/d); PS, phytosterols (1,000 mg/kg BW/d); RP, Ricewax (50 mg/kg BW/d) plus phytosterols (1,000 mg/kg BW/d).

Consistent with the effects of Octa-6 and Ricewax on lipid levels, FC-FSR was not perturbed by any of the treatments involving Octa-6 or Ricewax. Cholesterol biosynthesis has been measured using deuterium incorporation, which has been validated against the sterol balance and been shown to be sensitive to diet-induced changes in cholesterol synthesis rates (33). The results of the deuterium experiments did not support observations reported in previous studies that examined the effect of policosanols on cholesterol biosynthesis using indirect methods (5,16,17). For example, Menendez *et al.* (16) reported an inhibitory effect of policosanols on cholesterol synthesis using ^{14}C-acetate incorporation into the cellular cholesterol of human fibroblasts. A similar result was obtained in rabbits using tritiated water incorporation into liver sterols (5). A more recent study (17) demonstrated that the inhibition of policosanols on cholesterol biosynthesis pathway happened

before mevalonate generation, indicating that policosanols might not be acting as a direct HMG-CoA reductase inhibitor.

Oral administration of phytosterols reduced plasma T-C and HDL-C concentrations in the plasma without changing non-HDL-C or TG levels in the present study. Decreases in T-C have been observed previously in hamsters (24,34,35) and rabbits (36) fed a similar atherogenic diet supplemented with the same level of phytosterol mixture, compared with the controls. The non-HDL-C concentrations were calculated as the difference between T-C and HDL-C. Because hamsters have high levels of plasma HDL-C, which represents over 75% of the cholesterol pool, the absence of any differences between the control and phytosterol-treated hamsters in non-HDL-C may be due to the large proportion of cholesterol carried by HDL particles in this species.

The mechanisms of cholesterol-lowering action of phytosterols have been thought to be a result of inhibiting intestinal absorption and increasing fecal excretion of cholesterol (24,34). Meanwhile, upregulation in cholesterol biosynthesis has been observed in hamsters fed phytosterols, which reduce cholesterol absorption (24). The results of the current study did not show a significant effect of phytosterols on the FC-FSR, which is not consistent with previously studies in the same species (24,34). The authors consider that large variations observed within treatments may have contributed to the ineffectiveness of phytosterols on the cholesterol biosynthesis.

Results of the histopathological examination did not show any adverse effects of consuming Octa-6, Ricewax, or phytosterols on hamster organs including brain, liver, heart, and kidney. These data are in agreement with results of other safety and toxicity studies conducted in various animal models (14,31,32,37–42). Short- and long-term studies conducted in rats (43–45) or dogs (39) have not shown any policosanol-related toxic symptoms or histopathological changes in the organs. Reproductive performance and fetal/neonatal development have been assessed in rats, and no toxic effect was observed (46,47). Investigations also have shown policosanols from sugar cane wax or bee wax not to be genotoxic or mutagenic in mice (48), rats, or rabbits (46). Preclinical studies of acute, subchronic, and chronic toxicity of policosanols in cell and whole-body systems similarly have shown no discernible toxicity (37,38,40,49). These data suggest longer-term safety of these naturally occurring substances for use in functional foods.

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Effects of High-γ-Linolenic Acid Canola Oil Compared with Borage Oil on Reproduction, Growth, and Brain and Behavioral Development in Mice

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ABSTRACT: Previous research in rats and mice has suggested that γ-linolenic acid (GLA) derived from borage oil (BO: 23% GLA) may be an appropriate source for increasing levels of long-chain n-6 FA in the developing brain. Recently, transgenic technology has made available a highly enriched GLA seed oil from the canola plant (HGCO: 36% GLA). The first objective of this study was to compare the effects of diets containing equal levels of GLA (23%) from either BO or HGCO on reproduction, pup development, and pup brain FA composition in mice. The second objective was to compare the effects of the HGCO diluted to 23% GLA (GLA-23) with those of undiluted HGCO containing 36% GLA (GLA-36). The diets were fed to the dams prior to conception and throughout pregnancy and lactation, as well as to the pups after weaning. The behavioral development of the pups was measured 12 d after birth, and anxiety in the adult male offspring was assessed using the plus maze. The findings show that despite equivalent levels of GLA, GLA-23 differed from BO in that it reduced pup body weight and was associated with a slight increase in neonatal pup attrition. However, there were no significant effects on pup behavioral development or on performance in the plus maze. An increase in dietary GLA resulted in an increase in brain 20:4n-6 and 22:4n-6, with a corresponding decrease in 22:6n-3. Again, despite their similar levels of GLA, these effects tended to be larger in GLA-23 than in BO. In comparison with GLA-23, GLA-36 had larger effects on growth and brain FA composition but no differences with respect to effects on reproduction and behavioral development. These findings suggest that the HGCO can be used as an alternative source of GLA.

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The central nervous system is high in long-chain PUFA (LCPUFA), particularly arachidonic acid (AA, 20:4n-6) and DHA (22:6n-3). Their accretion in mammals is greatest during the period of most rapid brain development, i.e., during the pre- and early postnatal brain growth spurt (1). It has been suggested that, whereas AA is necessary for growth, DHA may be necessary for optimal brain and behavioral development. However, although dietary supplementation of DHA increases DHA in the brain, it also has a reciprocal effect in decreasing AA (2–4). The reduction in AA is, in turn, associated with growth deficiencies (5). Thus, dietary DHA supplementation is best undertaken in a context that also includes a source of long-chain n-6 FA. This can be accomplished by adding a small amount of AA or, alternatively, γ-linolenic acid (GLA, 18:3n-6) to the diet. GLA differs from AA in that, unlike the 20-carbon FA, it is not an immediate precursor of eicosanoids but rather allows for the metabolic regulation of AA levels. We showed previously in a study in which pregnant mice were fed diets containing fish oil with high levels of long-chain n-3 FA that GLA could serve as an alternate source of AA (6). In a subsequent study, using the artificial rearing system in rats, we showed that GLA, supplied directly to the developing rat during the brain growth spurt in the form of borage oil (BO), offset to some extent the effects of DHA on brain FA composition by increasing 22:4n-6 (7). This suggests that GLA may be an appropriate alternative source for increasing levels of long-chain n-6 FA in the developing brain.

GLA is available from various sources, including evening primrose oil, BO, and black currant oil. More recently, the use of transgenic technology has yielded a more economical source of GLA by using the canola plant as host. The yield of GLA from the modified canola plant ranges from 22 to 45% of total FA. The first objective of the present study was to evaluate in pregnant and lactating mice the effects of diets containing equal levels of GLA (23%), expressed as wt% FA, from high-GLA canola oil (HGCO) vs. borage oil (BO) on reproduction, pup growth, and brain and behavioral development. This is a standard animal model used in experiments of potential teratogens, and one that we have used before to investigate the developmental effects of both high n-6 FA (GLA) and n-3 FA (DHA) (8,9).

We showed previously that diets very high in GLA (35%), with 6% of n-3 FA provided in the form of α-linolenic acid (ALA, 18:3n-3), increased brain 20:3n-6 and 22:4n-6 while at the same time reducing DHA (8). The high-GLA diet also reduced birth weight and was associated with increased running wheel activity. Thus, the second objective of this study

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; BO, borage oil; GLA, γ-linolenic acid; HGCO, high-GLA canola oil; LA, linoleic acid; LCPUFA, long-chain PUFA; PC, choline phosphoglycerides; PE, ethanolamine phosphoglycerides; PS/PI, serine/inositol phosphoglycerides.

was to compare the effects of the diluted HGCO (GLA-23) with those of undiluted HGCO containing 36% GLA (GLA-36). In addition to the normal developmental measures, the behavior of adult male pups was assessed on the elevated plus maze, using standard measures of anxiety and activity. Although n-3 deficiency has been associated with increased anxiety on the plus maze in mice (10), there do not appear to have been similar studies of GLA effects. However, findings in humans suggest that GLA supplementation may reduce behavioral manifestations of stress reactivity (11). The experimental hypotheses were (i) that diets containing equal levels of GLA (23%) from HGCO vs. BO would have similar effects relative to the control group on reproduction, brain and behavioral development, and brain FA composition in mice, and (ii) that the effects of supplementation with HGCO containing 36% GLA would be larger than those of HGCO diluted to 23% GLA.

MATERIALS AND METHODS

Animals. This research protocol was reviewed by the Animal Care Committee at the University of Waterloo, and approved to be in accordance with requirements of the Canadian Council for Animal Care. Based on our previous work (4), it was determined that a sample size of 20 litters per group would allow the detection of a difference in terms of behavioral development of 1 SD between groups with a power of 90% and an α level of 0.05.

B6D2F_1 female mice were obtained from Harlan Sprague Dawley (Indianapolis, IN). They were housed in standard opaque-plastic shoebox mouse cages and kept under a reversed 12:12 light/dark cycle (lights off at 6:00 A.M.) at 22 ± 1°C with free access to food (see diets below) and tap water. The mice were 8 wk old at the start of the study and were assigned randomly to one of the five diets, starting feeding 2 wk prior to the commencement of breeding. The study was conducted in two cohorts over a 6-mon period, with all groups represented equally in both cohorts. Prepared diets were stored refrigerated under nitrogen, and the mice were fed three times weekly. Food intake and body weight were monitored for the first cohort for the first 2 wk of feeding; no consistent group differences were apparent on either of these measures (data available on request).

Diets. The dietary groups were constituted by adding specific oil mixtures to modified semisynthetic fat-free powdered diet (AIN-93G; Harlan-Teklad, Madison, WI) with a fat content of 10% (w/w). Diet composition is provided in Table 1 and the formulation and FA composition of the dietary oils is shown in Table 2.

FA profiles of the prepared diet were consistent with those of the dietary oils, with reductions in the percentage of individual PUFA due to dilution in the diet in the order of approximately 3%; these data are available on request. α-Tocopherol supplementation was not considered necessary in this study since the oil content and degree of unsaturation were similar among groups. The first objective of the study, i.e., to evaluate GLA-23 (from HGCO) vs. BO, was addressed by the first three dietary groups: (i) control (CON)—0% GLA, (ii) BO—22.5% GLA, and (iii) GLA-23—23.2% GLA. In each of these groups the n-3 FA were available as 1.6% ALA, and total PUFA (n-6 + n-3) were 60%. The second objective, i.e., the dose–response relationship, was addressed by comparing group (iv) GLA-36—36.2% GLA from HGCO + 1.4% ALA with GLA-23 and CON.

Breeding and early raising. Animals were mated daily, starting at the beginning of the dark phase, and were checked for the presence of copulatory plugs 7 h later. The day on which a vaginal plug was detected was considered day 0 of

TABLE 1
Diet Composition (TD 99040)

Component	g/kg
Oil	100
Casein	200
L-Cystine	3
Cornstarch	367.5
Maltodextrin	132
Sucrose	100
Cellulose	50
Mineral mix[a], AIN-93G-MX (TD 94046)	35
Vitamin mix[b], AIN-93-VX (TD 94047)	10
Choline bitartrate	2.5
TBHQ (antioxidant)	0.02

[a]Mineral mix (g/kg): calcium carbonate (357.0), potassium phosphate, monobasic KH_2PO_4 (196.0), potassium citrate, monohydrate (70.78), sodium chloride NaCl (74.0), potassium sulfate K_2SO_4 (46.6), magnesium oxide MgO (24.3), ferric citrate (6.06), zinc carbonate (1.65), manganous carbonate (0.63), cupric carbonate (0.31), potassium iodate KIO_3 (0.01), sodium selenate Na_2SeO_4 (0.01025), ammonium paramolybdate $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (0.00795), sodium metasilicate $Na_2SiO_3\cdot 9H_2O$ (1.45), chromium potassium sulfate $CrK(SO_4)_2\cdot 12H_2O$ (0.275), lithium chloride LiCl (0.0174), boric acid H_3BO_3 (0.0815), sodium fluoride NaF (0.0635), nickel carbonate, hydroxide, tetrahydrate (0.0318), ammonium vanadate NH_4VO_3 (0.0066), sucrose, finely ground (220.716).

[b]Vitamin mix (g/kg): nicotinic acid (3.0), calcium pantothenate (1.6), pyridoxine HCl (0.7), thiamin HCl (0.6), riboflavin (0.6), folic acid (0.2), D-biotin (0.02), vitamin B_{12} (0.1% in mannitol) (2.5); DL-α-tocopheryl acetate (500 IU/g) (15.0), vitamin A palmitate (500,000 IU/g) (0.8), vitamin D_3 (cholecalciferol, 500,000 IU/g) (0.2), vitamin K (phylloquinone) (0.075), sucrose, finely ground (974.705).

TABLE 2
FA Composition of Experimental Oils[a]

	CON	BO	GLA-23	GLA-36
16:0	10.9	10.7	11	5.2
18:0	2	3.9	2.4	2.6
18:1n-9	25.7	16.3	22.6	23
18:2n-6	58.4	36.4	34.6	27.5
18:3n-6	ND	22.5	23.2	36.2
18:3n-3	1.6	1.6	1.6	1.4
20:0	0.3	0.3	0.4	0.8
20:1	0.2	3.6	0.5	0.8
22:0	ND	ND	0.2	ND
22:1	ND	2.4	ND	0.1

[a]CON, corn oil (92.7%) + soybean oil (7.3%) = control; BO, borage oil (97.9%) + flaxseed oil (2.1%); GLA-23, palm oil (12%) + corn oil (29.5%) + flaxseed oil (0.5%) + high γ-linolenic acid canola oil (HGCO) (58%); GLA-36 HGCO (100%). ND, not detectable; GLA, γ-linolenic acid.

gestation, and all subsequent days refer to "days postconception." The pregnant dams were weighed on days 0, 7, and 14. Starting on day 18 they were checked twice daily for births, and the number of live and dead pups recorded. Births occurred on days 19 or 20, and on day 21 dams and litters were weighed and litter size recorded again. At this time, litters were then culled to six (three males and three females where possible). Litters were weighed on days 25, 32, 39, and 46 (weaning). Pups were also weighed individually at weaning to compare animals by sex. On day 32 (approximately 12 d after birth), one male and one female from each litter was assessed on a battery of tests of sensorimotor development, described in detail previously (4). All behavioral testing was done independently of knowledge of the treatment group. As a positive control, two additional females from each litter were assessed on day 30. Evidence of retarded development in the day 30 compared with the day 32 animals supports the reliability of the behavioral measure. A positive control group such as this is particularly helpful in interpreting results when there are no apparent effects of the experimental manipulation. Animals were weaned between days 43 and 48 postconception (about 23 to 28 d after birth) and ear-notched for identification. One male and one female, selected randomly from each litter (excluding the animals previously tested), were weighed, then anesthetized using halothane, and decapitated. The brains were extracted, weighed, and frozen immediately in liquid nitrogen. Tissue samples from two mice in each litter were pooled and stored at −80°C. Samples were coded so that FA analyses were conducted with no knowledge of the dietary group or of the relationship between different samples.

Brain FA composition. Tissue FA were extracted using the method of Folch *et al.* (12). Aliquots of total tissue lipid extracts were separated into different phospholipid fractions by TLC using chloroform/methanol/water/triethylamine (4:5:1:4 by vol) as the developing system. The FA in the PC, PE, and PI + PS fractions were methylated under nitrogen according to the method of Morrison and Smith (13). Heptadecanoic acid was added as the internal standard. FAME were analyzed by capillary GC (Hewlett-Packard 5890 II Plus; Hewlett-Packard, Palo Alto, CA) equipped with a 30-m (0.32 mm i.d.) capillary column (Omegawax, 0.25 μm film thickness; Supelco, Bellefonte, PA) and integrated by a Hewlett-Packard Chem Station. The oven temperature was programmed to increase from 120 to 200°C at 4°C/min and to hold a final 15 min. The identification of each FA was made with authentic standard mixtures (Nu-Chek-Prep, Elysian, MN).

Plus maze testing. At weaning one male from each litter was selected randomly for behavioral testing, ear-notched for identification, and group-housed with animals of the same dietary group. Testing began when the animals were 7 wk old. The testing apparatus was the standard elevated plus-maze used to measure activity and anxiety in mice (14). Briefly, this comprised two opposing open arms and two closed arms, connected by a central platform (5 × 5 cm). The floor was white Plexiglas, whereas the open (30 × 5 × 0.5 cm) and closed (30 × 5 × 15 cm) arms were made of clear Plexiglas (length × width × height). The maze was elevated 45 cm above the ground, and the area around the maze was enclosed by a white screen to provide a uniform background. Red light with an intensity of 3 lux was the only illumination provided during testing. Behavior was recorded by a video camera positioned directly above the maze and monitored by the experimenter, who was positioned behind the screen. A mouse was placed on the central platform, facing an open arm, and its behavior was videotaped for 5 min. An additional group of control animals were treated with 15 mg/kg pentobarbitone sodium, administered intraperitoneally 30 min prior to testing. This is known to increase the number of open-arm entries and was used as a "positive" control, for reasons similar to those described for behavioral development above. There were insufficient males to be able to include a control saline-injected group, but previous research has indicated that saline injection decreases open-arm exploration (15). As this effect is opposite that expected of drug-treated animals, a positive response in this group would be indicative of a drug effect. The maze was cleaned between mice with 70% ethanol.

The following measures were recorded on each trial: latency to first arm entry, type of arm first entered, and number of entries into open and closed arms. An arm entry was defined as the crossing of the entire body (minus the tail) into an arm, an exit as two paws onto the central platform. The length of time spent in the central platform and in the open and closed arms was determined by later viewing of the videotape. The standard measures analyzed included: the percentage of time spent on the open arms [time open/(time open + time closed)], percentage of entries into the open arms [# entries open/(# entries open + # entries closed)], and the total number of entries.

Statistical analyses. Data were analyzed by ANOVA using SAS with the Student–Neuman–Keuls test (SNK) used for *post hoc* comparisons among groups. Where data were obtained from more than one pup per litter, the litter was considered the unit of analysis. Statistical significance was set at α level < 0.05, except for the brain FA analyses, where the more stringent α level < 0.0001 was used for the overall ANOVA.

RESULTS

Maternal variables. There were no significant differences among the groups in terms of number of successful pregnancies, gestation length, or maternal weight gain during pregnancy and lactation (data not shown). Although litter size did not differ significantly either at birth (day 19) or on day 21, there was a small difference in pup loss. As shown in Table 3, both GLA-23 and GLA-36 lost slightly more pups in the first 2 d post-parturition than CON.

Pup growth. The pattern of preweaning pup growth is shown in Figure 1, with individual values for birth and weaning shown in Table 3. Growth was analyzed using a repeated measures analysis on the average litter weight over time, which indicated a significant Group × Time interaction. Subsequent simple effects analyses indicated that GLA-36 weighed less

TABLE 3
Litter Size and Pup Weight[a]

	CON ($n = 22$)[b]	BO ($n = 22$)	GLA-23 ($n = 21$)	GLA-36 ($n = 23$)
Litter size at birth (day 19)	9.86 ± 1.73	9.27 ± 1.70	10.43 ± 1.03	10.13 ± 1.46
Litter size on day 21	9.45 ± 1.82	8.41 ± 1.87	8.95 ± 1.77	8.65 ± 1.82
Pup loss between birth and day 21	0.41 ± 0.59^{y}	0.86 ± 0.99^{x,y}	1.48 ± 1.47^{x}	1.48 ± 1.41^{x}
Pup weight day 21[c]	1.61 ± 0.18^{x}	1.54 ± 0.16	1.52 ± 0.18	1.45 ± 0.15^{y}
Pup weight day 46 (weaning)	16.75 ± 1.10^{x}	16.65 ± 1.27^{x}	15.31 ± 1.24^{z}	15.65 ± 1.27^{y,z}
Pup brain weight[d] (weaning)	0.349 ± 0.058	0.351 ± 0.053	0.363 ± 0.044	0.354 ± 0.069

[a]Values represent mean ± SD (litter mean scores, three male and three female pups). Groups that do not share a superscript letter are significantly different [Student–Newman–Keuls (SNK) test, $P < 0.05$]. For abbreviations see Table 2.
[b]n = number of litters.
[c]Days postconception, day 0 = conception; birth normally occurred on day 19–20.
[d]Based on one male and one female per litter.

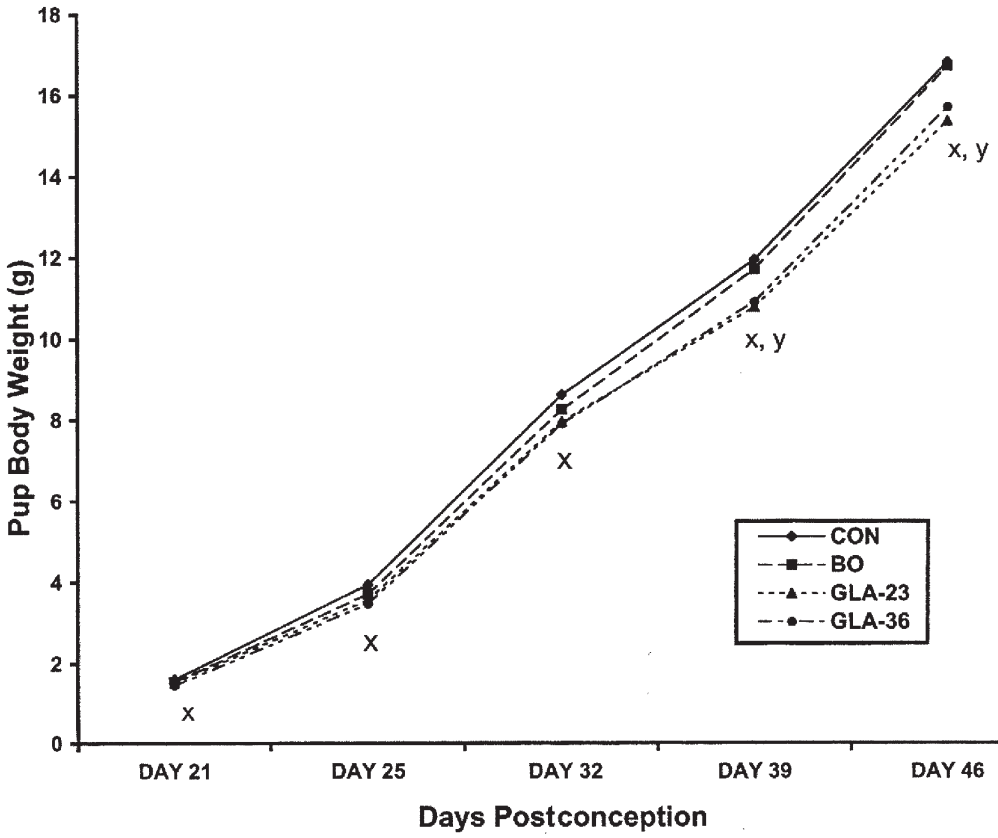

FIG. 1. Pre-weaning pup growth. Day 0 = conception, and birth normally occurred day 19–20. n = 19–23 litters per group. ^{x}GLA-36 weighed less than CON on all days. ^{y}GLA-23 weighed less than CON on days 39 and 46 (weaning), $P < 0.05$.GLA, γ-linolenic acid; CON, control (0% GLA); BO, borage oil (containing 22.5% GLA); GLA-23, diet containing 23.2% GLA from high-GLA canola oil (HGCO); GLA-36, diet containing 36.2% GLA from HGCO.

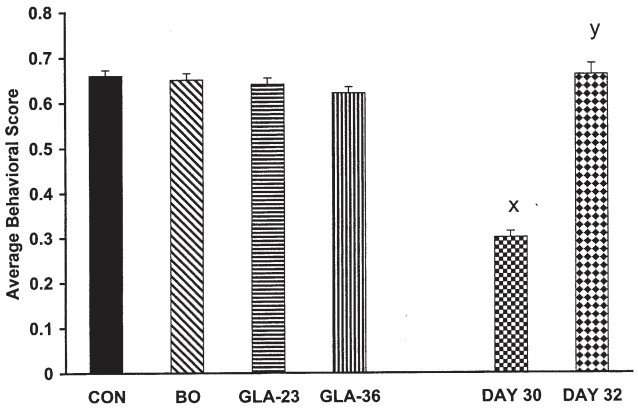

FIG. 2. Pup behavioral development (averaged across one male and one female per litter). n = 19–23 litters per group. ^{x,y}Positive control (day 30) and day 32 were significantly different ($P < .05$). There were no other significant differences; error bars represent SEM. For abbreviations see Figure 1.

than CON at all times from birth to weaning. On days 39 and 46 (weaning) both GLA-36 and GLA-23 weighed less than both CON and BO, which did not differ. As also shown in Table 3, there were no significant effects on brain weight.

Pup behavioral development. As shown in Figure 2, there were no significant differences among the groups tested at day 32. However, the positive control group tested on day 30 did show significant behavioral retardation, as expected, compared with 32-d-old animals, thereby confirming the reliability of the behavioral scale.

Plus maze. There were no significant differences among the dietary groups on any of these behavioral measures (data not shown). However, although the other groups did not differ from each other or CON, the positive control did show the expected effects, i.e., when compared with CON, mice treated with pentobarbitone showed significantly more open-arm entries and, as shown in Figure 3, spent a higher percentage of time on the open arms.

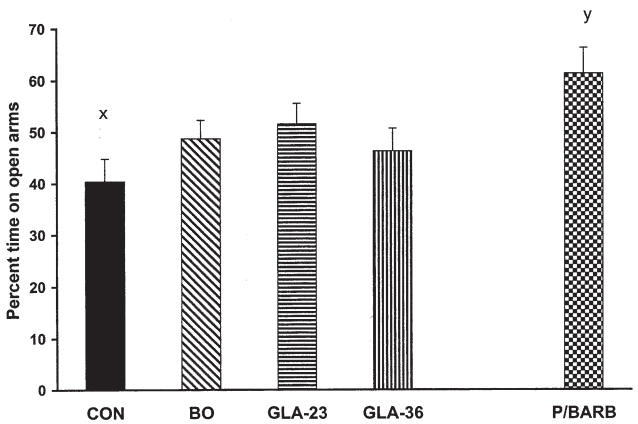

FIG. 3. Percentage of time spent on the open arms of the plus maze. n = 20–23 male pups per group. ^{x,y}Positive control (pentobarbitone: P/BARB) and CON were significantly different ($P < .05$). No other groups differed significantly. Error bars represent SEM. For other abbreviations see Figure 1.

Brain FA composition. The following section presents the findings with respect to FA composition for the following brain fractions: ethanolamine phosphoglycerides (PE), choline phosphoglycerides (PC), and serine/inositol phosphoglycerides (PS/PI). The FA data shown in Tables 4 (PE), 5 (PC), and 6 (PS/PI) represent the weight percentages of total FA for selected n-6 and n-3 FA whose values exceeded 1%. The following describes the significant effects in terms of total saturated, monounsaturated, and polyunsaturated FA as well as selected individual n-6 and n-3 FA: 20:3n-6, 20:4n-6, 22:4n-6, 22:5n-6, and 22:6n-3. The latter are reported separately for each fraction. It should be noted that in the brain the relative levels of 18:2n-6 and 18:3n-3 were below 1% in all fractions, and 18:3n-6 was not detected.

(i) Saturates. Total saturates did not differ in any of the fractions.

(ii) Monounsaturates. In PE, neither BO nor GLA-23 differed from each other or CON. In contrast, although GLA-36 did not differ from GLA-23, it was lower than CON. There were no significant effects in either PC or PS/PI.

(iii) Polyunsaturates. The groups did not differ in terms of total polyunsaturates in any of the fractions. It is, however, clearly apparent from the data that the fractions differ among themselves, with much lower levels of PUFA in PC.

(iv) PE. Both BO and GLA-23 showed increased levels of 20:3n-6 relative to CON, with this effect being greater in GLA-23 than in BO and largest in GLA-36. BO did not differ from CON in terms of 20:4n-6 but did have higher levels of 22:4n-6, whereas GLA-23 had higher levels of 20:4n-6 and 22:4n-6 than both BO and CON. GLA-36 had higher levels of each of these n-6 FA than any of the other groups. Levels of 22:5n-6 were lower in CON than any of the other groups, which did not differ. For 22:6n-3 the groups differed in the following order: CON > BO > GLA-23 > GLA-36. Total n-6 FA differed among all groups and were ordered as follows: GLA-36 > GLA-23 > BO > CON. Total n-3 FA also differed among all groups, with the order being reversed: CON > BO > GLA-23 = GLA-36. The summary measure represented by the n-6/n-3 ratio showed the groups differing in the following order: GLA-36 > GLA-23 > BO > CON.

(v) PC. Effects on 20:3n-6 and 22:4n-6 were the same as those just described in PE. In contrast, there were no significant effects on 20:4n-6. Also as seen in PE, levels of 22:5n-6 were lower in CON than all other groups, but in PC levels in BO were higher than in GLA-23 and GLA-36. BO did not differ from CON with respect to levels of 22:6n-3, but both GLA-23 and GLA-36 had similarly reduced levels when compared with BO and CON. Total n-6 FA did not differ among the groups; in terms of total n-3, CON = BO > GLA-23 = GLA-36. The n-6/n-3 ratio was ordered as follows: GLA-36 > GLA-23 = BO = CON.

(vi) PS/PI. Effects on 20:3n-6 and 22:4n-6 were also the same as seen in PE and PC: GLA-36 > GLA-23 > BO > CON. The only effect seen on 20:4n-6 was an increase in GLA-23 compared with BO. Both BO and GLA-23 had higher levels of 22:5n-6 than CON, and GLA-36 had higher levels than

Table 4
Selected FA Composition of Brain Ethanolamine Phosphoglycerides (wt% FA)[a]

	CON (n = 19)	BO (n = 21)	GLA-23 (n = 20)	GLA-36 (n = 21)
Total sat	37.25 ± 1.93	37.36 ± 1.94	37.69 ± 2.1	37.26 ± 2.59
Total mono	18.01 ± 1.40^{p}	17.59 ± 1.35^{p,q}	17.04 ±1.34^{p,q}	16.65 ± 1.29^{q}
Total poly	39.80 ± 2.68	40.30 ± 2.36	39.97 ± 2.22	41.37 ± 2.93
20:3n-6	0.69 ± 0.05^{y}	1.37 ± 0.15^{q}	1.15 ± 0.14^{x}	1.59 ± 0.20^{p}
20:4n-6	14.35 ± 1.47^{x}	14.67 ± 1.40^{x}	16.17 ± 0.97^{q}	17.73 ± 1.94^{p}
22:4n-6	4.43 ± 0.29^{y}	4.86 ± 0.40^{x}	5.97 ± 0.36^{q}	6.34 ± 0.60^{p}
22:5n-6	2.34 ± 0.30^{q}	2.87 ± 0.35^{p}	2.86 ± 0.42^{p}	2.80 ± 0.36^{p}
Total n-6	22.84 ± 1.47^{y}	24.48 ± 1.41^{x}	26.88 ± 1.39^{q}	29.12 ± 2.14^{p}
22:6n-3	16.70 ± 1.43^{p}	15.53 ± 1.34^{q}	12.33 ±1.37^{x}	11.19 ± 1.34^{y}
Total n-3	16.96 ±1.43^{p}	15.83 ± 1.31^{q}	13.09 ± 1.30^{x}	12.25 ± 1.29^{x}
Ratio n-6 to n-3	1.35 ± 0.08^{y}	1.55 ± 0.11^{x}	2.07 ± 0.18^{q}	2.40 ± 0.23^{p}

[a]Values represent mean ± SD (litter mean scores, one male and one female pup, where n = number of litters). Groups that do not share a superscript are significantly different (SNK). Total sat, total saturated FA; Total mono, total monounsaturated FA; Total poly, total PUFA; for other abbreviations see Tables 2 and 3.

Table 5
Selected FA Composition of Brain Choline Phosphoglycerides (wt% FA)[a]

	CON (n = 19)	BO (n = 21)	GLA-23 (n = 20)	GLA-36 (n = 21)
Total sat	63.67 ± 1.25	64.14 ± 1.00	64.35 ± 1.67	64.55 ± 2.38
Total mono	26.47 ± 0.73	26.25 ± 0.76	26.32 ± 0.96	26.07 ± 1.71
Total poly	9.87 ± 1.20	9.61 ± 0.76	9.32 ± 1.16	9.37 ± 1.27
20:3n-6	0.21 ± 0.02^{y}	0.47 ± 0.03^{q}	0.38 ± 0.04^{x}	0.54 ± 0.08^{p}
20:4n-6	5.17 ± 0.65	5.21 ± 0.46	5.38 ± 0.69	5.53 ± 0.78
22:4n-6	0.33 ± 0.06^{y}	0.36 ± 0.04^{x}	0.44 ± 0.05^{q}	0.47 ± 0.05^{p}
22:5n-6	0.36 ± 0.06^{x}	0.49 ± 0.11^{p}	0.43 ± 0.08^{q}	0.42 ± 0.06^{q}
Total n-6	7.52 ± 0.76	7.45 ± 0.51	7.63 ± 0.85	7.82 ± 0.94
22:6n-3	2.31 ± 0.45^{p}	2.10 ± 0.33^{p}	1.56 ± 0.38^{q}	1.37 ± 0.36^{q}
Total n-3	2.35 ± 0.46^{p}	2.16 ± 0.31^{p}	1.70 ± 0.38^{q}	1.56 ± 0.37^{q}
Ratio n-6 to n-3	3.27 ± 0.41^{q}	3.50 ± 0.39^{q}	4.64 ± 0.75^{q}	5.19 ± 0.89^{p}

[a]Values represent mean ± SD (litter mean scores, one male and one female pup, where n = number of litters). Groups that do not share a superscript are significantly different (SNK). For abbreviations see Tables 2–4.

Table 6
Selected FA Composition of Brain PS/PI (wt% FA)[a]

	CON (n = 19)	BO (n = 21)	GLA-23 (n = 20)	GLA-36 (n = 21)
Total sat	48.54 ± 3.69	49.42 ± 4.04	48.06 ± 3.48	49.18 ± 3.99
Total mono	18.31 ± 1.03	17.86 ± 1.25	18.04 ± 1.60	17.73 ± 1.23
Total poly	33.15 ± 3.88	32.72 ± 4.06	33.89 ± 3.86	33.09 ± 4.53
20:3n-6	0.58 ± 0.06^{y}	1.12 ± 0.11^{q}	0.97 ± 0.12^{x}	1.29 ± 0.17^{p}
20:4n-6	11.79 ± 1.23^{p,q}	11.48 ± 1.20^{q}	12.74 ± 1.44^{p}	12.17 ± 1.73^{p,q}
22:4n-6	2.52 ± 0.17^{y}	2.77 ± 0.23^{x}	3.45 ± 0.44^{q}	3.97 ± 0.27^{p}
22:5n-6	2.79 ± 0.43^{x}	3.37 ± 0.52^{p}	3.59 ± 0.69^{p,q}	3.73 ± 0.42^{q}
Total n-6	18.31 ± 1.57^{q}	19.23 ± 1.71^{q}	21.33 ± 2.17^{p}	21.63 ± 2.08^{p}
22:6n-3	14.71 ± 2.40^{p}	13.37 ± 2.50^{p,q}	12.08 ± 1.85^{q,x}	10.74 ± 2.56^{x}
Total n-3	14.83 ± 2.41^{p}	13.49 ± 2.50^{p,q}	12.56 ± 1.93^{q,x}	11.46 ± 2.59^{x}
Ratio n-6 to n-3	1.25 ± 0.12^{y}	1.45 ± 0.15^{x}	1.72 ± 0.18^{q}	1.94 ± 0.28^{p}

[a]Values represent mean ± SD (litter mean scores, one male and one female pup, where n = number of litters). Groups that do not share a superscript are significantly different (SNK). For abbreviations see Tables 2–4.

BO. In terms of total n-6, GLA-36 = GLA-23 > BO > CON. Levels of 22:6n-3 in BO and CON did not differ but were decreased in GLA-23 relative to CON and in GLA-36 relative to both CON and BO; the same pattern of findings pertained to total n-3. The n-6/n-3 ratio was ordered as follows: GLA-36 > GLA-23 > BO > CON.

DISCUSSION

The first objective of the present study was to compare the effects on reproduction, growth, and brain FA composition of feeding mice equivalent amounts of GLA, provided either as BO or as HGCO. The second objective was to compare the

effects of varying levels of GLA from HGCO. The following discusses the findings in relation to each of these objectives.

There were no significant effects on maternal weight gain, either pre- or postnatally, and there also were no significant differences in litter size, either at birth or 2 d later. Thus, the small effect on neonatal pup attrition, with the GLA-23 losing slightly more pups than CON, appears paradoxical. This finding can probably be attributed to the fact that the low GLA-23 group tended toward slightly higher litter size than CON at birth, and slightly lower litter size than CON 2 d later, neither of which was significant in itself but which did result in a significant difference score. The same effect was seen in GLA-36. It is worth noting that litter size at birth may not be a completely accurate assessment of litter size, because the dams were monitored every 24 h, and thus some time may have elapsed between the actual time of birth and the taking of the measurement. Nonetheless, these litter sizes are consistent with those in other studies we have done using this mouse strain (6,8,9).

There were significant effects on the growth of the pups. Neither BO nor GLA-23 differed from CON at birth, but by weaning GLA-23 weighed less than both CON and BO. Based on our previous findings with high levels of GLA, we had predicted that GLA-36 would be associated with lower pup body weight, and this was supported by the finding that GLA-36 pups weighed less than CON from birth to weaning and also weighed less than GLA-23 in the week before weaning. However, as GLA-23 and BO both contained the same amount of GLA, the finding of lower growth in GLA-23 was unexpected; this is discussed later in the context of the results on brain FA composition. There were no significant effects on brain weight. Because of the large sample size, this study has considerable power to identify small effects; in this case the body weight difference of 1.4 g between GLA-23 and CON at weaning translates into an effect size of approximately 1 SD, which is by convention considered large (16). As body composition was not measured in this study, the specific factors contributing to this reduced body weight cannot be identified. However, a study in rats showed that feeding GLA in the diet was associated with reduced body fat rather than lean body mass (17).

There were no significant effects either on the behavioral development scale or on the plus maze. This study was designed to detect an effect of 1 SD on behavioral development with 90% power. The findings with respect to each of the positive control groups attest to the reliability of the measurement. Thus, one can be reasonably confident that if there were an effect of any of these treatments on behavioral development, it would probably be small.

Consistent with our previous findings, the overall effects of supplementation with GLA were to increase levels of specific n-6 FA and to decrease those of n-3 FA, predominantly 22:6n-3 (DHA). With respect to the individual n-6 FA, comparison of the different fractions showed similar effects on 22:4n-6 but not on 20:4n-6 (AA), where there were the expected effects in PE, but not PC and PS/PI. Similarly, comparison of GLA-23 with GLA-36 showed that in all fractions levels of 22:4n-6 were higher in GLA-36, but only in PE were there differences between these groups in terms of AA and DHA. This is consistent with our previous work showing that brain 22:4n-6 appears to be more sensitive than 20:4n-6 to the effects of dietary supplementation with GLA (7). Although the effects of both BO and GLA-23 relative to CON were in the same direction, the effects of GLA-23 were larger than BO. For example, in some instances GLA-23 differed from both CON and BO, as seen in both AA and DHA in the PE fraction. Here BO did not increase AA relative to CON, whereas GLA-23 increased AA relative to both CON and BO. Similarly, the findings for DHA indicated that while BO decreased DHA relative to CON, GLA-23 decreased DHA relative to both CON and BO.

On account of their equivalent GLA content, the greater effect of GLA-23 compared with BO was again unexpected and was similar to those seen on growth. These effects may be related to some characteristic of HGCO other than its FA composition. For example, one way in which the oils in the present study vary is in the positional distribution of FA in TAG molecules, which may in turn contribute to differences in digestion, absorption, and uptake into the tissues. During digestion, dietary TAG are hydrolyzed by pancreatic lipase to FA and 2-MAG. After absorption, FA and 2-MAG are reesterified to form TAG and secreted as chylomicrons into circulation. Lipoprotein lipase in the peripheral tissues hydrolyzes the TAG molecules to FA and 2-MAG. FA are then taken up by the peripheral tissues. Lipoprotein lipase is an enzyme that cleaves the FA groups from the *sn*-1 and *sn*-3 positions of the TAG moiety. We have shown recently that approximately 75% of 18:3n-6 in HGCO is located at the *sn*-1/*sn*-3 positions and only 25% is at the *sn*-2 position (18). This is significantly different from borage oil in which 18:3n-6 is preferentially located at the *sn*-2 position (54.2% at *sn*-2 and 45.8% at the *sn*-1/*sn*-3 position) (19). Thus, the difference in bioavailability of 18:3n-6 in HGCO compared with borage oil may be due to the difference in positional distribution of 18:3n-6 in TAG molecules. This could be confirmed in the future through a dose–response study using lower doses of GLA from HGCO and a smaller range than the present study. The prediction would be that levels of HGCO that provided sufficient GLA to give tissue FA values similar to that of BO would not be associated with the effects on growth seen at higher levels.

In summary, this study is one of a series designed to evaluate the bioequivalence and safety of the newly developed high-GLA-content canola oil with traditional GLA-rich borage oil (20,21). The present findings indicated that some of the effects of GLA provided as HGCO differed from those of equivalent amounts of GLA provided as BO. Specifically, 23% GLA from HGCO reduced pup body weight and was associated with a slight increase in neonatal pup attrition. There were no significant effects on behavioral development or on performance in the plus maze. An increase in dietary GLA resulted in an increase in brain n-6 FA and a corresponding decrease in brain n-3. Moreover, despite their similar levels of

GLA, the effects on brain FA composition were greater in the mice provided 23% GLA as HGCO than with those receiving the same amount from BO. Comparison of the group receiving 23% GLA from HGCO with that receiving 36% indicated that at the higher level the effects on growth were greater, as were those on brain FA composition, particularly in the PE fraction. These findings support the use of HGCO as an alternative source of GLA.

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Natural Abundance Stable Carbon Isotope Evidence for the Routing and *de novo* Synthesis of Bone FA and Cholesterol

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ABSTRACT: This research reported in this paper investigated the relationship between diet and bone FA and cholesterol in rats raised on a variety of isotopically controlled diets comprising 20% C_3 or C_4 protein (casein) and C_3 and/or C_4 nonprotein or energy (sucrose, starch, and oil) macronutrients. Compound-specific stable carbon isotope analysis ($\delta^{13}C$) was performed on the FA (16:0, 18:0, 18:1, and 18:2) and cholesterol isolated from the diet ($n = 4$) and bone ($n = 8$) of these animals. The dietary signals reflected by the bone lipids were investigated using linear regression analysis. $\delta^{13}C$ values of bone cholesterol and stearic (18:0) acid were shown to reflect whole-diet $\delta^{13}C$ values, whereas the $\delta^{13}C$ values of bone palmitic (16:0), oleic (18:1), and linoleic (18:2) acids reflected dietary FA $\delta^{13}C$ values. Dietary signal differences are a result of the balance between direct incorporation (or routing) and *de novo* synthesis of each of these bone lipids. Estimates of the degree of routing of these bone lipids gleaned from correlations between $\Delta^{13}C_{dlipid-wdiet}$ ($= \delta^{13}C_{diet\ lipid} - \delta^{13}C_{whole\ diet}$) spacings and $\Delta^{13}C_{blipid-wdiet}$ ($= \delta^{13}C_{bone\ lipid} - \delta^{13}C_{whole\ diet}$) fractionations demonstrated that the extent of routing, where 18:2 > 16:0 > 18:1 > 18:0 > cholesterol, reflected the relative abundances of these lipids in the diet. These findings provide the basis for more accurate insights into diet when the $\delta^{13}C$ analysis of bone fatty FA or cholesterol is employed.

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Since the 1970s, stable isotope analysis ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) has provided a direct method with which to explore trophic interactions in modern and ancient food webs. The rationale behind using stable isotope analysis for dietary reconstruction is based on two well-established observations: (i) different food groups have characteristically different isotope ratios, and (ii) when these food groups are consumed by an organism, they influence the isotopic composition of its tissues. Hence, measured isotope values of a consumer's tissues serve as a natural tracer for its dietary intake. The dietary information or dietary *signal* obtained from the $\delta^{13}C$ analysis of different consumer tissues reflects different aspects of the diet (1–5). The relationship between dietary macronutrient components and consumer tissue types is complex and is thought to depend on several factors, including the nutritional status and digestive physiology of the animal, the turnover rate of the tissue, and its biosynthetic pathway (6).

Insights into the relationship between the isotopic composition of specific dietary macronutrients and body tissues have been gleaned from isotopically controlled animal feeding experiments (7–11). Important findings from these studies include: (i) an enrichment of 0.8 ± 1.1‰ is observed between the carbon isotopic composition of whole animals (nematodes, insects, shrimps, snails) and that of their respective diets (7); (ii) the isotopic relationships among the dietary biochemical components of foodstuffs, namely, $\delta^{13}C_{total\ organic\ matter} > \delta^{13}C_{lipid}$, $\delta^{13}C_{carbohydrates} > \delta^{13}C_{lipid}$, and $\delta^{13}C_{protein} > \delta^{13}C_{lipid}$, is inherited by the tissues of animals raised on them (7); (iii) bone collagen $\delta^{13}C$ values are biased toward that of the dietary protein (10,11); and (iv) bone apatite $\delta^{13}C$ values reflect that of the whole diet (10,11). On the molecular level, Hare *et al.* (9) measured the $\delta^{13}C$ and $\delta^{15}N$ values of individual collagenous amino acids isolated from modern (including laboratory-raised) pigs and archaeological bone using preparative ion-exchange HPLC, followed by off-line combustion and isotope ratio mass spectrometry (IRMS). This study showed that a characteristic pattern existed among the carbon and nitrogen isotope values of the amino acids. Moreover, the comparison of the stable isotope values of essential and nonessential amino acids derived from collagen to those present in the diet of the pigs gave insights into the metabolic pathways that govern these amino acids.

The technique of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS), originally reported by Matthews and Hayes (12), allows the separation and measurement of the stable isotope ratios of individual compounds in a sample mixture. GC/C/IRMS requires only nanograms of individual compounds to be introduced to achieve a precision of ±0.3‰ for carbon isotope determinations. It is therefore a much more sensitive and less laborious technique to use than preparative HPLC for tracing dietary carbon into consumer tissues at the molecular level. Adopting a molecular approach not only increases the specificity of dietary investigations but also can circumvent many of the problems associated with the effects of contamination encountered in bulk isotope determinations. GC/C/IRMS has allowed dietary insights to be gleaned using individual amino acids (13,14), FA (15–17), and cholesterol (16,18–20; Jim, S., Evershed, R.P., and Ambrose, S.H., unpublished data) as indicators of diet.

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Abbreviations: BSTFA, bis(trimethylsilyl)trifluoroacetamide; GC/C/IRMS, gas chromatography/combustion/isotope ratio mass spectrometry; TLE, total lipid extract; TMS, trimethylsilyl.

The aim of this paper was to use GC/C/IRMS to assess the relative importance of routing and synthesis *de novo* for each bone lipid to gain a better understanding of the dietary signal reflected in bone FA and cholesterol $\delta^{13}C$ values. To this end, it is necessary to consider the different metabolic pathways that affect their occurrence in bone. Linoleic acid (18:2) is an EFA that cannot be synthesized *de novo* in higher mammals (21,22). It must therefore be directly incorporated or routed from the diet, and thus bone linoleic acid $\delta^{13}C$ values are expected to reflect dietary values. Non-EFA (16:0, 18:0, and 18:1) and cholesterol can be both absorbed directly from the diet and synthesized *de novo* in the body from acetyl-CoA. Acetyl-CoA is the common metabolite formed from the catabolism of dietary lipids, carbohydrates, and proteins (or from tissue glycogen and fat stores). All the carbon atoms of the common FA, two-thirds of the carbon in carbohydrates, and approximately half of the carbon skeleton of amino acids contribute to the acetyl-CoA pool (23). Hence, $\delta^{13}C$ values of synthesized FA and cholesterol are expected to reflect whole-diet $\delta^{13}C$ values with a bias toward dietary lipid and carbohydrate values. However, the overall dietary signal of bone non-EFA and cholesterol is dependent on the relative importance of the processes of routing vs. *de novo* synthesis of these lipids. In humans, it has been estimated that the amount of cholesterol synthesized per day (typically 1.0 to 1.5 g) is at least twice that of the daily dietary intake for an average Western diet (24). Approximately half of the dietary cholesterol will be absorbed by the intestine and the other half excreted; thus, dietary cholesterol can be estimated to contribute *ca.* 20% of total body cholesterol. Dietary FA compositions have been shown to greatly influence the FA compositions in rat bone marrow (25), human serum and plasma lipids (26,27), and human bone and blood phospholipids (28,29), providing semiquantitative evidence for the direct incorporation of dietary FA into consumer tissues. Stable isotopic evidence for the influence of dietary FA on consumer tissues has been reported by Rhee *et al.* (30) and Stott *et al.* (16). These two studies showed that the $\delta^{13}C$ values of non-EFA in human serum and in pig bone are enriched with respect to their dietary sources. Stott *et al.* (16) also demonstrated that bone linoleic acid $\delta^{13}C$ values, as expected, were highly consistent with dietary values. However, Rhee *et al.* (30) observed a 3‰ depletion in serum linoleic acid $\delta^{13}C$ values with respect to dietary values.

MATERIALS AND METHODS

Sample description. Holtzman albino rats were raised on a variety of purified and pelletized diets comprising 20.0% protein, 50.2% sucrose, 15.5% starch, 5.0% oil, 5% fiber, 3.5% minerals, and 1% vitamins. One day after insemination, sperm-positive, 90-d-old female rats were placed on diets that their offspring would consume. Birth occurred 21 d after insemination, and weaning occurred 21 to 23 d later. The sexes were separated prior to sexual maturity. Normal room temperature (20°C) was maintained, and food and water were provided *ad libitum*. Male and female pairs were sacrificed at 91, 131, and 171 d after birth. Eight rat forelimbs from four distinct diets were sampled for lipid analysis, in addition to the whole diets and dietary oils. The dietary compositions and the $\delta^{13}C$ values of individual dietary components comprising the diets are shown in Table 1. Table 2 lists all the animals that were studied.

Bulk $\delta^{13}C$ analysis of whole diet, dietary macronutrients, bone collagen, and bone apatite. Bone collagen and apatite extraction procedures and $\delta^{13}C$ measurements summarized here are described in detail in Ambrose and Norr (10). Lipids were extracted from clean ground bone using petroleum ether. Collagen was extracted by demineralization with 0.1 M HCl, treated with 0.125 M NaOH, solubilized at 95°C, and freeze-dried. Whole diet, dietary macronutrients, and bone collagen were combusted at >800°C with Cu, CuO, and Ag foil in evacuated sealed quartz tubes. Bone apatite carbonate was prepared by deproteinization with NaOCl, treated with 1 M acetic acid to remove adsorbed carbonate, freeze-dried, and reacted under vacuum with 100% H_3PO_4 at 25°C. CO_2 was cryogenically distilled off-line and analyzed on dual inlet isotope ratio mass spectrometers at the Anthropology Department, University of Illinois (Nuclide 6-60 RMS), or the Illinois State Geological Survey (Finnegan MAT Delta E).

Chemicals and precautions. All solvents used were of HPLC grade and purchased from Rathburn Chemicals (Walkerburn, Scotland). The internal standard *n*-tetratriacontane, cholesterol standard, sodium hydroxide pellets, and derivatizing agent *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% vol/vol trimethylchlorosilane were purchased from the Sigma Chemical Company (Dorset, England). All glassware and ceramics employed were washed with Decon 90, dried in an oven, and rinsed with chloroform/

TABLE 1
Rat Dietary Compositions and Their Macronutrient Components[a,b]

Diet code	Composition		Components and $\delta^{13}C$ values (‰)			
	Protein	Energy[c]	Protein 20.0%	Sucrose 50.2%	Starch 15.5%	Oil 5.0%
D2A4	C_3	C_3	Milk casein (–24.5)	Beet (–24.2)	Rice (–26.4)	Cottonseed (–27.9)
D3G	C_3	C_4	Milk casein (–26.3)	Cane (–11.0)	Corn (–10.3)	Corn (–14.9)
D4H	C_4	C_4	Milk casein (–14.6)	Cane (–11.4)	Corn (–10.6)	Corn (–14.9)
D5I	C_4	C_3	Milk casein (–14.6)	Beet (–24.2)	Rice (–26.4)	Cottonseed (–27.9)

[a]Reference 9; Ambrose, S.H., unpublished data.
[b]One percent of vitamins, 3.5% of minerals, and 5% cellulose (wood and/or corn) were added to each diet.
[c]Energy = Σ (sucrose, starch, and oil components).

TABLE 2
Dietary Compositions, Sample Codes, Sex, and Pair of Individual Animals[a]

Dietary composition		Sample		
Protein	Energy[b]	Code	Sex	Pair
C_3	C_3	C3	M	2
C_3	C_3	C3	F	2
C_3	C_4	C3P/C4	M	3
C_3	C_4	C3P/C4	F	3
C_4	C_4	C4	F	1
C_4	C_4	C4	M	2
C_4	C_3	C4P/C3	F	2
C_4	C_3	C4P/C3	M	3

[a]First, second, and third pair animals were sacrificed at 91, 131, and 171 d old, respectively. P = protein; M = male and F = female.
[b]Energy = Σ (sucrose, starch, and oil components).

methanol (2:1 vol/vol) prior to use. Disposable rubber gloves were worn throughout the whole experimental procedure, from sample preparation to instrumental analysis, to prevent the contamination of samples with finger lipids. Extraction, filtration, and saponification (neutral and acid) blanks were used to monitor and locate any contamination that might be introduced during the experimental procedure.

Preparation of diet and bone samples for lipid extraction. Rat forelimbs were dissected into three different tissue types: bone (ulna and radius), skin, and flesh. Bones were manually cleaned of adhering flesh, cartilage, and tendons by scraping with a scalpel. Half a bone was used for the extraction procedure, typically the upper ulna. Rat bone and diet pellets were ground in a pestle and mortar prior to extraction. Liquid nitrogen was employed to aid the bone crushing process. Ranges of rat sample weights are as follows: 0.03 to 0.13 g of powdered bone and 0.83 to 1.41 g of powdered diet pellets.

Extraction of lipids from diet and bone. Samples were transferred into large screw-capped vials and known quantities of *n*-tetratriacontane (1 mg mL^{-1} in chloroform) added as an internal standard. The samples were extracted with chloroform/methanol (2:1 vol/vol, 5 to 10 mL) by ultrasonication (3 × 1 h, Decon F5200b), where the supernatant was removed and replaced intermittently. The total lipid extract (TLE) was then concentrated to *ca.* 5 mL under a gentle stream of nitrogen (5 psi) in an evaporation unit (TurboVap LV; Zymarck Corporation, Hopkinton, MA) with the thermostatic bath set at 40°C. Suspended particulates were removed from the TLE by centrifugation (1800 rpm, 20 min; MSE Mistral 1000) and filtration through a short pipette column packed with activated alumina.

Saponification of TLE. Aliquots of TLE were transferred into screw-capped test tubes, blown down to dryness under nitrogen gas, and hydrolyzed with 0.5 M methanolic NaOH (2 mL) at 70°C in a water bath for 1 h. After cooling, the mixture was extracted using hexane (3 × 2 mL), yielding the neutral cholesterol-containing fraction. The mixture was then acidified to pH 3 with 1 M HCl and extracted again using hexane (3 × 2 mL) to yield the FA fraction. Neutral fractions were converted to their trimethylsilyl (TMS) ether derivatives using BSTFA containing 1% vol/vol trimethylchlorosilane. Twenty microliters of BSTFA were added to each sample followed by heating at 70°C (Multiblok Lab-Line) for *ca.* 1 h. Excess BSTFA was removed under a gentle stream of nitrogen. FA were converted to their methyl ester derivatives. FA fractions were transferred into fresh screw-capped test tubes and blown down to dryness under nitrogen gas. The samples were methylated by adding 100 µL of 14% wt/vol boron trifluoride/methanol complex and heating in a water bath at 70°C for 1 h. After cooling, 2 mL of double-distilled water was added and the FAME extracted using diethyl ether (3 × 2 mL).

GC. High-temperature GC analyses of the TLE and neutral fractions were carried out using a Hewlett-Packard (HP) 5890 Series II gas chromatograph fitted with a fused-silica capillary column (15 m × 0.32 mm i.d.) coated with a dimethyl polysiloxane stationary phase (DB-1, J&W Scientific, Folsom, CA; Agilent Technologies, Palo Alto, CA; 0.1 µm film thickness). The temperature of the oven was held isothermally at 50°C (2 min) and then increased to 350°C (20 min) at a rate of 10°C min^{-1}. FAME GC analyses were performed using the chromatograph described above fitted with a fused-silica capillary column (50 m × 0.32 mm i.d.) coated with a polyethylene glycol stationary phase (CP-WAX 52 CB, Varian; Chrompack, Middleberg, The Netherlands; 0.25 µm film thickness). The temperature of the oven was held isothermally at 40°C (2 min) and then increased to 200°C (10 min) at a rate of 5°C min^{-1}. Hydrogen was used as the carrier gas, and FID was used to monitor the column effluent. Data were acquired and analyzed using HP Chemstation software.

GC/MS. GC/MS analyses were performed using a Finnigan 4500 quadrupole mass spectrometer (source temperature, 280°C; electron voltage, 35 eV) interfaced to a Carlo Erba HRGC 5160 Mega series gas chromatograph. The same columns and temperature programs as described above were used for the TLE, neutral, and FAME fractions. Hydrogen was used as the carrier gas. Data were acquired using an INCOS data system and processed using Interactive Chemical Information Software (ICIS) package.

GC/C/IRMS. GC/C/IRMS analyses were carried out using a Varian 3500 gas chromatograph coupled to a Finnigan MAT Delta-S isotope ratio mass spectrometer *via* a Finnigan MAT combustion interface (Pt/CuO) maintained at 850°C. For the neutral fractions, the GC was fitted with a fused-silica capillary column (50 m × 0.32 mm i.d.) coated with a dimethyl polysiloxane stationary phase (CP-SIL 5 CB, 0.25 µm film thickness). The temperature of the oven was held at 50°C (2 min) and then increased to 250°C at a rate of 10°C min^{-1}, then to 300°C (20 min) at 4°C min^{-1}. For the FAME fractions, the same column and temperature program as described above for GC and GC/MS analyses were used. Helium was used as the carrier gas and the mass spectrometer source pressure was maintained at 9×10^{-5} Pa. Data were collected and processed using Finnigan MAT Isobase software. Secondary standards (C_{19} *n*-alkane or 18:0 FAME) of known $\delta^{13}C$ value were measured every seventh run to monitor any fluctuations in instrumental measurements with time. The precision of triplicate

GC/C/IRMS analyses carried out on each sample was shown to be ±0.3‰. FA and cholesterol $\delta^{13}C$ values were corrected for the addition of derivatizing carbon.

RESULTS AND DISCUSSION

Diet and bone lipid compositions. Figure 1 presents partial gas chromatograms of the TLE, neutral, and FAME fractions of the four diets and of bone sample C4P/C3 (C_4 protein with C_3 energy; see Table 2 for sample codes), female, third pair. Dietary lipid compositions reflect that of the oil (cottonseed or corn oil) and casein constituting the diets. The cottonseed and corn oil exhibited very similar lipid distributions characterized by the presence of C_{32} and C_{36} DAG and C_{48} to C_{54} TAG. Milk fat displays a TAG distribution in the range of C_{28} to C_{54} but is characterized by a greater abundance of the C_{36} to C_{44} TAG (31). Differences in dietary lipid distributions can be explained by the differential lipid extraction of the C_3 and C_4 caseins during processing, prior to the formulation of the diets. The C_4 casein retained a significant proportion of its lipids and this resulted in the presence of C_{30} to C_{44} TAG, and 10:0 and 12:0 FA in the TLE and FAME, respectively, of diets D4H (Figs. 1g and 1i) and D5I (Figs. 1j and 1l). Bone TLE (Fig. 1m) were characterized by the presence of FFA, cholesterol, and C_{48} to C_{54} TAG.

Trends in the $\delta^{13}C$ values of diet and bone components. Figure 2a compares whole diet and dietary lipid with bone lipid, collagen, and apatite $\delta^{13}C$ values for the C3 and C4 animals. All bone lipid $\delta^{13}C$ values are depleted with respect to collagen or apatite values, and this finding is consistent with the relative depletion in ^{13}C that occurs during lipid biosynthesis with respect to other biochemical pathways (32,33). The majority of bone lipid $\delta^{13}C$ values are highly consistent with dietary lipid values. Small, positive diet-to-bone fractionations ($\Delta^{13}C_{b\text{lipid}-d\text{lipid}} = \delta^{13}C_{\text{bone lipid}} - \delta^{13}C_{\text{diet lipid}}$) are observed for 16:0, 18:1, 18:2, and cholesterol, where mean $\Delta^{13}C_{b\text{lipid}-d\text{lipid}}$ fractionations are +0.9, +0.7, +1.5, and –0.5‰, respectively. For 18:0, a larger $\Delta^{13}C_{b\text{lipid}-d\text{lipid}}$ fractionation of +3.6‰ toward whole-diet $\delta^{13}C$ values is observed, which suggests that a greater degree of *de novo* synthesis may have occurred with 18:0 when compared to the other non-EFA or cholesterol. However, a true assessment of

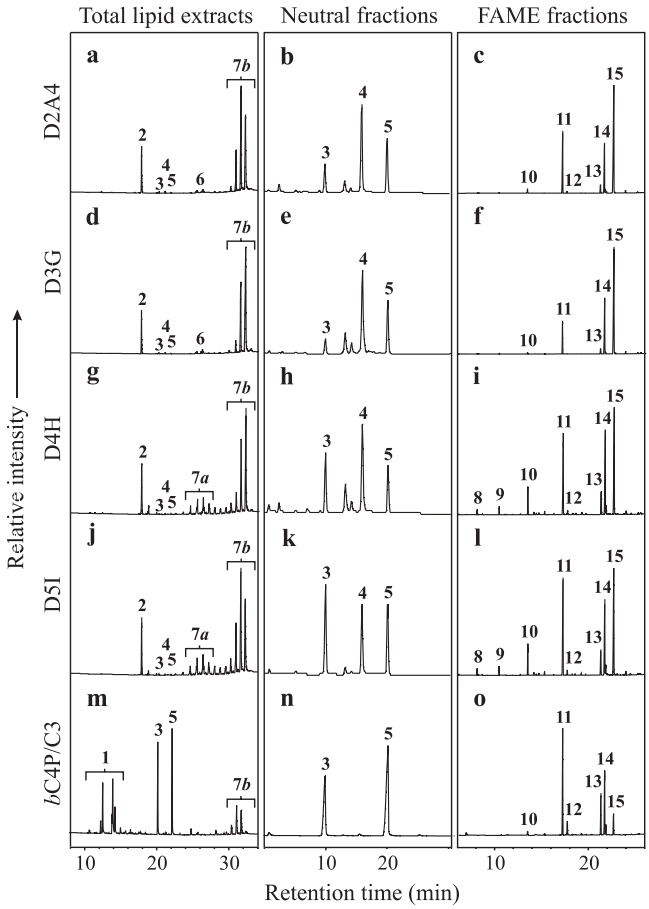

FIG. 1. Partial gas chromatograms of the total lipid extracts (a, d, g, j, and m), saponified trimethylsilylated neutral (b, e, h, k, and n), and FAME fractions (c, f, i, l, and o) of diets D2A4 (a, b, and c), D3G (d, e, and f), D4H (g, h, and i), D5I (j, k, and l) and bone *b*C4P/C3 (m, n, and o). **1** = FFA; **2** = sugar; **3** = cholesterol; **4** = β-sitosterol; **5** = internal standard; **6** = C_{32} and C_{36} DAG; **7*a*** = C_{30} to C_{44} TAG; **7*b*** = C_{46} to C_{54} TAG; **8** = $C_{10:0}$ FAME; **9** = $C_{12:0}$ FAME; **10** = $C_{14:0}$ FAME; **11** = $C_{16:0}$ FAME; **12** = $C_{16:1}$ FAME; **13** = $C_{18:0}$ FAME; **14** = $C_{18:1}$ FAME; and **15** = $C_{18:2}$ FAME.

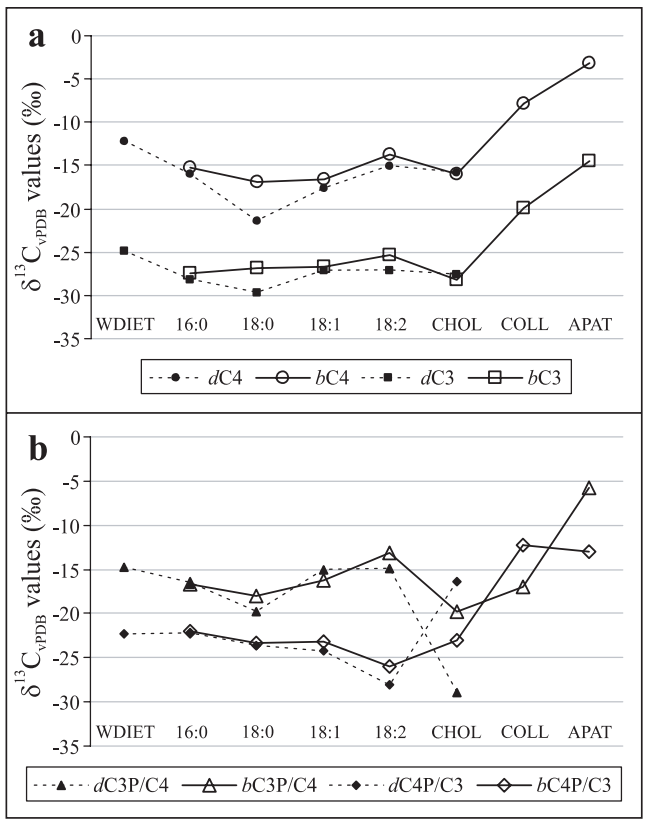

FIG. 2. Whole diet, diet and bone lipid, collagen, and apatite $\delta^{13}C$ values for: (a) C3 and C4 animals, and (b) C3P/C4 and C4P/C3 animals. Each bone data point represents the mean value for the analyses of two animals. WDIET = whole diet; CHOL = cholesterol; COLL = collagen; APAT = apatite; *d* = diet and *b* = bone.

the extent of routing or *de novo* synthesis can only be gleaned when the $\delta^{13}C$ values from the C3P/C4 and C4P/C3 animals are also taken into consideration. The small but significant $\Delta^{13}C_{b\text{lipid}-d\text{lipid}}$ fractionation of +1.5‰ shown for the EFA 18:2 must be caused by isotopic fractionation occurring during assimilation, transport, or catabolism.

Figure 2b presents the $\delta^{13}C$ values for the C3P/C4 and C4P/C3 animals. Here, bone lipid $\delta^{13}C$ values are depleted with respect to collagen or apatite values for the C4P/C3 animals only; for the C3P/C4 animals, bone collagen displayed a comparable $\delta^{13}C$ value to the bone FA. The reasons for this will become apparent when we consider the diet-to-bone lipid isotopic relationships in more detail. For both diets and in accordance with the C3 and C4 animals, small although not necessarily positive $\Delta^{13}C_{b\text{lipid}-d\text{lipid}}$ fractionations are seen for 16:0, 18:1, 18:2 where absolute mean values are 0.1, 1.2, and 1.9‰, respectively. In contrast to the C3 and C4 animals, a small absolute mean $\Delta^{13}C_{b\text{lipid}-d\text{lipid}}$ fractionation of 1.1‰ is observed for 18:0, and a large absolute mean $\Delta^{13}C_{b\text{lipid}-d\text{lipid}}$ fractionation of 7.9‰ is observed for cholesterol. When these findings are considered alongside those shown for the C3 and C4 animals, a clearer assessment of the relative importance of routing and *de novo* synthesis for each of the bone lipids can be gained. Comparable $\Delta^{13}C_{b\text{lipid}-d\text{lipid}}$ fractionations are shown for 18:2 in all four diets, demonstrating that bone 18:2 $\delta^{13}C$ values are independent of macronutrient isotopic differences. This finding is expected for linoleic acid, which must be derived solely from the diet. Comparable $\Delta^{13}C_{b\text{lipid}-d\text{lipid}}$ fractionations are also shown for 16:0 and 18:1, suggesting that the majority of these nonessential bone lipids are also directly incorporated from the diet. Conversely, $\Delta^{13}C_{b\text{lipid}-d\text{lipid}}$ fractionations for 18:0 and cholesterol have been shown to be influenced by macronutrient isotopic differences, suggesting that a major proportion of these lipids are derived from *de novo* synthesis. For all four diets, absolute mean $\Delta^{13}C_{b\text{lipid}-d\text{lipid}}$ fractionations for 16:0, 18:0, 18:1, 18:2, and cholesterol are 0.5, 2.3, 0.9, 1.7, and 4.2‰, respectively. If the magnitudes of these spacings are considered to be a measure of the extent of routing of the non-EFA and cholesterol, then this can be estimated to be of the order 16:0 > 18:1 > 18:0 > cholesterol. The $\Delta^{13}C_{b\text{lipid}-d\text{lipid}}$ fractionation observed for linoleic acid is significant, demonstrating that bone 18:0 $\delta^{13}C$ values are consistently more enriched by 1.7‰ with respect to dietary values. This fractionation obviously cannot be taken as a measure of the extent of routing and must arise due to isotopic fractionation occurring during metabolic processes other than *de novo* synthesis. Linoleic acid is the most abundant FA in these diets but is one of the minor FA observed in bone (Figs. 1c, 1f, 1i, 1l, 1o). If we postulate that the diet provides more than enough linoleic acid for these animals, then the enrichment observed in bone linoleic $\delta^{13}C$ acid values is consistent with a kinetic isotope effect occurring during its catabolism or conversion to other metabolites, e.g., prostaglandins and eicosanoids. Certainly, the formation of prostaglandins and eicosanoids from linoleic acid involves enzyme-catalyzed chain elongation and desaturation reactions (21,22) where isotopic fractionation may be introduced.

Correlations between diet and bone lipid $\delta^{13}C$ values. The relationships between diet (whole diet, protein, energy, FA, and cholesterol) and bone (collagen, apatite, FA, and cholesterol) $\delta^{13}C$ values were investigated using linear regression. Table 3 summarizes the R^2 values observed between each diet and bone component, and the most significant correlations are highlighted in bold. Collagen and apatite $\delta^{13}C$ values were shown to correlate best with dietary protein ($R^2 = 0.77$, $P \leq 0.01$) and whole diet ($R^2 = 0.99$, $P = 0.001$) values, respectively [as was also demonstrated for the data presented in Ambrose and Norr (10)], and these findings are consistent with those from the Tieszen and Fagre (11) study. The most significant correlations observed for the bone lipids are consistent with the interpretations of the $\Delta^{13}C_{b\text{lipid}-d\text{lipid}}$ fractionations above and are shown in Figure 3. As expected, bone linoleic $\delta^{13}C$ values correlated extremely well with dietary values ($R^2 > 0.99$, $P \leq 0.001$), providing further evidence for its direct incorporation from the diet. It was postulated that the majority of bone 16:0 and 18:1 FA were also routed directly from the diet, and indeed, the highest R^2 values of 0.97 ($P \leq 0.001$) and 0.95 ($P = 0.001$), respectively, were shown with respect to their corresponding dietary FA. Conversely, the majority of bone 18:0 FA and cholesterol was postulated to result from *de novo* synthesis, and this hypothesis is corroborated by a higher degree of correlation with whole diet $\delta^{13}C$ values rather than with their corresponding dietary FA values. Certainly, the low R^2 of 0.21 ($P > 0.05$) observed between diet and bone cholesterol indicates that the direct incorporation of cholesterol from the diet is not the dominant pathway.

Correlations between $\Delta^{13}C_{b\text{lipid}-w\text{diet}}$ fractionations and $\Delta^{13}C_{d\text{lipid}-w\text{diet}}$ spacings. Greater insight into the extent of routing of dietary lipids into bone lipids can be gleaned from plotting $\Delta^{13}C_{b\text{lipid}-w\text{diet}}$ fractionations against their corresponding $\Delta^{13}C_{d\text{lipid}-w\text{diet}}$ spacings ($= \delta^{13}C_{\text{diet lipid}} - \delta^{13}C_{\text{whole diet}}$). We focus here on the R^2 values and gradients (m) of the regression equations in Figure 4 to interpret diet-to-bone lipid relationships. R^2 values ranged from 0.67 to 0.96 ($P \leq 0.05$), showing that these relationships correlated well/very well with each other. Significant differences in the gradients of these regression lines are observed, demonstrating the varying degrees to

TABLE 3
R^2 Values from Linear Correlations of Diet and Bone $\delta^{13}C$ Values[a]

	R^2 values			
	Diet component			
Bone component	Whole diet	Protein	Energy	Lipid[b]
16:0	0.92	0.07	0.80	**0.97**
18:0	**0.95**	0.04	0.87	0.84
18:1	0.93	0.01	0.90	**0.95**
18:2	0.91	0.02	0.99	**0.996**
Cholesterol	**0.91**	0.18	0.73	0.21
Collagen	0.38	**0.77**	0.15	NA
Apatite	**0.99**	0.02	0.94	NA

[a]Boldfaced values indicate the most significant correlations.
[b]Lipid refers to corresponding lipid in the diet. NA, not applicable.

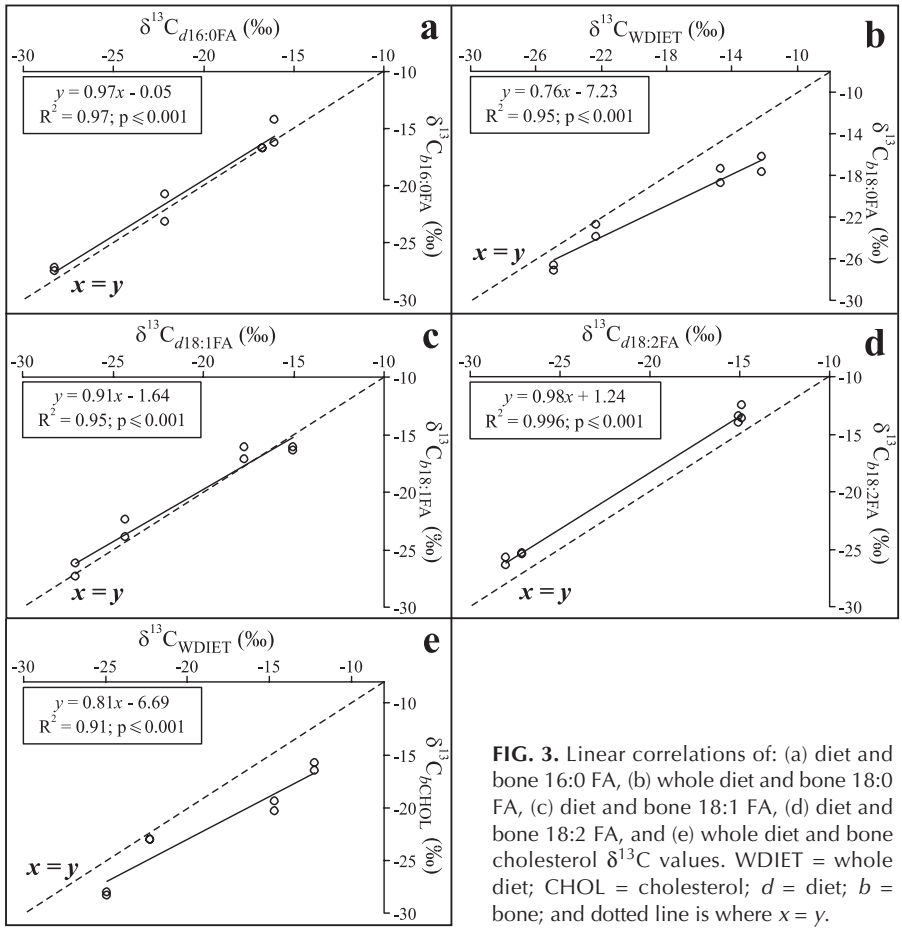

FIG. 3. Linear correlations of: (a) diet and bone 16:0 FA, (b) whole diet and bone 18:0 FA, (c) diet and bone 18:1 FA, (d) diet and bone 18:2 FA, and (e) whole diet and bone cholesterol $\delta^{13}C$ values. WDIET = whole diet; CHOL = cholesterol; *d* = diet; *b* = bone; and dotted line is where $x = y$.

which $\Delta^{13}C_{b\text{lipid}-w\text{diet}}$ fractionations depend on the difference between dietary lipid and whole-diet $\delta^{13}C$ values. The largest gradient is observed for linoleic acid where $m = 0.97$, and this can be interpreted as indicating that 97% of the carbon atoms in bone 18:2 are derived from the diet, reflecting the direct incorporation of this EFA from the diet. Thus, from Figure 4, the degree of routing of diet-to-bone lipids can be estimated to be of the order 18:2 (97%) > 16:0 (80%) > 18:1 (64%) > 18:0 (48%) > cholesterol (21%). Again, these estimates are entirely consistent with previous interpretations of routing as discussed above and, moreover, reflect the relative abundances of these lipids in the diet. The estimated percentage of routing for cholesterol corresponds to the predicted 20% discussed above and compares well with an estimate of 16% (34) where a further set of animals raised on marine protein were also considered.

In summary, the $\delta^{13}C$ values of the major lipids found in bone were shown to reflect different dietary signals. $\delta^{13}C$ values of bone cholesterol and stearic (18:0) acid reflected whole-diet $\delta^{13}C$ values, whereas the $\delta^{13}C$ values of bone palmitic (16:0), oleic (18:1), and linoleic (18:2) acids reflected dietary FA $\delta^{13}C$ values. Differences in their dietary signals were attributed to the different extents to which dietary lipids were routed to bone. Linoleic acid is an EFA, and the routing estimate of 97% confirms its direct incorporation from the diet. Significantly, the extent of routing of the non-EFA and cholesterol reflected their relative abundances in the diet. Although tissue lipid compositions have been shown to be greatly influenced by dietary lipids (24–28), this is the first stable isotopic experimental evidence at natural abundance to show that FA are absorbed proportionately from the diet. The potential of using bone FA and cholesterol $\delta^{13}C$ values as indicators of diet has therefore been shown in this study. However, the interpretation of lipid $\delta^{13}C$ values from ecological and archaeological studies, where there are likely to have been temporal changes in diet, requires knowledge of their turnover rates. Bone FA and cholesterol turnover rates have been estimated from the study of rats that were subjected to diet-switch experiments (Jim, S., Evershed, R.P., and Ambrose, S.H., unpublished data).

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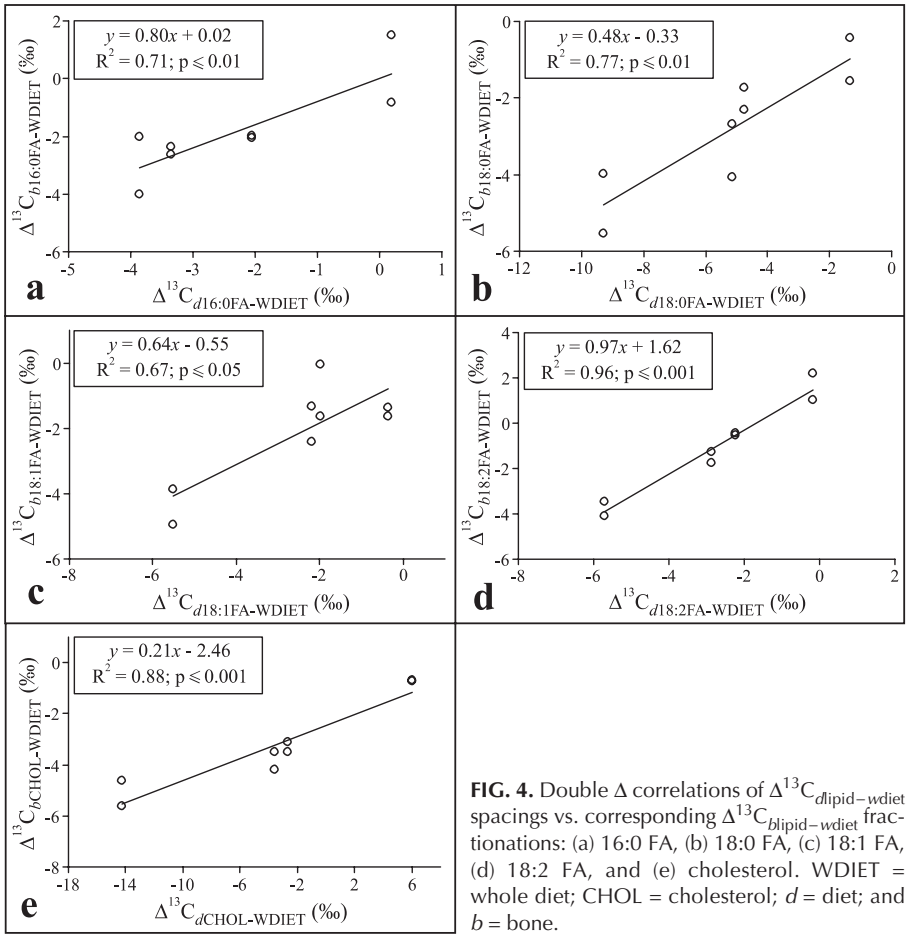

FIG. 4. Double Δ correlations of $\Delta^{13}C_{dlipid-wdiet}$ spacings vs. corresponding $\Delta^{13}C_{blipid-wdiet}$ fractionations: (a) 16:0 FA, (b) 18:0 FA, (c) 18:1 FA, (d) 18:2 FA, and (e) cholesterol. WDIET = whole diet; CHOL = cholesterol; *d* = diet; and *b* = bone.

trolled diet experiment protocols were approved by the Office of Laboratory Animal Care (OLAC), University of Illinois, Urbana-Champaign.

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COMMUNICATION

Whole-Body Utilization of n-3 PUFA in n-6 PUFA-Deficient Rats

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ABSTRACT: We evaluated the utilization of α-linolenic acid (18:3n-3) in growing rats consuming a diet deficient in n-6 PUFA. After 90 d, whole-body 18:3n-3 accumulation was 55% lower, total n-3 PUFA accumulation was 21% lower, and 18:3n-3 disappearance was 14% higher in n-6 PUFA-deficient rats. Part of the reduction of whole-body 18:3n-3 in n-6 PUFA-deficient rats was due to the 25% increase in net conversion of 18:3n-3 to long-chain n-3 PUFA. Despite adequate 18:3n-3 intake, n-6 PUFA deficiency decreased the accumulation of 18:3n-3 and total n-3 PUFA.

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Elevated intake of n-3 PUFA decreases tissue levels of n-6 PUFA, and high linoleic acid (18:2n-6) diets are used to exacerbate n-3 PUFA deficiency (1). α-Linolenic acid (18:3n-3) competes with 18:2n-6 and oleic acid (18:1n-9) for the desaturases, chain-elongases and enzymes of eicosanoid synthesis (2–4). Hence, n-3 and n-6 PUFA are widely and quite reasonably regarded as competitive. However, several studies show that 18:3n-3 can also reduce the requirement for 18:2n-6 (5–7) or act synergistically with 18:2n-6. Most, but not all, of this work had diets with very low to deficient intakes either of n-6 PUFA alone or of n-3 and n-6 PUFA together. For instance, Greenberg *et al.* (6) showed that after 11 wk on a fat-free diet, rats supplemented for an additional 11 wk with 10 mg/d of both 18:3n-3 and 18:2n-6 gained 93 g, whereas rats supplemented with 20 mg/d of 18:2n-6 but no 18:3n-3 gained 86 g. Hence, the presence of 18:3n-3 reduced by 50% the amount of 18:2n-6 required to regain about 90 g during the supplementation period. Other reports using comparable designs drew similar conclusions (5,7).

We recently showed that pigs normally β-oxidize about 90% of the 18:2n-6 they consume at 30–40 d old and that raised intake of 18:3n-3 in pigs reduces β-oxidation of 18:2n-6 by 60% (8). Together these studies (5–8) show a potential sparing effect of 18:3n-3 on the requirements for and metabolism of 18:2n-6. This sparing effect seems to occur at very low or adequate intakes of 18:2n-6. These observations raise questions about the suitability of studying n-6 PUFA requirements or metabolism using diets that are simultaneously deficient in both n-3 and n-6 PUFA, i.e., EFA deficiency (9).

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Abbreviations: 18:0, stearic acid; 18:1n-9, oleic acid; 18:2n-6, linoleic acid; 18:3n-3, α-linolenic acid; 20:3n-9, Mead acid; 20:4n-6, arachidonic acid; 22:5n-3, docosapentaenoic acid; 22:6n-3, docosahexaenoic acid; LCPUFA, long-chain polyunsaturated fatty acid.

We previously reported the effects of n-6 PUFA deficiency on growth and utilization of n-6 PUFA in rats provided with an adequate intake of fat, 18:1n-9, and 18:3n-3 (10). Our objective here is to report that diets adequate in 18:3n-3 but extremely deficient in n-6 PUFA significantly alter the whole-body utilization of n-3 PUFA, i.e., despite adequate n-3 PUFA intake, n-6 PUFA deficiency increases the oxidation and reduces whole-body storage of 18:3n-3 itself and n-3 PUFA as a whole.

MATERIALS AND METHODS

Animals and diets. Twenty-one-day-old male Sprague-Dawley rats were housed individually in wire-bottomed, stainless-steel cages as described previously (10). For the first 7 d, they were fed *ad libitum* a semipurified control diet containing 18:2n-6 at 2 energy%. Two energy% 18:2n-6 was chosen as the control level based on published 18:2n-6 nutrient requirements of the rat (11). After the acclimatization period, 6 rats were sacrificed to serve as the baseline reference group for the whole-body FA analysis. The remaining 12 rats were divided into two groups, with 6 rats continuing to consume the control diet (2 energy% 18:2n-6) and 6 rats switched to an n-6 PUFA-deficient diet (0.01 energy% from 18:2n-6) for a 90-d study period. The diets contained 200 g casein (the lipid content of the casein was 12 mg/g) supplemented with 400 mg DL-methionine, 555 g sucrose, 100 g cellulose, and 10 g AIN-76 vitamin mix, the latter supplemented with 10 mg choline and 35 g AIN-76 mineral mix. The casein contained 12 mg/g of fat. Both diets had the same micronutrient and macronutrient composition and differed only in the amount of 18:2n-6 and stearic acid (18:0) present (Table 1). Body weight and food intake were determined three times each week, and fecal collections were done near the beginning and end of the study period to determine n-3 PUFA balance by the whole-body FA balance method (10,12). After the 90-d test period, the remaining rats were killed for whole-body FA analysis. FA disappearance was calculated over a 90-d balance period as previously described (10,12) according to the following equation:

$$\text{Disappearance}_{18:3n-3} = \text{Intake}_{18:3n-3} - (\text{Accumulation}_{\sum n\text{-}3\text{PUFA}} - \text{Excretion}_{\sum n\text{-}3\text{PUFA}}) \quad [1]$$

This applies when the intake of n-3 long-chain PUFA (LC-PUFA; ≥20 carbons) is negligible.

Experimental and analytical methods. FA analysis was done on total lipid extracts prepared from the intact whole body of rats consuming the control and n-6 PUFA-deficient diets. Rat

TABLE 1
Fat Composition and FA Profile of the Control and n-6 PUFA-Deficient Diets

	Control	n-6 PUFA-deficient
Fat composition (g/kg diet)		
Hydrogenated soybean oil	71	84
Pure oleic acid	15	15
Pure 18:3n-3	1	1
Safflower oil	13	0
FA profile (% of total FA)[a]		
Palmitic acid	10.3	10.8
Stearic acid	62.1	72.4
Oleic acid	16.1	15.1
18:2n-6	10.0	0.05
∑n-6 PUFA[b]	10.0	0.05
18:3n-3	1.5	1.6
∑n-3 PUFA[b]	1.5	1.6

[a]FA analysis was performed on the complete diet. 18:3n-3, α-linolenic acid.
[b]FA ≥20 carbons were not detected (≤0.01%). 18:2n-6, linoleic acid.

carcasses were first diced and homogenized in a saline solution (0.9% NaCl in distilled water) after which the whole-body homogenate was extracted into chloroform/methanol (2:1, vol/vol) containing 0.01% BHT following the method of Folch *et al.* (13). An aliquot of the total lipid extract was then saponified twice with methanolic potassium hydroxide for 4 h at 100°C. Total lipid extracts were acidified with HCl, and FA were extracted in triplicate with 5 mL of diethyl ether. The total lipids were then transmethylated using 14% boron trifluoride in methanol (Sigma Chemical Co., St. Louis, MO) for 40 min at 90°C. FAME profiles were acquired by GC using a 30 m × 0.23 mm capillary column (Durabond 23; J&W Scientific, Folsom, CA) and a three-stage temperature program from 2 min at 50°C, to 180°C at 10°C/min, to 220°C at 5°C/min for 5 min. Measurements of fecal total lipids were obtained during the last 2 d of the study as described previously (10).

Statistical analysis. Data are expressed as the mean ± SD of six rats per group. Statistical comparisons were done using the Student's *t*-test where appropriate.

RESULTS

Body weight and feed intake. As reported previously (10), there was no significant difference in food intake over the 90-d balance period (2413 ± 97 g for the control group vs. 2445 ± 234 g for the n-6 PUFA-deficient rats, $P > 0.05$). Body weights did not differ until day 90 of the balance period, where rats consuming the n-6 PUFA-deficient diet weighed 15% less than controls (475 ± 30 g for the control vs. 405 ± 45 g for the n-6 PUFA-deficient rats, $P < 0.05$).

Whole-body FA concentration. Whole-body lipids of the n-6 PUFA-deficient rats had no change in saturated FA, 57% higher 18:1n-9, 300% higher Mead acid (20:3n-9), and 66% higher DHA (22:6n-3) (mg/g; Table 2). Rats consuming the n-6 PUFA-deficient diet had 33% lower 18:3n-3, 50% lower docosapentaenoic acid (22:5n-3), 96% lower 18:2n-6, and 77% lower arachidonic acid (20:4n-6) whole-body lipids.

TABLE 2
Whole-Body FA Concentration in Rats Consuming a Control or n-6 PUFA-Deficient Diet[a]

	Control	n-6 PUFA-deficient
∑SFA	46.7 ± 19.5[b]	49.7 ± 16.5
18:1n-9	53.0 ± 3.0	83.3 ± 9.3**
20:3n-9	0.3 ± 0.1	0.9 ± 0.02**
∑MUFA[c]	53.3 ± 5.0	84.2 ± 8.3**
18:3n-3	0.9 ± 0.2	0.6 ± 0.1**
20:5n-3	<0.01	<0.01
22:5n-3	0.1 ± 0.04	0.04 ± 0.03*
22:6n-3	0.6 ± 0.1	1.0 ± 0.2**
∑n-3 PUFA[c]	1.6 ± 0.3	1.5 ± 0.2
18:2n-6	14.8 ± 3.4	0.6 ± 0.1**
20:4n-6	1.8 ± 0.2	0.4 ± 0.04**
∑n-6 PUFA	17.0 ± 7.6	1.0 ± 0.04**

[a]Whole-body FA content was determined at the end of a 90-d balance period. SFA, saturated FA; 18:1n-9, oleic acid; 20:3n-9, Mead acid; MUFA, monounsaturated FA; 20:5n-3, EPA; 22:5n-3, docosapentaenoic acid; 22:6n-3, DHA; 18:2n-6, linoleic acid; 20:4n-6, arachidonic acid. For other abbreviation see Table 1. *$P < 0.05$ vs. **$P < 0.01$ vs. control diet.
[b]mg/g of whole body, mean ± SD; $n = 6$ rats per group.
[c]Includes more FA than shown.

Whole body n-3 PUFA balance. Rats consuming the n-6 PUFA-deficient diet had 7% higher 18:3n-3 intake (Table 3). Whole-body accumulation of 18:3n-3 (difference in whole-body content over the balance period) was 55% lower in the

TABLE 3
Whole-Body 18:3n-3 Balance in Rats Consuming a Control or n-6 PUFA-Deficient Diet[a]

	Control	n-6 PUFA-deficient
18:3n-3		
Intake (mg)	3619 ± 13[b]	3904 ± 160**
Excretion (mg)[c]		
18:3n-3	43	47
n-3 LCPUFA	36	39
18:3n-3 whole-body content (mg)		
29 d old[d]	50 ± 4	50 ± 4
119 d old	449 ± 117	226 ± 52**
18:3n-3 accumulation (mg)	399 ± 103	176 ± 24**
n-3 LCPUFA		
Intake	0	0
Whole-body content (mg)		
29 d old[d]	70 ± 2	70 ± 2
119 d old	330 ± 31	416 ± 44**
n-3 LCPUFA accumulation (mg)	260 ± 30	346 ± 39**
18:3n-3 conversion to n-3 LCPUFA (%)	7.2 ± 1.0	9.0 ± 1.0**
Total n-3 PUFA accumulation (mg)	659 ± 99	522 ± 48**
18:3n-3 disappearance (mg)	2880 ± 277	3296 ± 356*

[a]90-d balance period. LCPUFA, long-chain polyunsaturated FA. For other abbreviation see Table 1. *$P < 0.05$, **$P < 0.01$ vs. control diet.
[b]Mean ± SD, $n = 6$ rats per group.
[c]Calculated from Reference 12.
[d]Rats consumed the control diet for 7 d prior to serving as the baseline reference group.

n-6 PUFA-deficient group. The total n-3 PUFA accumulation was 21% lower in rats consuming the n-6 PUFA-deficient diet. Net conversion of 18:3n-3 to LCPUFA was 25% higher in the n-6 PUFA-deficient group compared to the control group. Actual disappearance (mg) of 18:3n-3 was 14% higher in rats consuming the n-6 PUFA-deficient diet.

DISCUSSION

In the present study, despite 1.6% of dietary FA as 18:3n-3, rats consuming the n-6 PUFA-deficient diet accumulated significantly less 18:3n-3. This is a novel observation indicating that deficiency of n-6 PUFA affects homeostasis of n-3 PUFA. The reduction in whole-body 18:3n-3 in the n-6 PUFA-deficient rats in the present study is partially explained by the increased disappearance of 18:3n-3. The diets had no detectable n-3 LCPUFA, yet whole-body 22:6n-3 was increased in the n-6 PUFA-deficient rats. Thus, some of the lower whole-body content of 18:3n-3 was explained by the 25% increase in conversion of 18:3n-3 to LCPUFA (Table 3). The increase in 22:6n-3 is consistent with reports that a low n-6 PUFA intake or a decrease in the n-6/n-3 PUFA ratio increases the desaturation/chain elongation of 18:3n-3 to 22:6n-3 (2,3). Therefore, it appears that 18:3n-3 metabolism is increased, possibly to contribute to energy demands but also to maintain the overall LCPUFA content of the growing n-6 PUFA-deficient rat. Nevertheless, increased net synthesis of n-3 LCPUFA accounted for only 38% of the lower 18:3n-3; the rest was due to increased disappearance.

Since 20:4n-6 was 82% lower in the n-6 PUFA-deficient rats, the higher whole-body concentrations 22:6n-3 and 20:3n-9 may be important for maintaining the LCPUFA content of membranes. As expected, the whole-body content of all n-6 PUFA was decreased in rats consuming the n-6 PUFA-deficient diet. In our previous report (10), rats consuming an n-6 PUFA-deficient diet for 90 d had a 29-fold increase in whole-body 18:2n-6 disappearance compared to the 14% increase in 18:3n-3 disappearance observed in this study. Because whole-body FA balance methodology cannot be used to study the disappearance of FA synthesized *de novo*, it is not known whether the disappearance of other FA was also increased in response to n-6 PUFA deficiency.

We conclude that, despite an adequate 18:3n-3 PUFA intake, increased disappearance and conversion of 18:3n-3 to LCPUFA contribute to lower the accumulation of 18:3n-3 and total n-3 PUFA during 90 d of n-6 PUFA deficiency.

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Cloning of an Alkaline Lipase Gene from *Penicillium cyclopium* and Its Expression in *Escherichia coli*

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ABSTRACT: The gene encoding an alkaline lipase of *Penicillium cyclopium* PG37 was cloned with four steps of PCR amplification based on different principles. The cloned gene was 1,480 nucleotides in length, consisted of 94 bp of promoter region, and had 6 exons and 5 short introns ranging from 50 to 70 nucleotides. The open reading frame encoded a protein of 285 amino acid residues consisting of a 27-AA signal peptide and a 258-AA mature peptide, with a conserved motif of Gly-X-Ser-X-Gly shared by all types of alkaline lipases. However, this protein had a low homology with lipases of *P. camembertii* (22.9%), *Humicola lanuginosa* (25.6%), and *Rhizomucor miehei* (22.3%) at the amino acid level. The mature peptide-encoding cDNA was cloned and expressed in *Escherichia coli* on pET-30a for confirmation. A distinct band with a M.W. of 33 kDa was detected on SDS-PAGE. Results of a Western blot analysis and an enzyme activity assay verified the recombinant 33-kDa protein as an alkaline lipase. Its catalytic properties were not changed when compared with its natural counterpart.

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Lipases (EC 3.1.1.3) catalyze the hydrolysis of TAG. They can catalyze the hydrolysis reaction at the interface between water and oil, as well as the synthesis of lipids in organic solvents (1–3). Recently, lipases have been widely used in detergents (4), cosmetics (5), drugs (6), and the food industry (7). Lipases are produced by almost every living organism. During the past decades, lipases have been purified from mammalian, bacterial, fungal, and plant sources (8–11), and the characteristics of the enzymes such as M.W., amino acid composition, metal ion-binding capacity, and substrate specificity have also been studied in detail. Despite wide differences in their M.W., amino acid sequences, and substrates, most of the lipases share the same core topology of a 3-D structure, known as the α/β hydrolase fold (12,13). The primary structure shared by all lipases studied thus far is the conserved motif Gly-X-Ser-X-Gly, which is essential for enzymatic activity. Serine residue is considered to be the catalytic residue, and mutation at this position will lead to the loss of enzymatic activity (14). The two flanking glycine residues are essential for topology. For most of the lipases, the active center also requires the participation of a histidine and an acidic amino acid (Asp or Glu). This catalytic triad sits in a hydrophobic cleft of the enzyme, where the lipid fatty acyl chain inserts and gets cleaved.

Although lipases have been widely used, their further commercialization is hindered by low stability, low activity, or low specificity of the enzyme. Therefore, more attention is being focused on research into modifying their structure and improving their catalytic properties by chemical or physical means and, most recently, through genetic engineering. Recombinant DNA and genetic engineering technology not only provide the most powerful tools for deciphering the relationship between the structure and function of lipases but also make it possible for their rational modification. Boston *et al.* (15) used error-prone PCR to introduce mutation near one of the catalytic residues (His206) of the *Pseudomonas mendocina* lipase. After screening, one of the mutant enzymes was 2.5 times more active than the wild-type enzyme. Patkar *et al.* (16) gained a mutated B lipase of *Candida antarctica* that was fivefold more stable at 60°C by changing the original pentapeptide Thr-X-Ser-X-Gly into the conservative Gly-X-Ser-X-Gly. Much more work has been done in this field, either to increase the pH stability of lipases (17) or to reduce their protease sensitivity (18). In 1989, Huge-Jensen *et al.* (19) successfully increased lipase production in *Aspergillus oryzae* by introducing a gene encoding an alkaline lipase from *Humicola lanuginosa*.

In previous studies, we successfully isolated a strain of *Penicillium cyclopium* PG37 that is able to secrete a high activity of alkaline lipase (20). The lipase was purified to homogeneity and its enzymatic characteristics were determined. Ibrik *et al.* (21) and Chahinian *et al.* (22) purified a similar lipase from another *P. cyclopium* strain. In this work, we report the cloning of the alkaline lipase gene from *P. cyclopium* PG37 based on four steps of PCR amplification and its expression in *Escherichia coli*. Some of its catalytic properties were compared with the natural

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Abbreviations: Primer AP, GGATCCCTTCACTCTCAAGTGC; primer CE3, GCGCGGCCGCTCAGCTCAGATAGCCAC; primer CE5, GGAATTCGCAACTGCTGACGCCG; primer G3, AACTGCAGTCAGCTCAGATAGCCAC; primer G5, ATGTTGTTCAACTACCAATC; primer N1, GA(C/T)GC(C/T)GC(C/T)GC(C/T)TTCCC; primer N2, CC(C/T)GA(C/T)CT(G/C/T)CA(C/T)CG(C/T)GC(A/G/C/T)GC; primer OT, oligo dT-M13 primer M4; primer PR1, GAAGGCTGCTGGACCGTTGT; primer PR2, CCGTTGTCTTTGGCTGC; primer RM1, CTCTCATGATCTTCACATCAG; primer S5, AAGCAGTGGTAACAACGCAGCAGTACGCGGG; PVA, polyvinyl alcohol; RACE, rapid amplification of cDNA end.

lipase. Our results provide a basis for further study of lipase structure and function, as well as improvement of enzymatic activity, thermal stability, and other properties.

MATERIALS AND METHODS

Bacterial strains and plasmids. Penicillium cyclopium PG37 was isolated from soil as reported previously (20). *Escherichia coli* strains XL_1-Blue and BL21 were used as host cells for gene cloning and expression, respectively. T-vector for cloning of PCR products was purchased from Promega (Madison, WI). Expression vector pET-30a was obtained from Novagen (Madison, WI).

Reagents. Enzymes used for DNA cloning were purchased from Bio-Labs (Beverly, MA), Gibco/BRL (Grand Island, NY), and Roche (Stockholm, Sweden). TRIZOL reagent and culture media were obtained from Gibco/BRL. IPTG, X-gal, and M-MLV reverse transcriptases were products of Promega. Diethylpyrocarbonate and MOPS were purchased from Amresco (Solon, OH). Nitrocellulose membrane was obtained from Schleicher & Schuell (Dassel, Germany). The SMART™ PCR cDNA library construction kit was purchased from ClonTech (Palo Alto, CA). The Takara RNA PCR Kit (AMV version 2.1) was purchased from Takara Biotechnology (Dalian, China). The BCA-200 Protein Assay Kit was purchased from Pierce (Rockford, IL). All other chemicals were of analytical grade. The Hi-Trap affinity column for Ni^{2+}-chelating chromatography was from Amersham Pharmacia Biotech (Uppsala, Sweden).

Total RNA extraction. Penicillium cyclopium PG37 was cultured in liquid media containing 4% soybean meal, 3% corn steep liquor, 1.0% soybean phospholipids, 0.1% $MgSO_4 \cdot 7H_2O$, 1.0% K_2HPO_4, 0.05% sodium citrate, and 0.2% soybean oil for 24 to 30 h. The mycelia were collected by centrifugation at 4000 × *g* for 10 min. To 100 mg wet weight of pellet was added 1 mL TRIZOL in a 1.5-mL tube; the pellet was homogenized for 3–5 min then allowed to stand at room temperature for 5 min. Then 0.2 mL chloroform was added to the tube and the tube was subjected to vigorous vortexing for 15 s. The mixture was then centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatant was collected and 0.5 mL of isopropyl alcohol was added. The mixture was kept at room temperature for 10 min. RNA was recovered by centrifugation at 12,000 × *g* for 10 min at 4°C and washed twice with 75% ethanol. The RNA was kept at –80°C in 70% ethanol for long-term storage. For the reverse transcription reaction, the RNA pellet was air dried and dissolved in RNase-free water.

Genomic DNA extraction. Preparation of genomic DNA from *P. cyclopium* PG37 was carried out according to the method reported by Garber and Yoder (23). The mycelia were washed three times with SE buffer (0.15 mol/L NaCl, 0.1 mol/L EDTA; pH 8.0) and then homogenized in 5 vol (wt/vol) of the same buffer. The homogenate was supplemented with SDS and proteinase K to final concentrations of 0.6% and 100 µg/mL, respectively. The mixture was then incubated at 37°C for 2 h with shaking at 100 rpm. SDS was again added to the mixture to reach a final concentration of 2%, and the mixture was incubated for another 20 min at 60°C. The mixture was chilled on ice and mixed with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1), followed by shaking at 150 rpm for 30 min. The supernatant was collected after centrifugation at 10,000 × *g* for 10 min at 4°C and extracted with an equal volume of chloroform. DNA was precipitated by adding 2 vol of ethanol, and the precipitate was dissolved in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) supplemented with 50 µg/mL RNase.

Primers for PCR amplification. Two degenerated primers, N1 [GA(C/T)GC(C/T)GC(C/T)GC(C/T)TTCCC] and N2 [CC(C/T)GA(C/T)CT(G/C/T)CA(C/T)CG(C/T)GC(A/G/C/T)GC], were designed to correspond to the lipase N-terminal amino acid residues DAAAFPD and PDLHRAA, respectively. Primer OT (original name, oligo dT-M_{13} primer M_4) was provided by the Takara RT-PCR Kit (version 2.1). Primers RM1 (CTCTCATGATCTTCACATCAG) and S5 (AAGCAGTGGTAACAACGCAGAGTACGCGGG were provided by ClonTech's SMART™ PCR cDNA library construction kit and were used for 5′ rapid amplification of cDNA end (RACE) of the lipase cDNA. Primers G5 (ATGTTGTTCAACTACCAATC) and G3 (AACTGCAGTCAGCTCAGATAGCCAC) were used for amplification of the full-length cDNA and genomic DNA encoding the alkaline lipase. For cloning of the promoter region, the oligonucleotides CGGCAC and GGATCCCTTCACTCTCAAGTGC were mixed and used as an adaptor to be ligated to the genomic DNA digested with restriction enzyme MspI. Primers AP (the same sequence as the long oligonucleotide in the adapter), PR1 (GAAGGCTGCTGGACCGTTGT), and PR2 (CCGTTGTCTTTGGCTGC) were used for amplification of the promoter region. For expression of lipase cDNA on pET-30a in *E. coli*, forward primer CE5 (GGAATTCGCAACTGCTGACGCCG, with the *Eco*RI site) and reverse primer CE3 (GCGCGGCCGCTCAGCTCAGATAGCCAC, with the *Not*I site) were used for the introduction of restriction sites.

Reverse transcription of cDNA. For cloning of partial cDNA by using degenerated primers, the total RNA was reverse transcribed with AMV reverse transcriptase by using primer OT provided by the Takara RNA PCR Kit (version 2.1). For cloning the 5′-end of the lipase cDNA, reverse transcription was performed with the SMART PCR cDNA library construction kit with AMV reverse transcriptase. The S5 primer was added to the reaction solution after the end of reverse transcription and incubated at 42°C for another 10 min so that the derived cDNA molecules could have the S5 primer sequence at their 3′-end. All the reverse transcription reactions were performed following instructions provided by the manufacturers.

Protein purification and amino acid sequencing. Purification of alkaline lipase from *P. cyclopium* PG37 was reported previously (20). The N-terminal amino acid sequence was analyzed on a 470A automatic sequencer obtained from Applied Biosystems (Foster City, CA). The sequence of 20 N-terminal amino acid residues was determined to be ATADAAAFPDLHRAAKLSSA (20).

Expression and purification of recombinant alkaline lipase in E. coli. Molecular cloning was carried out according to stan-

dard methods (24). The cDNA fragment encoding the mature peptide of the alkaline lipase was amplified with CE5 and reverse primer CE3. The DNA fragment was ligated into pET-30a with restriction sites of *BamH*I and *EcoR*I to generate construct pET-Lip. The expression construct was transformed into *E. coli* BL21 for expression. The transformant was inoculated in LB broth supplemented with 100 μg/mL ampicillin and cultured with shaking at 37°C overnight. Twenty microliters of seed culture was transferred into fresh medium and cultured until O.D. at 600 nm reached 1.0. IPTG (final concentration 0.25 mM) was then added for induction. The bacterial cells were cultured for another 5 h before collection by centrifuge. SDS-PAGE and Western blotting combined with immunostaining were applied for confirmation of the expressed product.

For lipase purification, *E. coli* cells from 100 mL culture were collected by centrifugation and washed once with PBS, then suspended in 10 mL PBS containing 4 mg/mL lysozyme followed by incubation at 37°C for 30 min. The suspension was subjected to ultrasonication treatment, and the cell debris was removed by centrifugation at 450 × *g* for 10 min at 4°C. The supernatant was further centrifuged at 12,000 × *g* for 15 min at 4°C. The inclusion bodies were recovered in the pellet, washed once with PBS containing 2 M urea, and then solublized with 1 mL denaturing buffer (8 M urea in PBS). The protein was refolded by rapid dilution into 20 mL of PBS containing oxidized (0.306 g/L) and reduced (0.307 g/L) glutathione under stirring. The solution was kept at 4°C for 20 h and then centrifuged to remove the precipitates. Finally, the recombinant alkaline lipase was purified by Ni^{2+}-chelating chromatography according to the instructions provided by the manufacturer.

Determination of protein concentration. Protein concentration was determined by using a BCA-200 Protein Assay Kit.

Enzyme activity assay. A qualitative enzyme activity assay for alkaline lipase was carried out according to the method described by Mourey and Kilbertus (25) with minor modifications. Briefly, 2.5 g agar, 6.25 mL tributyrin, 18.75 mL 3% polyvinyl alcohol (PVA), 2.5 mL 1% Victoria blue, and 225 mL 0.05 M glycine-NaOH (pH 10.0) were added to a 500 mL flask and mixed under heating until homogeneity. The mixture was poured into 15-cm plates and allowed to cool to room temperature. Wells of 1 cm in diameter were punched into the plates. Different amounts of sample were introduced into the wells and incubated at 37°C for 24 h. Enzyme activity was estimated by the diameters of darkened plaque.

A quantitative enzyme assay of lipase was carried out at 30°C and pH 10.0 with emulsified olive oil. Briefly, 1 vol of olive oil and 3 vol of 3% PVA were mixed well with a blender to form an emulsified substrate. Five milliliters of this substrate was mixed with 4 mL of 0.05 M glycine-NaOH (pH 10.0) and adjusted to pH 10.0 again. An appropriate amount of lipase diluted in 1 mL of the same buffer was added and incubated at 30°C for 10 min. The reaction was stopped by adding 10 mL of a 1:1 acetone/ethanol mixture. After further agitation for 10 min for total extraction of FA, titration was performed with 25 mM NaOH in a pH-stat until an end point of 10.0 was reached. The effect of temperature on lipase activity was measured as above except that the reaction was carried out at different temperatures ranging from 20 to 40°C. The effect of pH on lipase activity was measured as described above except that the reaction was carried out in 25 mM Tris buffer containing 100 mM NaCl at different pH from 6 to 11. For recombinant lipase, the concentration of the protein was determined after purification and diluted with 0.05 M glycine-NaOH (pH 10.0) to 1 μg/mL for the quantitative enzyme activity assay as described above. One milliliter of 0.05 M glycine-NaOH (pH 10.0) was used as a blank control. The same amount of denatured recombinant lipase was used as a negative control, and the same amount of purified natural lipase was used as a positive control. The volume of the 25 mM NaOH used in the sample subtracted from that used in the blank was proportional to the enzyme activity.

Antisera preparation. Alkaline lipase purified from *P. cyclopium* PG37 was used as an antigen to immunize Balb/C mice as described previously (26). The serum of immunized mice was used as the antibody in the Western blot assay. All studies involving animals were approved by an Institutional Review Board.

Western blot analysis. After SDS-PAGE, the protein was transferred to nitrocellulose membrane on Bio-Rad semidry transfer (Hercules, CA). The antibody prepared as described above was used in this assay. All procedures were carried out as described previously (26).

RESULTS

Cloning of cDNA encoding the alkaline lipase of P. cyclopium. Although we determined the sequence of 20 N-terminal amino acids of an alkaline lipase purified from culture medium of *P. cyclopium* PG37 (20), because of the high degeneracy of the genetic codons of those amino acids, it was difficult to design specific oligonucleotides as probes for DNA hybridization. To clone the cDNA and the gene with limited amino acid sequence information, we developed a procedure using a PCR amplification approach based on different principles.

For the first step, we tried to obtain a partial cDNA sequence of the alkaline lipase starting from the N-terminal amino acid sequence. Two degenerated primers were synthesized corresponding to the different parts of the 20 amino acid residues. Primer N1 corresponded to DAAAFP (residues 4–9), whereas N2 corresponded to PDLHRAA (residues 9–15). The degeneracy of the primers was designed based on statistical data of codon usage of other genes of the *Penicillium* genus. Primer OT was used as the primer for reverse transcription of the first strand of cDNA from total RNA, so that PCR amplification could be carried out using OT as one of the two primers. The first strand of cDNA was used as the template for amplification with primers N1 and OT for 35 thermal cycles of 94 (30 s), 53 (30 s), and 72°C (45 s). A 1-kb DNA fragment and several other bands were amplified as shown in Figure 1. Each band was agarose gel-purified and subjected to a second round of PCR amplification with primers N2 and OT. Only the 1-kb fragment was again ampli-

fied, as shown in Figure 1A. Together with the size of the purified protein, the results strongly implied that the 1-kb fragment was the cDNA of alkaline lipase. The 1-kb fragment amplified with OT and N1 was cloned on T-vector and the nucleotide sequence was subsequently determined. The insert was 971 bp in length and consisted of 945 bp of lipase cDNA and 26 bp of primer OT. The inserted DNA fragment contained the nucleotide sequence of the N2 primer and the cDNA coding for 17 amino acid residues of the N-terminus, as well as a stop codon on the corresponding reading frame of the alkaline lipase. Therefore, the 945-bp fragment was confirmed to be the cDNA encoding the mature peptide of the purified alkaline lipase of *P. cyclopium* PG37 (Figs. 2 and 3).

Next, we moved on to obtain the full-length cDNA of the alkaline lipase by a modified 5′ RACE approach based on the characteristics of M-MLV reverse transcriptase (27). The total RNA was reverse transcribed with a random-hexamer primer. After 30 min of reverse transcription reaction, the S5 primer was added to the reaction solution and incubated at 42°C for another 10 min so that part of the derived cDNA molecules had the S5 primer sequence at their 3′-end. The cDNA was used as the template for PCR amplification with primers S5 and RM1 (which was complementary to the 21-bp nucleotide sequence located 262 bp downstream from the N1 primer on the 1-kb fragment). A 470-bp fragment was obtained as the major PCR product, as shown in Figure 1B. The PCR products were cloned on T-vector, and the nucleotide sequence was subsequently determined. The insert contained a 283-bp nucleotide sequence identical to that between primer N1 and primer RM1 of the 945-bp fragment and a new 163-bp sequence in which an ATG initiator codon and DNA sequence encoding for a signal peptide of 27 amino acids were recognized. By aligning the nucleotide sequence of the two fragments, the full-length cDNA of the alkaline lipase was obtained. The open reading frame coinciding with the N-terminal amino acids was reduced to encode a protein of 285 amino acid residues consisting of a signal peptide of 27 amino acids and a mature peptide of 258 amino acids. This protein had a M.W. (33 kD) close to that of the purified alkaline lipase (28 kD). The amino acid sequences of two other peptides determined by protein chemistry were also recognized in the reading frame.

Cloning of the alkaline lipase gene. To confirm the identity of the full-length cDNA of the alkaline lipase, efforts were made to clone the genomic DNA of the alkaline lipase gene. The alkaline lipase gene was amplified from genomic DNA of *P. cyclopium* PG37 with primers G5 and G3, which were located at the 5′- and 3′-ends of the full-length cDNA sequence. A 1.1-kb DNA fragment was amplified (Fig. 1C). The sequence of the 1.1-kb DNA fragment contained the complete nucleotide sequence of the full-length cDNA with five short intervening introns ranging from 50 to 70 nucleotides, as indicated in Figures 2 and 3.

Cloning of the promoter region. Cloning of the promoter region of the alkaline lipase gene was based on a ligation-mediated PCR amplification approach (Wang, Y., unpublished data). The genomic DNA of *P. cyclopium* PG37 was first digested with restriction enzyme *Msp*I. The derived DNA was ligated with an adapter, which was an equimolar mixture of two oligonucleotides, CGGCAC and GGATCCCTT-CACTCTCAAGTGC. The ligated DNA sample was first amplified with primers AP and PR1, and a 150-bp DNA

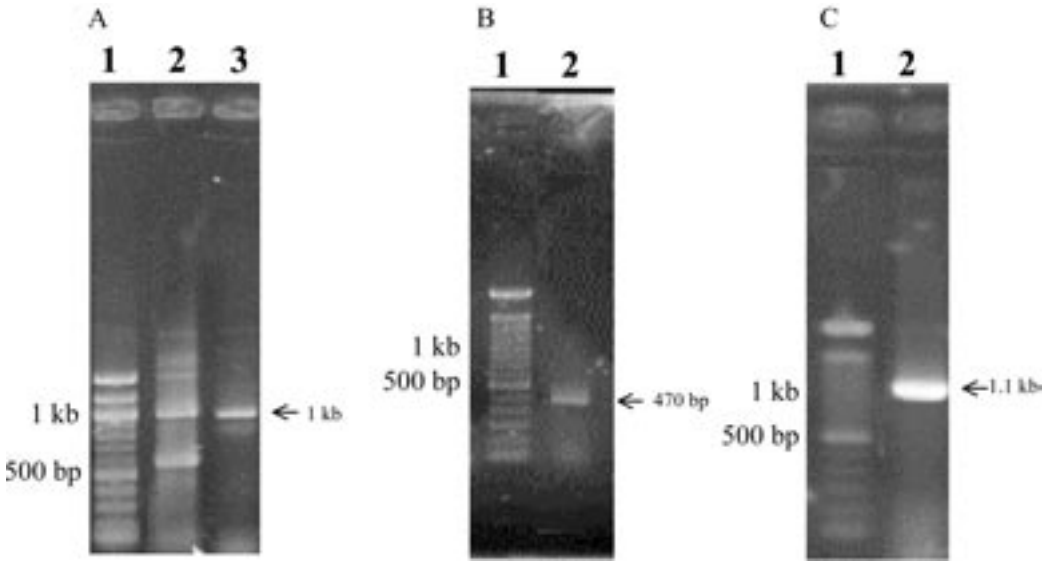

FIG. 1. Cloning of an alkaline lipase gene from *Penicillium cyclopium* PG37. (A) Amplification of a DNA fragment encoding the mature peptide. Lane 1, DNA marker. Lane 2, cDNA transcribed with primer OT was amplified with degenerative primer N1 together with primer OT. Only the 1-kb fragment in lane 2 could be amplified again in a second round of PCR with primer OT and degenerative primer N2, as shown in lane 3. (B) Cloning of the 5′ region of the alkaline lipase mRNA by the 5′ rapid amplification of cDNA end technique. Lane 1, DNA marker. The 470-bp DNA fragment in lane 2 was obtained by PCR amplification with primers S5 and RM1. (C) A 1.1-kb DNA fragment was amplified from the genomic DNA of *P. cyclopium* by primers G5 and G3 located at the 5′- and 3′-ends of the alkaline lipase cDNA. Lane 1, DNA marker. See text for descriptions of primers used.

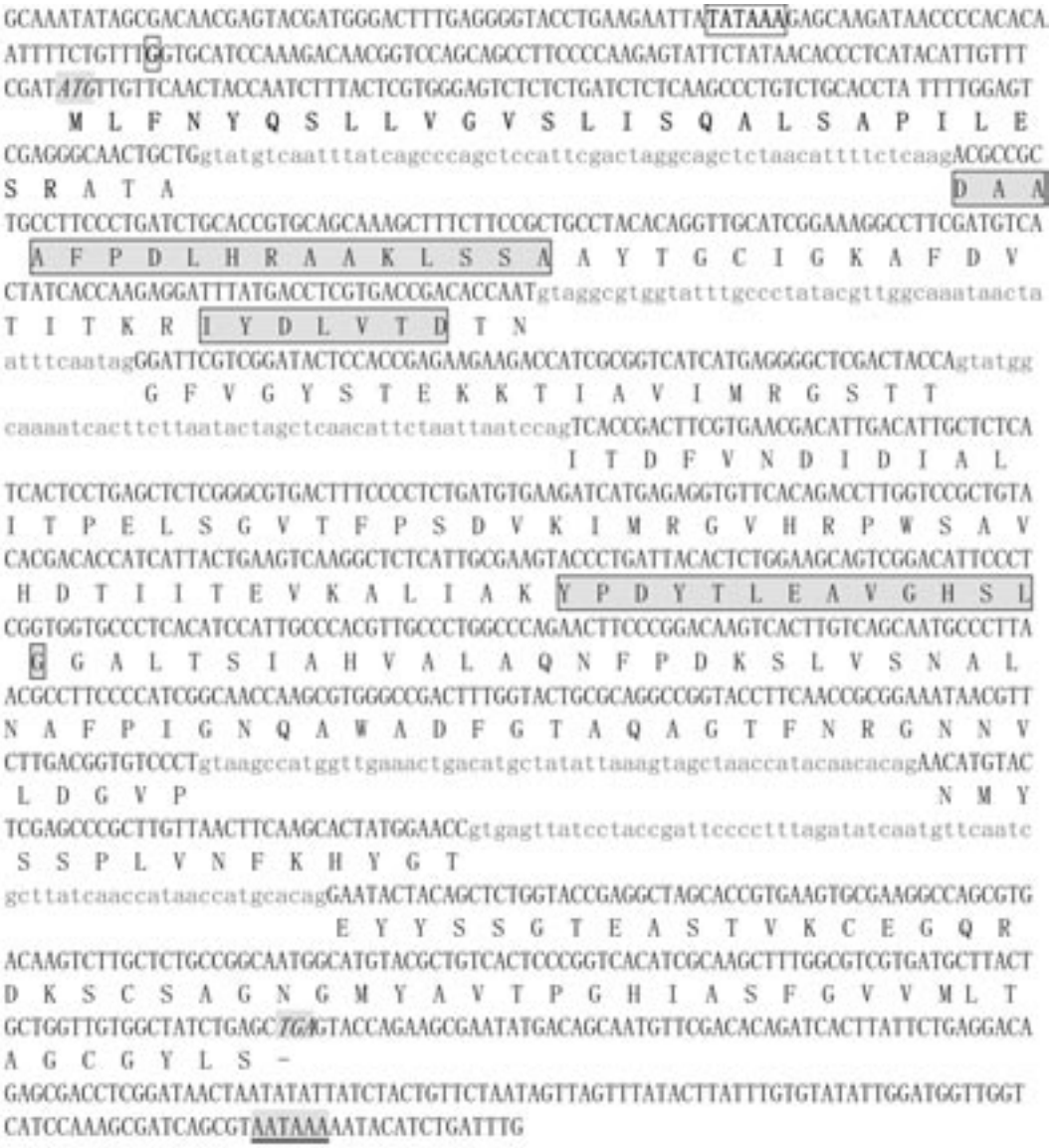

FIG. 2. Nucleotide sequence of the alkaline lipase gene of *P. cyclopium* PG37 and its deduced amino acid sequence. DNA sequences of introns are shown in lowercase letters. The amino acid sequences determined by sequencing are indicated in gray boxes. The signal peptide is in boldface letters. The bold letters in boxes, **TATAAA** and **G,** indicate the putative TATA box and the starting point of transcription, respectively. The start codon and stop codons are shown in grayed italic. The polyadenylation site, AATAAA, is shown as grayed underlined letters.

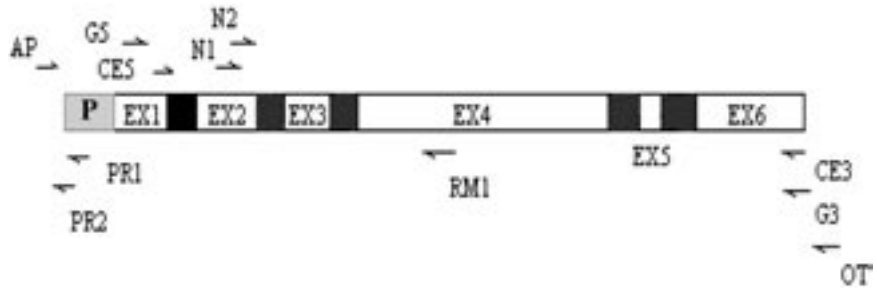

FIG. 3. Schematic drawing of the structure of the alkaline lipase gene of *P. cyclopium* and the positions of primers used in this work. Dark boxes and white boxes (indicated with EX) represent introns and exons, respectively. Arrows indicate the positions of primers. The gray box indicates the promoter region.

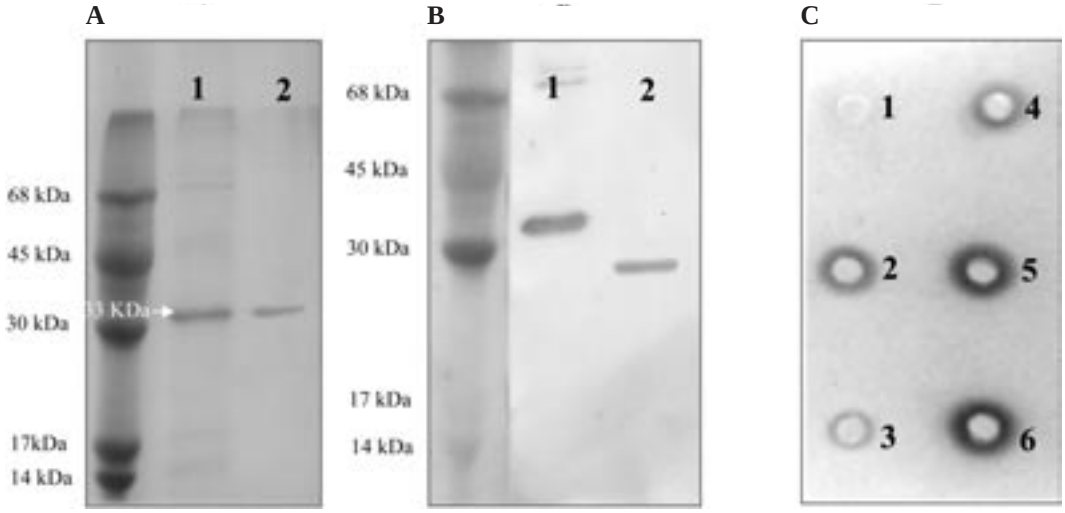

FIG. 4. Expression of the alkaline lipase of *P. cyclopium* PG37 in *Escherichia coli.* (A) The mature peptide of the alkaline lipase was expressed with pET-30a as a fusion protein in BL21 cells. The inclusion body was isolated and applied directly onto SDS-PAGE (1) or purified by Ni^{2+}-chelating chromatography (2). (B) Western blot analysis of the expressed fusion protein (1) and the natural alkaline lipase purified from fermented broth of *P. cyclopium* (2). (C) The enzyme activity assay of the recombinant alkaline lipase by Victoria blue as described in the Materials and Methods section. Well 1, 20 µL of PBS as blank; well 2, 20 µg of natural lipase as the positive control; wells 3 to 6, 30 , 40, 120, and 240 µg of the refolded recombinant lipase were applied.

fragment was obtained as the major PCR product. The fragment was agarose gel-purified and subjected to a second round of PCR amplification with primers AP and PR2 for confirmation. As a result, a 130-bp DNA fragment was amplified—the nucleotide with a putative TATA box. To further confirm the sequence of the promoter region, a primer at the 5′-end of the promoter (GCAAATATAGCGACAACGAGTACG) was synthesized and used for PCR amplification together with primer RM1. A 750-bp fragment was amplified from genomic DNA, the size and nucleotide sequence of which were identical to the assembled data in Figures 2 and 3.

Sequence characterization. The complete gene of the alkaline lipase of *P. cyclopium* PG37 was assembled from the nucleotide sequences obtained in four steps of PCR amplification, as shown in Figures 2 and 3. The gene was 1480 bp in length, and consisted of a 94-bp promoter region and 5 introns. The 5 introns were relatively short, 58, 49, 50, 56, and 69 bp in length, respectively. The putative TATA box was located 32 bp upstream from the transcription start point. The open reading frame encoded a protein of 285 amino acid residues. The predicted mature peptide consisted of 258 amino acids with the theoretical M.W. of 27.3 kDa, which matches the value measured by the mass spectrum (20). The mass spectral data also imply that the lipase protein is not glycosylated. The conserved motif Gly-X-Ser-X-Gly in all lipase proteins studied thus far was also recognized in the predicted sequence. Amino acid homology alignment of the predicted lipase with three other lipases from *P. camembertii*, *H. lanuginosa*, and *Rhizomucor miehei* was carried out. The overall similarity between the four enzymes was around 40%, which was not as high as expected. Alignment with each of the three lipases was also carried out. The similarities between *P. cyclopium* and *P. camembertii*, *H. lanuginosa*, and *R. miehei* were 22.9, 25.6, and 22.3%, respectively.

Expression of lipase in E. coli. Owing to the low homology between the cloned gene and other reported lipase genes, it was necessary to determine whether the recombinant protein of the cloned gene did have the enzymatic activity of the lipase. To this end, primers CE5 and CE3 were applied to amplify the mature peptide-encoding cDNA with adequate restriction enzyme sites so that the cDNA could be inserted into expression vector pET-30a in the right reading frame. Because of the His-tag fusion peptide in expression vector pET-30a, the recombinant protein had 46 extra amino acids. As shown in Figure 4A, a high efficiency of lipase protein expression was achieved with pET-30a. The recombinant protein showed a M.W. of about 33 kDa as indicated by the arrow, which was close to the theoretical value. The 33-kDa protein band was confirmed by Western blotting with antiserum, as shown in Figure 4B. The protein was purified by Ni^{2+}-chelating chromatography, then refolded as described above and subjected to an enzymatic activity assay. As shown in Figure 4C, the refolded protein had about 20% the activity of the native alkaline lipase purified from culture media, as five times the amount of refolded protein was needed to form a plaque with the same size of native lipase. The results demonstrated that the product of the cloned cDNA was an alkaline lipase.

Catalytic properties of the lipases. After purification, the catalytic properties of the lipases (both recombinant and native) were determined as described in the Materials and Methods section. As shown in Table 1, the specific enzyme activity of the purified recombinant lipase was 79.0% that of the natural lipase, but the total enzyme activity per liter medium

TABLE 1
Catalytic Properties of Lipases

Character of lipases	Specific activity after purification (U/mg)	Quantitative activity (U/L medium)	Optimal temperature (°C)	Optimal pH
Native	5,000	450,000	30	10
Recombinant	4,000	2,000,000	30	10

was 4.4 times that of the natural lipase. The effect of temperature on enzyme activity was measured as described in the Materials and Methods section. The purified recombinant lipase showed the highest enzyme activity at 30°C, as did the natural lipase, but it was slightly more sensitive to temperature than its natural counterpart (data not shown). The purified recombinant lipase and the natural lipase had the same type of pH activity dependence curve (data not shown). The optimal pH for the hydrolysis reaction was 10; more than half of their enzyme activity was lost at pH 9.0 and 11. We also measured the substrate specificity of the recombinant lipase, and no difference was seen when compared with the natural lipase (data not shown). All of these results confirm that the cDNA we cloned encodes the alkaline lipase of *P. cyclopium*.

DISCUSSION

The cloning of a novel gene depends largely on the combination of DNA library construction and the screening of candidate genes. Various methods have been developed to simplify the process, such as screening of an expression cDNA library or genomic DNA library with antibodies or by detecting specific enzyme activity of the target protein; however, all the present methods are still time-consuming and laborious.

The 20 amino acid residues of the N-terminus of the alkaline lipase determined previously were not adequate for designing a specific oligonucleotide probe. Therefore, extra effort was necessary to clone this gene. In this work, cloning the cDNA and the gene of the alkaline lipase from *P. cyclopium* PG37 was achieved by using four steps of PCR amplification based on different principles. The first step was to use degenerated primers for amplification of mature peptide-coding cDNA. The second step was to obtain the full-length cDNA by a modified 5′ RACE approach. The third step was to obtain the intron sequences in genomic DNA corresponding to the full-length cDNA. And finally, the promoter region was obtained by a ligation-mediated PCR approach. The first two steps are a modified application of the principles used by ClonTech's SMART PCR cDNA library construction kit. The third step was a conventional PCR amplification. The final step of PCR amplification was originally developed in our lab and is to be published elsewhere (Wang, Y.R., Hong, J., Ming, M., Ding, J.D., Li, Q.G., and Huang, W.D., unpublished data).

Lipases are ubiquitous enzymes that are expressed by nearly all living organisms. There is tremendous diversity at the primary structure level among lipases of different species. It is now known that bacterial lipases with low homologies at the amino acid level share very similar 3-D structures. Therefore, bacterial lipases provide a good model for studying the relationship between the diversity of amino acid sequences and the similarity of 3-D structures, as well as the similar enzymatic functions.

It has been reported that the lipases can be divided into four major families based on their 3-D structures, the *R. miehei* family, the *C. rugosa* family, the *Burkholderia cepacia* family, and the *Staphylococcus* family (28). The first family includes lipases from *R. miehei* and all species of *Rhizopus*, as well as the enzymes from related organisms such as *Thermomyces lanuginosa* and *Penicillium*. From a BLAST search, we found that the 3-D structure of *P. camemberti*, *R. nieveus*, *T. lanuginosa*, and *R. miehei* were most similar to the lipase of *P. cyclopium*. Therefore, we concluded that the purified lipase belonged to the *R. miehei* family. Alignment by Cn3D 3.0 software also showed high homologies between the alkaline lipase of *P. cyclopium* and those of the first family (Fig. 5). The amino acids in capital letters present the conserved secondary structures in these lipases. The catalytic triad consisting of Ser 159, Asn 215, and His 268 of *P. cyclopium* lipase can be predicted based on this alignment according to the catalytic triad of *R. miehei* lipase, which is S144, D203, and H257 (29). The G-X-S-X-G pentapeptides in all five lipases are identical (G-H-S-L-G), which may reflect their close relationship in evolution.

Of the *Penicillum* species, the high similarity among TAG-hydrolyzing lipases from *P. cyclopium*, *P. expansum*, and *P. solitum* (sp. UZLM-4) was reported earlier (21). We have amplified the lipase gene of *P. expansum* using primers based on the DNA sequence of the *P. cyclopium* lipase gene (Wu, M.C., Sun, C.R., Huang, W.D., and Sun, C.R., unpublished data), which support their conclusion. There are no published reports that *P. camembertii* can produce this type of lipase, but *P. cyclopium* and *P. expansum* are able to produce MAG- and DAG-hydrolyzing lipases, as *P. camembertii* does. Chahinian *et al*. (30) purified a partial acylglycerol lipase from *P. cyclopium*, whose sequence of the 20 first amino acid residues was similar to the *P. camembertii* lipase but which differed from *P. cyclopium* lipase I (the TAG-hydrolyzing lipase).

Although the first 20 amino acids of the alkaline lipase we purified from *P. cyclopium* were identical to those reported by Ibrik *et al.* (21), many other properties were different. In our previous work (20), we measured the pH dependence of the natural lipase and found that the enzyme activity decreased sharply at pH 9.0 and 11. Almost all the enzyme activity was lost below pH 8.0, which was much different from that reported by Ibrik *et al.* (21). Considering that the differences in substrate and the assay method we used may have led to the discrepancy, we tried to measure the effect of pH on lipase activity under the conditions Ibrik *et al*. had used. This failed, most probably because of the low concentrations of the substrate. After modifying their methods by increasing the tributyrin concentration as described in the Materials and Methods section, we successfully determined the pH dependence curve, which was the same as we reported earlier (20) when using olive oil as substrate and 0.05 M glycine-NaOH as buffer. The isoelectric point of our lipase was 5.4, whereas

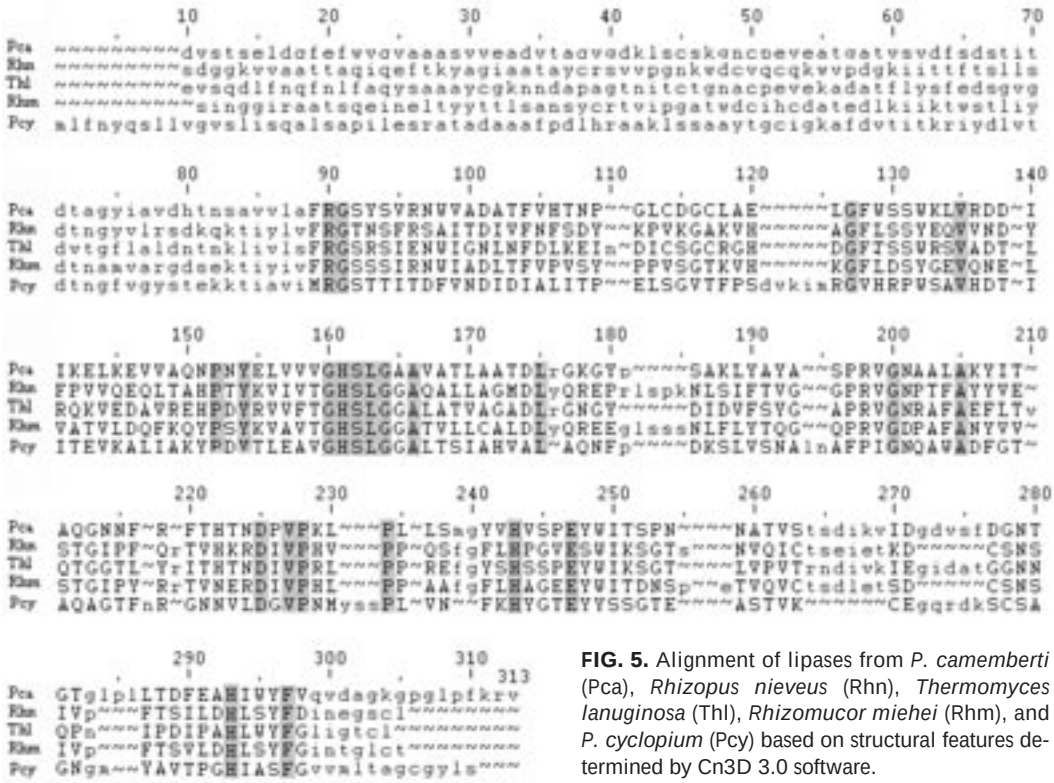

FIG. 5. Alignment of lipases from *P. camemberti* (Pca), *Rhizopus nieveus* (Rhn), *Thermomyces lanuginosa* (Thl), *Rhizomucor miehei* (Rhm), and *P. cyclopium* (Pcy) based on structural features determined by Cn3D 3.0 software.

that reported by Ibrik *et al.* (21) was 5.1. The amino acid composition was also different. It is well documented that at least three extracellular lipases have been produced from *P. cyclopium* (31–33). The amount of each lipase produced depends on the culturing conditions. We detected another fraction containing lipase activity when purifying the alkaline lipase from a *P. cyclopium* culture medium (Wu, M.C., Wang, S., Huang, W.D., and Sun, C.R., unpublished data). It had the same M.W. and substrate specificity but a different isoelectric point of 5.0, which was much more similar to Ibrik's lipase. All these data support the conclusion that they are different enzymes.

One of our goals in cloning this gene was to obtain a thermostable and alkaline-stable lipase by using a protein engineering approach. The elucidation of the primary structure of the alkaline lipase of *P. cyclopium* provided us with a solid basis for protein engineering, considering the rapid accumulation of amino acid sequences of lipases from different sources. We are now studying improvements in their enzyme properties *via* a point-mutation approach.

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Conjugated Methyl Sulfide and Phenyl Sulfide Derivatives of Oxidosqualene as Inhibitors of Oxidosqualene and Squalene-Hopene Cyclases

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ABSTRACT: Various (1*E*,3*E*)- and (1*Z*,3*E*)-conjugated methylthio derivatives of oxidosqualene (OS) and conjugated and nonconjugated phenylthio derivatives of OS were obtained. These compounds, designed as inhibitors of pig liver and *Saccharomyces cerevisiae* 2,3-oxidosqualene-lanosterol cyclases (OSC) (EC 5.4.99.7) and of *Alicyclobacillus acidocaldarius* squalene-hopene cyclase (SHC) (EC 5.4.99.-), contain the reactive function adjacent to carbons involved in the formation of the third and the fourth cycle during OS cyclization. All the new compounds are inhibitors of OSC and SHC, with various degrees of selectivity. The conjugated methylthio derivatives behaved as potent inhibitors of *S. cerevisiae* OSC, whereas most of the phenylthio derivatives were especially active toward SHC.

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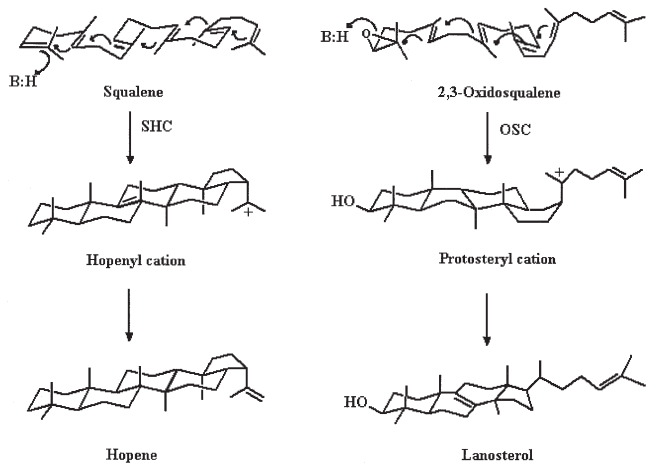
2,3-Oxidosqualene-lanosterol cyclase (OSC) (EC 5.4.99.7) and squalene-hopene cyclase (SHC) (EC 5.4.99.-) are two enzymes converting (3*S*)-2,3-oxidosqualene (OS) and squalene to tetra- and pentacyclic triterpenes, respectively (Scheme 1). These carbocyclizations proceed regio- and stereospecifically with protonation, cyclization, elimination, and, in the case of OSC, also rearrangement. OSC are membrane-associated enzymes that catalyze the cyclization of OS to lanosterol in mammals and yeasts and to cycloartenol in higher plants (1–3). SHC is a bacterial enzyme that catalyzes the cyclization of squalene to hopene and hopanoids (3–5); it is also able to cyclize squalene and OS, whereas OSC does not cyclize squalene. The two enzymes show many similarities in their cyclization mechanisms, SHC behaving as a more primitive enzyme.



SCHEME 1

OSC binds OS in a chair-boat-chair conformation; the cyclization of OS starts with the protonation of the epoxide by an electrophilic residue of the enzyme, and the cyclization then mediates sequential ring formation through double-bond addition of partially cyclized carbocationic intermediates, giving a tetracyclic cation, the protosteryl ion, which undergoes a series of 1,2-methyl and hydride shifts, and a terminal proton abstraction to lanosterol (1–3,6,7). SHC binds squalene in all-chair conformation, and the cyclization of squalene starts with the protonation of one of the terminal double bonds by an electrophilic residue of the enzyme. The cyclization proceeds through a different folding of the substrate, giving a pentacyclic cation, the hopenyl cation, which undergoes the loss of the 29-proton without skeletal rearrangement, yielding hopene as the main product (3).

OSC have been purified and cloned from different species: *Candida albicans*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* among the fungi; higher plants; the rat; the pig; and human (8–17). SHC from *Alicyclobacillus acidocaldarius* has recently been overexpressed in *Escherichia coli*, purified, and crystallized (18–20). Rat liver OSC is an 83-kDa protein possessing 733 amino acids, whereas SHC is a 71-kDa protein with 631 amino acids that shows 17–27% homogeneity with the cloned OSC. Since no OSC has yet been crystallized, SHC is so far the only suitable model for studying the mechanism of triterpene formation and inhibition from a structural standpoint (21).

OSC and more recently SHC have been the targets of inhibition studies. Acyclic squalene-derived inhibitors, in which the positively charged carbocation was replaced by a nitrogen, were initially developed (22,23). Various mono-, bi-, and tricyclic aza derivatives, analogs of partially cyclized sterol

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Abbreviations: HR-MS, high resolution MS; OS, 2,3-oxidosqualene; OSC, 2,3-oxidosqualene-lanosterol cyclase; SHC, squalene-hopene cyclase.

intermediates, some of which display high activity, have been obtained (24). Among the substrate mimics, 2,3;18,19-dioxidosqualene shows strong inhibitory potency (25).

The first potent, mechanism-based inactivator of animal OSC was (18*Z*)-29-methylidene-2,3-oxidosqualene (26), whereas (18*E*)-29-methylidenehexanor-2,3-oxidosqualene (27,28) behaved as a potent, selective, and time-dependent inhibitor of the yeast enzyme. Other 2,3-oxidosqualenoid dienes and vinyl epoxides were found to be inhibitors of yeast and animal OSC (29). Various series of sulfur-containing OS derivatives, in which sulfur has replaced a carbon in the squalene skeleton, have been developed, some of which behave as potent inhibitors of OSC, particularly of the *C. albicans* enzyme (30–33).

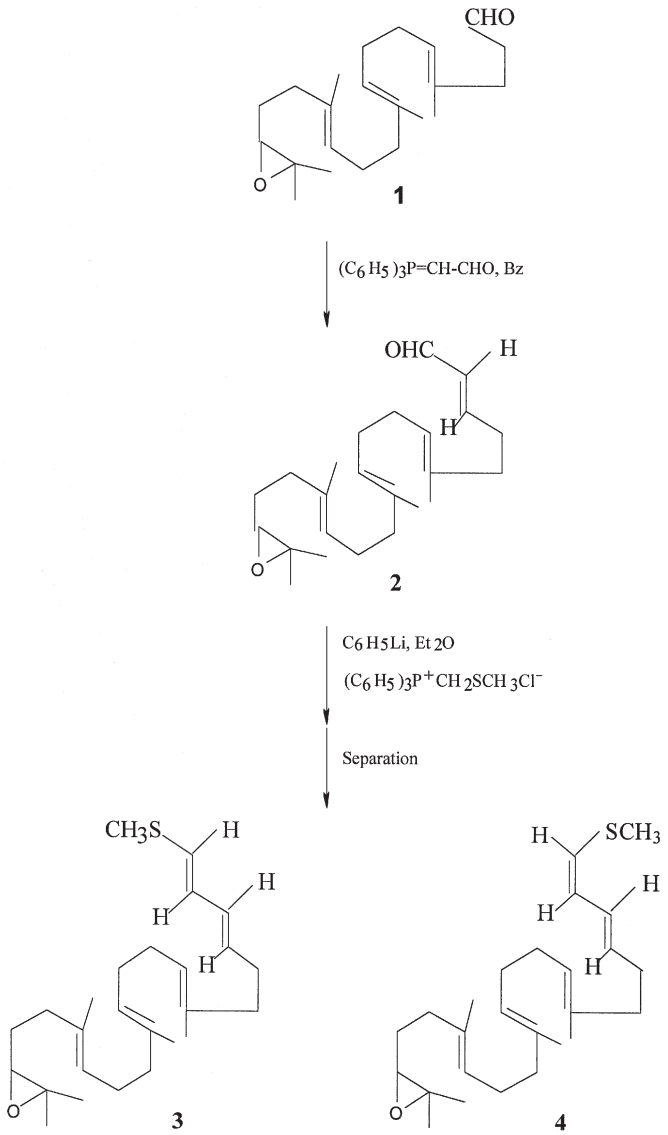
Few specific SHC inhibitors have been developed, among the most potent being amidrazone and amidoxime derivatives (34), but some inhibitors of OSC have been studied for their inhibitory activity on SHC (35,36). It has been shown that derivatives lacking the terminal isoprenic unit and possessing a correctly located double bond near the reactive function are often selective inhibitors of yeast OSC or SHC but are less active against animal OSC.

In the present study, new analogs of truncated OS, in which the reactive function has been placed adjacent to carbons of OS involved in the formation of the third or fourth cycle, were obtained and studied as inhibitors of pig and *S. cerevisiae* OSC and *A. acidocaldarius* SHC. In isomers **3**, **4**, **7**, **8**, and **9** (Scheme 2 and Fig. 1), the conjugated sulfide system would greatly increase the stability of the carbocation formed by the enzyme. The positive charge is presumably delocalized through the conjugated system and the adjacent sulfur, and the thiocarbenium ion might react with the nucleophilic residues of the enzyme active site.

MATERIALS AND METHODS

Chemicals. ^{1}H NMR spectra were recorded on a Bruker AC 200 instrument (Karlsruhe, Germany) for samples in $CDCl_3$ solution at room temperature, with Me_4Si (TMS) as internal standard. Coupling constants (*J*) are given in Hz. IR spectra were recorded on a PE 781 (PerkinElmer, Palo Alto, CA) spectrophotometer. Mass spectra were recorded on a Finnigan MAT TSQ 700 spectrometer (San Jose, CA). Microanalyses were determined on an elemental analyzer 1106 (Carlo Erba Strumentazione, Milano, Italy) and were within ±0.3% of the theoretical values. Reactions were monitored by TLC on F_{254} silica gel precoated sheets (Merck, Darmstadt, Germany); after development, the sheets were exposed to iodine vapor. Flash-column chromatography was performed on 230–400 mesh silica gel. Diethyl ether was dried over sodium benzophenone ketyl. All solvents were distilled prior to flash chromatography.

Squalene, lanosterol, and polyoxyethylene 9 lauryl ether were obtained from Sigma Chemical Co. (St. Louis, MO). [^{14}C]Squalene and [^{14}C]-(3*S*)-2,3-oxidosqualene were obtained through biological synthesis, by incubating a pig liver



SCHEME 2

S_{10} fraction with [2-^{14}C]mevalonate (NEN, Boston, MA; specific activity 2.04 GBq/mmol = 55 mCi/mmol) (36).

C_{22} and C_{17} squalene aldehyde epoxides **1** and **5** and C_{22} and C_{17} squalene aldehyde bromohydrins **10** and **12** (Schemes 2 and 3 and Fig. 1) were obtained as previously described (27,37).

*(2E,6E,10E,14E)-18,19-Epoxy-6,11,15,19-tetramethyl-2,6,10,14-eicosatetraenal (**2**, Scheme 2).* C_{22} squalene aldehyde epoxide **1** (320 mg, 0.962 mmol) was dissolved in benzene (20 mL) and triphenylphosphanylidene acetaldehyde (1.2 equiv., 350 mg, 1.15 mmol) was added in portions. The mixture was stirred under reflux for 4 d under dry argon. After evaporation of benzene *in vacuo*, the oil was dissolved in diethyl ether (50 mL), washed with saturated brine (1 × 30 mL), dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The resulting oil was purified by flash chromatography with petroleum ether/diethyl ether, 98:2, then 94:6, to give (2*E*)-aldehyde **2** (120 mg, 35% yield from **1**), as a color-

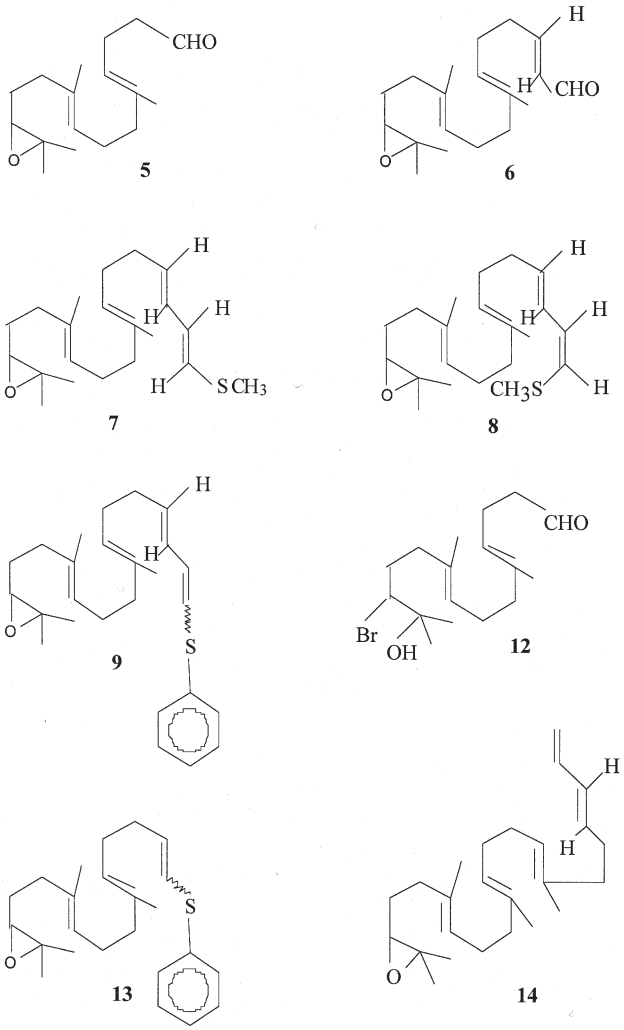
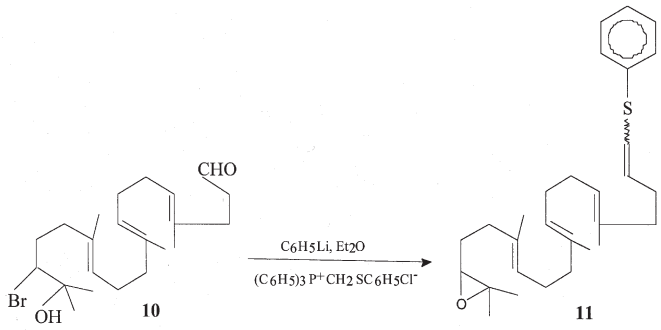


FIG. 1. Structures of various methyl sulfide and phenyl sulfide derivatives of oxidosqualene cyclase, intermediates, and (18*E*)-29-methylidenehexanor-2,3-oxidosqualene (**14**).

less oil. ^{1}H NMR ($CDCl_3$): δ 1.26 and 1.30 (2 *s*, 6H, epoxidic CH_3), 1.56–1.66 (*m*, 11H, allylic CH_3 and C*H*$_2$-epoxide), 1.97–2.24 (*m*, 12H, allylic CH_2), 2.33–2.45 (*m*, 2H, C*H*$_2$CH=CHCHO), 2.70 (*t*, 1H, epoxidic CH, *J* = 6.2 Hz), 5.02–5.22 (*m*, 3H, vinylic CH), 6.12 (*dd*, 1H, α-aldehyde vinylic CH, *J* = 15.6, 7.8 Hz), 6.84 (*dt*, 1H, β-aldehyde vinylic CH, *J* = 15.6, 6.6 Hz), 9.50 (*d*, 1H, C*H*O, *J* = 7.8 Hz); IR (liquid film) 2970, 2930, 2860, 1700 (CO), 1450, 1380 cm^{-1}; CI-MS (isobutane) *m/z* 359 (95), 358 (1), 341 (100); high resolution (HR)-MS *m/z* 358.2870 (calc. for $C_{24}H_{38}O_2$, 358.2872; found: C, 80.36; H, 10.69; O, 8.90. Calc. for $C_{24}H_{38}O_2$: C, 80.39; H, 10.68; O, 8.92%).

*(1*E*,3*E*,7*E*,11*E*,15*E*)-19,20-Epoxy-7,12,16,20-tetramethyl-1-methylthio-1,3,7,11,15-heneicosapentaene (**3**, Scheme 2) and (1*Z*,3*E*,7*E*,11*E*,15*E*)-19,20-epoxy-7,12,16,20-tetramethyl-1-methylthio-1,3,7,11,15-heneicosapentaene (**4**, Scheme 2).* In a two-necked flask, anhydrous diethyl ether (5 mL) and phenyllithium (1.6 M solution in hexane, 2.4 equiv., 360 µL, 0.58 mmol) were placed and stirred at room temperature under dry nitrogen. (Methylthiomethyl)triphenylphosphonium chloride



SCHEME 3

(1.2 equiv., 104 mg, 0.29 mmol), dissolved in anhydrous diethyl ether (3 mL), was then added, and the red solution turned pale yellow. After stirring for 10 min at room temperature, (2*E*)-aldehyde **2** (1.0 equiv., 86 mg, 0.24 mmol), in anhydrous diethyl ether (2 mL), was added and left for 30 min with stirring. The mixture was then poured into cold diethyl ether/saturated aqueous NaCl (1:1, 50 mL) and extracted with diethyl ether (3 × 30 mL). The combined extracts were washed with saturated brine (1 × 30 mL), dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The resulting oil was purified by flash chromatography with petroleum ether/diethyl ether, 98:2, then 94:6, to give first 14.5 mg of 1*E*,3*E* isomer **3** in 15% yield, and then 30.9 mg of 1*Z*,3*E* isomer **4** in 32% yield (47% overall yield, *E/Z* = 1:2), as colorless oils. 1*E*,3*E* isomer **3**: ^{1}H NMR ($CDCl_3$): δ 1.26 and 1.30 (2 *s*, 6H, epoxidic CH_3), 1.56–1.72 (*m*, 11H, allylic CH_3, and C*H*$_2$-epoxide), 1.97–2.16 (*m*, 14H, allylic CH_2), 2.27 (*s*, 3H, SC*H*$_3$), 2.70 (*t*, 1H, epoxidic CH, *J* = 6.2 Hz), 5.05–5.18 (*m*, 3H, vinylic CH), 5.54 (*dt*, 1H, C*H*=CH–CH=CHS, *J* = 14.5 and 6.5 Hz), 5.87–6.17 (*m*, 3H, CH=C*H*–C*H*=C*H*S); IR (liquid film) 2970, 2930, 2860, 1550, 1450, 1380 cm^{-1}; EI-MS *m/z* 402 (6%), 387 (1), 355 (2), 315 (1), 249 (3), 188 (5), 149 (5), 145 (10), 133 (15), 113 (100); CI-MS (isobutane) *m/z* 403 (100), 385 (55), 355 (18); HR-MS *m/z* 402.2960 (calc. for $C_{26}H_{42}OS$, 402.2956; found: C, 77.53; H, 10.49; O, 3.95; S, 7.95. Calc. for $C_{26}H_{42}OS$: C, 77.55; H, 10.51; O, 3.97; S, 7.96%). 1*Z*,3*E* isomer **4**: ^{1}H NMR ($CDCl_3$): δ 1.26 and 1.30 (2 *s*, 6H, epoxidic CH_3), 1.56–1.72 (*m*, 11H, allylic CH_3 and C*H*$_2$-epoxide), 1.97–2.20 (*m*, 14H, allylic CH_2), 2.30 (*s*, 3H, SC*H*$_3$), 2.70 (*t*, 1H, epoxidic CH, *J* = 6.2 Hz), 5.05–5.18 (*m*, 3H, vinylic CH), 5.68 (*dt*, 1H, C*H*=CH–CH=CHS, *J* = 14.5 and 6.6 Hz), 5.78 (*d*, 1H, CH=CH–CH=C*H*S, *J* = 9.6 Hz), 6.05 (*t*, 1H, CH=CH–C*H*=CHS), 6.30 (*dd*, 1H, CH=C*H*–CH=CHS, *J* = 14.5 and 9.9 Hz); IR (liquid film) 2970, 2930, 2860, 1550, 1450, 1380 cm^{-1}; EI-MS *m/z* 402 (8), 355 (2), 315 (1), 293 (4), 249 (4), 202 (6), 167 (10), 149 (28), 133 (20), 113 (100); CI-MS (isobutane) *m/z* 403 (100), 385 (38), 355 (18); HR-MS *m/z* 402.2955 (calc. for $C_{26}H_{42}OS$, 402.2956; found: C, 77.56; H, 10.53; O, 3.97; S, 7.94. Calc. for $C_{26}H_{42}OS$: C, 77.55; H, 10.51; O, 3.97; S, 7.96%).

*(2*E*,6*E*,10*E*)-14,15-Epoxy-7,11,15-trimethyl-2,6,10-hexadecatrienal (**6**, Fig. 1).* (2*E*)-aldehyde **6** was obtained starting from C_{17} squalene aldehyde epoxide **5**, using the method described for aldehyde **2**, in 32% yield. ^{1}H NMR ($CDCl_3$): δ

1.26 and 1.30 (2 *s*, 6H, epoxidic CH_3), 1.55–1.64 (*m*, 8H, allylic CH_3 and CH_2-epoxide), 2.02–2.23 (*m*, 8H, allylic CH_2), 2.33–2.44 (*m*, 2H, CH_2CH=CHCHO), 2.70 (*t*, 1H, epoxidic CH, *J* = 6.2 Hz), 5.05–5.18 (*m*, 2H, vinylic CH), 6.12 (*dd*, 1H, α-aldehyde vinylic CH, *J* = 15.6, 7.8 Hz), 6.85 (*dt*, 1H, β-aldehyde vinylic CH, *J* = 15.6, 6.6 Hz), 9.50 (*d*, 1H, C*H*O, *J* = 7.8 Hz); IR (CCl_4) 2970, 2930, 2860, 1700 (CO), 1450, 1380 cm^{-1}; CI-MS (isobutane) *m/z* 291 (100), 290 (0.3), 273 (68), 255 (12); HR-MS *m/z* 290.2244 (calc. for $C_{19}H_{30}O_2$, 290.2246; found: C, 78.59; H, 10.43; O, 11.00. Calc. for $C_{19}H_{30}O_2$: C, 78.57; H, 10.41; O, 11.02%).

*(1*E*,3*E*,7*E*,11*E*)-15,16-Epoxy-8,12,16-trimethyl-1-methylthio-1,3,7,11-heptadecatetraene (**7**, Fig. 1) and (1*Z*,3*E*,7*E*,11*E*)-15,16-epoxy-8,12,16-trimethyl-1-methylthio-1,3,7,11-heptadecatetraene (**8**, Fig. 1).* Compounds **7** and **8** were obtained starting from (2*E*)-aldehyde **6**, using the method described for compounds **3** and **4**, in 53% overall yield. 1*E*,3*E* isomer **7** was separated first in 18% yield, followed by 1*Z*,3*E* isomer **8** in 35% yield (53% overall yield, *E/Z* = 1:2), as colorless oils. 1*E*,3*E* isomer **7**: ^{1}H NMR ($CDCl_3$): δ 1.26 and 1.30 (2 *s*, 6H, epoxidic CH_3), 1.56–1.68 (*m*, 8H, allylic CH_3 and CH_2-epoxide), 2.00–2.16 (*m*, 10H, allylic CH_2), 2.28 (*s*, 3H, SCH_3), 2.71 (*t*, 1H, epoxidic CH, *J* = 6.2 Hz), 5.02–5.18 (*m*, 2H, vinylic CH), 5.55 (*dt*, 1H, C*H*=CH–CH=CHS, *J* = 14.5 and 6.5 Hz); 5.86–6.17 (*m*, 3H, CH=C*H*–C*H*=C*H*S); IR (liquid film) 2970, 2930, 2850, 1550, 1450, 1380 cm^{-1}; EI-MS *m/z* 334 (6), 279 (3), 223 (3), 167 (6), 149 (27), 126 (10), 113 (100); CI-MS (isobutane) *m/z* 335 (100), 317 (63), 287 (35); HR-MS *m/z* 334.2328 (calc. for $C_{21}H_{34}OS$ 334.2330; found: C, 75.42; H, 10.24; O, 4.76; S, 9.55. Calc. for $C_{21}H_{34}OS$: C, 75.39; H, 10.24; O, 4.78; S, 9.58%).1*Z*,3*E* isomer **8**: ^{1}H NMR ($CDCl_3$): δ 1.26 and 1.30 (2 *s*, 6H, epoxidic CH_3), 1.56–1.68 (*m*, 8H, allylic CH_3 and CH_2-epoxide), 2.00–2.16 (*m*, 10H, allylic CH_2), 2.28 (*s*, 3H, SCH_3), 2.70 (*t*, 1H, epoxidic CH, *J* = 6.2 Hz), 5.02–5.18 (*m*, 2H, vinylic CH), 5.66 (*dt*, 1H, C*H*=CH–CH=CHS, *J* = 14.5 and 6.6 Hz), 5.77 (*d*, 1H, CH=CH–CH=C*H*S, *J* = 9.6 Hz), 6.04 (*t*, 1H, CH=CH–C*H*=CHS), 6.30 (*dd*, 1H, CH=C*H*–CH=CHS, *J* = 14.5 and 9.9 Hz); IR (liquid film) 2970, 2930, 2850, 1550, 1445, 1380 cm^{-1}; EI-MS *m/z* 334 (4), 294 (2), 254 (1), 183 (2), 167 (2), 149 (8), 126 (15), 113 (100); CI-MS (isobutane) *m/z* 335 (100), 317 (65), 287 (28); HR-MS *m/z* 334.2334 (calc. for $C_{21}H_{34}OS$, 334.2330; found: C, 75.39; H, 10.22; O, 4.75; S, 9.55. Calc. for $C_{21}H_{34}OS$: C, 75.39; H, 10.24; O, 4.78; S, 9.58%).

*(3*E*,7*E*,11*E*)-15,16-Epoxy-8,12,16-trimethyl-1-phenylthio-1,3,7,11-heptadecatetraene (**9**, Fig. 1).* Compound **9** was obtained starting from (2*E*)-aldehyde **6**, following the method described for compounds **3** and **4**, using (phenylthiomethyl)-triphenylphosphonium chloride instead of (methylthiomethyl)triphenylphosphonium chloride. It was obtained as a mixture (about 1:1, by ^{1}H NMR analysis) of 1*E* and 1*Z* isomers, in 28% yield. ^{1}H NMR ($CDCl_3$): δ 1.26 and 1.30 (2 *s*, 6H, epoxidic CH_3), 1.56–1.66 (*m*, 8H, allylic CH_3 and CH_2-epoxide), 1.98–2.25 (*m*, 10H, allylic CH_2), 2.70 (*t*, 1H, epoxidic CH, *J* = 6.2 Hz), 5.05–5.18 (*m*, 2H, vinylic CH), 5.78–6.50 (*m*, 4H, C*H*=C*H*–C*H*=C*H*S), 7.18–7.38 (*m*, 5H, arom. CH); IR (CCl_4) 2975, 2940, 2860, 1585, 1550, 1480, 1380 cm^{-1}; EI-MS *m/z* 396 (100), 316 (20), 293 (8), 287 (18), 279 (15), 243 (26); CI-MS (isobutane) *m/z* 397 (100), 379 (55); HR-MS *m/z* 396.2483 (calc. for $C_{26}H_{36}OS$, 396.2487; found: C, 78.75; H, 9.12; O, 4.01; S, 8.10. Calc. for $C_{26}H_{36}OS$: C, 78.73; H, 9.15; O, 4.03; S, 8.08%).

*(5*E*,9*E*,13*E*)-17,18-Epoxy-5,10,14,18-tetramethyl-1-phenylthio-1,5,9,13-nonadecatetraene (**11**, Scheme 3).* Anhydrous diethyl ether (3 mL) and phenyllithium (1.6 M solution in hexane, 3.5 equiv, 350 µL, 0.56 mmol) were placed in a two-necked flask and stirred at room temperature under dry nitrogen. (Phenylthiomethyl)triphenylphosphonium chloride (1.2 equiv, 80 mg, 0.19 mmol), dissolved in anhydrous diethyl ether (3 mL), was then added and the red solution turned orange. After stirring for 10 min at room temperature, C_{22} squalene aldehyde bromohydrin **10** (1.0 equiv, 66 mg, 0.16 mmol), in anhydrous diethyl ether (1 mL), was added and left for 30 min under stirring. The mixture was then poured into cold diethyl ether/saturated aqueous NaCl (1:1, 50 mL) and extracted with diethyl ether (3 × 30 mL). The combined extracts were washed with saturated brine (1 × 30 mL), dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The resulting oil was purified by flash chromatography with petroleum ether/diethyl ether, 99:1, 98:2, then 97:3, to give compound **11** as a colorless oil. It was obtained as a mixture (about 1:1, by ^{1}H NMR analysis) of 1*E* and 1*Z* isomers, in 42% yield. ^{1}H NMR ($CDCl_3$): δ 1.26 and 1.30 (2 *s*, 6H, epoxidic CH_3), 1.56–1.66 (*m*, 11H, allylic CH_3 and CH_2-epoxide), 1.98–2.40 (*m*, 14H, allylic CH_2), 2.70 (*t*, 1H, epoxidic CH, *J* = 6.2 Hz), 5.00–5.18 (*m*, 3H, vinylic CH), 5.78–6.21 (*m*, 2H, C*H*=C*H*S), 7.16–7.37 (*m*, 5H, arom. CH); IR (KBr pellet) 2970, 2930, 2860, 1585, 1480, 1380 cm^{-1}; EI-MS *m/z* 438 (1), 328 (1), 285 (5), 242 (1.8), 216 (1.8), 202 (4), 149 (100); HR-MS *m/z* 438.2958 (calc. for $C_{29}H_{42}OS$ 438.2956; found: C, 79.38; H, 9.66; O, 3.63; S, 7.29. Calc. for $C_{29}H_{42}OS$: C, 79.40; H, 9.65; O, 3.65; S, 7.31%).

*(5*E*,9*E*)-13,14-Epoxy-6,10,14-trimethyl-1-phenylthio-1,5,9-pentadecatriene (**13**, Fig. 1).* Compound **13** was obtained starting from C_{17} squalene aldehyde bromohydrin **12**, using the method described for compound **11**, as a mixture (about 1:1, by ^{1}H NMR analysis) of 1*E* and 1*Z* isomers, in 40% yield. ^{1}H NMR ($CDCl_3$): δ 1.26 and 1.30 (2 *s*, 6H, epoxidic CH_3), 1.57–1.65 (*m*, 8H, allylic CH_3 and CH_2-epoxide), 1.97–2.41 (*m*, 10H, allylic CH_2), 2.70 (*t*, 1H, epoxidic CH, *J* = 6.2 Hz), 5.02–5.20 (*m*, 2H, vinylic CH), 5.77–6.20 (*m*, 2H, C*H*=C*H*S), 7.15–7.37 (*m*, 5H, arom. CH); IR (KBr pellet) 2970, 2930, 2860, 1585, 1480, 1380 cm^{-1}; EI-MS *m/z* 370 (1), 279 (5), 260 (0.9), 243 (1.2), 215 (9.8), 203 (1), 149 (100); HR-MS *m/z* 370.2329 (calc. for $C_{24}H_{34}OS$, 370.2330; found: C, 77.78; H, 9.26; O, 4.30; S, 8.66. Calc. for $C_{24}H_{34}OS$: C, 77.78; H, 9.25; O, 4.32; S, 8.65%).

Enzyme assays. (i) Solubilization and purification of OSC. Solubilized and partially purified pig liver and yeast OSC were obtained as previously described (38).

(ii) Assay of OSC and SHC activities and kinetic determination. The enzyme activity of OSC was determined by incu-

bating 2,000 cpm of [^{14}C]-(3*S*)-2,3-oxidosqualene and evaluating the amount of lanosterol formed, as previously described (38). [^{14}C]-(3*S*)-2,3-Oxidosqualene and [^{14}C]squalene were obtained by biological synthesis, incubating 1 μCi of [^{14}C]mevalonolactone with an S_{10} supernatant of a pig liver homogenate (25 mg of proteins) in the presence of the OSC inhibitor U-18666A, following the method of Popják (39). Recombinant squalene-hopene cyclase was kindly provided by Prof. Karl Poralla (Tübingen University, Germany).

To determine the enzyme activity of SHC, 10 μM squalene and 2,000 cpm of [^{14}C]squalene were dissolved in ethanol in the presence of polyoxyethylene 9 lauryl ether (final concentration 0.05%) in test tubes. The solvent was evaporated under nitrogen and the enzyme (3 μg in 1 mL of citrate buffer, 0.1 M, pH 6.0, containing 0.1% polyoxyethylene 9 lauryl ether) was added to the test tubes and incubated for 30 min at 55°C. The reaction was stopped by adding methanolic KOH (10% concentration, 1 mL) to each test tube and heating at 80°C for 30 min in a water bath. After extraction with 2 mL of petroleum ether, the solvent was evaporated. The extracts were redissolved in small amounts of CH_2Cl_2 and spotted on TLC plates developed with petroleum ether. Conversion of squalene to labeled hopene was quantified by a radio-TLC scanner (Packard System 2000 Imaging Scanner; Hewlett-Packard, Palo Alto, CA), and the percentage of transformation was calculated by integration. Alternatively, bands corresponding to squalene and hopene were scraped off the plate and counted with a liquid scintillation counter (Beckman LS500 TD; Beckman Instruments, Fullerton, CA). In these conditions the amount of hopan-22-ol formed was negligible (less than 1%) and was ignored.

IC_{50} inhibition values were determined by adding the inhibitors, in ethanol solution, to the mixture of nonradiolabeled and radiolabeled substrates and incubating with SHC, as described above, using (18*E*)-29-methylidenehexanor-2,3-oxidosqualene **14** as control compound.

(iii) Time-dependent inactivation of OSC and SHC. Time-dependent inactivation of OSC was determined at 37°C as previously described (37). Time-dependent inactivation of SHC was determined at 55°C by adding the inhibitors to enzyme solution in the absence of substrate. Aliquots were withdrawn at suitable intervals and diluted 50-fold by transfer to test tubes containing nonradiolabeled and radiolabeled substrate squalene (10 μM) plus polyoxyethylene 9 lauryl ether (0.1%) in 0.1 M citrate buffer, pH 6. Residual activity was determined by comparison with an enzyme solution preincubated under the conditions above.

RESULTS AND DISCUSSION

Chemistry. The synthesis of (1*E*,3*E*)- and (1*Z*,3*E*)-conjugated methylthio derivatives of OS was developed, starting from C_{24} and C_{19} (2*E*)-squalene aldehyde epoxides **2** and **6**. Thus, C_{22} and C_{17} squalene aldehyde epoxides **1** and **5** were submitted to a Wittig reaction with triphenylphosphanylidene acetaldehyde in benzene (40). The *E* olefins **2** and **6** were stereospecifically obtained, in 35 and 32% yield, respectively, without detection of the *Z* olefin. The *E* configuration of conjugated aldehydes **2** and **6** was assigned by ^{1}H NMR analysis (41). In particular, coupling constants (*J*) of the olefinic proton of (2*E*)-derivatives **2** and **6** were 15.6 Hz, and the chemical shifts of the aldehydic protons of (2*E*) derivatives **2** and **6** were 9.50 δ. The synthesis of (2*E*)-squalene aldehyde epoxides **2** and **6** was also performed starting directly from C_{22} and C_{17} squalene aldehyde bromohydrins **10** and **12**. In this way, contemporary closure of bromohydrin to epoxide occurred, but the reaction proceeded in lower overall yields.

The synthesis of (1*E*,3*E*)- and (1*Z*,3*E*)-conjugated methylthio derivatives of OS, **3**, **4** and **7**, **8** was then developed. A survey of the literature showed no methods applicable for the present purposes. (2*E*)-Squalene aldehyde epoxides **2** and **6** were submitted to a Wittig reaction with (methylthiomethyl)-triphenylphosphonium chloride and phenyllithium in diethyl ether, affording methylthio derivatives **3**, **4** and **7**, **8**, in *E/Z* = 1:2 ratio, and in 47 and 53% overall yields, respectively. The mixture was purified and separated by flash chromatography, eluting first the (1*E*,3*E*)-isomer and then the (1*Z*,3*E*)-isomer. The structure was assigned by ^{1}H NMR analysis, by comparison with spectra of 1-ethylthio-1,3-hexadienes (42) and 1-methylthio-1,3-pentadienes (43). In particular, the structure was assigned on the basis of the ^{1}H NMR shifts and of the *cis* and *trans* coupling constants, which were in the 8–10 and 14–15 Hz ranges, respectively. Similarly, Wittig reactions of the suitable aldehydes **6**, **10**, and **12**, with (phenylthiomethyl)triphenylphosphonium chloride and phenyllithium, afforded phenylthio derivatives of OS **9**, **11**, and **13**, as 1:1 mixtures of *E* and *Z* isomers. For the synthesis of nonconjugated phenylthio derivatives of OS, **11** and **13**, C_{22} and C_{17} squalene aldehyde bromohydrins **10** and **12** were used directly. In this case, simultaneous closure of the bromohydrin to epoxide was achieved during the Wittig reaction. All the new derivatives possessed the epoxidic function in racemic form.

Biological activity. Analogs of OS, with a sulfur atom in the position α to a carbon bearing a positive charge during cyclization of the substrate, have been shown to be potent inhibitors of OSC, particularly if the sulfur is placed at C-20 (30–33). Recently, it has been shown that the lack of a terminal isoprenic chain, as in various truncated methylthio derivatives, affords potent and selective inhibition of *S. cerevisiae* OSC, but not of animal OSC (36). These derivatives may be expected to cyclize, and the charge of the carbocation formed would presumably be distributed between the carbon and the adjacent sulfur. These stabilized carbocations probably then react with a nucleophilic residue of the enzyme, which normally would stabilize the natural carbocation.

Based on these considerations, conjugated methylthio and phenylthio derivatives of OS bearing the reactive functions at crucial positions were developed (Schemes 2 and 3, Fig. 1). Once partially cyclized by the enzyme, the positive charge was expected to be highly delocalized between the carbon normally bearing the double bond, the additional double bond, and the adjacent sulfur.

TABLE 1
Inhibition Values of Pig Liver and *Saccharomyces cerevisiae* OSC and *Alicyclobacillus acidocaldarius* SHC by Methylthio and Phenylthio Derivatives of Truncated Oxidosqualene

	IC_{50} (μM)[a]		
Compound[b]	OSC pig liver	OSC *S. cerevisiae*	SHC *A. acidocaldarius*
3	1.2	0.4	1.4
4	1	0.9	1.8
7	3.6	6	1
8	2.6	2	4
9	20	12	2.2
11	7	1	3
13	7	10	7
14 (control)	3.5	1.5	0.2

[a]OSC, 2,3-oxidosqualene-lanosterol cyclase; SHC, squalene-hopene cyclase.
[b]For compound structures, see Schemes 2 and 3, and Figure 1.

Table 1 reports the IC_{50} inhibition values obtained with a solubilized and partially purified OSC of pig liver, a microsomal suspension of *S. cerevisiae* OSC, and a purified recombinant SHC of *A. acidocaldarius*; (18*E*)-29-methylidenehexanor-2,3-oxidosqualene **14** was taken as control compound. The compounds tested are inhibitors of OSC and SHC, the most active being (*E*)- and (*Z*)-conjugated methylthio derivatives **3** and **4**. Unlike (18*E*)-29-methylidenehexanor-2,3-oxidosqualene **14**, which behaved as a specific and potent inhibitor of yeast OSC (26) whereas the (18*Z*) isomer was almost inactive (27), there was little difference in inhibition activity on yeast OSC vs. animal OSC between isomers **3** and **4**. Methylthio conjugated isomers **7** and **8**, although less active, also showed similar inhibition activity. Possibly, once cyclized by the enzyme, the highly stabilized carbocation is less reactive toward the nucleophilic residues of the enzyme, stabilizing the natural carbocations. The phenylthio derivatives **9**, **11**, and **13** were less active on animal and yeast OSC than the methylthio derivatives, probably because of the steric bulk of the phenyl group. On the contrary, the phenylthio derivatives **9**, **11**, and **13** maintained good activity against SHC, as SHC probably can withstand more bulky groups in its active site.

Time-dependency was studied on the three enzymes with the more active inhibitors **3** and **4** at concentrations up to 10-fold higher than the IC_{50}. No time-dependency was observed at the concentrations tested. We think compounds **3**, **4**, **7**, and **8** may be cyclized up to the reactive function by the enzymes OSC and SHC. Determination of the structure of the cyclized metabolites should provide important information on the enzyme mechanisms.

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Evaluation of Apolipoprotein A-I Kinetics in Rabbits *in vivo* Using *in situ* and Exogenous Radioiodination Methods

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ABSTRACT: The kinetics of *in vivo* clearance of apolipoprotein (apo) A-I radioiodinated by the iodine monochloride (ICl) method of McFarlane [McFarlane, A.S. (1958) Efficient Trace-Labelling of Proteins with Iodine, *Nature 182*, 53] as modified by Bilheimer and co-workers [Bilheimer, D.W., Eisenberg, S., and Levy, R.I. (1972) The Metabolism of Very Low Density Lipoprotein Proteins. I. Preliminary *in vitro* and *in vivo* Observations, *Biochim. Biophys. Acta 260*, 212–221] and by using the IODO Beads Iodination Reagent were evaluated in rabbits. Both human apoA-I and rabbit HDL radioiodinated by the IODO Beads Iodination Reagent were cleared faster from plasma of rabbits than those radiolabeled by the ICl method. However, the different radiolabeling procedures in the ICl method, i.e., apoA-I radiolabeled either exogenously or *in situ* as a part of intact HDL, were not associated with a significant difference in the *in vivo* kinetics of apoA-I in rabbits if apoA-I was prepared by the guanidine HCl method and used fresh. ^{125}I-ApoA-I subjected to delipidation and lyophilization was cleared only slightly faster from the plasma of rabbits than fresh ^{125}I-apoA-I. We also found that apoA-I separated by the guanidine HCl method and used fresh was cleared faster from the plasma of rabbits when it was injected as free apoA-I without adding serum albumin or after *in vitro* incubation with rabbit HDL than when injected after reassociation with rabbit plasma. We conclude that the ICl method is a more appropriate radioiodination method for studying the *in vivo* kinetics of HDL than the IODO Beads Iodination Reagent and that the *in vitro* incubation conditions before injection are important factors that affect the *in vivo* kinetics of apo A-I.

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Considerable attention has been paid to the role of HDL and its major protein, apolipoprotein (apo) A-I, in protecting against coronary heart disease (CHD) (1–6). The antiatherogenic effects of HDL have been directly evidenced by studies of the over- or underexpression of apoA-I using genetic animal models of reverse cholesterol transport (7–10). Furthermore, the increased synthesis of apoA-I as a result of drug administration has actually reduced coronary events in epidemiological studies (11,12).

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Abbreviations: Apo, apolipoprotein; CHD, coronary heart disease; FCR, fractional catabolic rate; HPCE, high-performance capillary electrophoresis; LpA-I, human reconstituted HDL; LPL, lipoprotein lipase; RMT, relative migration time; SDS-CGE, SDS-capillary gel electrophoresis.

Since increased levels of apoA-I may be due to an increased rate of synthesis and/or reduced fractional catabolic rate (FCR), examining the turnover of apoA-I is important for studying HDL metabolism. Most investigations of the *in vivo* metabolism of plasma lipoproteins have been conducted by making use of iodine-labeled plasma lipoproteins (12–18). Endogenous stable isotope labeling of apoA-I with amino acids also has been used as a valid approach to study the metabolism of HDL constituents in humans (19–21), having the theoretical advantage that there is no possibility of altering the nature of the apoprotein through isolation and labeling. However, the technique has major limitations in kinetics studies of proteins with slow turnover rates, such as apoA-I, because the tracer can be recycled and reincorporated into newly synthesized proteins. In addition, the kinetic analysis of endogenous labeling studies relies on several assumptions (22,23) and is much more complex than that of exogenous radiolabeling studies.

For decades, McFarlane's iodine monochloride (ICl) method, as modified by Bilheimer *et al.,* and two basic procedures that use different radioiodination techniques of the ICl method (*in situ* and exogenous apolipoprotein radiolabeling) have been used to investigate the turnover of apoA-I (24). One approach is to trace-label a density fraction of HDL isolated by ultracentrifugation (*in situ* apoprotein radiolabeling) and inject the intact trace-labeled lipoprotein into the human or animal subject. ApoA-I is then isolated from the HDL in plasma drawn at different intervals to construct specific radioactivity decay curves that are used in kinetic analysis. An alternative and simpler approach is to trace-label purified apoA-I and intercalate trace-labeled apoA-I into HDL by incubation with HDL (exogenous apoprotein radiolabeling). The plasma clearance of apoA-I can then be observed simply by measuring the rate of tracer decay in whole plasma. This procedure has become quite popular since frequent and repeated cumbersome subfractionation of HDL apoA-I is no longer required. However, we and other authors have reported that the clearance of apoA-I radioiodinated by the ICl method and incorporated into the HDL separated by ultracentrifugation is not strictly comparable to that of apoA-I radioiodinated *in situ* on whole HDL (12,13,17,24–26): Exogenously labeled apoA-I is consistently catabolized slightly but significantly faster. Osborne *et al.* (27), who were able to separate radioiodinated apoA-I into fractions (a fraction that was indistinguishable in its physiological properties from unlabeled apoA-I and a fraction that was catabolized faster), suggest

that iodination of free apoA-I may modify tyrosine residues in a way that prevents the normal reassociation of apoA-I with HDL.

Although no definite explanation exists for the discrepancy in the kinetic behavior of apoA-I radioiodinated by the *in situ* and by the exogenous radiolabeling technique of the ICl method, we were encouraged to test the hypothesis that the process for separation and storage of apoA-I and *in vitro* incubation condition of apoA-I may affect the *in vivo* kinetics of apoA-I by the findings of Vega *et al.* (16), who provided a simple method for isolating apoA-I in large quantities using guanidine HCl and showed that apoA-I isolated freshly, radioiodinated without storage, and injected with human albumin without *in vitro* incubation with ultracentrifuged HDL has a kinetic behavior similar to that of apoA-I radiolabeled as part of intact HDL in human subjects.

Kinetics studies in humans are difficult if not impossible to perform in many countries. Rabbits, especially transgenic rabbits with human LCAT or lipoprotein lipase (LPL) overexpression, are useful experimental animals for examining lipoprotein, especially HDL, metabolism and the mechanism of atherosclerosis. Therefore, we evaluated in rabbits the kinetics of exogenously radioiodinated free apoA-I, isolated by the guanidine HCl method used by Vega *et al.* (16), and apoA-I radioiodinated *in situ* as part of the intact HDL, to clarify whether free apoA-I could also be a reliable tracer for whole plasma apoA-I in animal models.

Recently, Braschi *et al.* (28) used the commercially available IODO Beads Iodination Reagent, which is purportedly a gentle yet efficient means of labeling protein tyrosine residues with ^{125}I, for a kinetics study in rabbits and reported that human ^{125}I-apoA-I iodinated with the IODO Beads Iodination Reagent exhibits a normal secondary structure and surface charge (29). However, Braschi *et al.* (29) also reported that apoA-I radiolabeled by the IODO Beads Iodination Reagent was catabolized much faster than native apoA-I. They suggested that specific modification of tyrosine residues affects the conformation and *in vivo* metabolism of iodinated apoA-I (29). Structural properties of apoA-I ^{125}I-labeled by the ICl method also have been shown to be different from those of the unlabeled protein (27,30). However, it is not clear whether radioiodination by the IODO Beads Iodination Reagent and the ICl method may affect the *in vivo* kinetics of apoA-I differently, and no study has directly compared the two radioiodination methods.

Therefore, in the present study we also compared the kinetics of HDL radiolabeled by the novel IODO Beads method and by the conventional ICl method of McFarlane (31) as modified by Bilheimer *et al.* (32).

MATERIALS AND METHODS

Animals. Forty-four female normolipidemic Japanese White rabbits obtained from Kyudo Co., Ltd. (Fukuoka, Japan) were used in this study. The rabbits were housed individually in an environmentally controlled room (Animal Center, Fukuoka University School of Medicine, Fukuoka, Japan) with a 12-h light/dark cycle (light 7 A.M.–7 P.M.) and fed standard rabbit chow LRC-4 (Oriental Yeast, Tokyo, Japan) at 80 g/d per animal at 10:30 A.M. (33). This experimental animal study was approved by the Ethics Committee of Fukuoka University.

Study design. All of the rabbits were randomly divided into nine groups (Groups 1–8), as shown in Table 1. A kinetics study was performed in each group of rabbits separately.

Materials. Commercial lyophilized human apoA-I were obtained from Cosmo Bio Co., Ltd. (Tokyo, Japan). IODO Beads Iodination Reagent, BCA Protein Assay Reagent,

TABLE 1
Groups of Rabbits Used in the Kinetics Study[a]

Group	Material injected	Separation method	Storage	Labeling method	*In vitro* incubation
Group 1	Human apoA-I	Commercially available	Lyophilized	IODO Beads	With rabbit HDL
Group 2	Human apoA-I	Commercially available	Lyophilized	ICl	With rabbit HDL
Group 3	Rabbit HDL	Sequential ultracentrifugation	Fresh	IODO Beads	With rabbit HDL
Group 4 (Group 4.A)	Rabbit HDL	Sequential ultracentrifugation	Fresh	ICl	With rabbit HDL
Group 5	Rabbit apoA-I	Guanidine HCl method	Fresh	ICl	With rabbit plasma
Group 6.A	Rabbit apoA-I	Guanidine HCl method	Fresh	ICl	No[b]
Group 6.B	Rabbit apoA-I	Guanidine HCl method	Fresh	ICl	With rabbit HDL
Group 7	Rabbit apoA-I	Guanidine HCl method	Lyophilized	ICl	With rabbit plasma
Group 8	Rabbit apoA-I	Guanidine HCl method	Delipidized and lyophilized	ICl	With rabbit plasma

[a]ICl, iodine monochloride method of McFarlane (31) as modified by Bilheimer *et al.* (32).
[b]Group 6.A was injected with free apoA-I. Apo, apolipoprotein.

Micro BCA™ Protein Assay Reagent, and Slide-A-Lyzer Dialysis Cassette were purchased from Pierce (Rockford, IL). $Na^{125}I$ was obtained from Amersham Pharmacia Biotech (Tokyo, Japan).

Isolation of HDL from rabbit plasma. Blood was freshly drawn in EDTA from the marginal auricular veins of two rabbits after an overnight fast, and HDL was isolated from the pooled plasma (about 36 mL) by sequential ultracentrifugation. The plasma was first adjusted with solid KBr to a density of 1.063 g/mL and subjected to ultracentrifugation in a TLA-100.3 rotor in a Beckman TL-100 Tabletop Ultracentrifuge for 5 h at 541,000 × *g* and 4°C. The bottom fraction was collected by cutting the tubes, resuspended using liquid KBr (*d* = 1.063 g/mL), and subjected to ultracentrifugation again for 5 h at 541,000 × *g* and 4°C to remove any contaminating lipoproteins. The density of the bottom fraction was raised to 1.21 g/mL, and the solution was overlaid with an equal volume of KBr solution of similar density and ultracentrifuged for 10 h at 541,000 × *g* and 10°C. The top fraction was carefully collected using a syringe with a 27G needle, washed once by carefully overlaying a half-volume of liquid KBr (*d* = 1.21 g/mL), and ultracentrifuged for another 10 h to remove contaminating plasma albumin. The top fraction was dialyzed in a Slide-A-Lyzer Dialysis Cassette against a solution of 0.85% NaCl, 0.01 M Tris-HCl (pH = 7.4), and 0.01% EDTA in preparation for labeling with ^{125}I.

Separation of rabbit apoA-I from HDL. Rabbit apoA-I was separated by the guanidine HCl method reported by Vega *et al.* (16) with minor modifications. In brief, fresh HDL preparation was diluted with 0.85% NaCl to ~3 mg/mL HDL protein, and guanidine hydrochloride was added to bring the solution to 3 M. Protein was measured using the BCA Protein Reagent. Vega *et al.* (16) used a final concentration of 4 M guanidine HCl to separate apoA-I. We reduced the concentration of guanidine HCl to 3 M to further improve the purity of apoA-I (34). The solution was incubated for 3 h at 37°C. ApoA-I will dissociate from HDL under these conditions (16,34). After incubation, the solution was extensively dialyzed against a solution of 0.85% NaCl, 0.01 M Tris-HCl (pH = 8.0), and 0.01% EDTA. The HDL solution was then adjusted to a density of 1.21 g/mL by adding solid KBr, carefully overlain with a half-volume of liquid KBr of similar density, and subjected to ultracentrifugation in a TLA-100.3 rotor for 15 h at 541,000 × *g* and 10°C. One-fourth of the bottom fraction was collected by cutting the tubes and was dialyzed against a solution of 0.85% NaCl, 0.01 M Tris-HCl (pH = 7.4), and 0.01% EDTA. The purity of the isolated rabbit apoA-I was examined by SDS-PAGE in a slab gel apparatus and by capillary gel electrophoresis. The apoA-I preparation was concentrated and buffer-exchanged with 1 M glycine-NaOH buffer (pH = 8.5) by using a Microcon Centrifugal Filter Device YM-10 (Millipore Corporation, Bedford, MA) and used immediately for radioiodination.

Part of the apoA-I preparation was stored after being lyophilized in a Labconco Freeze Dry System (Lyph-Lock 4.5 Liter Benchtop Model 77500; Labconco Corp., Kansas City, MO) or delipidated with acetone/ethanol (1:1, vol/vol) before lyophilization.

Slab gel SDS-PAGE. Conventional slab gel SDS-PAGE (35) was performed on a vertical gel apparatus (AE-6400, Atto Corporation, Tokyo, Japan) according to the manufacturer's instructions.

SDS-capillary gel electrophoresis separation. The isolated rabbit apoA-I was characterized by applying the recent technology of high-performance capillary electrophoresis (HPCE) systems. Protein separation by HPCE is analogous to that achieved with slab gel systems except that no support media are used, electrophoresis is carried out in free solution, and results are obtained in minutes rather than hours with the attendant advantages of automation, precise control of operating conditions, and direct on-line quantification (36). The technique of using an SDS-containing UV-transparent polymer network, usually termed SDS-capillary gel electrophoresis (SDS-CGE), gives results similar to those obtained with SDS slab gel electrophoresis (36) and has been successfully used to resolve apoA-I in human HDL (36,37).

SDS-CGE was performed using an eCAP SDS 14-200 kit (Beckman Coulter, Fullerton, CA) on a PACE/MDQ Glycoprotein System controlled by the 32 Karat Software (version 4.0) and equipped with a UV detector (Beckman Coulter). The capillaries used were coated Beckman SDS capillaries, 37 or 57 cm long (30 or 50 cm to the detector) × 100 μm internal diameter (i.d.). They were rinsed at high pressure (20 psi) for 10 min with 1 M HCl and for 10 min with non-polyacrylamide, SDS-containing linear gel buffer (eCAP SDS 14-200 gel buffer) before each run. For electrophoresis, the protein sample was dissolved in 100 μL of diluted SDS sample buffer (final conc.: 60 mM Tris-HCl, pH = 6.6, containing 0.5% wt/vol SDS). After the addition of 10 μL Orange G marker (0.1% wt/vol) and 5 μL mercaptoethanol (Sigma), the tubes were heated at 100°C for 10 min in a Block Incubator (Astec, Fukuoka, Japan), cooled in an ice bath for 3 min, and centrifuged for 5 min at 12,879 × *g* and 4°C in a microtube centrifuge (MRX-151; Tomy Seiko Co., Ltd., Tokyo, Japan). Forty microliters of the supernatant was then placed in a 0.2-μL microvial prior to injection. Each sample was injected automatically by low pressure (0.5 psi) for 60 s into a capillary thermostated at 20°C and filled with SDS gel buffer, which was replaced between each run. Separation was run at 20°C with reverse polarity (i.e., cathode at the detector end of the capillary) and at a constant voltage of 11 or 17 kV (300 V/cm), ramped over 0.5 min. The migration of separated proteins past the UV detector was monitored by absorbance at 214 nm. The chromatographic data were collected at a rate of 4 Hertz, and analyzed by the 32 Karat Software to obtain relative migration times (RMT).

Radioiodination of apoA-I and HDL by the IODO Beads Iodination Reagent. Commercially available lyophilized human apoA-I or purified rabbit HDL was iodinated with $Na^{125}I$ in 0.1 N NaOH using the IODO Beads Iodination Reagent and manufacturer-recommended protocols. Excess $Na^{125}I$ was removed by gel filtration using a PD-10 column

(Amersham Pharmacia Biotech, Tokyo, Japan) and dialyzed against a solution of 0.85% NaCl. Human ^{125}I-apoA-I (specific activity: 2114 dpm/ng) was associated with unlabeled rabbit HDL overnight at 4°C and purified by ultracentrifugation before injection into the marginal auricular vein of Group 1 rabbits, as we described previously (25,38). Briefly, the mixture of human ^{125}I-apoA-I and rabbit HDL was adjusted to a density of 1.21 g/mL using solid KBr and ultracentrifuged using a TLA-100.3 rotor for 10 h at 541,000 × *g* and 4°C. The 1.21 g/mL supernatant was isolated by tube slicing, and the unbound iodide was removed by extensive dialysis against a solution of 0.85% NaCl. Rabbit ^{125}I-HDL (specific activity: 295 dpm/ng) was mixed with unlabeled rabbit HDL to reduce radiation damage before dialysis and injected into the marginal ear veins of Group 3 rabbits.

Radioiodination of apoA-I and HDL by the ICl method. Purified rabbit HDL or apoA-I was radioiodinated with ^{125}I-Na according to the detailed procedure of Goldstein *et al.* (14), which was based on Bilheimer's modification (32) of the iodine monochloride method of McFarlane (31).

ICl was made according to Goldstein's procedure as a stock solution of 33 mM at 4°C in a brown bottle and diluted 30-fold with 2 M NaCl before use (14).

HDL was dialyzed against 0.5 M glycine-NaOH buffer (pH = 10) for about 30 min. Carrier-free Na^{125}I in 0.1 M NaOH was added, and this was immediately followed by the rapid and forceful injection of diluted ICl using a syringe. The quantity of ICl added was calculated so that the efficiency of iodination was about 50% (31). TCA precipitation was performed as previously described (12). Radiolabeled HDL (specific activity: 251 dpm/ng) was diluted with unlabeled rabbit HDL to reduce radiation damage. HDL was dialyzed against a solution of 0.85% NaCl to remove unbound radioactivity, and then injected into the marginal auricular veins of Group 4 rabbits.

ApoA-I was radioiodinated as in the method used for HDL labeling except that commercial lyophilized human apo A-I or concentrated rabbit apoA-I isolated by the guanidine HCl method was mixed with an equal volume of 1 M glycine NaOH buffer (pH = 8.5) before adding Na^{125}I and ICl. Human ^{125}I-apoA-I (specific activity: 604 dpm/ng) was associated with unlabeled rabbit HDL at 4°C overnight and purified by ultracentrifugation (25,38) before injection into the marginal auricular veins of Group 2 rabbits. Rabbit ^{125}I-apoA-I (specific activity: 613 dpm/ng) separated by the guanidine HCl method was reassociated with rabbit plasma for 30 min at 37°C, as described by Brousseau *et al.* (39), and injected into Group 5 rabbits. The same rabbit apoA-I (specific activity: 425 dpm/ng) was also injected as free apoA-I (Group 6.A), or after *in vitro* incubation with unlabeled rabbit HDL (Group 6.B). Rabbit ^{125}I-apoA-I (specific activity: 654 dpm/ng) isolated by the guanidine HCl method but stored directly in a lyophilized form was injected into Group 7 rabbits, and rabbit ^{125}I-apoA-I (specific activity: 671 dpm/ng) that was stored in a lyophilized form after extraction by organic solvents was injected into Group 8 rabbits.

In vivo *kinetics studies in rabbits.* After sterile-filtration through a 0.22-μm Millipore filter, which absorbs very little protein, 10 to 20 μCi of radiolabeled apoA-I was injected into the marginal ear vein in each group of rabbits. To prevent sequestration of radioiodide resulting from apo catabolism, 3 mg of NaI was injected into each rabbit 30 min prior to the injection of labeled apoA-I. Blood samples were taken from the marginal auricular vein on the other side at 10 min, 3 h, 6 h, 12 h, and 24 h, and daily for 6 d. The radioactivity decay curves were constructed by counting plasma radioactivity at each time point. Specific radioactivity decay curves were constructed for six of the Group 4 rabbits (Group 4.A) by measuring specific activity of apoA-I separated from plasma at each time point by SDS-PAGE in a disc gel apparatus as previously described (12,17,25,40).

Data analysis and calculation of kinetic parameters. Data are presented as mean ± SD unless otherwise indicated. All of the data analysis was performed using the SAS Software Package (Version 6.12, Statistical Analysis System, SAS Institute, Cary, NC) at Fukuoka University (Fukuoka, Japan). The radioactivity decay curves of apoA-I required two exponentials for adequate fitting, which was performed using a custom SAS program. The FCR was calculated as the reciprocal of the area under the decay curves, as described previously (12). The FCR values were compared among groups of rabbits by an ANOVA using the general linear model procedure (41). The significance level was considered to be 5% unless indicated otherwise.

RESULTS

Determination of the in vivo *fractional catabolic rates of human apoA-I and rabbit HDL radioiodinated by the ICl method and IODO Beads Iodination Reagent.* Figure 1 shows the mean radioactivity decay curves (fraction of initial plasma radioactivity) of human apoA-I (Fig. 1A) and rabbit HDL (Fig. 1B) radioiodinated using the IODO Beads Iodination Reagent and the ICl method. As shown in Figure 1A, human apoA-I labeled with ^{125}I by the IODO Beads method (Group 1) apparently was cleared much faster from rabbit plasma than that labeled by the ICl method (Group 2). As shown in Figure 1B, intact HDL ^{125}I-labeled by the IODO Beads Iodination Reagent (Group 3) was also apparently cleared much faster from rabbit plasma than that labeled by the ICl method (Group 4). The FCR values are shown in Figure 2. As shown, Group 1 (1.576 ± 0.094 pools/d) and Group 3 (1.678 ± 0.619 pools/d) rabbits had significantly higher FCR values than Group 2 (0.976 ± 0.031 pools/d) and Group 4 (0.583 ± 0.033 pools/d) rabbits, respectively, as assessed by ANOVA.

The FCR data of human apoA-I radioiodinated by the ICl method in Group 2 rabbits (0.976 ± 0.031 pools/d) are similar to the value of 1.2 ± 0.1 pools/d for unlabeled human apoA-I in rabbits as reported by Braschi *et al.* (29), suggesting that the ICl method is a more appropriate radioiodination method for the *in vivo* kinetics study of apoA-I.

To evaluate the *in situ* and exogenous apoprotein radiolabeling techniques of the ICl method, we isolated rabbit

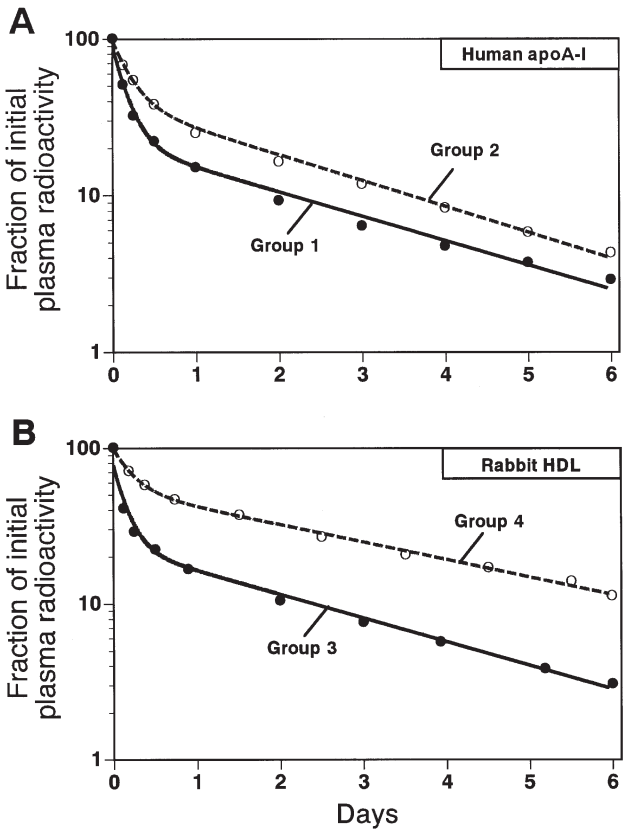

FIG. 1. Mean radioactivity decay curves (fraction of initial plasma radioactivity) of (A) human apolipoprotein (apo) A-I and (B) rabbit HDL radioiodinated by the iodine monochloride (ICl) method (○) of McFarlane (31) as modified by Bilheimer *et al.* (32) and the commercial IODO Beads Iodination Reagent (●).

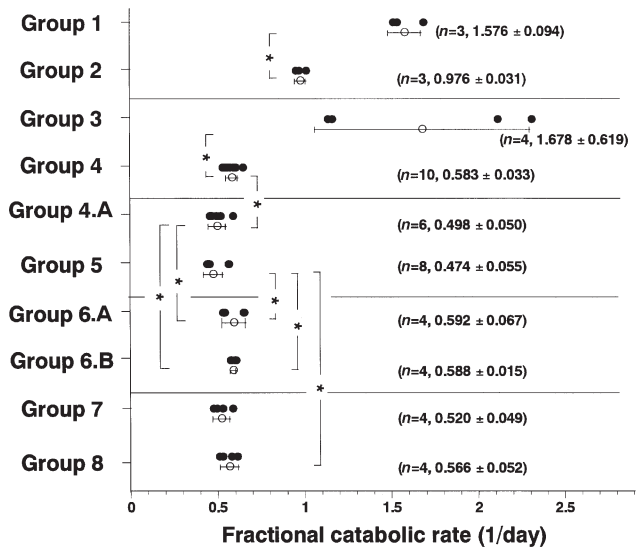

FIG. 2. Fractional catabolic rates (FCR) of human apoA-I (Groups 1 and 2), rabbit HDL (Groups 3 and 4), and rabbit apoA-I (Groups 5, 6.A, 6.B, 7, and 8) in rabbits. Each group of rabbits is defined in Table 1. In Group 4.A, the FCR was determined from the fractional specific radioactivity decay curve of apoA-I. Solid circles (●) indicate FCR in each individual rabbit. Open circles (○) and error bars indicate mean values and SD of FCR in each group of rabbits; the actual data are also given in parentheses. An asterisk (*) indicates a significant difference ($P < 0.05$) in FCR between groups of rabbits as assessed by ANOVA. For other abbreviation see Figure 1.

apoA-I using the guanidine HCl method of Vega *et al.* (16), and examined the isolated rabbit apoA-I by gel electrophoresis.

Electrophoretic characterization of rabbit apoA-I. The apparent M.W. of rabbit apoA-I freshly isolated by the guanidine HCl method was determined by co-injecting eCAP protein M.W. markers with rabbit apoA-I into a 57-cm-long capillary for separation. The apparent molecular mass of rabbit apoA-I calculated from the calibration curve based on its RMT (data not shown) was 22.4 kDa, which is similar to the value of 25 kDa reported by Borresen and Kindt (42) and smaller than that (28.3 kDa) reported for human apoA-I by Schaefer *et al.* (43).

Contamination of the isolated HDL and apoA-I samples was checked by SDS-CGE. The separation of apoA-I was completed within 15 min using a 37-cm-long capillary, and each of the three preparations of rabbit apoA-I presented a single homologous peak (data not shown), indicating that apoA-I isolated by the guanidine HCl was very pure and no apparent degradation occurred during the isolation process.

The same samples were also examined by the conventional SDS-PAGE: Each apoA-I preparation showed one homologous band, and the contaminating serum albumin was not detectable (data not shown).

Rabbit apoA-I preparations radioiodinated with ^{125}I by the ICl method and IODO Beads Iodination Reagent were checked by autoradiography (Fig. 3). As shown in Figures 3A and 3B, all of the preparations of radioiodinated apoA-I showed a unique band at the position of apoA-I, and we detected no apparent aggregation of apoA-I in each radioiodinated apoA-I preparation. Autoradiography of apoA-I radioiodinated by the IODO Beads Iodination Reagent also showed a minor band that migrated in front of the SDS-PAGE gel but after the tracking dye (Fig. 3B, lane 3). This minor band could represent degraded proteins produced during iodination of apoA-I and subsequent storage, since apoA-I has been reported to undergo disaggregation during radioiodination by chloramine T (44), a stronger oxidizing agent than IODO Beads Iodination Reagent; and this process will continue if the material is stored. However, for apoA-I radioiodinated by the ICl method (Fig. 3, lanes 1, 2, and 4), only a very faint band was observed at the same position. Therefore, the IODO Beads Iodination Reagent could give less accurate results than the ICl method in metabolic studies since it produced an unacceptable amount of oxidative denaturants.

Determination of the in vivo *fractional catabolic rates of rabbit HDL apoA-I radioiodinated* in situ *and exogenously by the ICl method.* The *in situ* and exogenous radiolabeling techniques of the ICl method were compared between Group 4.A rabbits, in which the specific radioactivity decay curve of *in situ* ^{125}I-labeled HDL apoA-I was constructed by isolating apoA-I from the HDL in plasma, and Group 5 rabbits (Fig. 4A). As shown in Figure 4A, the mean radioactivity decay curve of exogenously ^{125}I-labeled apoA-I isolated and used

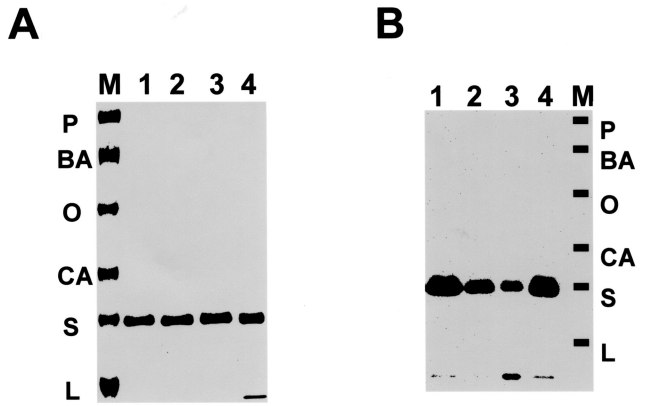

FIG. 3. SDS-PAGE (A) and autoradiograms (B) of rabbit apoA-I radioiodinated by the IODO Beads Iodination Reagent (lane 3) and the ICl method (lanes 1, 2, and 4). Lane 1: freshly isolated apoA-I subjected to lyophlization; 2, freshly isolated apoA-I subjected to organic solvent extraction and lyophilization; 3 and 4, fresh rabbit apoA-I isolated by the guanidine HCl method. M, SDS protein markers (L = lysozyme, S = soybean trypsin inhibitor, CA = carbonic anhydrase, O = ovalbumin, BA = bovine serum albumin, P = phosphorylase) ranging from M_r 21.3 to 112.0 kDa. For abbreviations see Figure 1.

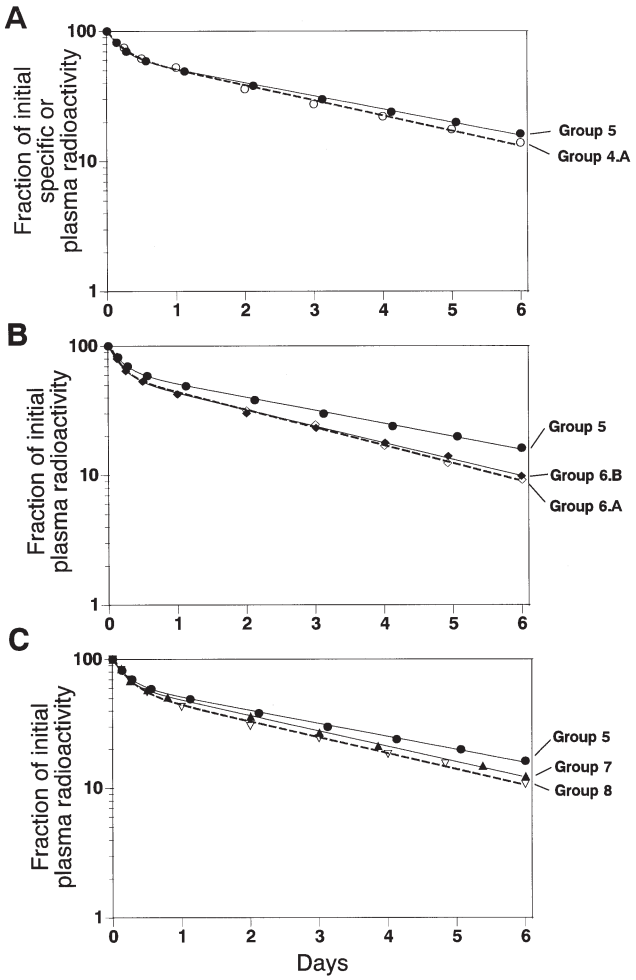

FIG. 4. (A) Mean specific radioactivity decay curve of rabbit apoA-I radioiodinated *in situ* as part of intact HDL (Group 4.A, ○), and mean radioactivity curves of exogenously radioiodinated rabbit apoA-I freshly isolated by the guanidine HCl method and reassociated with rabbit plasma before injection (Group 5, ●) (B). Mean radioactivity decay curves of ^{125}I-apoA-I freshly isolated and injected as free apoA-I (Group 6.A, ◇) or incubated with unlabeled rabbit HDL before injection (Group 6.B, ◆). (C) Mean radioactivity decay curves of ^{125}I-labeled apoA-I freshly isolated but lyophilized (Group 7, ▲) or delipidated by organic solvent extraction before lyophilization (Group 8, ▽).

fresh (Group 5) was initially superimposed on the specific radioactivity decay curve of apoA-I that was labeled *in situ* as part of intact HDL (Group 4.A) and differed only slightly after 3–6 d. As shown in Figure 2, the FCR values in Group 5 rabbits (0.474 ± 0.055 pools/d) were very similar to those in Group 4.A rabbits (0.498 ± 0.050 pools/d), suggesting that free ^{125}I-labeled rabbit apoA-I prepared by the guanidine HCl method and injected after reassociation with rabbit plasma has kinetic characteristics similar to those of apoA-I as a part of intact HDL in rabbits. This result confirms the finding of Vega *et al.* in human subjects (16).

Since the secondary structure of apoA-I changes dramatically upon interaction with lipids, plasma lipoproteins, and other nonpolar interfaces (27,45), which may affect the metabolic properties of apoA-I, and since Shepherd *et al.* (24) suggested that the differences in metabolism of ^{125}I-apoA-I intercalated into HDL *in vitro* and its counterpart labeled *in situ* in lipoprotein may be a function of the intercalation process, we compared the metabolism of rabbit ^{125}I-labeled (by the ICl method) apoA-I freshly isolated by guanidine HCl method but injected as free apoA-I (Group 6.A) or after *in vitro* incubation with rabbit HDL (Group 6.B) with that of rabbit ^{125}I-labeled apoA-I freshly isolated and reassociated with rabbit plasma (Group 5). As shown in Figure 4B, ^{125}I-apoA-I injected as free apoA-I (Group 6.A) or after association with rabbit HDL (Group 6.B) was catabolized slightly faster than that injected after reassociation with rabbit plasma (Group 5). As shown in Figure 2, FCR values in Group 6.A (0.592 ± 0.067 pools/d) and Group 6.B (0.588 ± 0.015 pools/d) rabbits were significantly ($P < 0.05$), albeit only slightly, greater than those in Group 5 (0.474 ± 0.055 pools/d) or Group 4.A rabbits (0.498 ± 0.050 pools/d), suggesting that *in vitro* incubation affects the metabolism of exogenously radioiodinated apoA-I.

To examine the effects of the storage and isolation process of apoA-I on its *in vivo* kinetics, we compared the metabolism of exogenously radioiodinated (by the ICl method) rabbit apoA-I freshly isolated but stored as a lyophilized form (Group 7) or extracted by organic solvent before lyophilization (Group 8) with that of rabbit apoA-I freshly isolated and radioiodinated without storage (Group 5). As shown in Figure 4C, the catabolism of ^{125}I-apoA-I in Groups 7 and 8 was very slightly faster than that in Group 5. As shown in Figure 2, although differences in the FCR values between Group 7 (0.520 ± 0.049 pools/d) and Group 5 rabbits (0.474 ± 0.055 pools/d) did not reach statistical significance, the FCR values in Group 8 (0.566 ± 0.052 pools/d) were very slightly but

significantly higher than those in Group 5 (0.474 ± 0.055 pools/d) rabbits, suggesting that care should be taken in isolating and storing apoA-I when performing *in vivo* kinetics studies.

DISCUSSION

Since apoA-I is the major protein of HDL, most previous studies have investigated the kinetics of HDL using trace-labeled apoA-I. The ICl radioiodination method of McFarlane (31), as modified by Bilheimer *et al.* (32), has been widely used to radiolabel apoproteins and lipoproteins. Very recently, Braschi *et al.* (28) used a commercially available radioiodination reagent, IODO Beads Iodination Reagent, to study the kinetics of reconstituted HDL particles. Their later study (29) compared the catabolic rates of unlabeled apoA-I and apoA-I radioiodinated by IODO Beads Iodination Reagent. The present study directly compared the catabolic rates of apoA-I radioiodinated by the IODO Beads method and ICl method to clarify whether radioiodination itself may be responsible for the higher turnover of apoA-I radioiodinated by the IODO Beads method as compared to unlabeled apoA-I, as reported by Braschi *et al.* (29).

Our results, that both human apoA-I and rabbit HDL radioiodinated using IODO Beads Iodination Reagent were cleared much faster from plasma of rabbits than those radio iodinated by the ICl method (Figs. 1,2), agree with those of Braschi *et al.* (29), who reported that human apoA-I, human reconstituted HDL (LpA-I), and exchange-labeled human HDL_3 were cleared much faster than unlabeled proteins from plasma of rabbits if radioiodinated by IODO Beads Iodination Reagent. Although radioiodination by both the IODO Beads Iodination Reagent (29) and ICl method (27,30) modifies the structural properties of apoA-I, our result that human apoA-I radioiodinated by the ICl method (Fig. 2, Group 2) gave kinetic parameters similar to those of unlabeled human apoA-I in rabbits (29) suggests that the *in vivo* kinetics of apoA-I radioiodinated by the ICl method is affected to a much lesser extent than that of apoA-I radioiodinated by the IODO Beads method. Our autoradiography result (Fig. 3), which suggests that oxidative degradation of apoA-I during radioiodination by the IODO Beads Iodination Reagent may contribute to the accelerated metabolism of apoA-I, supports this notion.

It is possible that the higher turnover of human apoA-I radioiodinated by the IODO Beads method (Fig. 1A) may simply reflect the increased oxidation of apoA-I and its removal by reticuloendothelial cells caused by overiodination of the protein, since the specific activity of apoA-I radioiodinated by the IODO Beads method was about 3.5-fold greater than that of apoA-I radioiodinated by the ICl method. This is supported by the fact that the specific activity of ^{125}I-labeled apoA-I in the study by Braschi *et al.* (29) (2200 dpm/ng) was also much greater than the criterion of Goldstein *et al.* (14) (200–600 dpm/ng). However, our finding that HDL radioiodinated by the IODO Beads method was also catabolized faster from rabbit plasma than that radioiodinated by the ICl method, even though the specific activities of the radiolabeled proteins were similar (295 and 251 dpm/ng), suggests that overradioiodination of the protein may not be the only cause of the difference in the two radioiodination methods.

By using a procedure in which apoA-I is radiolabeled exogenously by the ICl method in its lipid-free form and then incorporated into plasma HDL by *in vitro* or *in vivo* incubation (15,24,39), the kinetics of the metabolism of individually labeled apoA-I can be quantified much more easily than with a procedure in which whole HDL is labeled directly and decay curves of specific activity of apoA-I are constructed by SDS-PAGE. Thus, the former approach is preferred. Although we and other authors demonstrated that the kinetic parameters of apoA-I radioiodinated by the two approaches are not strictly comparable (12,13,17,24–26), the study of Vega *et al.* (16) in a relatively large number of human subjects did show that human apoA-I exogenously radioiodinated by the ICl method has a kinetic behavior similar to that of apoA-I radioiodinated *in situ* as a part of HDL. Therefore, the present study is performed to examine whether the method of Vega *et al.* (16) can be an effective approach to study the *in vivo* kinetics of apoA-I in animals.

In the study of Vega *et al.* (16), the authors reported a simple but high-yield technique to isolate human apoA-I that uses the fact that only apoA-I dissociates from HDL at a low concentration of guanidine HCl, as first reported by Nichols *et al.* (34). We isolated rabbit apoA-I using the guanidine HCl method of Vega *et al.* (16), and checked our rabbit apoA-I preparations by using the recent technique of capillary SDS gel electrophoresis, which is useful for estimating the quality and integrity of an apoA-I preparation (37). The electropherograms indicate that apoA-I isolated by the guanidine HCl method is of high purity (data not shown). The yield of apoA-I is also high: We stably obtained about 500 μg apoA-I protein from 36 mL of normal rabbit plasma.

Our finding, that the metabolism of rabbit apoA-I isolated by the guanidine HCl method, radioiodinated freshly, and injected into rabbits after reassociation with rabbit plasma was similar to that of rabbit apoA-I radioiodinated *in situ* on whole HDL, agrees with the results of Vega *et al.* (16) and others (15,18) in human studies. Our FCR data of apoA-I in Japanese White rabbits (Fig. 2, Group 5, 0.474 ± 0.055 pools/d) were similar to those reported by Brousseau *et al.* (39) in New Zealand White rabbits (0.528 ± 0.036 pools/d). Our results are consistent with the experiment of Shepherd *et al.* (24), who found that apoA-I directly labeled with ^{131}I was not catabolized differently from ^{125}I-apoA-I labeled on HDL and isolated before injection, supporting the notion that radioiodination by the iodine monochloride method itself may not account for the faster catabolism of exogenously radiolabeled apoA-I as reported by Shepherd *et al.* (24).

The discrepancies among the results of the previous studies using different labeling procedures (12–16) are not yet clearly explainable. We investigated the effects of *in vitro* incubation and storage and isolation process of apoA-I on the

in vivo kinetics of apoA-I exogenously radioiodinated by the ICl method in rabbits.

The finding that the metabolism of ^{125}I-apoA-I intercalated into HDL by *in vitro* incubation was slightly faster than that of apoA-I radioiodinated *in situ* on whole HDL (Figs. 2, 4B) agrees with that of Shepherd *et al.* (24) in human subjects and confirms our previous finding in rabbits that rabbit apoA-I_4 and apoA-I_5, the major isoforms of rabbit apoA-I, when radioiodinated exogenously by the ICl method and injected after *in vitro* incubation with unlabeled rabbit HDL, had FCR values (25) that were greater than that of HDL apoA-I radioiodinated *in situ* as whole HDL (17).

Since apoA-I dissociates from HDL during ultracentrifugation (46) and the conformation and surface charge of lipoprotein containing apoA-I are suggested to affect the clearance of LpA-I from plasma (28), it is possible that compositional changes of HDL may occur during the isolation process and thus affect the catabolic rates of the exchange-labeled HDL apoA-I. In fact, our results show that apoA-I exogenously radioiodinated by the ICl method and reassociated with rabbit plasma before injection was cleared from plasma similar to that radioiodinated *in situ* as a part of HDL (Fig. 2).

Our result, that ^{125}I-apoA-I injected as free apoA-I was catabolized faster than apoA-I radioiodinated *in situ* on whole HDL (Figs. 2 and 4B), is consistent with that of Malmendier *et al.* (47). However, Schaefer *et al.* (15) and Vega *et al.* (16), who diluted the free radioiodinated apoA-I with human serum albumin to minimize radiation damage and injected the radioiodinated apoA-I as a mixture consisting of the tracer and human serum albumin, reported a similar metabolism for apoA-I radioiodinated exogenously or *in situ* as intact HDL. In addition, Malmendier *et al.* (47) reported that the metabolism of radioiodinated apoA-I complexed with PC was not different from that of apoA-I labeled *in situ* on whole HDL. Although there is still no clear explanation for these phenomena, our finding, together with those of other authors (15,24,47), indicates that *in vitro* incubation before injection affects the metabolism of exogenously radioiodinated apoA-I.

Since our preparation of rabbit apoA-I by the guanidine HCl method was used fresh without storage, and it was not necessary for it to be treated with organic solvents as in the delipidation of HDL, a step that is generally needed in the separation process of apoA-I by other methods, we tested the effects of lyophilization and both the delipidation and lyophilization on the metabolism of our isolated apoA-I in rabbits.

Our findings, that rabbit apoA-I freshly isolated but subjected to delipidation and lyophilization was catabolized only slightly faster from the plasma of rabbits than apoA-I isolated and used fresh without storage (Figs. 2 and 4C), suggest that the preparation and storage of apoA-I may not be major factors responsible for the discrepancies among results of previous studies using different labeling procedures for the ICl iodination method (12,13,17,24–26), since the small difference could also be due to interexperimental variations. That there was no significant difference in the turnover of fresh and lyophilized ^{125}I-apoA-I suggests that lyophilization may not significantly change the conformation of apoA-I, since Braschi *et al.* (28) demonstrated that the conformation of apoA-I radioiodinated with IODO Beads Reagent and reconstituted into HDL plays a central role in regulating the catabolism of reconstituted HDL.

Therefore, the guanidine HCl method should be a preferable method for isolating apoA-I for metabolic studies since it is simple and the time needed in the isolation process is relatively short. In fact, the guanidine HCl treatment has also been shown to be a simple and less time-consuming method for delipidation of HDL as compared to the delipidation method by organic solvents (48). ApoA-I dissociates extensively upon exposure of human HDL to guanidine HCl, and the dissociated apoA-I did not recombine with HDL after removal of guanidine HCl (34). We expect that this method for isolating apoA-I should be applicable in other animal models.

This is the first direct comparison of radioiodination using the IODO Beads Iodination Reagent with the ICl method of McFarlane (31) as modified by Bilheimer *et al.* (32) and shows that the latter is more suitable for metabolic studies. Our results suggest that apoA-I prepared by the guanidine HCl method (16) and freshly radiolabeled is an appropriate marker for *in vivo* kinetics studies in rabbits and that the *in vitro* incubation conditions of radioiodinated apoA-I may be the most important factors that affect the *in vivo* kinetics of apoA-I. Our findings should contribute greatly in evaluating the effects of drugs on HDL metabolism, investigating the mechanism of atherosclerosis, and studying the *in vivo* kinetics of other apoproteins.

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Protective Action on Human LDL Against Oxidation and Glycation by Four Organosulfur Compounds Derived from Garlic

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ABSTRACT: Human LDL were used to study the protective action of four organosulfur compounds (diallyl sulfide, DAS; diallyl disulfide, DADS; *S*-ethylcysteine, SEC; *N*-acetylcysteine, NAC) derived from garlic against oxidation and glycation. The four organosulfur compounds significantly inhibited superoxide production by xanthine-xanthine oxidase ($P < 0.05$) and showed marked copper-chelating capability. DAS and DADS exhibited greater antioxidant activities against copper- and amphotericin B-induced LDL oxidation ($P < 0.05$) than SEC and NAC. However, SEC and NAC were more effective in sparing LDL α-tocopherol ($P < 0.05$). When oxidation was minimized, SEC was the most powerful agent against LDL glycation ($P < 0.05$); however, DADS was superior to other agents in suppressing both oxidation and glycation when LDL oxidation occurred simultaneously with glycation. These results suggest that the four organosulfur compounds derived from garlic are potent agents for protecting LDL against oxidation and glycation, and that they may benefit patients with diabetes mellitus or cardiovascular diseases by preventing complications.

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Both LDL oxidation and glycation are strongly related to diabetic complications, atherosclerosis, and other cardiovascular diseases (1–4). Glycated LDL reportedly is more susceptible to oxidation than unglycated LDL, and oxidized LDL is more prone to modification by glycation (4–6). Since LDL oxidation and glycation are closely interrelated, and both are important factors contributing to the development of diabetic and cardiovascular pathogenesis, the development of proper agents with both antioxidative and antiglycative properties may provide medical benefits.

Several studies have reported that organosulfur compounds derived from garlic and onion possess marked antioxidant and antimicrobial activities (7–10). Earlier, we observed that two lipophilic organosulfur compounds, diallyl sulfide (DAS) and diallyl disulfide (DADS), and two hydrophilic organosulfur compounds, *S*-ethylcysteine (SEC) and *N*-acetylcysteine (NAC), show marked antioxidant protection for isolated human erythrocytes *via* nonenzymatic actions (11). Whether these natural agents are able to protect LDL against oxidation and glycation remains unknown. On the other hand, our study found DAS and DADS show greater antifungal effects than the antibiotic amphotericin B (AmB) (12), which is often used for invasive fungal infection therapy (13–15). The use of AmB often enhances LDL oxidation and atherosclerotic development (14,15). Therefore, our present study also examined the antioxidant effects of these organosulfur agents against AmB-induced LDL oxidation.

Our objective was to study the protective effects on human LDL against oxidation and glycation by four organosulfur compounds derived from garlic. The results will provide information regarding the possible medical benefits of using these agents to prevent diabetic and cardiovascular complications.

MATERIAL AND METHODS

LDL preparation. Blood was drawn from healthy graduate students in Chungshan Medical University (Taichung City, Taiwan) after an overnight fast. The LDL fractions with density 1.006–1.063 were isolated from plasma by sequential ultracentrifugation (16). Native LDL was dialyzed against 1.5 mM PBS and sterilized with a porous filter (0.22 μM). The protein concentration of LDL was determined by the method of Lowry *et al.* (17) using BSA as a standard. In all experiments, the LDL fraction was diluted to a final concentration of 500 μg protein/mL using PBS.

α-Tocopherol analysis. The concentration of α-tocopherol in LDL was measured by the HPLC method of Palozza and Krinsky (18). In freshly prepared LDL, α-tocopherol content was 30.16 nmol/mg LDL protein.

Chelating effects on copper. The method described in Dillon *et al.* (19) was used to determine the chelating effect of DAS, DADS, SEC, and NAC on copper. This method is based on restoring the activity of xanthine oxidase, which is inhibited in the presence of copper. Thus, if these organosulfur agents could chelate copper, the xanthine oxidase activity would be restored.

Superoxide production and xanthine oxidase activity. The restoring activity of xanthine oxidase was assayed by monitoring the production of superoxide ions. In this study, superoxide

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Abbreviations: AmB, amphotericin B; CD, conjugated diene; DADS, diallyl disulfide; DAS, diallyl sulfide; MDA, malondialdehyde; NAC, *N*-acetylcysteine; SEC, *S*-ethylcysteine; TBA, thiobarbituric acid.

production and xanthine oxidase activity were measured as cytochrome C reduction. Xanthine oxidase at 107 mU/mL and xanthine solution at 1.6 mM were prepared in PBS (pH 7.4). Superoxide ions were generated in a reaction volume of 1 mL containing 160 μM xanthine and 1.25 mg cytochrome C. Xanthine oxidase at 10.7 mU was added to initiate this reaction, and the production of superoxide ion was continuously monitored at 550 nm at 37°C for 2 h. Each of the four organosulfur agents was added at 10 μM along with or without 50 μM $CuSO_4$, and EDTA at 60 μM was added to 50 μM $CuSO_4$ as a positive control.

Organosulfur compounds and treatments. DAS (purity 97%) and DADS (purity 80%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). DADS was further purified by fractional distillation. The purity of each diallyl sulfide was then examined by an HPLC method (20) using a high-performance liquid chromatograph (Hitachi) equipped with a Supelco (Bellefonte, PA) LC18 column. Diallyl sulfides with purity greater than 95% were used in this study. NAC (99%) and SEC (99.5%) were purchased from Sigma Chemical Co. (St. Louis, MO). For determination of individual effectiveness of antioxidant action, the concentrations of DAS, DADS, NAC, and SEC were 5 and 10 μM. On the basis of lipid solubility, DAS or DADS was first dissolved in methanol and then added to an LDL suspension for final concentration preparation. The influence of residual methanol upon lipid oxidation in LDL was determined to be not significant (data not shown). NAC and SEC were added directly to the LDL solution.

AmB treatment. AmB (95%) was purchased from Sigma Chemical Co. AmB was dissolved in methanol first and then added to an LDL solution, with or without organosulfur compound treatment, for final concentrations of 5 and 10 μM AmB.

LDL oxidation measurement. One milliliter of $CuSO_4$ (10 μM) was used to initiate LDL oxidation in 10 mL of an LDL solution sample. After incubating the LDL solution at 37°C for 72 h, the method of Jain and Palmer (21) was used to measure malondialdehyde (MDA) formation (nmol/mg LDL protein). Briefly, 0.2 mL LDL solution was suspended in 0.8 mL PBS. Then 0.5 mL TCA (30%) was added. After vortexing and standing in ice for 2 h, samples were centrifuged at $1500 \times g$ for 15 min. Supernatant (1 mL) was mixed with 0.25 mL thiobarbituric acid (TBA) (1%), and the mixture was heated in a boiling water bath for 15 min. The concentration of MDA-TBA complex was assayed using a high-performance liquid chromatograph equipped with a reversed-phase Shodex KC-812 column (Showa Denko, Tokyo, Japan) with a UV-vis detector at 532 nm.

The formation of conjugated diene (CD), a lipid oxidation product, in LDL also was determined according to the method described by Esterbauer *et al.* (22). The lipid oxidation of an LDL solution containing 5 or 10 μM of each organosulfur agent was initiated at 37°C by 0.1 mM $CuCl_2$. Absorbance at 234 nm was continuously recorded for 60 min at 37°C by a Hitachi U-2001 spectrometer with a constant temperature recirculator. The lag phase, expressed in minutes, was defined as the period where no oxidation occurred. A longer lag phase indicated less CD formation.

In vitro *glycation of LDL.* LDL glycation was performed according to the method described in Li *et al.* (3). Briefly, 50 mM glucose in PBS (pH 7.4) was added to an LDL solution with and without organosulfur compound treatment. Sodium azide at 0.02% was used as antibiotic to prevent bacterial growth. This solution was sterile filtered, covered with N_2, and stored for 6 d at 37°C in the dark. After glycation, the solutions were dialyzed against PBS (20 mL against 4 L) at 4°C for 40 h. Then glycated LDL was separated from nonglycated LDL by applying a GlycoGel II column (Pierce, Rockford, IL), in which 500 μL LDL solution was loaded on the column, and glycated LDL was eluted with 2 mL sorbitol buffer, pH 10.25. Neither copper nor any other oxidant was used for the experiments on LDL glycation.

Measurement of LDL glycation. The method of Duell *et al.* (23) was used to measure LDL glycation level. LDL solution (200 μL) was mixed with 200 μL 4% $NaHCO_3$ and 200 μL 0.1% trinitrobenzoic acid. This mixture was flushed with N_2, sealed, and incubated at 37°C in the dark. After 2 h, the absorbance at 340 nm was measured spectrophotometrically. The blank was a mixture of LDL and $NaHCO_3$ in PBS. LDL glycation is reported as relative reduction in the level of free ε-amino groups of L-lysine when compared with LDL solution in the absence of glucose. During LDL glycation, samples were treated with or without EDTA (0.5 mM), and LDL oxidation level was also determined.

Statistical analysis. The effect of each treatment was analyzed from eight different preparations ($n = 8$). Data were subjected to ANOVA and computed using the SAS General Linear Model (GLM) procedure (24). Differences among means were determined by the least significant difference test with significance defined at $P < 0.05$.

RESULTS

The antioxidant activities for each organosulfur agent are presented in Table 1. The four organosulfur agents showed dose-dependent antioxidant protection on LDL, in which DADS had the greatest antioxidant activity, compared to DAS and the other two hydrophilic agents, against Cu^{2+}-induced MDA and CD formation ($P < 0.05$). The relationship between the four organosulfur agents and LDL α-tocopherol level is presented in Figure 1. The presence of SEC and NAC at 5 μM significantly increased the retention of α-tocopherol in LDL ($P < 0.05$), and SEC was more effective than NAC ($P < 0.05$).

The inhibitory effects on superoxide production and the copper-chelating effects of these agents are shown in Table 2. In the upper part of this table, where copper was absent, the four agents significantly inhibited the generation of superoxide ion by xanthine-xanthine oxidase ($P < 0.05$) DADS was a more effective superoxide scavenger than DAS, and these were followed by NAC and SEC ($P < 0.05$). In the lower part of this table, copper was present and inhibited the activity of xanthine oxidase. Thus, control groups showed no superoxide production. The presence of EDTA significantly restored the activity of xanthine oxidase and the production of superoxide because

TABLE 1
Antioxidant Activity[a] of Diallyl Sulfide (DAS), Diallyl Disulfide (DADS), *S*-Ethylcysteine (SEC), and *N*-Acetylcysteine (NAC) Against Cu^{2+}-Induced (A) Malondialdehyde (MDA) Formation (nmol/mg LDL protein) After a 72-h Incubation at 37°C, and (B) Conjugated Diene (CD) Formation at 37°C

Treatment	Conc. (μM)	MDA formation[b] (nmol/mg LDL protein)	CD formation lag phase (min)
Control[c]		45.4 ± 5.3[f]	8 ± 1.2[a]
DAS	5	26.7 ± 1.6[c]	15 ± 1.5[b]
	10	20.1 ± 2.1[b]	29 ± 1.0[d]
DADS	5	19.8 ± 2.4[b]	18 ± 2.1[c]
	10	14.3 ± 1.7[a]	38 ± 2.5[e]
SEC	5	32.6 ± 2.0[d]	12 ± 1.7[b]
	10	28.4 ± 2.8[c]	18 ± 2.3[c]
NAC	5	35.8 ± 2.5[e]	13 ± 2.0[b]
	10	31.3 ± 2.3[d]	20 ± 2.7[c]

[a]Least square means with a common superscript roman letter within a column are not different at the 5% level.
[b]MDA level of LDL at the beginning of incubation was 2.1 ± 1.3 nmol/mg LDL protein.
[c]Controls contained no antioxidant agent.

EDTA chelated copper ($P < 0.05$). The four organosulfur agents *via* their copper-chelating effects restored partial xanthine oxidase activity and superoxide production. SEC and NAC were the weaker copper chelators. Thus, more unchelated copper inhibited xanthine oxidase activity and resulted in less superoxide production ($P < 0.05$). Among these four compounds, DADS, as a more effective copper chelator, restored more xanthine oxidase activity, and thus more superoxide ions were produced.

AmB treatments at 5 and 10 μM significantly increased LDL oxidation levels as by determined MDA formation (Fig. 2, $P < 0.05$). However, the presence of the four organosulfur agents at 5 μM significantly reduced 10 μM AmB-induced LDL oxidation (Fig. 2, $P < 0.05$). Of these, DADS was the most effective agent.

LDL treated with 50 mM glucose and EDTA significantly increased glycation level (Table 3, $P < 0.05$). Under EDTA protection, the presence of any of the four organosulfur agents at 5 and 10 μM significantly reduced LDL glycation, and the most powerful antiglycative agent was SEC ($P < 0.05$). On the other hand, both LDL oxidation and LDL glycation significantly increased when LDL was treated with 50 mM glucose without EDTA protection (Table 3, $P < 0.05$). Although the presence of any of the four organosulfur agents at 5 and 10 μM significantly reduced both LDL oxidation and glycation when compared with controls ($P < 0.05$), the most antioxidative and antiglycative agent was DADS.

DISCUSSION

Several studies have reported that garlic extract can suppress LDL oxidation (19,23,26). The results of this study agree with those previous studies and verify that the four organosulfur

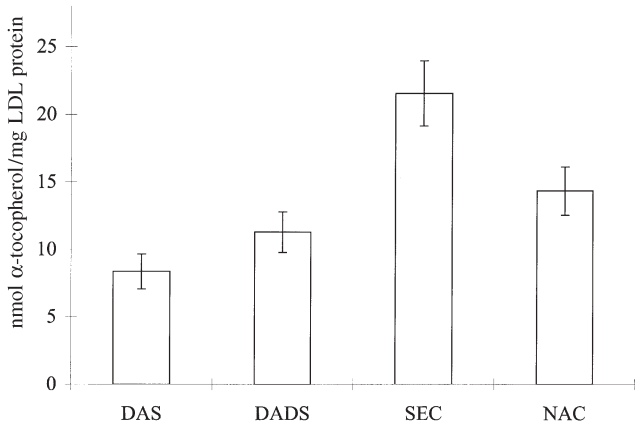

FIG. 1. The retention level of α-tocopherol in LDL treated with 5 μM DAS, DADS, SEC, and NAC after a 72-h incubation at 37°C. DAS, diallyl sulfide; DADS, diallyl disulfide; SEC, *S*-ethylcysteine; NAC, *N*-acetylcysteine. In freshly prepared LDL, α-tocopherol content was 30.16 nmol/mg LDL protein. Data are expressed as mean ± SD.

TABLE 2
Effect of 10 μM DAS, DADS, SEC, and NAC on Superoxide Production[a] by Xanthine/Xanthine Oxidase Without and With the Addition of 50 μM $CuSO_4$

Agent	$CuSO_4$	Superoxide production (ΔA 550 nm/min)[b]
Control[c]	–	0.042 ± 0.003[d]
DAS	–	0.029 ± 0.001[b]
DADS	–	0.025 ± 0.001[a]
SEC	–	0.033 ± 0.002[c]
NAC	–	0.035 ± 0.002[c]
Control[c]	+	0[a]
EDTA	+	0.039 ± 0.004[d]
DAS	+	0.024 ± 0.002[c]
DADS	+	0.023 ± 0.001[c]
SEC	+	0.015 ± 0.003[b]
NAC	+	0.019 ± 0.004[b]

[a]Least square means with a common superscript roman letter within a column are not different at the 5% level. Data are expressed as mean ± SD.
[b]ΔA 550 nm = absorbance difference at 550 nm as measured at time = 0 and at 120 min. For other abbreviations see Table 1.
[c]Control group contained no agent.

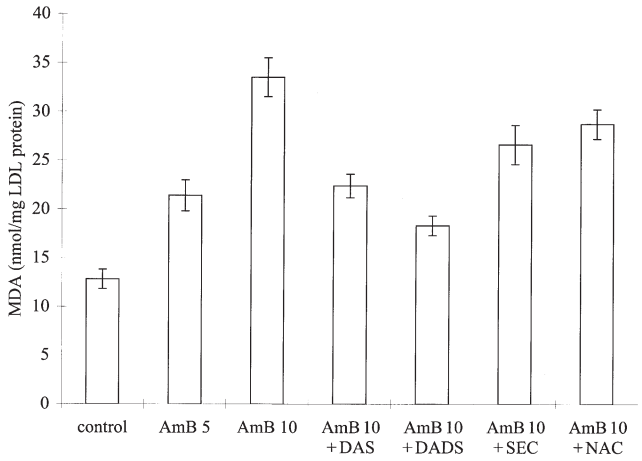

FIG. 2. The prooxidant effect of amphotericin B (AmB) at 5 and 10 μM (AmB 5, AmB 10), and the antioxidant protection of 5 μM DAS, DADS, SEC, and NAC against 10 μM AmB-induced malondialdehyde (MDA) formation (nmol/mg LDL protein) after a 48-h incubation at 37°C. For other abbreviations see Figure 1.

compounds contributed to the antioxidant protective activity of garlic on LDL (Table 1). Our previous study (11) indicated that these four agents possessed marked reducing power and iron-chelating capability. The present study further indicates that these agents are able to scavenge superoxide ions generated by xanthine-xanthine oxidase, chelate copper, and spare α-tocopherol in LDL (Table 2, Fig. 1). Based on these antioxidant characteristics, these organosulfur compounds are potent protective agents for LDL against oxidative damage.

Lynch and Frei (27) indicated that LDL oxidation is a causal step in atherosclerosis and that both superoxide and redox-active transition metal ions are implicated in this process. Dillon *et al.* (19) reported that garlic extract can inhibit superoxide production by xanthine-xanthine oxidase. Our study further indicates that DAS and DADS, two organosulfur compounds from garlic, inhibit superoxide production (Table 2, upper part). Swain and Gutteridge (28) also indicated that catalytic metal divalent ions such as copper and iron contribute to the prooxidant environment of the atherosclerotic lesion where LDL oxidation takes place extensively. Our previous study (11) as well as the present one indicate that DAS and DADS are effective chelators for both copper and iron. Since DAS and DADS can effectively scavenge superoxide ion and chelate transition metal ions, the use of these two compounds may be helpful in retarding LDL oxidation and in reducing the prooxidant effects of superoxide and metal divalent ions in the environment of the atherosclerotic lesion.

The dose-dependent prooxidant effect of AmB on LDL observed in our present study (Fig. 2) is in agreement with other studies (13–15) that indicated that AmB, with a great affinity to the lipid part of the LDL particle, could extract cholesterol from LDL and sensitize the modified LDL to oxidation. The four organosulfur compounds in this report exhibited marked antioxidant protection against AmB-induced oxidation (Table 1), and the reducing power and superoxide scavenging ability of these agents partially explains their antioxidant activities. These results support the use of these agents with AmB for fungal infection therapy in order to prevent AmB-induced oxidative damage in LDL. Like AmB, DAS and DADS also possess marked antifungal capability (12,29). In our previous study (12) the combined use of AmB with DAS or DADS showed synergistic anti-*Candida* and anti-*Aspergillus* effects. Therefore, the use of DAS or DADS with AmB may not affect the antifungal efficiency of AmB.

Nonenzymatic glycation of LDL is accompanied by oxidative, radical-generating reactions (4,6,30,31). In the presence of EDTA, oxidation was not responsible for the observed glycation; in this instance, glycation may be due simply to the interaction between LDL protein and glucose. Both SEC and NAC are hydrophilic amino acid derivatives. These proper-

TABLE 3
Protective Effect of 5 and 10 μM DAS, DADS, SEC, and NAC on LDL Against 50 mM Glucose-Induced Glycation and Oxidation With or Without 0.5 mM EDTA Treatment[a]

	With EDTA		Without EDTA	
Treatment	Glycation[b]	Oxidation[c]	Glycation	Oxidation
LDL	2.8 ± 1.0^{a,b}	2.1 ± 0.2^{a}	3.2 ± 1.1^{a}	20.4 ± 1.5^{a}
LDL + glucose	16.2 ± 2.5^{f}	2.3 ± 0.1^{a}	21.3 ± 3.2^{f}	57.3 ± 4.3^{h}
DAS, 5 μM	12.2 ± 1.8^{e}	2.0 ± 0.2^{a}	15.5 ± 1.6^{d}	41.5 ± 2.6^{e}
DAS, 10 μM	10.3 ± 1.4^{d,e}	1.9 ± 0.2^{a}	12.1 ± 1.0^{c}	36.2 ± 2.3^{d}
DADS, 5 μM	9.2 ±1.5^{d}	2.1 ± 0.1^{a}	11.4 ± 0.5^{c}	33.3 ± 1.8^{c}
DADS, 10 μM	7.2 ± 1.0^{c}	1.9 ± 0.2^{a}	8.8 ± 0.8^{b}	28.6 ± 2.0^{b}
SEC, 5 μM	5.2 ± 0.5^{b,c}	2.2 ± 0.2^{a}	17.0 ± 2.0^{e}	47.4 ± 3.1^{f}
SEC, 10 μM	2.0 ± 0.5^{a}	2.0 ± 0.3^{a}	14.3 ± 1.4^{d}	43.9 ± 2.7^{e}
NAC, 5 μM	7.1 ± 0.8^{c}	1.8 ± 0.1^{a}	18.2 ± 1.7^{e}	50.3 ± 3.5^{g}
NAC, 10 μM	4.3 ± 1.0^{b}	2.1 ± 0.2^{a}	14.4 ± 1.2^{d}	46.2 ± 3.0^{f}

[a]Least square means with a common roman letter superscript within a column are not different at the 5% level. Data are expressed as mean ± SD. For abbreviations see Table 1.
[b]Glycation is expressed as a percentage.
[c]Oxidation is expressed by MDA formation (nmol/mg LDL protein). MDA level of LDL at the beginning of incubation was 2.0 ± 1.1 nmol/mg LDL protein.

ties may allow them to exhibit a great affinity to glucose and/or LDL protein. Thus, these two agents are more efficient in protecting LDL protein and in interfering with the nonenzymatic Maillard reactions that occur between glucose and LDL protein. It has been reported that NAC can suppress the generation of N^6-(carboxymethyl)lysine, a product of glycation, and is a potent agent for glycoxidative injury that occurs in continuous ambulatory peritoneal dialysis (32). Our results verify that NAC is able to retard glycation, and we further found that SEC is a powerful agent against glycation, especially LDL glycation.

On the other hand, elevated levels of LDL glycation were observed when LDL oxidation was not suppressed. In this condition, the oxidation from LDL should be an important contributor toward the elevated glycation because DADS, a lipophilic agent, was the most powerful antioxidative and antiglycative agent. That is, DADS might first retard the oxidation that occurred in LDL lipid, and then retard the subsequent oxidation-related glycation. Such results bear out that LDL glycation is strongly related to its oxidation (2, 31), and also support the idea that delaying LDL oxidation is helpful in retarding LDL glycation.

The four agents are naturally formed in *Allium* foods such as garlic, Chinese leek, and onion. The content of DAS, DADS, and SEC was 250–480, 2600–4100, and 1900–2250 μg/kg garlic, respectively (12). Based on their natural properties and dietary availability, the use of these agents as antioxidative and/or antiglycative agents should be safe and acceptable.

In conclusion, four organosulfur agents that chelate copper and scavenge superoxide ions had marked dose-dependent antioxidative and antiglycative properties. These could benefit patients with diabetes or cardiovascular disease.

ACKNOWLEDGMENT

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Antioxidant Action of a Lipophilic Nitroxyl Radical, Cyclohexane-1-spiro-2′-(4′-oxyimidazolidine-1′-oxyl) -5′-spiro-1″-cyclohexane, Against Lipid Peroxidation Under Hypoxic Conditions

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ABSTRACT: Nitroxyl radicals are known to act as radical scavenging antioxidants. In the present study, a lipophilic nitroxyl radical, cyclohexane-1-spiro-2′-(4′-oxyimidazolidine-1′-oxyl)-5′-spiro-1″-cyclohexane (nitroxyl radical I) was synthesized and its antioxidant capacity was assessed in comparison with a hydrophilic nitroxyl radical, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (Tempol). Both nitroxyl radical I and Tempol inhibited methyl linoleate oxidation induced by free radicals, and the efficacy increased with decreasing partial pressure of oxygen, the effect being more pronounced for nitroxyl radical I than Tempol. Their hydroxylamines inhibited lipid peroxidation more effectively than their corresponding parent nitroxyl radicals. In liposomal membranes, a synergistic effect was observed in the combination of nitroxyl radical I with ascorbic acid, whereas only an additive effect was observed between Tempol and ascorbic acid. The present study suggests that nitroxyl radical I and its hydroxylamine may act as potent antioxidants, especially in combination with ascorbic acid under hypoxic conditions.

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Increasing experimental and clinical evidence exists showing that active oxygen species and free radicals are involved in the pathogenesis of various diseases, cancer, and aging (1). Aerobic organisms are protected from oxidative stress induced by active oxygen species by an array of defense systems, among them antioxidants having different functions. Obviously, one of the important functions of antioxidants is to scavenge active radicals, thereby inhibiting lipid peroxidation, protein modification, and DNA damage. Numerous natural and synthetic antioxidants have been explored and tested. Nitroxyl radicals are known to scavenge alkyl and peroxyl radicals effectively (2); they have been applied in a variety of biochemical studies (3–6) and have been widely used as spin labels and ESR imaging agents. Nitroxyl radicals of the quinoline and indoline types in particular were reported to scavenge not only alkyl radicals but also alkoxyl or peroxyl radicals (7,8).

The potency of antioxidants *in vivo* is known to be determined by many factors, such as the chemical reactivity toward the radical, distribution, mobility within the microenvironment, and interaction with other antioxidants (9). With these considerations in mind, we synthesized a lipophilic nitroxyl radical, cyclohexane-1-spiro-2′-(4′-oxyimidazolidine-1′-oxyl)-5′-spiro-1″-cyclohexane (nitroxyl radical I) as a potent antioxidant candidate. To elucidate the effects of the localization of this nitroxyl radical on its antioxidant activity, a hydrophilic nitroxyl radical, 4-hydroxy-2,2, 6,6-tetraethylpiperidine-*N*-oxyl (Tempol), was also studied for comparison.

In this study, the inhibitory effects of the nitroxyl radical and its reduced form, hydroxylamine, on lipid peroxidation were evaluated under different oxygen partial pressures. It is well known that oxygen concentration is maintained at low levels in living organisms. Thus, the reactivities of nitroxyl radicals in solution toward peroxyl and carbon-centered radicals were examined. Furthermore, the effects of ascorbic acid (vitamin C), on the antioxidative effect of nitroxyl radicals was assessed.

MATERIALS AND METHODS

Reagents. Nitroxyl radical I was prepared as reported previously (10). Briefly, 1-amino-1-cyanocyclohexane and cyclohexanone were stirred in a methanol solution at room temperature, after which an appropriate amount of 40% NaOH solution was added to this solution. White crystal cyclohexane-1-spiro-2′-(4′-oxyimidazolidine)-5′-spiro-1″-cyclohexane was obtained after continuous stirring for several hours at room temperature, and it was oxidized by hydrogen peroxide and sodium tungstate to give the nitroxyl radical I (Scheme 1). Tempol, L-α-phosphatidylcholine,β-linoleoxyl- γ-palmitoyl (PLPC), and L-α-phosphatidylcholine dimyristoyl (14:0 PC) were obtained from Sigma Chemical Co. (St. Louis, MO) and used as received. Methyl linoleate (MeLH) was obtained

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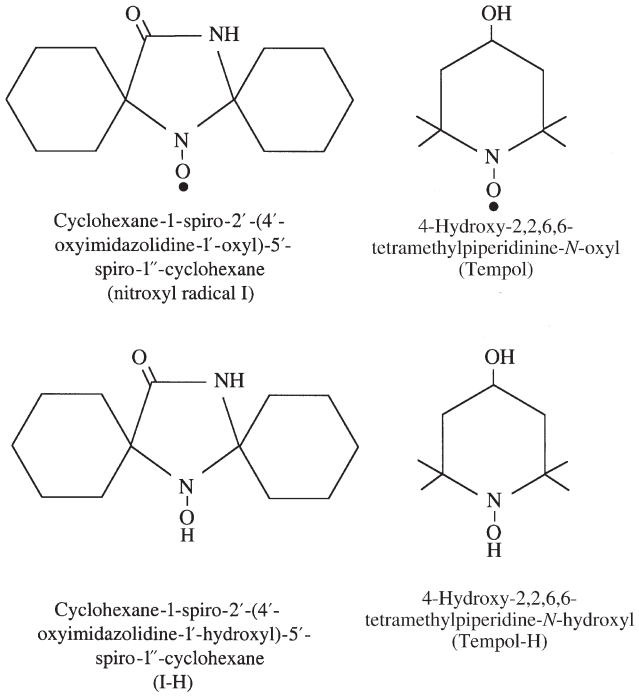
Abbreviations: 14:0 PC, L-α-phosphatidylcholine dimyristoyl; AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); I-H, cyclohexane-1-spiro-2′-(4′-oxyimidazolidine-1′-hydroxyl)-5′-spiro-1″-cyclohexane; MeLH, methyl linoleate; nitroxyl radical I, cyclohexane-1-spiro-2′-(4′-oxyimidazolidine-1′-oxyl)-5′-spiro-1″-cyclohexane; PLPC, L-α-phosphatidylcholine,β-linoleoxyl-γ-palmitoyl; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl; Tempol-H, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-hydroxyl.



SCHEME 1

from Tokyo Kasei (Tokyo, Japan). The azo compounds used as radical initiators, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) and 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), were obtained from Wako Pure Chemical Ind. (Osaka, Japan) and used as received. Other chemicals were of the highest grade available commercially. Mixed nitrogen/oxygen gas was purchased from Sumitomo Seika Chemicals Co. (Chiba, Japan). The nitroxyl radicals used in this study are shown in Scheme 1.

Hydroxylamine was isolated by the following method. A nitroxyl radical solution (50 μM in acetonitrile) was mixed with ascorbic acid (5 mM, 1% volume of nitroxyl radical solution), and the mixture was incubated for 2 h at room temperature. Three times by volume of chloroform/H_2O (2:1 by vol) was then added to the mixture. After vigorous mixing with a vortex mixer for 1 min, the chloroform layer was separated and evaporated to obtain a powder. Hydroxylamine was identified using an HPLC equipped with a photodiode array detector (SPD-M10AVP; Shimadzu, Kyoto, Japan) with an ODS column (particle size 5 μm; 4.6 × 250 mm; Wako), and acetonitrile/H_2O (6:4 by vol) was used as the eluent at 1.0 mL/min. The structure of these reduced forms was identified by LC–MS.

Competitive reactions between galvinoxyl and nitroxyl radicals. The reactions of nitroxyl radicals with alkyl and peroxyl radicals in the presence of galvinoxyl were performed at 37°C under air and nitrogen. AAPH and AMVN were used as water-soluble and lipophilic radical initiators, respectively. The experiments were carried out under nitrogen using a glass apparatus equipped with vacuum cock, two glass side arms, and a quartz cell for spectrophotometric analysis as follows. The galvinoxyl and nitroxyl radical solutions were taken into one of the side arms and the AMVN or AAPH solution into the other side arm. After both solutions were frozen with liquid nitrogen, the air in the vessel was evacuated by a rotary vacuum pump, and then nitrogen was introduced. Air was replaced by nitrogen in three freeze–thaw cycles, and the two solutions were mixed and introduced into the quartz cell. The decrease in galvinoxyl (initial concentration: 10 μM) was analyzed spectrophotometrically (UV-2450; Shimadzu, Kyoto, Japan) by following the maximum absorption of galvinoxyl at 428 nm.

Reduction of nitroxyl radicals by ascorbic acid. The reduction of nitroxyl radicals by ascorbic acid was measured in methanol or 14:0 PC liposome suspensions under air by following the ESR spectra of the nitroxyl radicals. Equal volumes of methanol solutions of 100 μM nitroxyl radical and ascorbic acid were mixed. When the reaction was carried out in the 14:0 PC liposome suspension, the 14:0 PC liposomal membrane incorporated with the nitroxyl radical was prepared first, then the aqueous ascorbic acid solution (1:100 by vol) was added to the liposomal solution. The initial concentrations of the nitroxyl radical, ascorbic acid, and 14:0 PC were 50 μM, 50 μM, and 2.83 mM, respectively, at the onset of mixing. ESR spectra were recorded on an X-band JEOL JES-TE 100 spectrometer under the following conditions: magnetic field, 335.3 mT; sweep time, 5 mT/min; microwave power, 1 mW; modulation frequency, 9.4 GHz; and modulation amplitude, 0.4 mT.

Inhibiting effect of nitroxyl radicals and hydroxylamine under hypoxic conditions. The oxidation of MeLH induced by AMVN was carried out in the presence of either the nitroxyl radical or hydroxylamine in an acetonitrile solution at 37°C under air and in hypoxic conditions. The air in the test tube was replaced with three different concentrations of oxygen, 1, 0.1, and 0.02%, following the procedure described above. The absorbance at 234 nm was followed spectrophotometrically to measure the formation of MeLH hydroperoxides.

Reaction of hydroxylamine with galvinoxyl. The reaction of hydroxylamine with galvinoxyl was carried out in acetonitrile at 37°C under air, and the rate was measured by following the decrease in absorbance at 428 nm with a spectrophotometer. The galvinoxyl (10 μM) was reacted with 5, 10, or 20 μM hydroxylamine.

Oxidation of PLPC liposomal membranes in the presence of nitroxyl radicals. The oxidation of PLPC was carried out in liposomal membranes as described previously (11). The PLPC hydroperoxides were measured with an HPLC equipped with a UV detector with an ODS column (particle size 5 μm; 4.6 × 250 mm; Wako), and methanol/40 mM phosphate buffer (9:1 by vol) was delivered as the eluent at 1.0 mL/min.

Partition coefficient of nitroxyl radicals and the corresponding hydroxylamines. To assess the lipophilicity of each nitroxyl radical and its corresponding hydroxylamine, the partition coefficient in 1-octanol and water was measured. 1-Octanol and water (1:1 by vol) were thoroughly mixed for

at least 12 h, and a known amount of nitroxyl radical or hydroxylamine was then added. The mixture was mixed with a vortex mixer for 2 min and then centrifuged for 10 min. Aliquots of the 1-octanol and water layer were measured by HPLC equipped with a photodiode array detector (SPD-M10A VP; Shimadzu).

All of the experiments in this paper were repeated several times, in general, and the results were reproducible within an experimental error of ±5%.

RESULTS

Reactivity of nitroxyl radicals toward alkyl and peroxyl radicals under air and nitrogen. Obviously, one of the most important factors determining antioxidant activity is reactivity of an antioxidant toward radicals; this can be measured by a competition method using a reference compound. In the present study, galvinoxyl was chosen as a reference, because it was bleached by the reaction with alkyl and peroxyl radicals. Galvinoxyl is reduced by the hydrogen-donating free radical scavengers and has been used to assess the antioxidant activity of various compounds (12). Galvinoxyl did not interact with nitroxyl radicals directly. Another well-known stable radical, 2,2-diphenyl-1-picrylhydrazyl, reacted with nitroxyl radicals spontaneously in solution (data not shown).

The reactivities of nitroxyl radical I and Tempol toward the alkyl and peroxyl radicals formed by the decomposition of azo compounds in the absence and presence of oxygen, respectively, were measured by following the decrease in maximum absorption of galvinoxyl at 428 nm. Figure 1 shows the decrease in absorption of galvinoxyl in the presence of either nitroxyl radical I or Tempol by the reaction with alkyl and peroxyl radicals formed by the decomposition of AMVN and AAPH at 37°C under nitrogen and air. As shown in Figure 1, neither nitroxyl radical I nor Tempol influenced the consumption rate of galvinoxyl under air. However, under nitrogen a remarkable decrease was observed for both nitroxyl radical I and Tempol: Nitroxyl radical I reduced the consumption rate more effectively than did Tempol with both AAPH and AMVN.

Reduction of nitroxyl radicals by ascorbic acid. The rate of reduction of nitroxyl radicals by ascorbic acid was measured by following the decrease in intensity of ESR. Nitroxyl radical I and Tempol decreased to less than 10% of the initial concentration in 2 h in the methanol solution (Fig. 2). In liposomal membranes, both nitroxyl radicals decreased at the same rate as in methanol at the initial phase, but the rate leveled off and half the nitroxyl radical I remained unreduced while 30% of the Tempol was not reduced.

Reaction of hydroxylamine with galvinoxyl. The reaction of hydroxylamine with galvinoxyl was carried out to assess the reactivity of the hydroxylamines of nitroxy radical I (I-H) and Tempol (Tempol-H), and the decrease in absorbance of galvinoxyl at 428 nm was followed. As shown in Figure 3, the rate of galvinoxyl consumption increased with the increase in hydroxylamine concentration. I-H reduced galvinoxyl more rapidly than Tempol-H.

Oxidation of MeLH in the presence of nitroxyl radicals or hydroxylamines under air and in hypoxic conditions. The antioxidant action of nitroxyl radical I and Tempol and the corresponding hydroxylamines was studied under air and in hypoxic conditions in the oxidation of MeLH. Oxidation was measured by following the increase in absorption at 234 nm attributable to the conjugated diene of MeLH hydroperoxides; the primary products formed quantitatively. The rates of MeLH oxidation did not differ appreciably under air or under 1 or 0.1% oxygen in nitrogen; however, oxidation was reduced considerably under 200 ppm oxygen (Fig. 4A). The antioxidant effects of nitroxyl radical I (Fig. 4B) and Tempol (Fig. 4C) were increased with a decreasing concentration of oxygen. Although nitroxyl radical I showed no antioxidant

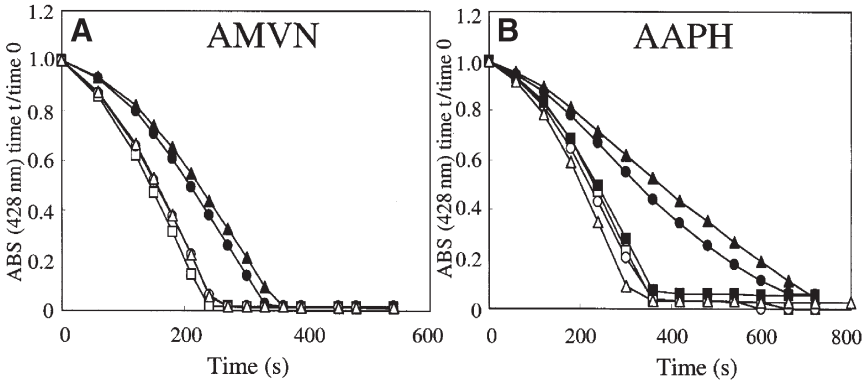


FIG. 1. Competitive reactions between 10 μM galvinoxyl and 10 μM nitroxyl radicals with alkyl and peroxyl radicals derived from (A) 10 mM 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) in acetonitrile or (B) 10 mM 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) in methanol under air or nitrogen at 37°C. □: without nitroxyl radical I under air; ■: without nitroxyl radical I under nitrogen; △: with nitroxyl radical I under air; ▲: with nitroxyl radical I under nitrogen; ○: with Tempol under air, ●: with Tempol under nitrogen. ABS, absorbance; see Scheme 1 for structures of nitroxy radical I and Tempol.

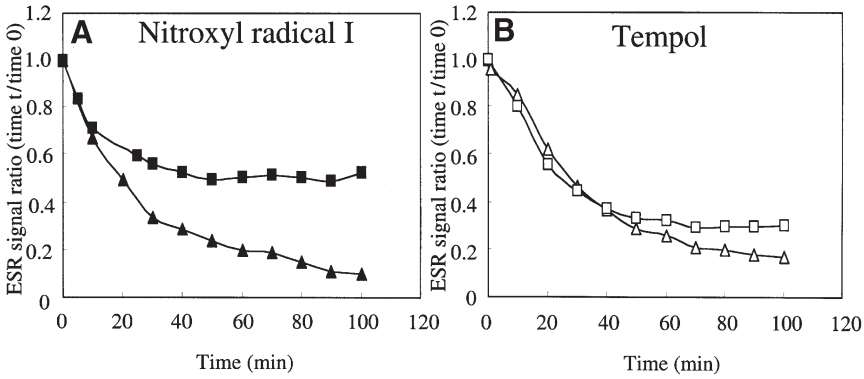


FIG. 2. The reduction of nitroxyl radicals (50 μM) by ascorbic acid (50 μM) in methanol or 14:0 PC liposome was followed. A, nitroxyl radical I; B, Tempol. ▲: nitroxyl radical I in methanol; ■: nitroxyl radical I in 14:0 PC liposome; △: Tempol in methanol; □: Tempol in 14:0 PC liposome.

effect under air, it reduced the rate of MeLH oxidation remarkably under hypoxic conditions.

With regard to the hydroxylamines, both I-H and Tempol-H inhibited oxidation much more effectively than did the corresponding nitroxyl radicals. Although the antioxidant effect of Tempol-H was not affected by oxygen concentration, I-H reduced the rate of the MeLH oxidation remarkably under 1% oxygen (Fig. 5).

Synergistic inhibition of lipid peroxidation by nitroxyl radicals and ascorbic acid. It has been well documented that the combination of α-tocopherol and ascorbic acid exerts a synergistic effect against lipid peroxidation in membranes, micelles, and LDL. Ascorbate reduces α-tocopheroxyl radicals to regenerate α-tocopherol, although ascorbate is not capable of effectively scavenging radicals within the lipophilic compartment (9,13,14). The effects of ascorbic acid with either nitroxyl radical or hydroxylamine in the oxidation of PLPC liposomal membranes are shown in Figures 6A and 6B, when ascorbic acid at higher concentrations suppressed the oxidation by itself. Interestingly, the combination of ascorbic acid with nitroxyl radical I and its hydroxylamine inhibited oxidation completely and the inhibition period was prolonged markedly, whereas the combination with Tempol and its hydroxylamine resulted simply in an additive effect.

Partition coefficient of nitroxyl radicals and the corresponding hydroxylamines. To assess the localization of the antioxidant in liposomal dispersions, the partition coefficient between water and 1-octanol was measured for nitroxyl radicals and hydroxylamines. The results summarized in Table 1 show that the log *P* values, hence the lipophilicity, decreased in the order of nitroxyl radical I > I-H > Tempol > Tempol-H. The log *P*-values were nearly the same between nitroxyl radicals I and I-H, whereas Tempol-H was more hydrophilic than Tempol.

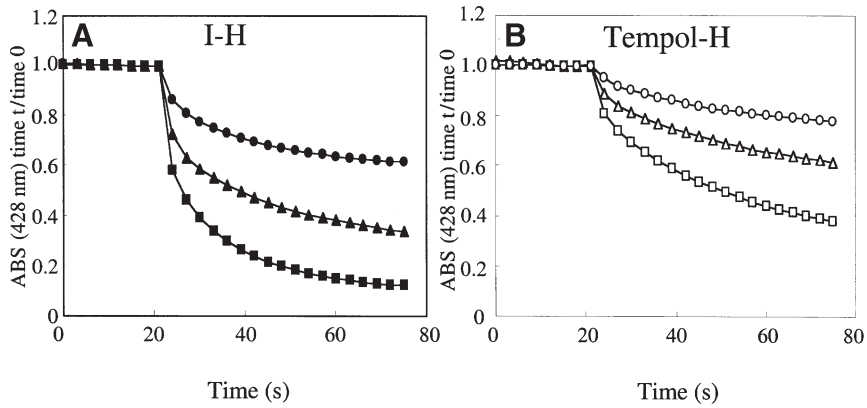


FIG. 3. The reaction of galvinoxyl with hydroxylamine. Galvinoxyl (10 μM) was reacted with hydroxylamine in an acetonitrile solution at 37°C under air. A: I-H; B: Tempol-H. ■: 20 μM I-H; □: 20 μM Tempol-H; ▲: 10 μM I-H; △: 10 μM Tempol-H; ●: 5 μM I-H; ○: 5 μM Tempol-H. See Scheme 1 for structures of I-H and Tempol-H; for other abbreviation see Figure 1.

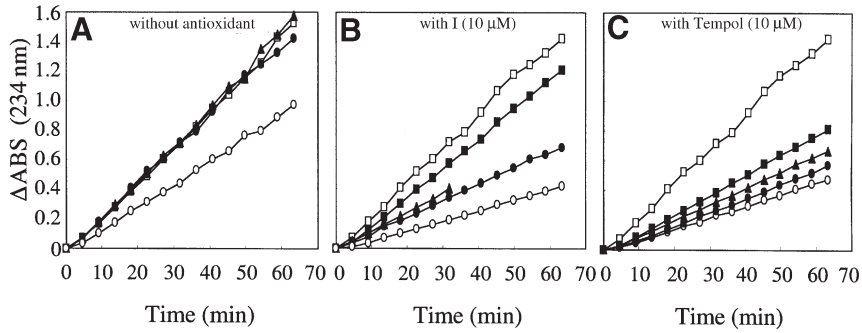


FIG. 4. Methyl linoleate (MeLH) (15.2 mM) oxidation induced by AMVN (0.2 mM) in the absence (A) and presence of either nitroxyl radical I (10 μM) (B) or Tempol (10 μM) (C) in acetonitrile solution at 37°C under hypoxic conditions. ○: 0.02% O_2; ●: 0.1% O_2; ▲: 1% O_2; □: under air without nitroxyl radical; ■: under air with nitroxyl radical. The increase in absorption at 234 nm (ΔABS) was followed as described in the Materials and Methods section. I, nitroxy radical I; for other abbreviations see Figure 1. See Scheme 1 for structures of nitroxy radical I and Tempol.

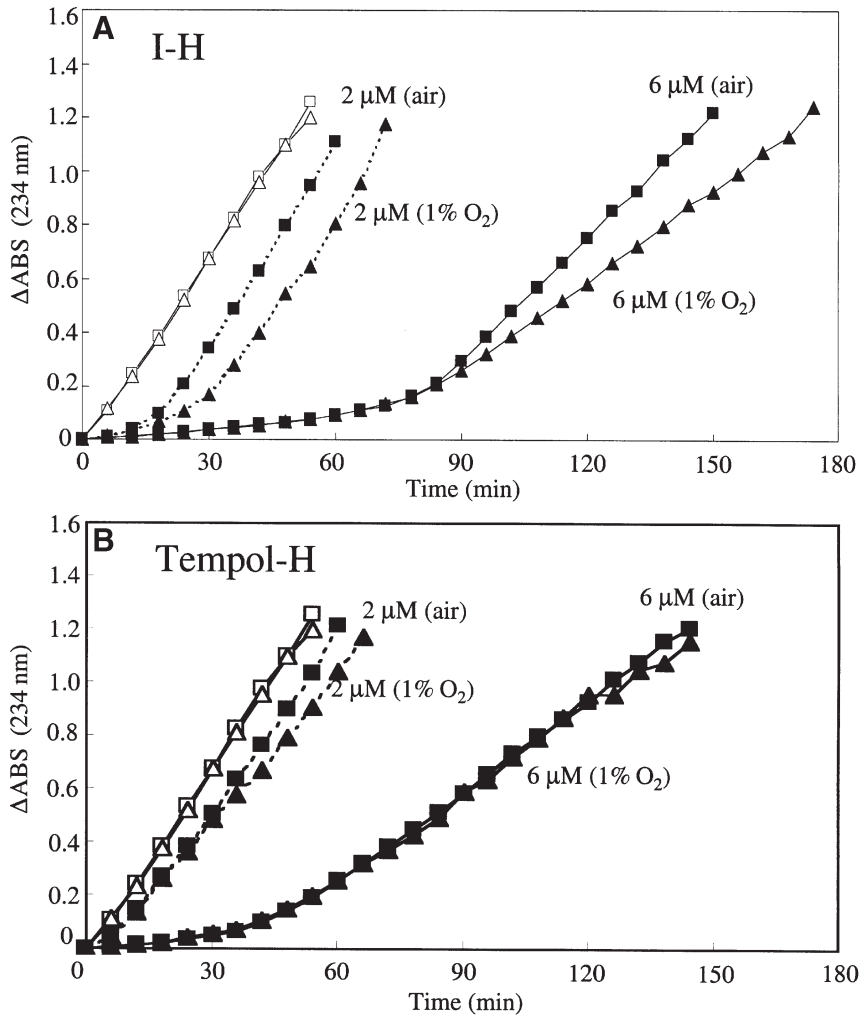


FIG. 5. Antioxidative effect of I-H (A) and Tempol-H (B) in the oxidation of MeLH (15.2 mM) induced by AMVN (0.2 mM) in acetonitrile solution at 37°C. □: Without hydroxylamine under air; ■: with hydroxylamine under air; △: without hydroxylamine under 1% O_2; ▲: with hydroxylamine under 1% O_2; (· · · ·): 2 μM of hydroxylamine; (—): 6 μM of hydroxylamine. See Scheme 1 for structures of I-H and Tempo-H; for other abbreviations see Figures 1 and 4.

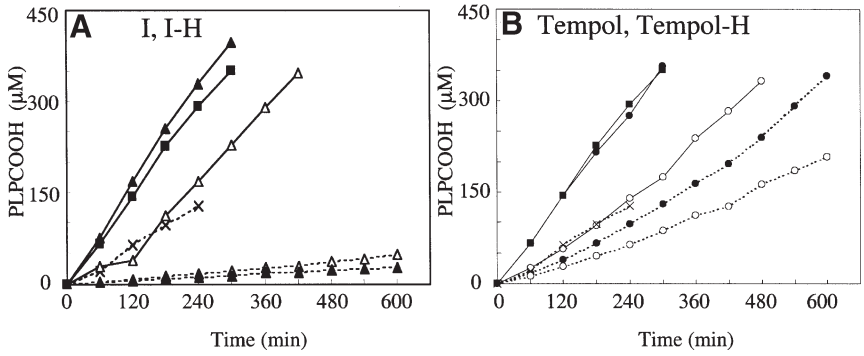


FIG. 6. Inhibition of L-α-phosphatidylcholine,β-linoleoxyl-γ-palmitoyl (PLPC) (2.83 mM) oxidation with AMVN (1 mM) by either nitroxyl radicals [(A), nitroxyl radical I; (B), Tempol] or the corresponding hydroxylamine in the absence (—) or presence (· · ·) of ascorbate in liposome membrane at 37°C. ■: Without antioxidant; ▲: nitroxyl radical I; ●: Tempol; △: I-H; ○: Tempol-H; ×: ascorbic acid. PLPCOOH, PLPC hydroperoxides.

TABLE 1
Partition Coefficients of Nitroxyl Radicals and the Corresponding Hydroxylamines Between Water and 1-Octanol at 37°C

Antioxidant	Partition coefficient log(*P*)[a]
Nitroxyl radical I	2.17
I-H	2.09
Tempol	0.24
Tempol-H	−1.43

[a]*P* = Co/Cw, where Co and Cw are the concentrations of nitroxyl radicals and hydroxylamines in 1-octanol (Co in M) and water (Cw in M). Nitroxy radical I, cyclohexane-1-spiro-2′-(4′-oxyimidazolidine-1′-oxyl)-5′-spiro-1″-cyclohexane; I-H, cyclohexane-1-spiro-2′-(4′-oxyimidazolidine-1′-hydroxyl)-5′-spiro-1″-cyclohexane; Tempol, 4-hydroxy-2,2,6,6,-tetramethylpiperidine-*N*-oxyl; Tempol-H, 4-hydroxy-2,2,6,6,-tetramethylpiperidine-*N*-hydroxyl.

DISCUSSION

With increasing evidence of the involvement of oxidative damage in various disorders and diseases, the role of antioxidants has received considerable attention. Polyunsaturated lipids are quite susceptible to oxidation, and the efficiency of antioxidants against lipid peroxidation has been explored. In the present study, the antioxidant activity of nitroxy radical I was evaluated against the oxidation of linoleic acid esters. These are a suitable substrate, since linoleate is the most abundant PUFA *in vivo* and its oxidation yields conjugated diene hydroperoxides quantitatively (15,16). Thus, the rate of oxidation and the antioxidant activity can be evaluated accurately.

We found that nitroxyl radical I was more reactive toward the alkyl radical than the alkylperoxyl radical (Fig. 1); consequently, its antioxidant capacity increased with decreasing oxygen pressure (Fig. 4). This is favorable for nitroxyl radical I, since the oxygen concentration *in vivo* is rather low. Nitroxyl radical I was reduced by ascorbate to yield the corresponding hydroxylamine. The reduction of the nitroxyl radical in the membrane by ascorbate was previously found to decrease as the radical went deeper into the interior of the membrane (17) and LDL (18). Interestingly, the nitroxyl radical incorporated into the PC liposomal membrane was reduced by ascorbate as rapidly as in the homogenous solution, implying that nitroxyl radical I is localized at the membrane surface and/or that it moves rapidly within the membrane. This is advantageous for exerting an antioxidant function.

The hydroxylamine was found to act as a potent antioxidant. More importantly, the combination of nitroxyl radical I and ascorbate resulted in a synergistic effect; they inhibited oxidation efficiently, both in rate and duration. Hydroxylamine reacts with the radical by donating a hydrogen atom to yield the nitroxyl radical, which is reduced by ascorbate to regenerate hydroxylamine. Thus, nitroxyl radical I and ascorbate may function as an efficient antioxidant network. On the other hand, Tempol and its hydroxylamine are hydrophilic (Table 1), and the combination with ascorbate results in an additive effect. The hydroxylamine of nitroxyl radical I was more reactive toward galvinoxyl than was Tempol-H (Fig. 3). The reason for this effect is not clear at present, but the difference in lipophilicity of the hydroxylamines may have contributed to their reactivity in the acetonitrile solution.

The reaction of alkyl radicals with oxygen to give alkylperoxyl radicals proceeds rapidly at a diffusion-controlled rate. However, the pentadienyl radical formed from polyunsaturated lipids is resonance-stabilized and has a longer life than the simple alkyl radical, especially at low oxygen concentrations. Nitroxyl radical I rapidly scavenges the lipid-derived carbon-centered radical and inhibits oxidation. In this case, the nitroxyl radical yields a stable radical–radical coupling product, and it is consumed. The hydroxylamine probably reacts with both the peroxyl and carbon-centered radicals by hydrogen atom transfer to give the nitroxyl radical, which may be reduced to give hydroxylamine, constituting a recycling network.

In conclusion, nitroxyl radical I was found to act as a potent antioxidant, especially under hypoxic conditions. Moreover, it exerted a clear synergistic effect with ascorbate, and the combination of nitroxyl radical I and ascorbate was quite effective in inhibiting lipid peroxidation in the membranes.

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Effect of Temperature and Addition of α-Tocopherol on the Oxidation of Trilinolein Model Systems

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ABSTRACT: The effects of temperature and addition of α-tocopherol were evaluated in trilinolein model systems through quantification of oxidized TAG monomers, dimers, and polymers following oxidation at different temperatures. Samples of trilinolein without and with 250 and 500 mg/kg α-tocopherol added were stored at 25, 60, and 100°C. Quantification of oxidized monomers, dimers, and polymers by a combination of adsorption and exclusion chromatography provided a useful measurement for studying the evolution of oxidation. Results showed that the amounts of primary oxidation compounds (trilinolein oxidized monomers) that accumulated during the induction period decreased as the temperature increased, indicating that the slope of the initial linear stage of oxidation depended on temperature. The end of the induction period was marked by a sharp increase in the levels of total oxidation compounds, the initiation of polymerization, and the loss of α-tocopherol. Addition of α-tocopherol did not prevent, but rather delayed, formation of trilinolein oxidized monomers and the initiation of polymerization.

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The complexity of the reactions involved in lipid oxidation and the wide range of compounds produced cause great difficulties in evaluating alterations in oxidation and justify the need for new analytical procedures with general application. The methods currently in use have been reviewed by several authors, who have generally concluded that, in spite of the multitude of assays available, no universal method allows the extent of oxidation to be evaluated throughout the entire process (1–7). Therefore, the need arises for improving methodologies to reevaluate aspects of particular concern, such as the effectiveness of antioxidants and, in general, the influence of different variables that modify the rate of oxidative reactions.

Previously, we developed and applied a methodology based on a combination of adsorption and size-exclusion chromatographies that enables quantification of oxidized and polymeric compounds, as well as hydrolytic products, i.e. DAG and FA (8,9). Application of this procedure has proved to be of great utility for quantification of oxidation compounds in fats and fatty foods at low temperatures (10–15). This analytical approach offers the great advantage of providing a good measurement for early and advanced stages of oxidation by concomitantly evaluating primary and secondary oxidation products. A modification of this methodology was introduced in which small quantities of samples and solvents were used together with an internal standard for purposes of quantification (16). The high reproducibility achieved for samples with low alteration levels added further possibilities for applying the methodology to initial stages of oxidation.

In a previous publication (17), we reported the value of this methodology for oxidation studies. Early stages of oxidation were characterized by a significant increase in monomeric oxidation compounds, whereas the presence of polymerization compounds in significant amounts indicated the end of the induction period (IP). The objective of the present study was to obtain complete quantitative data on the main groups of compounds formed during oxidation of trilinolein (LLL), used as model system, in order to gain insight into the effects of temperature and added α-tocopherol on oxidation kinetics. Unfortunately, in most of the oxidation studies published, the effects of tocopherol have been evaluated at a fixed level of oxidation, sometimes at very low oxidation points, e.g., at PV of 10–20 meq/kg; in fact, the degree of oxidation should be determined at appropriate time intervals, making one data point insufficient (18). Additionally, it is generally recommended that the formation of both primary and secondary products be measured together with the stability of tocopherols (7).

LLL has been used in this work as a model unsaturated TAG to avoid interference from potential prooxidant or antioxidant effects of minor compounds that could otherwise be present in oils. Linoleyl was selected as the FA constituent of the model system because this fatty acyl group is the most susceptible to oxidation in most natural fats and oils. α-Tocopherol was selected because it is the most important natural antioxidant in fats and oils. The concentrations of α-tocopherol used were 250 and 500 mg/kg, which are within the range naturally occurring in commercial seed oils (19). Experiments were undertaken at 25°C, and accelerated oxidative assays were carried out at 60 and 100°C, the latter two being temperatures commonly used in standard accelerated tests directed toward estimating the shelf life of oils (20,21).

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Abbreviations: DIM, trilinolein dimers; HPSEC, high-performance size-exclusion chromatography; IP, induction period; LLL, trilinolein; LLL-250, trilinolein with 250 mg/kg α-tocopherol added; LLL-500, trilinolein with 500 mg/kg α-tocopherol added; oxMON, oxidized monomers; POL, trilinolein polymers.

EXPERIMENTAL PROCEDURES

Samples and treatments. LLL was purchased from Nu-Chek-Prep (Elysian, MN), and α-tocopherol was obtained from Aldrich Chemical Co. (Milwaukee, WI). A solution of α-tocopherol (10 mg/mL) was prepared by weighing 50 mg of α-tocopherol into a 5-mL volumetric flask using diethyl ether stabilized with ethanol (Romil, Cambridge, United Kingdom). Samples of LLL with 500 (LLL-500) and 250 mg/kg (LLL-250) α-tocopherol added were prepared by adding 1 and 0.5 mL of α-tocopherol solution, respectively, to 20 g LLL. Samples were homogenized using a magnetic stirrer, purged of solvents at 30°C with a stream of nitrogen, and maintained at –40°C until the experiments were conducted. Samples of LLL, LLL-250, and LLL-500 were placed in open beakers (surface-to-volume ratio of 10 cm^{-1}) to ensure their accessibility to air and then either heated in an oven at 60 or 100°C, or stored at 25°C in the dark for different periods of time. Experiments were repeated at each temperature tested.

Analytical determinations. (i) Separation of polar fractions by adsorption chromatography. LLL samples were fractionated using silica cartridges for solid-phase extraction (Sep-Pak columns supplied by Waters Associates, Milford, MA). The methodology is described in detail (including precision, accuracy, and recovery data) in a previous publication (16). Briefly, 2 mL of the sample solution in *n*-hexane, containing 50 mg of sample and 1 mg of monostearin used as internal standard (Nu-Chek-Prep), was placed on the column and the solvent was passed through while the sample was retained on the column. Next, the nonpolar fraction was eluted with 15 mL of petroleum ether/diethyl ether (90:10). A second fraction containing polar compounds and the internal standard was eluted with 15 mL of diethyl ether. Nonpolar and polar fractions were evaporated under reduced pressure and redissolved in 1 mL of THF for further analyses by TLC (to check the efficiency of the separation) and high-performance size-exclusion chromatography (HPSEC).

(ii) HPSEC. Fractions of polar compounds from the LLL samples, obtained as outlined above, were analyzed by HPSEC using a Rheodyne 7725y injector with a 10-μL sample loop pump (Waters Associates), an HP 1037 A refractive index detector (Hewlett-Packard, Avondale, PA), and an HP 3392 A integrator. The separation was performed on two 100- and 500-Å Ultrastyragel columns (25 × 0.77 cm i.d.; Hewlett-Packard) packed with a porous, highly cross-linked styrene-divinylbenzene copolymer (film thickness < 10 μm) connected in series, with THF (1 mL/min) as the mobile phase (16). The groups of oxidized compounds separated were LLL oxidized monomers (oxMON), LLL dimers (DIM), and LLL polymers (POL).

(iii) α-Tocopherol content. α-Tocopherol levels were quantified by HPLC with fluorescence detection (22).

Statistical analysis. SigmaStat and SigmaPlot software packages (SPSS Science, Chicago, IL) were used for the kinetic study and plots.

RESULTS AND DISCUSSION

Figure 1 presents a representative HPSEC chromatogram of the polar fraction of an LLL sample stored at 60°C showing the groups of compounds quantified through the analytical procedure used. At that point of oxidation, the LLL sample showed a significant increase in oxMON (17.7% on total sample) and DIM (1.4% on total sample), and even POL could be detected (retention time: 11.4 min). The oxMON peak comprises a large number of monomeric LLL molecules containing one or more oxidized fatty acyl groups, either peroxide groups or other oxygenated functions such as epoxy, keto, or hydroxy groups. Quantification of this group of compounds can therefore be of great utility not only to detect the oxidation products initially formed, even before rancidity, but also to follow oxidation during further stages. Polymerization compounds, here separated into dimeric LLL molecules (DIM) and higher oligomeric LLL molecules (POL), are characteristic of advanced oxidation. The number of possibilities of different structures for the compounds formed during oxidation is enormous, even starting from LLL as a model compound, and this number increases exponentially with higher M.W. Therefore, great difficulties are still encountered in elucidating and quantifying the structures of such compounds, especially of DIM and POL (23). In this context, the main advantage provided by the methodology used in this study is that three groups of compounds, which include initial and decomposition oxidation products, can be quantified concomitantly. Thus, it is possible to determine the degree of oxidation at any time during the course of oxidation (17).

Tables 1 to 3 show the evolution of oxidation in LLL samples without and with added α-tocopherol at 25, 60, and

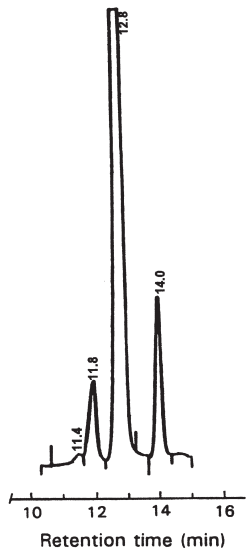

FIG. 1. High-performance size-exclusion chromatogram of a representative polar fraction isolated from trilinolein (LLL) oxidized at 60°C. Retention times (min): 11.4, LLL polymers; 11.8, LLL dimers; 12.8, LLL oxidized monomers; and 14.0, monostearin (internal standard).

TABLE 1
Evolution of Oxidation in Trilinolein (LLL) Samples at 25°C

Model system	Days	Oxidation compounds (%)				Remaining α-tocopherol (%)
		Total	oxMON	DIM	POL	
LLL	1	1.1	1.1	ND	ND	—
	2	2.2	2.2	ND	ND	—
	3	5.7	5.6	0.1	ND	—
	4	10.8	10.5	0.3	ND	—
	5	18.8	18.2	0.6	ND	—
	6	27.9	26.6	1.1	0.2	—
	8	39.7	34.9	3.8	1.0	—
	10	46.9	40.1	5.2	1.6	—
	13	65.4	47.4	11.1	6.9	—
	17	84.2	55.9	15.7	12.6	—
LLL-250	6	1.5	1.5	ND	ND	92.5
	12	2.9	2.8	0.1	ND	92.2
	17	4.0	3.6	0.4	ND	73.5
	23	8.8	8.6	0.2	ND	60.8
	26	8.9	8.7	0.2	ND	57.1
	27	9.2	9.1	0.1	ND	51.5
	29	9.7	9.6	0.1	ND	38.1
	33	9.0	8.8	0.2	ND	31.3
	41	10.9	10.5	0.4	ND	7.5
	44	12.0	11.6	0.4	0.1	ND
	47	16.3	15.4	0.7	0.2	—
	51	61.3	41.9	8.1	2.3	—
LLL-500	10	2.1	2.0	0.1	ND	92.1
	17	3.7	3.6	0.1	ND	52.7
	23	4.1	4.0	0.1	ND	48.4
	26	5.8	5.7	0.1	ND	38.1
	29	6.5	6.4	0.1	ND	31.3
	33	7.8	7.6	0.2	ND	28.5
	41	8.8	8.7	0.1	ND	24.0
	47	10.6	10.3	0.3	ND	4.5
	51	11.9	11.7	0.2	0.1	ND
	54	14.3	13.2	0.8	0.3	—
	58	43.4	34.5	7.2	1.7	—
	60	69.1	43.8	16.4	8.9	—

[a]LLL-250, trilinolein with 250 mg/kg α-tocopherol added; LLL-500, trilinolein with 500 mg/kg α-tocopherol added; oxMON; oxidized monomers; DIM, dimers; POL, polymers; ND, not detected.

100°C, respectively. For each sampling point, values for the total oxidation compounds and their distribution in oxMON, DIM, and POL are included, and levels of α-tocopherol are expressed as the percentage of remaining α-tocopherol. Oxidation compounds were not detected in samples at the start of the experiments. Results of duplicate experiments showed good reproducibility for the oxidation progress and distribution of specific groups of compounds under the conditions used (CV < 8% for total oxidation compounds).

To illustrate the general LLL oxidation profiles, total oxidation compounds are represented for all samples in Figure 2. Regardless of the temperature used, there was a rapid increase in total oxidation products in LLL samples without tocopherol from the beginning, whereas the presence of the antioxidant led to a considerable delay in the formation of oxidation compounds and allowed two stages to be clearly distinguished. The first period was characterized by the slow progression of oxidation and the second was characterized by an accelerated oxidation. The end of the induction period could therefore be defined in samples with added antioxidants as the time when a notable shift in oxidation rate was observed. For example, at 25°C the end of the induction period occurred between 47 and 51 d in LLL-250, corresponding to an increase in oxidation compounds from 16.3 to 61.3%. In LLL-500 it occurred between 54 and 58 d, corresponding to an increase in oxidation compounds from 14.3 to 43.4%. At 60°C, the end of the induction period for LLL-250 and LLL-500 was observed between 95 and 102 h, and between 111 and 114 h, respectively, at changes from 5.4 to 23.5% and from 8.1 to 17.7% oxidation compounds. At 100°C, the end of the induction period occurred between 8 and 10 h and between 10 and 12 h, respectively, in LLL-250 and LLL-500, corresponding to increases in oxidation compounds from 4.8 to 12.5% and from 5.2 to 8.8%. Also, regardless of temperature, the end of the induction period was indicated by the total loss of α-tocopherol in all samples (Tables 1 to 3). In other words, once the antioxidants were exhausted, the course of oxidation entered a second accelerated phase.

TABLE 2
Evolution of Oxidation in LLL Samples at 60°C

Model system	Hours	Oxidation compounds (%)				Remaining α-tocopherol (%)
		Total	oxMON	DIM	POL	
LLL	3	3.0	3.0	ND		—
	6	4.4	4.3	0.1	ND	—
	9	10.9	10.4	0.5	ND	—
	11	16.4	15.4	0.9	0.1	—
	13	19.4	17.7	1.5	0.2	—
	15	29.0	26.1	2.5	0.4	—
	17	34.0	29.7	3.6	0.7	—
	19	40.7	34.6	4.9	1.2	—
	21	46.0	37.5	6.4	2.1	—
	23	56.5	44.3	8.8	3.4	—
LLL-250	24	1.3	1.3	ND	ND	76.1
	46	2.1	2.1	ND	ND	60.6
	54	2.8	2.8	ND	ND	53.1
	71	3.4	3.4	ND	ND	38.9
	79	3.8	3.7	0.1	ND	24.3
	95	5.4	5.2	0.2	ND	ND
	102	23.5	20.9	2.2	0.4	—
	105	40.7	34.6	4.9	1.2	—
	108	56.1	44.1	8.4	3.6	—
	111	65.3	46.2	11.9	7.2	—
LLL-500	24	1.5	1.5	ND	ND	81.0
	46	2.8	2.8	ND	ND	68.6
	54	3.3	3.3	ND	ND	63.8
	71	4.4	4.4	ND	ND	53.8
	79	4.8	4.8	ND	ND	43.0
	95	5.4	5.3	0.1	ND	27.2
	102	6.1	5.9	0.2	ND	17.4
	108	7.2	6.9	0.3	ND	5.0
	111	8.1	7.7	0.4	ND	ND
	114	17.7	16.5	1.1	0.1	—

[a]For abbreviations see Table 1.

It is also interesting to note the profile of oxidation at 100°C. As can be observed, the increase in oxidation compounds in the accelerated phase was slower than that at 25 or 60°C in spite of the more rapid oxidation at 100°C. An explanation for these different profiles could be that the quantity of air was limited during the oxidation process at 100°C. Oxygen solubility decreases when the temperature increases, and, even at the high surface-to-volume ratio used in this study, it is possible that the availability of oxygen was reduced at 100°C. In fact, when oxygen was bubbled at 100°C in the Rancimat apparatus under the conditions specified for measuring the oil stability index (24), no induction period was found for LLL. The sample was more rapidly oxidized and hence dependent on the availability of oxygen. In contrast, IP obtained with the Rancimat apparatus for LLL-250 and LLL-500 were similar to those shown in Figure 2C for LLL-250 and LLL-500 (8.9 and 10.3 h, respectively). Furthermore, the lines obtained with the Rancimat apparatus for the accelerated stage of oxidation rose more sharply with time than those in Figure 2C, suggesting that oxygen had been limited in the latter case.

On the other hand, the main effect of the increase in temperature was, as expected, a decrease in the induction period. An additional important observation was the influence of temperature on the amount of oxidation compounds that accumulated at the end of the IP, which decreased as the temperature increased. This was probably related to the effect of temperature on antioxidant degradation.

From the results obtained at each temperature, it is clear that oxidation still proceeded, although it was delayed, in the presence of α-tocopherol. Especially at 25°C, considerable amounts of oxidation compounds were compatible with substantial levels of α-tocopherol remaining. For example, at 27 d, LLL with 250 mg/kg α-tocopherol added (Table 1) contained 9.2% total oxidation compounds, and approximately 50% of α-tocopherol was still present. We also concluded that the increase in antioxidant levels from 250 to 500 mg/kg did not substantially modify the stability against oxidation of LLL. This is not strange given the results obtained in numerous studies on the effect of antioxidant concentration, some of which have even indicated the prooxidant action of α-tocopherol at high concentrations (25–27).

Specific quantification of oxMON, DIM, and POL provided complementary information of great utility for elucidating oxidation kinetics (Tables 1 to 3). In general, the only group of compounds that increased during the early oxidation stage,

TABLE 3
Evolution of Oxidation in LLL Samples at 100°C

Model system	Hours	Oxidation compounds (%)				Remaining α-tocopherol (%)
		Total	oxMON	DIM	POL	
LLL	1.5	3.4	3.1	0.3	ND	—
	3.0	6.8	6.3	0.5	ND	—
	4.5	10.9	10.4	1.1	ND	—
	6.0	13.2	11.5	1.7	ND	—
	7.0	17.1	14.4	2.5	0.2	—
	8.0	22.8	18.6	3.8	0.4	—
	10.0	27.9	21.1	6.0	0.8	—
	12.0	33.0	23.5	8.0	1.5	—
	14.0	38.3	24.8	9.9	3.6	—
	16.0	46.1	27.2	12.8	6.1	—
	18.0	56.4	32.4	14.4	9.6	—
	20.0	76.5	40.3	19.5	16.7	—
	22.0	93.4	47.0	22.4	24.0	—
LLL-250	3.0	1.9	1.8	0.1	ND	65.7
	6.0	3.4	3.0	0.4	ND	39.2
	7.0	4.0	3.6	0.4	ND	22.5
	8.0	4.8	4.3	0.5	ND	8.8
	10.0	12.5	10.6	1.6	0.3	ND
	12.0	18.4	15.5	2.6	0.3	—
	14.0	24.4	19.2	4.6	0.6	—
	16.0	33.0	23.8	7.4	1.8	—
LLL-500	3.0	2.2	2.0	0.2	ND	82.5
	6.0	3.5	3.2	0.3	ND	75.7
	7.0	4.1	3.7	0.3	ND	62.4
	8.0	4.3	3.9	0.4	ND	45.6
	10.0	5.2	4.5	0.7	ND	19.9
	12.0	8.8	7.4	1.4	ND	3.1
	14.0	13.4	12.7	2.5	0.2	ND
	16.0	20.0	16.0	3.6	0.4	—

[a]For abbreviations see Table 1.

independent of temperature or the presence of antioxidants, was the group of oxMON, mainly composed of hydroperoxides during early oxidation (28). As can be observed in the tables, concentrations of oxMON were practically identical to those of total oxidation compounds before oxidation accelerated, independent of temperature and the amount of antioxidant. At the end of the induction period, oxidation was accelerated, as shown by the sharp increase in oxMON and the development of polymerization reactions, denoted by a significant rise in dimers. In general, increases of about 1% in dimer concentrations indicated the start of the accelerated phase at all temperatures tested.

However, the oxMON concentration at the end of the IP depended on temperature and was much lower as the temperature increased. For example, in LLL-500 samples, the end of the IP occurred when samples contained between 13.2 and 34.5%, 8.5 and 16.5%, and 4.3 and 7.4% oxMON at 25, 60, and 100°C, respectively. These results indicate that polymerization started at very different levels of primary oxidation products, depending on temperature. Such differences were clearly reflected in the ratio oxMON-to-polymerization compounds (DIM + POL) obtained at 25, 60, and 100°C. For example, for similar levels of total oxidation compounds (27.9–29.0%), that ratio was approximately 20:1, 9:1, and 3:1, respectively. On the other hand, overall results showed that, independent of temperature, initiation of accelerated oxidation was clearly marked by a rise in polymerization compounds and a total loss of antioxidants. The general oxidation pattern found for samples without and with antioxidants is illustrated in Figure 3.

Kinetic considerations. Since oxidized TAG monomers are, in practice, the only products formed during the early stages of oxidation, we can write

$$\mathrm{LLL} + \mathrm{O_2} \rightarrow \mathrm{oxMON} \qquad [1]$$

Assuming that oxMON do not participate in other side reactions during this period,

$$d[\mathrm{oxMON}]/dt = k[\mathrm{oxMON}]^n \qquad [2]$$

where k is the rate constant and n is the reaction order. Rearrangement and integration lead to the following equation (29):

$$[\mathrm{oxMON}]^{(1-n)} = [\mathrm{oxMON}]_0^{\ (1-n)} + (1-n)kt \qquad [3]$$

which represents the relationship between [oxMON] and t (time) during the early stages of oxidation, where $[\mathrm{oxMON}]_0$ is the initial concentration of oxMON, that is, 0.

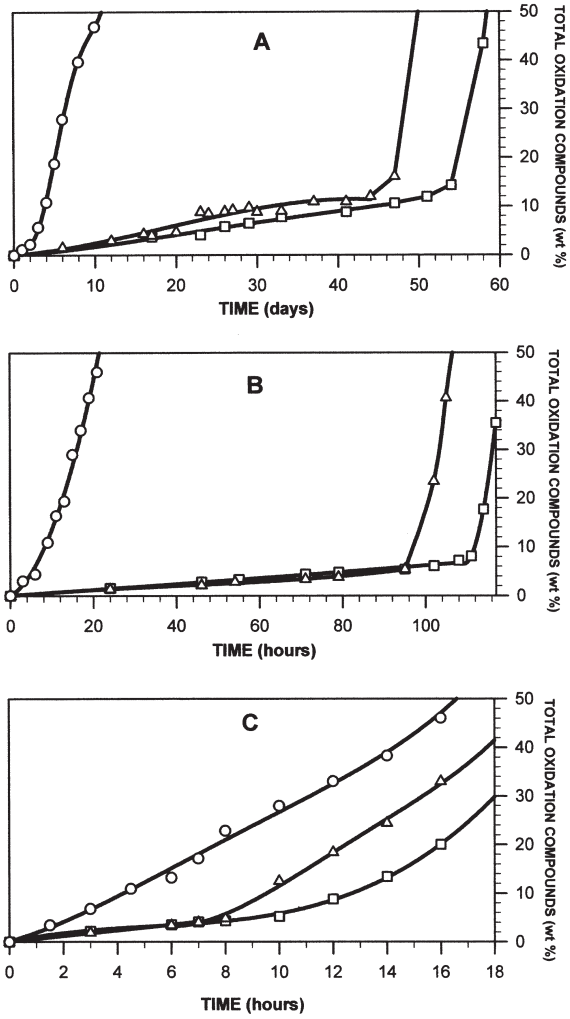

FIG. 2. Evolution of total oxidation compounds in samples of LLL without (-○-) and with the addition of 250 (-Δ-) and 500 (-□-) mg/kg α-tocopherol at 25 (A), 60 (B), and 100°C (C). For abbreviation see Figure 1.

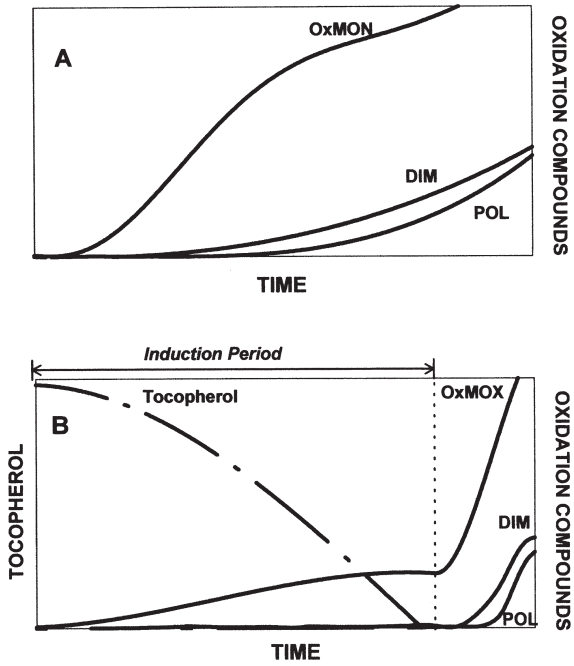

FIG. 3. General oxidation profile found for LLL without (A) and with added tocopherol (B). oxMON, oxidized monomers; DIM, dimers; POL, polymers. For other abbreviation see Figure 1.

Kinetic parameters were calculated from the experimental data by the least squares method. For experiments without α-tocopherol, no clear IP were obtained, and data corresponding to dimer concentrations lower than 1% were considered for kinetic studies.

Table 4 lists the values corresponding to the rate constants for oxMON formation (k), the reaction order (n), and the correlation coefficients (r). Values found for the reaction order were not significantly different from 0 when the antioxidant was present, thus indicating that the increase in oxMON was linear during the induction period. On the contrary, the reaction orders for LLL without antioxidant were different from 0 and close to 0.5 at 25 and 60ºC, as previously reported (30). As already mentioned, the lower value for the order of reaction obtained at 100ºC for LLL without antioxidant could be attributed to oxidation under conditions of limited air.

The influence of temperature on the oxidation rate during the IP can also be examined on the basis of the Arrhenius law:

$$k = A \exp(-E_a/RT) \quad [1]$$

where k is the rate constant, A is a pre-exponential factor, E_a is the activation energy, T is the absolute temperature, and R = 8.314 J/mol K.

A plot of ln k vs. T^{-1} should thus yield a straight line:

$$\ln k = \ln A - (E_a/RT) \quad [5]$$

Experimental values obtained for E_a were 41.8 ± 9.1, 46.1 ± 8.1, and 51.1 ± 2.0 kJ/mol, for LLL, LLL-250, and LLL-500, respectively. Such similarity in values would indicate that there was no substantial change in E_a when tocopherol was added.

On the other hand, k and the IP are inversely proportional and, consequently, ln IP would be directly proportional to E_a/RT. Values for the experimental IP were 8, 95, and 1128 h at 100, 60, and 25ºC, respectively, for LLL containing 250 α-tocopherol and were 10, 111, and 1296 h at 100, 60, and 25°C, respectively, for LLL containing 500 mg/kg α-tocopherol. The correlation coefficients obtained between ln k and ln IP were 0.994 and 0.999 for LLL containing 250 and 500 mg/kg α-tocopherol, respectively.

Figure 4 shows the linear relationship between ln IP and $1/T$ in samples with added α-tocopherol at 25, 60, and 100°C. Thus, assays at 60 or 100°C could be used to predict the IP at room temperature. Also, similar oxidation mechanisms for different α-tocopherol contents can be deduced from the parallel lines corresponding to 250 and 500 mg/kg α-tocopherol.

TABLE 4
Kinetic Parameters[a] for Formation of LLL Oxidized Monomers at 25, 60, and 100°C

T (°C)	α–Tocopherol (mg/kg)	*k*	*n*	*r*
25	0	$(67.4 \pm 6.6)\cdot10^{-3}$	0.568 ± 0.019	0.999
	250	$(12.2 \pm 1.7)\cdot10^{-3}$	0.005 ± 0.105	0.964
	500	$(7.8 \pm 0.5)\cdot10^{-3}$	0.108 ± 0.049	0.994
60	0	$(74.3 \pm 0.5)\cdot10^{-2}$	0.441 ± 0.087	0.991
	250	$(4.9 \pm 0.2)\cdot10^{-2}$	0.094 ± 0.090	0.992
	500	$(5.9 \pm 0.9)\cdot10^{-2}$	0.067 ± 0.089	0.989
100	0	1.96 ± 0.08	0.125 ± 0.040	0.999
	250	0.53 ± 0.02	0.0 ± 0.1	0.996
	500	0.49 ± 0.04	0.0 ± 0.2	0.982

[a]Mean values ± SE.

It is important to note that the results obtained here do not agree with those establishing that the mechanism of oil oxidation changes from ambient temperature to 100ºC (31) and that, consequently, oil shelf life cannot be deduced from accelerated tests. Such a conclusion could be easily assumed; however, it is important to point out two important considerations. First, oil shelf life may vary greatly depending on external parameters other than temperature. Oil storage in light or dark conditions and differences in availability of oxygen caused by packing and storage conditions are important enough to expect considerable differences in resistance of the oil to oxidation. Thus, we should define the specific conditions needed to predict the shelf life of the oil.

Second, deducing oil shelf life from accelerated tests also involves differences in the oxidation level measured and in the analytical method used, i.e., evaluating the IP by a sudden change in conductivity or oxygen depletion in the automated methods, the appearance of rancidity in the Schaal oven test, or the time to reach a certain PV beyond which the oil cannot be marketed as edible, in the case of room temperature tests.

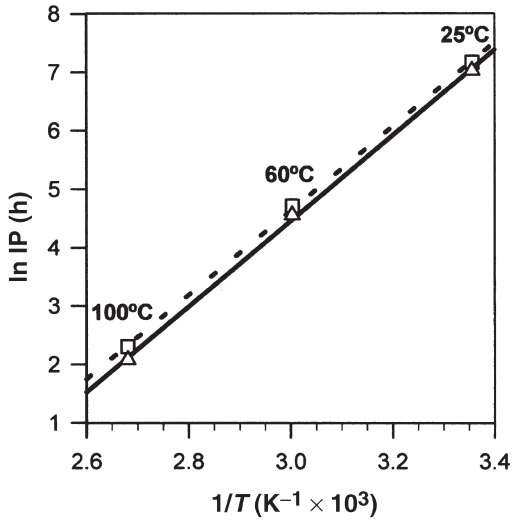

FIG. 4. Plot of ln induction period (IP) vs. 1/*T* in samples of LLL with the addition of 250 (-Δ-) and 500 (-□-) mg/kg α-tocopherol at 25, 60, and 100°C.

Nevertheless, with the exception of temperature, the same conditions of darkness and adequate air supply and the same criterion for evaluating oxidation (development of advanced oxidation either by the formation of polymerization compounds or the loss of antioxidants) were applied in this study. The results obtained indicate that temperature is not a variable that contributes significantly to changes in the reaction mechanism of oxidation under the conditions applied. Therefore, it would be useful in future studies to define whether the different oxidation mechanisms claimed are due to differences in variables other than temperature.

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Photochemical Oxidation and Autoxidation of Chlorophyll Phytyl Side Chain in Senescent Phytoplanktonic Cells: Potential Sources of Several Acyclic Isoprenoid Compounds in the Marine Environment

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ABSTRACT: Visible light-induced degradation of the chlorophyll phytyl side chain was studied in senescent cells of two phytoplanktonic strains (*Skeletonema costatum* and *Thalassiosira weissflogii*). Particular attention was paid to the induction of autoxidation processes on the phytyl chain and its photoproducts by photochemically produced hydroperoxides. The combination of photochemical oxidation and autoxidation reactions resulted in the production of several acyclic isoprenoid compounds that have been unambiguously identified by comparison of their retention times and mass spectra with those of appropriate standards. Various mechanisms are proposed to explain the formation of these oxidation products. These processes appear to be potential sources of numerous oxidized acyclic isoprenoids that previously have been detected in lacustrine and marine environments. Some oxidation products newly described or whose presence in natural samples was never reported in the literature were then sought in particulate matter, sediment, and microbial mat samples. The results obtained supported the significance of photochemical oxidation and autoxidation of phytoplanktonic chlorophyll phytyl side chain in the marine environment.

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The role played by photochemical processes during the degradation of phytoplankton in the euphotic layer of the oceans has been virtually ignored to date owing to the lack of adequate tracers. To address possible effects of stratospheric ozone depletion, some studies have recently examined the degradative effects of enhanced UV-B doses on phytoplanktonic lipids (1). However, photochemical damage in phytoplanktonic cells is not restricted to UV radiation (2). Irradiation of killed phytoplanktonic cells by light at wavelengths used for their growth in fact results in rapid degradation of chlorophylls (2,3). The photochemical degradation of chlorophylls has so far been studied almost exclusively with respect to the porphyrin moiety of the molecule, which is the more reactive portion. Despite some recent progress regarding intermediary photoproducts, no stable and specific markers for the photodegradation of the chlorophyll macrocycle could be characterized (4).

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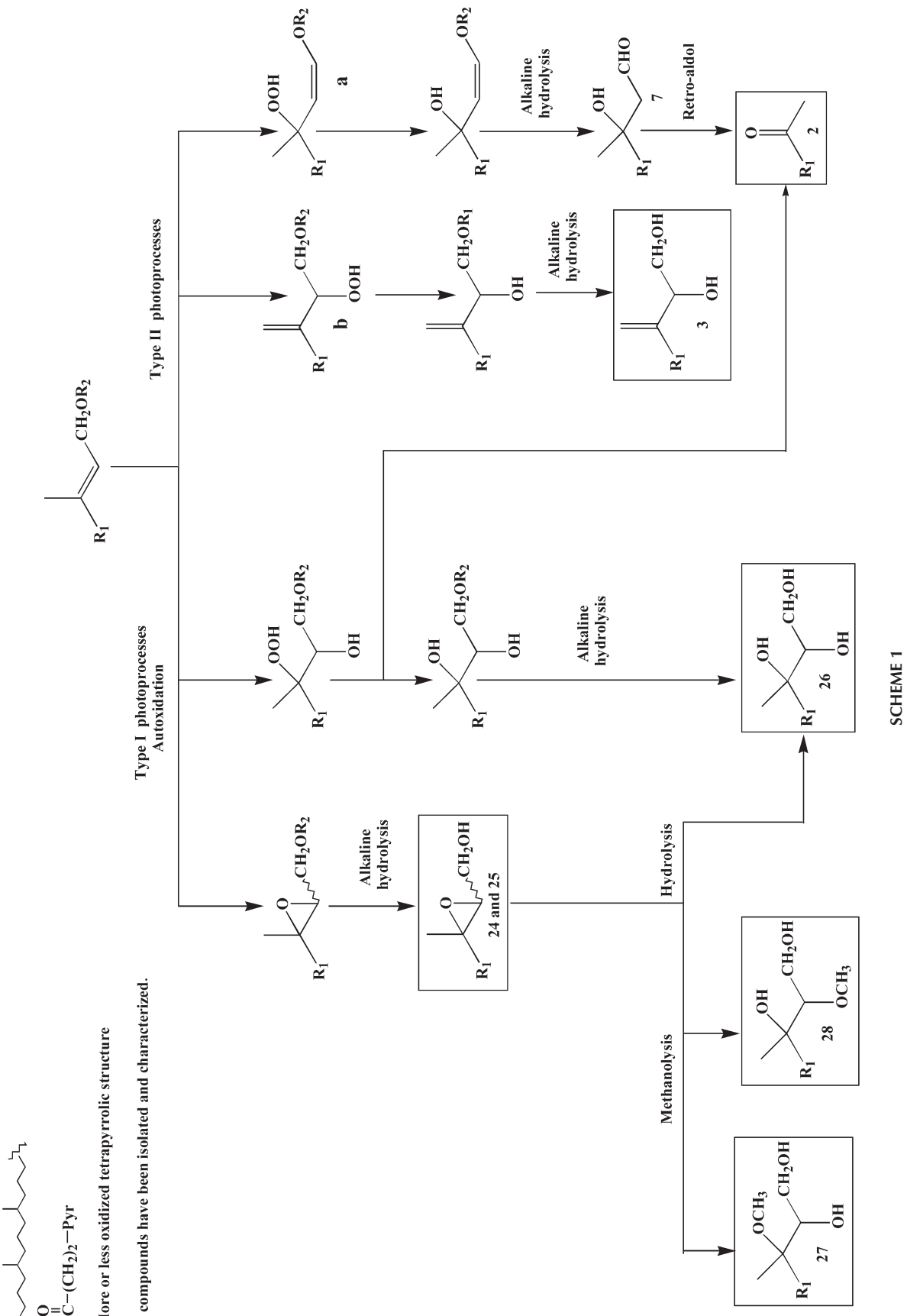
The isoprenoid phytyl side chain of chlorophyll is also very sensitive to photochemical processes. In fact, in phytodetritus, the visible light-dependent degradation rates were only three to four times higher for the chlorophyll tetrapyrrolic structure than for its phytyl side chain (5). It was previously demonstrated that the type II (i.e., involving $^1O_2$) photosensitized oxidation of the phytol moiety of chlorophyll *a* or *b* leads to the production of two main types of photoproducts, quantifiable after alkaline hydrolysis in the form of 6,10,14-trimethylpentadecan-2-one (phytone) and 3-methylidene-7,11,15-trimethylhexadecan-1,2-diol (phytyldiol) (6) (Scheme 1). In contrast, type I (i.e., involving oxy free radicals) photooxidation of the phytyl chain and subsequent alkaline hydrolysis affords mainly (*Z*)- and (*E*)-3,7,11,15-tetramethyl-2,3-epoxyhexadecan-1-ol, 3,7,11,15-tetramethylhexadecan-1,2,3-triol, and phytone (7) (Scheme 1). Analysis of isoprenoid photoproducts of chlorophyll after irradiation of different killed phytoplanktonic cells by visible light clearly established that the photodegradation of the chlorophyll phytyl side chain in phytodetritus involved mainly $^1O_2$ and to a small degree free radicals (3,8).

In phytodetritus, the photodegradation processes stop when all the photosensitizers (i.e., the tetrapyrrolic moiety of chlorophylls) have been destroyed or after the cells settle out of the euphotic zone. At this time, significant amounts of photochemically produced hydroperoxides are present. Although these compounds are generally considered to be very labile, significant amounts of hydroperoxides deriving from phytoplanktonic sterols (9) and monounsaturated FA (10) were recently detected in particulate matter and recent sediment samples. Under mild conditions, numerous organic compounds react with molecular oxygen to give different oxygen-containing species such as peroxides, hydroperoxides, alcohols, ketones, aldehydes, and acids (11). These autoxidation processes proceed by a radical chain mechanism and act mainly on compounds possessing hydrogens whose bond energies are relatively low (allylic, tertiary, α to oxygen, etc.). It is well known that autoxidation is enhanced by the presence of a peroxide or hydroperoxide initiator (11). In senescent phytoplanktonic cells, photochemically produced hydroperoxides could thus induce autoxidation of unsaturated lipid components such as phytol and its photoproducts.

The aim of the present work was to determine whether autoxidative processes may operate on the chlorophyll phytyl


R1 = (CH2)7–CH=CH–(CH2)7–CH3
R2 = —C(=O)—(CH2)2—Pyr
Pyr = More or less oxidized tetrapyrrolic structure
Framed compounds have been isolated and characterized.
Type I photoprocesses Autoxidation
Type II photoprocesses
Alkaline hydrolysis
Hydrolysis
Methanolysis
Retro-aldol
24 and 25
26
27
28
SCHEME 1

side chain in senescent phytoplankton cells. For this purpose, we irradiated killed cells of two strains of phytoplankton (*Skeletonema costatum* and *Thalassiosira weissflogii*) possessing clearly distinct chlorophyllase activities (12) with visible light for relatively long times (15 d). Then, to compare our *in vitro* observations with naturally occurring processes, some of the more characteristic lipid oxidation products identified were sought in particulate matter and sediment samples.

EXPERIMENTAL PROCEDURES

Biological material. *Skeletonema costatum* and *T. weissflogii* were obtained from the collection of living microalgae of the Centre d'Océanologie de Marseille. These two strains were grown in 500 mL f/2 medium (13) at 20°C using two 30 W fluorescent lamps (Osram, Fluora) and a 12 h/12 h light and dark cycle. The cultures were harvested by centrifugation ($8{,}000 \times g$) during the stationary phase for maximal yield. Prior to experiments the concentrated cells were taken through several freeze–thaw cycles to provide some disruption of cellular structure (2).

Photodegradation experiments. Broken phytoplanktonic cells were distributed in Pyrex flasks containing 50 mL of supernatant to which had been added 1 mL of a 0.1 M solution of mercuric chloride (to avoid bacterial growth). The flask-contents were irradiated (with magnetic stirring) using the same lamps as those used for growth at 20°C. Irradiance [as Photosynthetically Active Radiation (PAR)] was measured using a Licor LI 1000 data logger equipped with an LI 1935A spherical quantum sensor. Dark controls were carried out in parallel.

Treatment of phytoplanktonic cells. After filtration on GF/F (Whatman) paper, phytodetritus was chemically reduced (15 min) in methanol (25 mL) by excess $NaBH_4$ or $NaBD_4$. During this operation, hydroperoxides and ketones are reduced to the corresponding alcohols, and the possibility of some ester cleavage cannot be totally excluded. After reduction, 25 mL of water and 2.8 g of KOH were added, and the mixture was saponified by refluxing for 2 h. After cooling, the content of the flask was extracted with dichloromethane (3×20 mL), and the combined extracts were dried over Na_2SO_4, filtered, and concentrated by rotary evaporation to give fraction F_1. The aqueous phase was then acidified with hydrochloric acid (pH = 1) and extracted three times with dichloromethane. The combined dichloromethane extracts were dried over Na_2SO_4, filtered, and concentrated by rotary evaporation to give fraction F_2.

Particulate matter, sediment, and microbial mat sampling. The SOFI station is located in the Gulf of Lion (Mediterranean Sea) approximately 30 km from Marseilles at 43°04′N, 5°08′E. Two sediment traps (type PPS5; Technicap, La Turbie, France) with a 1 m^2 opening were deployed on December 10, 1997, along a fixed mooring under the euphotic layer (56.5 m) and 20 m from the bottom (142 m) and recovered on February 20, 1998. To avoid bacterial decomposition, the sample cups were filled before deployment with filtered (0.2 μm) seawater containing 5% of formaldehyde and 1.1 g L^{-1} of sodium tetraborate. The top layer of the bottom sediment (10 cm) was collected at 162 m depth with a multitube corer (Multicorer type Mark IV; Bowers and Connelly, Oban, United Kingdom; core diameter, 15 cm).

The DYFAMED station is located in the Ligurian Sea (northwestern Mediterranean Sea, 43°25′N, 7°52′E). Automated sediment traps (type PPS5; Technicap) were used to collect particulate matter samples at programmed intervals with a resolution of 4 and 7 d. The traps were deployed at 200 and 1000 m of depth.

The microbial mats were collected with manual corers in the Camargue (France) at sampling station no. 1 of the MATBIOPOL European program. This station is a pristine site located in a very large pond in the south of the Rhone delta, close to the sand barrier on the sea coast. The mats are constituted of laminated layers of about 5 to 10 cm depth (14).

The different samples were maintained in isothermal bags during transportation to Marseilles and then stored at –20°C for analysis.

Treatment of particulate matter, sediment, and microbial mat samples. All manipulations were carried out in foil-covered vessels in order to exclude photochemical artifacts. Particulate matter samples were filtered on preweighed GF/F (Whatman) paper and dried at 40°C, while sediment and microbial mat slices were cut under dim light and analyzed directly. Treatment involved reduction and alkaline hydrolysis as described above for phytoplanktonic cells.

Derivatization. The residues obtained were taken up in 300 μL of a mixture of pyridine and bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco, Bellefonte, PA) (2:1, vol/vol) and allowed to silylate at 50°C for 1 h. Following evaporation to dryness under nitrogen, the residue was taken up in ethyl acetate and analyzed by GC and GC–EI–MS.

Identification and quantification of oxidation products. These compounds were identified by comparison of retention times and mass spectra with those of standards and quantified (calibration with external standards) by GC–EI–MS. For low concentrations or in the case of coelutions, quantification was assessed by selected ion monitoring (SIM). GC–EI–MS analyses were carried out with a Hewlett-Packard (HP) 5890 series II plus gas chromatograph connected to an HP 5972 mass spectrometer. The following operating conditions were employed: 30 m × 0.25 mm (i.d.) columns coated with HP5 or HP1 (film thickness, 0.25 μm); oven temperature programmed from 60 to 130°C at 30°C min^{-1} and then from 130 to 300°C at 4°C min^{-1}; carrier gas (He) maintained at 1.04 bar until the end of the temperature program and then programmed from 1.04 bar to 1.5 bar at 0.04 bar min^{-1}; injector (splitless or on-column with a retention gap) temperatures, 300 or 50°C, respectively; electron energy, 70 eV; source temperature, 170°C; cycle time, 1.5 s.

Standard compounds. (*E*)-Phytol was purchased from Acros (Loughborough, England) and purified by column chromatography on silica gel (Kieselgel 60 + 0.5% H_2O) with

hexane/ethyl acetate (19:1, vol/vol) as eluant. 6,10,14-Trimethylpentadecan-2-one was obtained by oxidation of phytol with $KMnO_4$ in acetone (15). 3-Methylidene-7,11,15-trimethylhexadecan-1,2-diol was produced in two steps from (*E*)-phytol according to a previously described procedure (6). (*Z*)- and (*E*)-phytenic acids were synthesized in two steps: (i) oxidation of a mixture of (*Z*)- and (*E*)-phytols (Aldrich, St. Quentin Fallavier, France) with CrO_3/pyridine in dry methylene chloride (16), and (ii) oxidation of the resulting phytenals with sodium chlorite (17). 4,8,12-Trimethyltridecanoic acid was synthesized from isophytol (Interchim, Montlucon, France) by a previously described procedure (18). 5,9,13-Trimethyltetradecanoic acid was produced from 6,10,14-trimethylpentadecan-2-one (19). The synthesis of 3-hydroxy-3,7,11,15-tetramethylhexadecanoic acid required two steps: (i) sonically accelerated condensation of 6,10,14-trimethylpentadecan-2-one with α-bromoethyl acetate in the presence of zinc and iodine (Reformatsky reaction) (20), and (ii) saponification of the resulting β-hydroxyester. $AlLiH_4$ reduction of 4,8,12-trimethyltridecanoic acid, 6,10,14-trimethylpentadecan-2-one and 3-hydroxy-3,7,11,15-tetramethylhexadecanoic acid in dry diethyl ether afforded 4,8,12-trimethyltridecan-1-ol, 6,10,14-trimethylpentadecan-2-ol, and 3,7,11,15-tetramethylhexadecan-1,3-diol, respectively. Treatment of (*Z*)- and (*E*)-phytols with *meta*-chloroperoxybenzoic acid in dry methylene chloride yielded (*Z*)- and (*E*)-2,3-epoxy-3,7,11,15-tetramethylhexadecan-1-ols. Methanolysis of these glycidic aldehydes afforded a mixture of diastereoisomeric 3-methoxy-3,7,11,15-tetramethylhexadecan-1,2-diol and 2-methoxy-3,7,11,15-tetramethylhexadecan-1,3-diols. 3,7,11,15-Tetramethylhexadecan-1,2,3-triol, 2,3-dihydroxy-3,7,11,15-tetramethylhexadecanoic acid, and 3-hydroxymethyl-7,11,15-trimethyl hexadecan-1,2,3-triol were, respectively, obtained after oxidation of phytol, phytenic acids, and 3-methylidene-7,11,15-trimethylhexadecan-1,2-diol with OsO_4 in pyridine–dioxane (21). The synthesis of 2-methylidene-6,10,14-trimethylpentadecanoic acid required two steps: (i) cleavage of 3-methylidene-7,11,15-trimethylhexadecan-1,2-diol with lead tetraacetate in benzene, and (ii) oxidation of the resulting 2-methylidene-6,10,14-trimethylpentadecanal with sodium chlorite (17). Dihydrophytol, phytanic acid, and pristanic acid were obtained by hydrogenation of phytol, phytenic acid, and 2-methylidene-6,10,14-trimethylpentadecanoic acid, respectively, in methanol with Pd-$CaCO_3$ as a catalyst.

RESULTS AND DISCUSSION

Degradation of chlorophyll phytyl chain in killed cells of S. costatum *and* T. weissflogii. After exposure of *S. costatum* and *T. weissflogii* killed cells to 143 moles of photon m^{-2} (irradiation for 15 d), 98.5 and 84% of chlorophyll phytyl chain was degraded, respectively (Table 1), whereas no significant degradation was observed in dark controls. The higher degradation rate observed in the case of *S. costatum* could be attributed to the strong chlorophyllase activity of this diatom catalyzing the hydrolysis of chlorophyll to free phytol and chlorophyllide (12). Owing to its electron-withdrawing properties, the ester group in fact decreases electron density on the double bond of the phytyl chain. Thus, the reactivity of phytyl toward the electrophilic singlet oxygen is lower than that of phytol (8). However, despite the presence of a high proportion of free phytol in *Phaeodactylum tricornutum* killed cells, the photodegradation rate of the total (free + esterified) chlorophyll phytyl chain previously determined (8,22) was not significantly higher than in the case of algae with weak chlorophyllase activity (5). This surprising result was attributed to a decrease in the proximity of phytol to the tetra-

TABLE 1
Isoprenoid Compounds Detected After Irradiation of Senescent Phytoplanktonic Cells[a]

Compound	Code	*Skeletonema costatum*	*Thalassiosira weissflogii*
4,8,12-Trimethyltridecan-1-ol	**16**	1.6	3.2
4,8,12-Trimethyltridecanoic acid	**22**	1.9	1.2
6,10,14-Trimethylpentadecan-2-ol	**4**	16.4	19.1
5,9,13-Trimethyltetradecanoic acid	**37**	3.5	5.3
2,6,10,14-Tetramethylpentadecanoic acid	**15**	1.0	1.1
2-Methylidene-6,10,14-trimethylpentadecanoic acid	**19**	7.4	7.4
3,7,11,15-Tetramethylhexadecan-1-ol	**36**	0.8	Tr
3,7,11,15-Tetramethylhexadec-2-en-1-ol	**1**	1.5	16.0
3,7,11,15-Tetramethylhexadecanoic acid	**34**	0.2	Tr
3,7,11,15-Tetramethylhexadec-(*Z*)-2-enoic acid	**29**	0.7	Tr
3,7,11,15-Tetramethylhexadec-(*E*)-2-enoic acid	**30**	2.6	Tr
3-Methylidene-7,11,15-trimethylhexadecan-1,2-diol	**3**	33.4	28.0
3,7,11,15-Tetramethylhexadecan-1,3-diol	**5**	5.8	1.0
3-Hydroxy-3,7,11,15-tetramethylhexadecanoic acid	**6**	14.0	8.1
2-Methoxy-3,7,11,15-tetramethylhexadecan-1,3-diol	**28**	1.1	2.4
3-Methoxy-3,7,11,15-tetramethylhexadecan-1,2-diol	**27**	1.0	4.0
3-Methylidene-7,11,15-trimethylhexadecan-1,2,4-triol	**23**	1.6	1.1
3,7,11,15-Tetramethylhexadecan-1,2,3-triol	**26**	3.5	2.1
2,3-Dihydroxy-3,7,11,15-tetramethylhexadecanoic acid	**35**	1.1	Tr
3-Hydroxymethyl-7,11,15-trimethylhexadecan-1,2,3-triol	**20**	0.9	ND

[a]Relative percentages. Tr = traces (< 0.1%); ND = not detected.

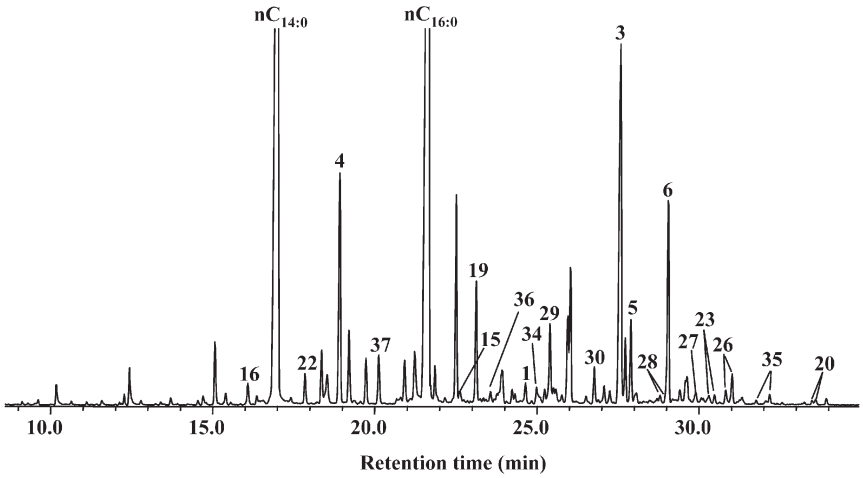
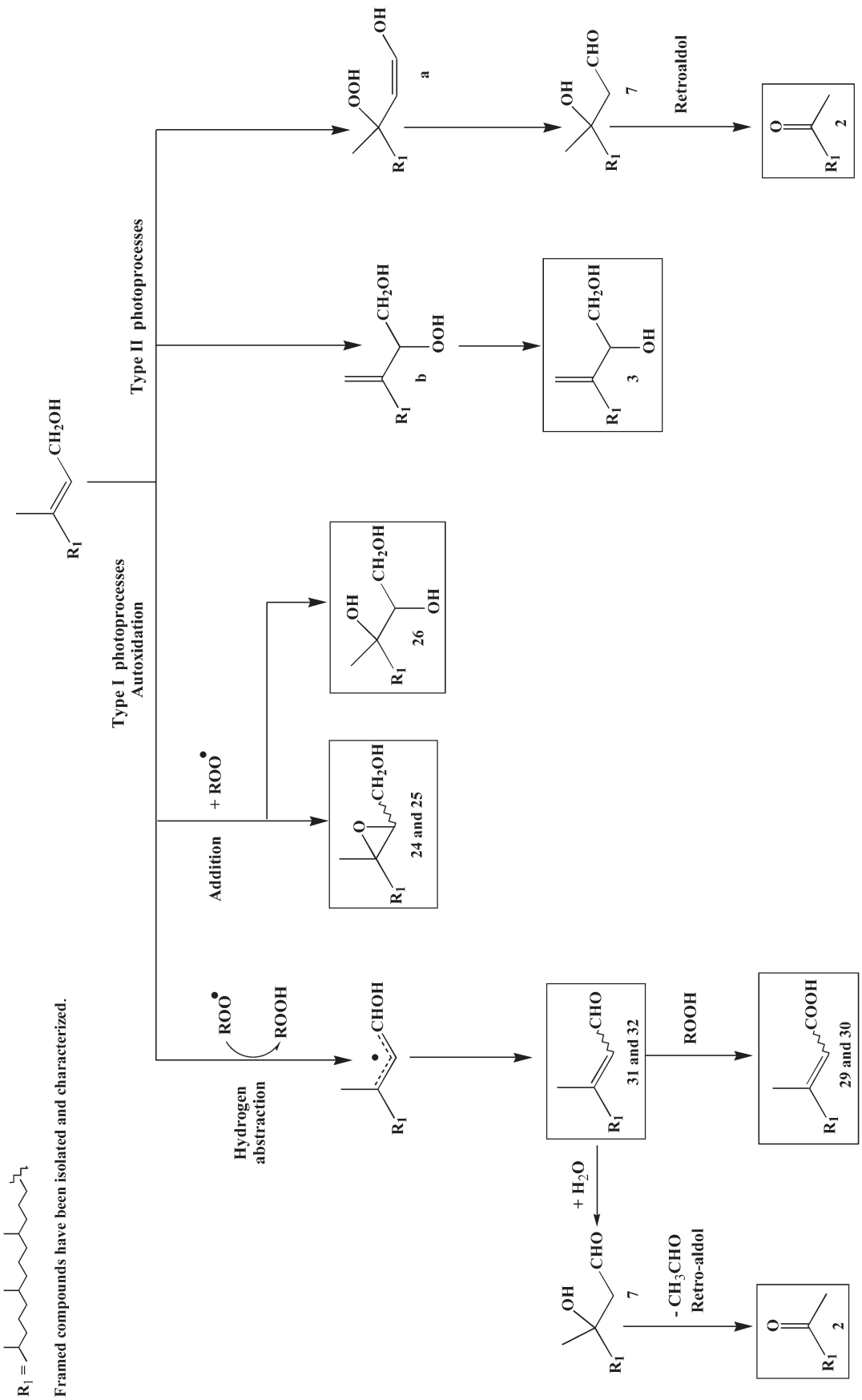


FIG. 1. Partial total ion chromatogram showing the chlorophyll phytyl chain oxidation product region after injection of the F_1 extract of *Skeletonema costatum* killed cells exposed to 143 mol of photons m^{-2} (*Mixtures of diastereoisomers). (**1**) 3,7,11,15-Tetramethylhexadec-2-en-1-ol (phytol); (**3**) 3-methylidene-7,11,15-trimethylhexadecan-1,2-diol (phytyldiol); (**4**) 6,10,14-trimethylpentadecan-2-ol; (**5**) 3,7,11,15-tetramethylhexadecan-1,3-diol; (**6**) 3-hydroxy-3,7,11,15-tetramethylhexadecanoic acid; (**15**) 2,6,10,14-tetramethylpentadecanoic acid (pristanic acid); (**16**) 4,8,12-trimethyltridecan-1-ol; (**19**) 2-methylidene-6,10,14-rimethylpentadecanoic acid; (**20**) 3-hydroxymethyl-7,11,15-trimethylhexadecan-1,2,3-triol*; (**22**) 4,8,12-trimethyltridecanoic acid; (**23**) 3-methylidene-7,11,15-trimethylhexadecan-1,2,4-triol*; (**26**) 3,7,11,15-tetramethylhexadecan-1,2,3-triol*; (**27**) 3-methoxy-3,7,11,15-tetramethylhexadecan-1,2-diol*; (**28**) 2-methoxy-3,7,11,15-tetramethylhexadecan-1,3-diol*; (**29**) 3,7,11,15-tetramethylhexadec-(*Z*)-2-enoic acid [(*Z*)-phytenic acid)] + *x*; (**30**) 3,7,11,15-tetramethylhexadec-(*E*)-2-enoic acid [(*E*)-phytenic acid)]; (**34**) 3,7,11,15-tetramethylhexadecanoic acid (phytanic acid); (**35**) 2,3-dihydroxy-3,7,11,15-tetramethylhexadecanoic acid*; (**36**) 3,7,11,15-tetramethylhexadecan-1-ol (dihydrophytol); (**37**) 5,9,13-trimethyltetradecanoic acid.

pyrrolic moiety of chlorophyll (and therefore from the singlet oxygen source) after the action of chlorophyllase. Consequently, the higher degradation rate observed in the case of *S. costatum* must probably be more the result of the greater efficiency of autoxidative processes toward free phytol. Indeed, it was previously demonstrated that autoxidation of the chlorophyll phytyl chain involves mainly the addition of hydroxyl or peroxyl radicals to the double bond (7), in a similar fashion to type I photoprocesses (Scheme 1), whereas the autoxidative degradation of free phytol involves additional abstraction of allylic hydrogen atoms (Scheme 2). This is in good agreement with the results of Huyser and Johnson (23), who demonstrated that esterification of allylic alcohols strongly favors addition processes rather than abstraction.

Characterization of oxidation products and mechanistic aspects. As has been previously demonstrated in the case of other algae (3,5), type II photodegradation of the chlorophyll phytyl chain in killed cells of *S. costatum* and *T. weissflogii* leads mainly to the production of photoproducts of type **a** and **b** (Schemes 1 and 2), quantifiable after alkaline hydrolysis, respectively, in the form of 6,10,14-trimethylpentadecan-2-one (**2**) and 3-methylidene-7,11,15-trimethylhexadecan-1,2-diol (phytyldiol) (**3**) (3). The almost total reduction of the ketone **2** to the corresponding alcohol **4** observed after $NaBH_4$ reduction and subsequent alkaline hydrolysis (Table 1, Fig. 1) clearly shows that photoproducts of type **a** are quickly hydrolyzed in phytodetritus. Irradiation of the two killed phytoplanktonic strains for relatively long times also results in the production of several other isoprenoid compounds (Table 1, Fig. 1). Most of these compounds were unambiguously identified by comparison of their chromatographic retention times and mass spectra (Figs. 2 and 3) with those of adequate standards.

The quick hydrolysis of photoproducts of type **a** in phytodetritus is well supported by the detection of significant amounts of 3,7,11,15-tetramethylhexadecan-1,3-diol (**5**) and 3-hydroxy-3,7,11,15-tetramethylhexadecanoic acid (**6**) (Table 1). Deuterium labeling (reduction with $NaBD_4$ instead of $NaBH_4$) demonstrated that compound **5** results from the reduction of 3-hydroxy-3,7,11,15-tetramethylhexadecanal (**7**) or 3-hydroperoxy-3,7,11,15-tetramethylhexadecanal (**8**) during the treatment (Fig. 3D). The β-aldol **7**, which is produced by homolytic cleavage of the O–O bond of photoproducts of type **a**, reduction of the foregoing alkoxyl radical, and subsequent hydrolysis (Scheme 3), cannot be directly characterized since it does not survive alkaline hydrolysis and is cleaved to the ketone **2** (retro-aldol reaction). Owing to the presence of hydroperoxides in phytodetritus, this compound is easily oxidized to the corresponding hydroxyacid **6**. It is interesting to note that the direct hydrolysis of photoproducts of type **a** may afford 3-hydroperoxy-3,7,11,15-tetramethyl-hexadecanal (**8**) (Scheme 3), which can then (i) undergo an intramolecular reaction to give an endoperoxide hemi-acetal (**9**) (24) affording the hydroxyacid **6** after homolytic cleavage of its O–O bond, or (ii) rearrange to the isomeric (*Z*)- and (*E*)-2,3-epoxy-


R1 =
Framed compounds have been isolated and characterized.
Type I photoprocesses
Autoxidation
Hydrogen abstraction
ROO•
ROOH
Addition
+ ROO•
24 and 25
26
Type II photoprocesses
a
b
3
7
Retroaldol
2
31 and 32
29 and 30
ROOH
+ H2O
- CH3CHO
Retro-aldol

SCHEME 2

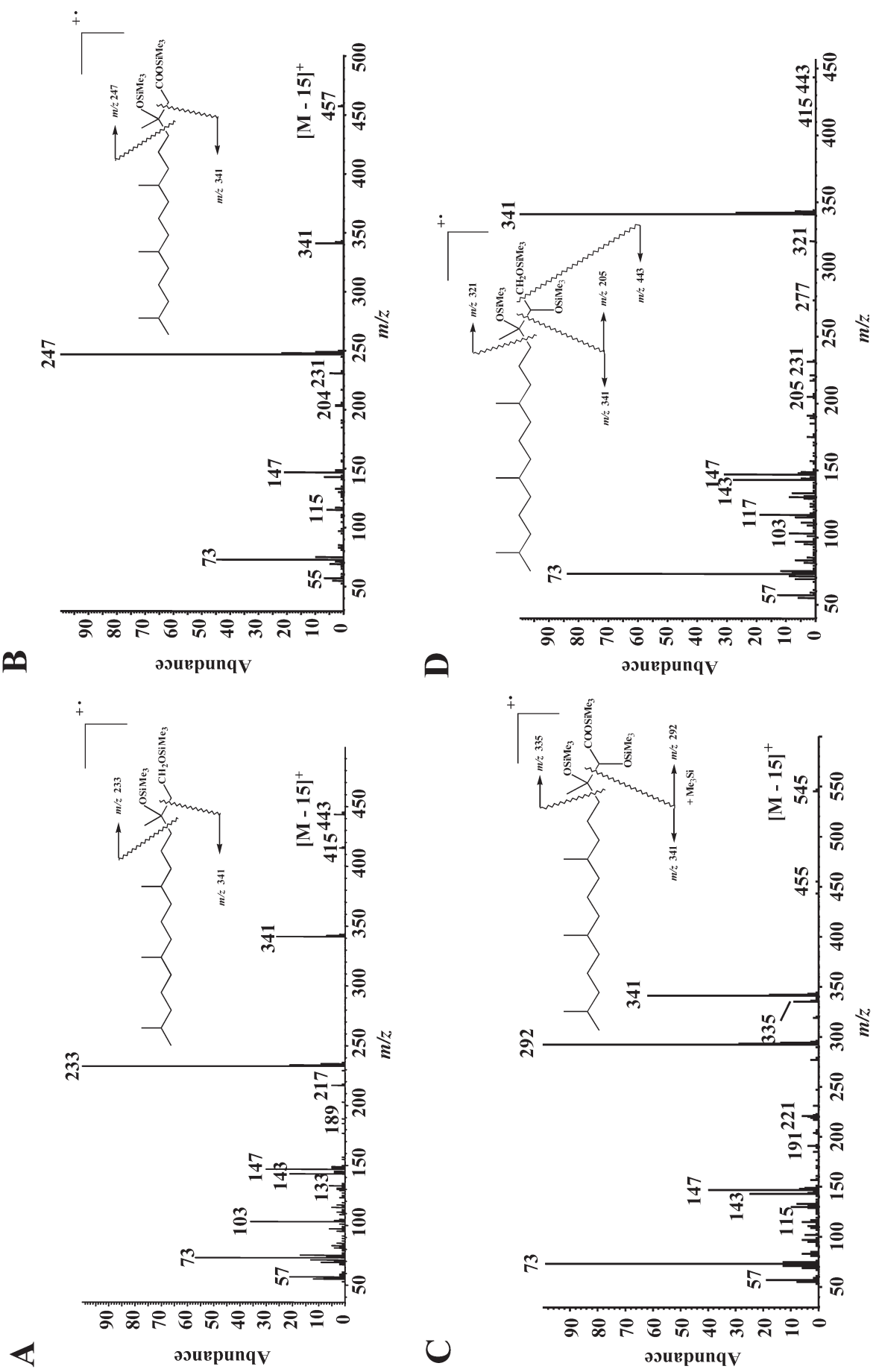


FIG. 2. EI mass spectra of (A) 3,7,11,15-tetramethylhexadecan-1,3-diol (**5**), (B) 3-hydroxy-3,7,11,15-tetramethylhexadecanoic acid (**6**), (C) 2,3-dihydroxy-3,7,11,15-tetramethylhexadecanoic acid (**35**), and (D) 3,7,11,15-tetramethylhexadecan-1,2,3-triol (**26**) (silylated).

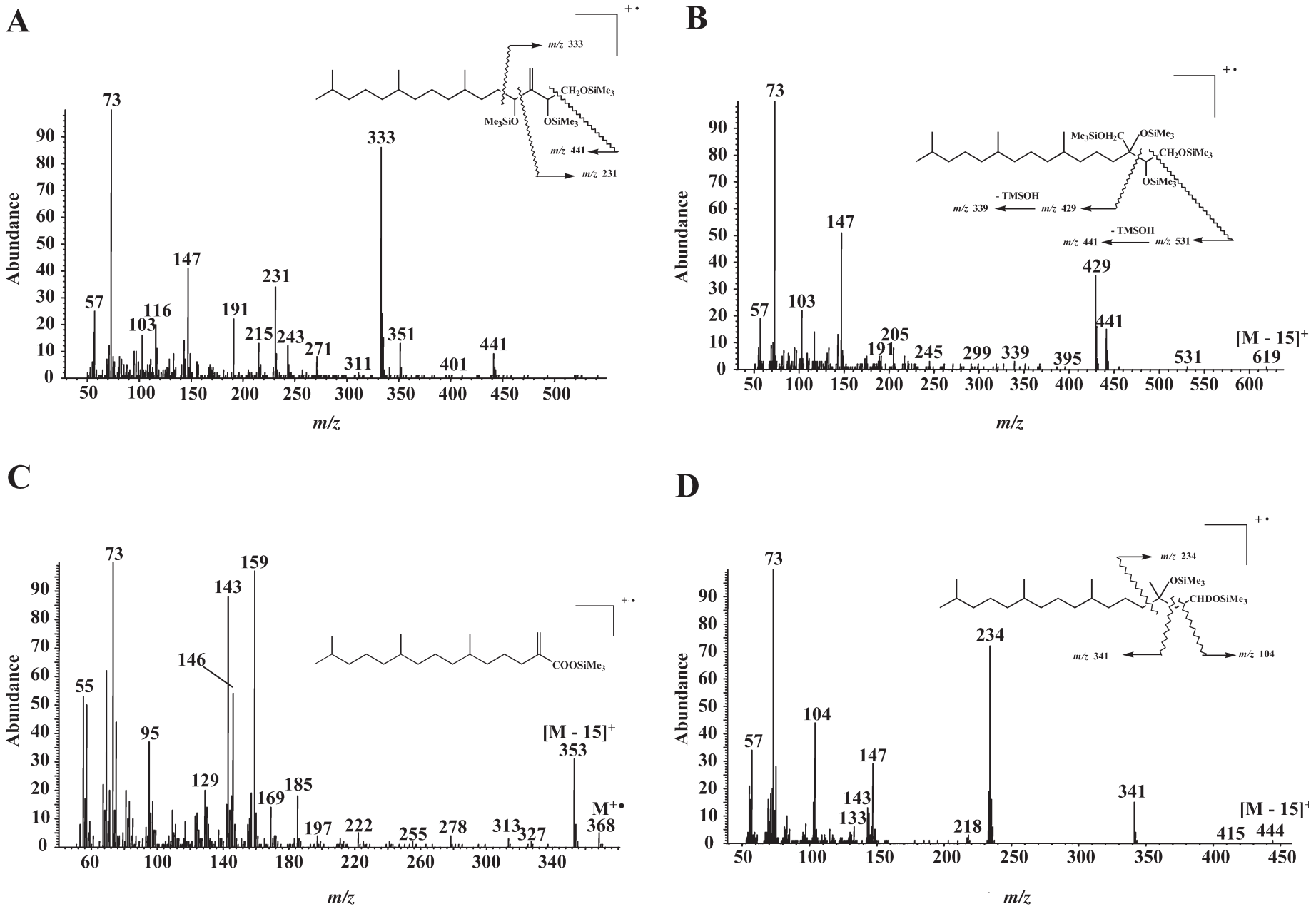
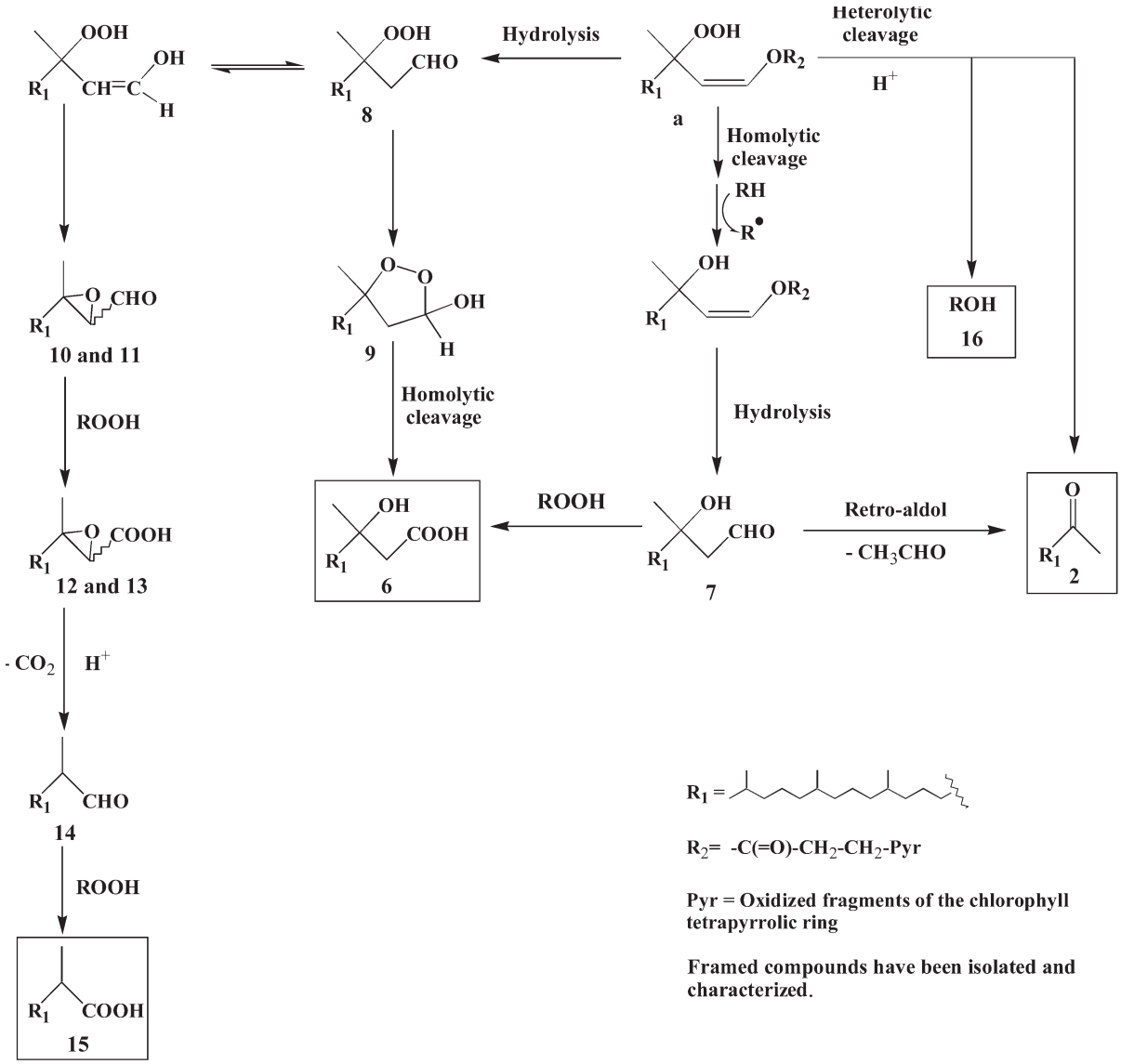


FIG. 3. EI mass spectra of (A) 3-methylidene-7,11,15-trimethylhexadecan-1,2,4-triol (**23**), (B) 3-hydroxymethyl-7,11,15-trimethylhexadecan-1,2,3-triol (**20**), (C) 2-methylidene-6,10,14-trimethylpentadecanoic acid (**19**), and (D) [1-D]-3,7,11,15-tetramethylhexadecan-1,3-diol (**5**) (silylated). TMSOH, trimethylsilanol.

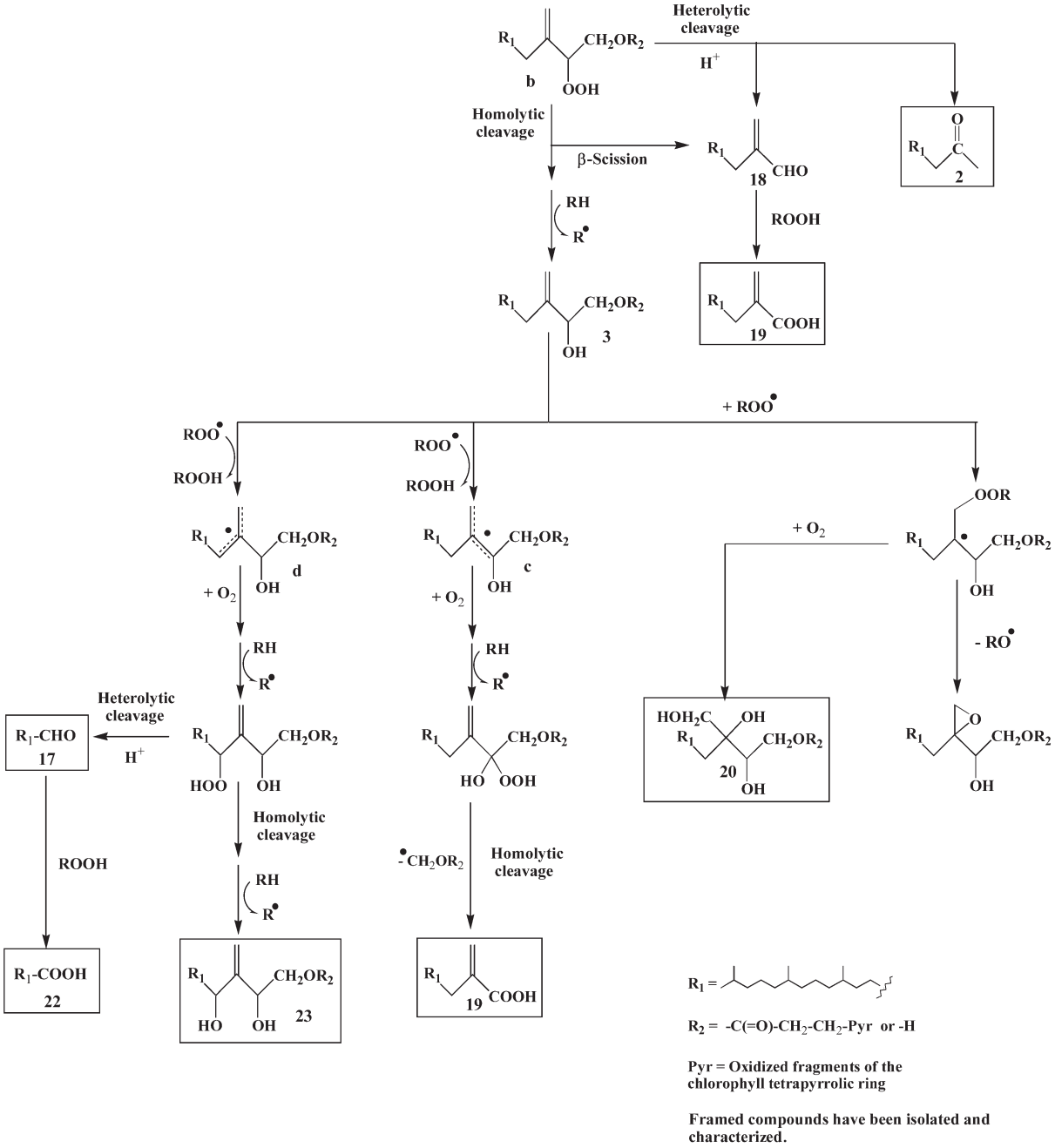
3,7,11,15-tetramethylhexadecanals (**10** and **11**) (25). In the presence of hydroperoxides, these glycidic aldehydes are oxidized to the corresponding acids (**12** and **13**), which can then undergo an easy proton-catalyzed decarboxylation (26) to give 2,6,10,14-tetramethylpentadecanal (pristanal) (**14**) and 2,6,10,14-tetramethylpentadecanoic acid (pristanic acid) (**15**) after subsequent oxidation (Scheme 3).

Small amounts of 4,8,12-trimethyltridecan-1-ol (**16**) were also detected (Table 1). Deuterium labeling demonstrated that approximately 50% of this compound was present before $NaBH_4$ reduction, the other 50% arising from the reduction of the corresponding aldehyde (**17**). The formation of this alcohol may be attributed to proton-catalyzed cleavage of photoproducts of type **a** (27), this process also affording phytone (**2**) (Scheme 3). Although these cleavages are generally acid-catalyzed, several have been reported to occur in the absence of any added acid (27). The detection of homologous series of $NaBH_4$ reduced ω-oxocarboxylic and α,β-dicarboxylic acids in the C_7–C_{11} range [resulting from heterolytic cleavage of photochemically produced allylic hydroperoxyacids (28)] in the fractions F_1 and F_2 supports the involvement of such processes in killed phytoplanktonic cells.

Proton-catalyzed cleavage can also act on photoproducts of type **b** producing phytone (**2**) and 2-methylidene-6,10,14-trimethylpentadecanal (**18**) (Scheme 4), this aldehyde then being quickly oxidized to the corresponding acid (**19**) in the presence of hydroperoxides. Homolytic cleavage of the O–O bond of these photoproducts affords alkoxyl radicals, which can either undergo subsequent β-scission reaction to give the aldehyde **18** or be reduced to the corresponding alcohols after abstraction of a hydrogen atom from other molecules (Scheme 4). Classical addition of a peroxyl radical to the double bond of free or esterified phytyldiol **3** thus formed (29) gives tertiary radicals (Scheme 4). These radicals can then (i) lead to epoxides by fast intramolecular homolytic substitution (11) or (ii) react with molecular oxygen affording free



SCHEME 3

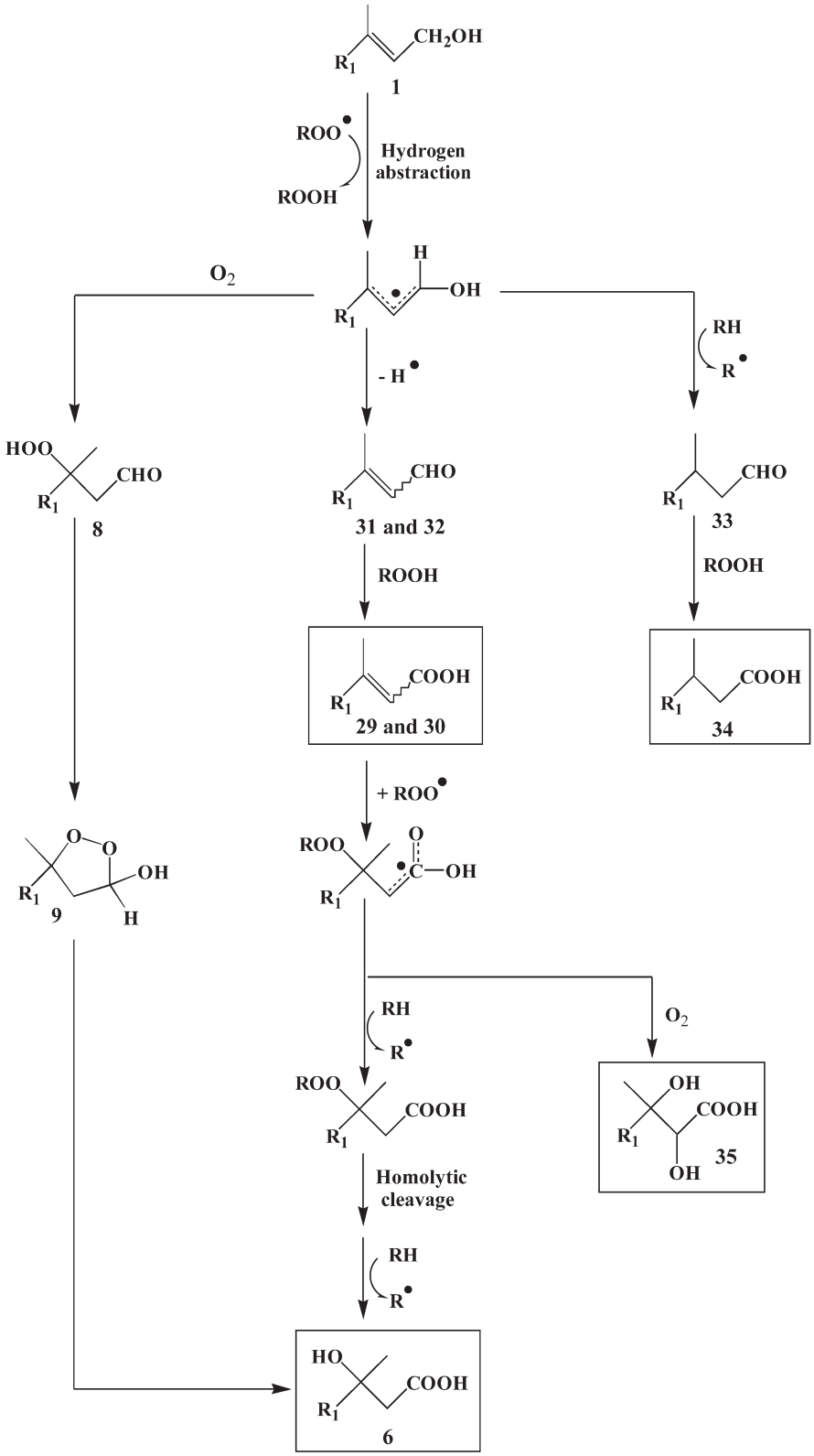


SCHEME 4

or esterified 3-hydroxymethyl-7,11,15-trimethylhexadecan-1,2,3-triol (**20**).

Peroxyl radicals may also induce allylic hydrogen abstraction on free or esterified phytyldiol **3** affording well-stabilized radicals of types **c** and **d** (Scheme 4). Oxidation of radicals of type **c** results in the formation of 2-methylidene-6,10,14-trimethylpentadecanoic acid (**19**), whereas that of radicals of type **d** affords hydroperoxides, which may undergo (i) heterolytic cleavage to give 4,8,12-trimethyltridecanal (**17**) and then the corresponding acid (**22**) or (ii) homolytic cleavage and subsequent reduction to give free or esterified 3-methylidene-7,11,15-trimethylhexadecan-1,2,4-triol (**23**) (Scheme 4).

It was previously demonstrated that autoxidation of the chlorophyll phytyl chain involves mainly the addition of hydroxyl or peroxyl radicals to the double bond, affording after subsequent gentle alkaline hydrolysis mainly (*Z*)- and (*E*)-3,7,11,15-tetramethyl-2,3-epoxyhexadecan-1-ols (**24** and **25**), 3,7,11,15-tetramethylhexadecan-1,2,3-triol (**26**), and phytone (**2**) (Scheme 1) (7). Indeed, we detected significant amounts of the triol **26** in phytodetritus (Table 1, Fig. 1); the detection of 3-methoxy-3,7,11,15-tetramethylhexadecan-1,2-diol (**27**) and 2-methoxy-3,7,11,15-tetramethylhexadecan-1,3-diol (**28**), resulting from the methanolysis of the epoxides **24** and **25** under the alkaline hydrolysis conditions used, attests to the



Framed compounds have been isolated and characterized.

SCHEME 5

TABLE 2
Reported Occurrence of Oxidized Acyclic Isoprenoid Compounds in Lacustrine and Marine Environments

Compound	Code	References
4,8,12-Trimethyltridecanoic acid[b,d]	**22**	31–33
6,10,14-Trimethylpentadecan-2-one[b,c,d,e]	**2**	33,34–38[a]
5,9,13-Trimethyltetradecanoic acid[b,d]	**37**	31,33
2,6,10,14-Tetramethylpentadecanoic acid[b,d]	**15**	39
3,7,11,15-Tetramethylhexadecanal[c,d]	**33**	40
(*Z* and *E*)-3,7,11,15-Tetramethylhexadec-2-enal[b,c,d,e]	**31,32**	30,31,40,41
3,7,11,15-Tetramethylhexadecanoic acid[b,d]	**34**	31,33,42
3,7,11,15-Tetramethylhexadec-(*Z*)-2-enoic acid[b,d]	**29**	33
3,7,11,15-Tetramethylhexadec-(*E*)-2-enoic acid[b,c,d]	**30**	31,33,40,43
3-Methylidene-7,11,15-trimethylhexadecan-1,2-diol[b,d,e]	**3**	5,6

[a]And numerous others.
[b]Marine environment.
[c]Lacustrine environment.
[d]Sediments.
[e]Particulate matter.

presence of such compounds in senescent phytoplanktonic cells before treatment. Deuterium labeling (reduction with $NaBD_4$) showed that a small proportion of compounds **26–28** also arises from reduction and hydrolysis or methanolysis of the glycidic aldehydes **10** and **11** (Scheme 3).

The presence of nonnegligible amounts of phytol oxidation products deriving from abstraction processes [e.g., (*Z*)- and (*E*)-3,7,11,15-tetramethylhexadec-2-enoic acids (**29** and **30**)] in senescent cells of *S. costatum* (Table 1, Fig. 1) can be attributed to the well-documented high chlorophyllase activity of this strain (12) catalyzing the hydrolysis of chlorophyll to free phytol and chlorophyllide. Allylic hydrogen abstraction at carbon 1 of free phytol forms a well-stabilized radical, which may then either lose a hydrogen atom to give (*Z*)- and (*E*)-3,7,11,15-tetramethylhexadec-2-enals (**31** and **32**), easily oxidized to the corresponding phytenic acids **29** and **30** in the presence of hydroperoxides (Scheme 2), or abstract a hydrogen atom to another molecule to give 3,7,11,15-tetramethylhexadecanal (**33**) and then 3,7,11,15-tetramethylhexadecanoic acid (**34**) (phytanic acid), or react with oxygen affording 3-hydroxy-3,7,11,15-tetramethylhexadecanoic acid (**6**) (Scheme 5). This β-hydroxy acid may also result from (i) hydration of phytenals **31** and **32** [which is a very easy process (30)] (Scheme 2) followed by the oxidation of the β-aldol **7** thus formed, or (ii) addition of peroxyl radicals to the double bond of phytenic acids **29** and **30**, this process also affording 2,3-dihydroxy-3,7,11,15-tetramethylhexadecanoic acid (**35**) (Scheme 5). Deuterium labeling clearly showed that 3,7,11,15-tetramethylhexadecan-1-ol (**36**) detected in the lipid extract of *S. costatum* (Table 1) arises from the reduction of the corresponding aldehyde **33** during the treatment.

Significance of photochemical oxidation and autoxidation of chlorophyll phytyl side chain in the marine environment. Photochemical oxidation and autoxidation of the phytyl chain of phytoplanktonic chlorophyll appear to be potential sources of numerous oxidized acyclic isoprenoids previously detected in lacustrine and marine environments (Table 2). The involvement of these processes must be taken into account in future works using such compounds as biogeochemical tracers (44).

To our knowledge, there is no report in the literature of the presence of 3,7,11,15-tetramethylhexadecan-1,3-diol (**5**), 3-hydroxy-3,7,11,15-tetramethylhexadecanoic acid (**6**), 2-methylidene-6,10,14-trimethylpentadecanoic acid (**19**), 3-hydroxymethyl-7,11,15-trimethylhexadecan-1,2,3-triol (**20**), 3-methylidene-7,11,15-trimethylhexadecan-1,2,4-triol (**23**), (*Z*)- and (*E*)-3,7,11,15-tetramethyl-2,3-epoxyhexadecan-1-ols (**24** and **25**), 3,7,11,15-tetramethylhexadecan-1,2,3-triol (**26**), and 2,3-dihydroxy-3,7,11,15-tetramethylhexadecanoic acid (**35**) in natural samples. We thus have searched for these compounds in $NaBH_4$-reduced total lipid extracts of particulate

TABLE 3
Concentration (%) of Newly Described Isoprenoids Relative to Intact Chlorophyll Phytyl Side Chain in Different Natural Samples

Compound	Code	Particulate matter (SOFI station)[a]	Particulate matter (DYFAMED station)[b]	Sediment (SOFI station)[c]	Microbial mats (Camargue)[d]
3,7,11,15-Tetramethylhexadecan-1,3-diol	**5**	—	2.7	—	—
3-Hydroxy-3,7,11,15-tetramethylhexadecanoic acid	**6**	—	—	0.2	0.3
3-Methylidene-7,11,15-trimethylhexadecan-1,2,4-triol	**23**	0.8	5.8	0.4	0.6
3,7,11,15-Tetramethylhexadecan-1,2,3-triol	**26**	1.7	2.5	0.7	1.3
3-Methoxy-3,7,11,15-tetramethylhexadecan-1,2-diol	**27**	0.4	6.4	—	—
2-Methoxy-3,7,11,15-tetramethylhexadecan-1,3-diol	**28**	0.3	1.4	—	—

[a]Sample collected between December 11 and 14, 1997, at 142 m.
[b]Sample collected between August 3 and 10, 1998, at 200 m.
[c]A 1-cm slice, representing the sediment between 2 and 3 cm below the surface of the 10-cm core.
[d]A 0.5-cm slice, representing the sediment between 1.0 and 1.5 cm below the surface of the 10-cm core.

matter, sediment, and microbial mat samples. The results obtained (Table 3) clearly show the presence of compounds **5**, **6**, **23**, and **26** in the extracts analyzed. We also detected 2-methoxy-3,7,11,15-tetramethylhexadecan-1,3-diol (**28**) and 3-methoxy-3,7,11,15-tetramethylhexadecan-1,2-diol (**27**) in particulate matter samples (Table 3), which attests to the presence of nonnegligible amounts of (*Z*)- and (*E*)-3,7,11,15-tetramethyl-2,3-epoxyhexadecan-1-ols (**24** and **25**) before the treatment. Although 2-methylidene-6,10,14-trimethylpentadecanoic acid (**19**) is strongly produced after irradiation of killed phytoplanktonic cells (Table 1, Fig. 1), we failed to detect this acid in particulate matter and sediment samples containing high amounts of phytyldiol **3**. It seems that heterolytic cleavage of chlorophyll photoproducts of type **b**, which must constitute the main source of this compound in phytodetritus (Scheme 4), does not occur significantly under natural conditions. We have previously demonstrated that the degradation of photoproducts of type **b** in seawater mainly involve homolysis of the O–O bond and subsequent reduction of the alkoxyl radicals thus formed (6). Consequently, it is assumed that the detection of large amounts of the acid **19** after *in vitro* incubation must result in enhancement of heterolytic cleavages (at the expense of homolytic processes) by some components of the f/2 medium. The detection of significant amounts of compounds **5** and **6** in the reduced samples shows that, as was demonstrated above *in vitro* in killed phytoplanktonic cells, hydrolysis of chlorophyll photoproducts of type **a** (Scheme 3) is also a quick process under environmental conditions.

Acyclic isoprenoid compounds are well suited as biological markers since they are often abundant and widely distributed. Moreover, the relatively stable isoprane skeletal unit is readily identified and allows these compounds to be used as tracers over long periods of geological time (45). Consequently, some isoprenoids have been proposed as indicators of oxic conditions during sedimentation (37,43). A difficulty with the use of these compounds as markers of oxic conditions is the possibility that they may be formed *via* microbially mediated reactions that may not reflect the redox conditions of the sediment as a whole (44,45). Chlorophyll phytyl side chain oxidation products **23–26**, which are present in the marine environment, might constitute interesting new tracers of oxic deposition conditions. The presence of pairs of diastereoisomers and of *Z* and *E* configurations, which is the result of nonstereospecific oxy-free radical processes, argues against enzymatic formation since enzymatic processes are generally highly stereospecific. There is a need to search for these compounds in sediments from contrasting sedimentation conditions in order to validate their role as tracers of oxic deposition conditions.

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Isolation of Unsaturated Diols After Oxidation of Conjugated Linoleic Acid with Peroxygenase

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ABSTRACT: Oat seeds are a rich source of peroxygenase, an iron heme enzyme that participates in oxylipin metabolism in plants. An isomer of CLA, 9(*Z*),11(*E*)-octadecadienoic acid (**1**), believed to have anticarcinogenic activity, was used as a substrate for peroxygenase in an aqueous medium using *t*-butyl hydroperoxide as the oxidant. After acidification of the reaction medium, the products were extracted with ethyl ether, converted to their methyl esters, and characterized using HPLC. Major products after reaction for 24 h showed resonances from ^{1}H NMR spectroscopy that were further downfield than the expected epoxides and were thought to be diol hydrolysis products. However, analyses by HPLC with atmospheric pressure chemical ionization MS (APCI–MS) of the putative allylic diols or their bis-trimethylsilyl ether derivatives gave incorrect M.W. The M.W. of the diols could be obtained by APCI–MS after removal of unsaturation by hydrogenation or by EI–MS after conversion of the allylic 1,2-diols to cyclic methyl boronic esters. Data from MS in conjunction with analyses using ^{1}H and ^{13}C NMR showed that the methylated products from **1** were methyl 9,10(*threo*)-dihydroxy-11(*E*)-octadecenoate, methyl 9,10(*erythro*)-dihydroxy-11(*E*)-octadecenoate, methyl 9,12(*erythro*)-dihydroxy-10(*E*)-octadecenoate, and methyl 9,12(*threo*)-dihydroxy-10(*E*)-octadecenoate. Solid-phase extraction without prior acidification and conversion of the products to methyl esters allowed identification of the following epoxides: methyl 9,10(*Z*)-epoxy-11(*E*)-octadecenoate (**6M**), methyl 9,10(*E*)-epoxy-11(*E*)-octadecenoate, and methyl 11,12(*E*)-epoxy-9(*Z*)-octadecenoate. At times of up to at least 6 h, **6M** accounted for approximately 90% of the epoxide product. Product analysis after the hydrolysis of isolated epoxide **6M** showed that hydrolysis of epoxide **6** could largely account for the diol products obtained from the acidified reaction mixtures.

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CLA is a term that refers to the positional and geometric conjugated dienoic isomers of linoleic acid. In the last two decades, studies have shown that CLA isomers can suppress cancer development, are antiatherogenic, and have both growth-promoting and body fat-reducing properties. Only very recently, however, have some of the sites of CLA action been elucidated on a molecular basis, and it has become apparent that different CLA isomers have different physiological effects and that metabolites of CLA are responsible for some of its actions (1). The oxidation of CLA has received only limited study. Yurawecz *et al*. (2) found that CLA in methanol/water mixtures was oxidized to furan FA when exposed to air, and a subsequent study investigated the autoxidation products of furan fatty esters derived from CLA (3). Hämäläinen *et al.* (4) obtained the ^{1}H and ^{13}C NMR spectra of autooxidized CLA methyl ester and showed that hydroperoxides were formed. Lie Ken Jie and Pasha (5) studied the action of *m*-chloroperoxybenzoic acid and potassium peroxymonosulfate on CLA isomer, methyl 9(*Z*),11(*E*)-octadecadienoate. A mixture of positional monoepoxides, but no diepoxide, was generated by chloroperoxybenzoic acid, whereas potassium peroxymonosulfate in the presence of trifluoroacetone gave 9,10(*Z*);11,12(*E*)-diepoxystearate. Recently, we have been investigating the iron heme enzyme peroxygenase from oat (*Avena sativa*) seeds for its ability to replace acid catalysts in the production of fatty epoxides, materials that are used as plasticizers and stabilizers of plastic polymers (6–8). Previously, we found that oleic, linoleic, linolenic, ricinoleic, and erucic acids gave epoxide derivatives when exposed to peroxygenase in the presence of the oxidant *t*-butyl hydroperoxide, and that *Z* configuration double bonds were preferred for epoxidation (8,9). When the CLA isomer 9(*Z*),11(*E*)-octadecadienoic acid **1** was subjected to the action of peroxygenase in the presence of *t*-butyl hydroperoxide, unexpected products were found after acidic workup, rather than the expected 9,10-monoepoxide. We report here that these products are isomeric 1,2- and 1,4-diol derivatives of CLA. In addition, we show that the likely major precursor of these diols is the epoxide formed with the *Z* configuration on the 9,10 double bond of **1**, and that this epoxide can be isolated without acidification by using solid-phase extraction.

MATERIALS AND METHODS

Materials. Sigma Chemical Co. (St. Louis, MO) was the source of *t*-butyl hydroperoxide (70%). 9(*Z*),11(*E*)-Octadecadienoic acid, 98+% (**1**) and 9(*E*),11(*E*)-octadecadienoic acid (**5**) were purchased from Matreya (Pleasant Gap, PA). Palladium on activated carbon, methyl boronic acid, and 3-chloroperoxybenzoic acid (CPBA) were purchased from Aldrich (Milwaukee, WI). *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce (Rockford, IL). C-18 Mega Bond Elut columns were purchased from Varian (Palo Alto, CA). Methyl (±)-*threo*-9,10-dihydroxyoctadecanoate and methyl (±)-*erythro*-9,10-dihydroxyocta-

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Abbreviations: APCI–MS, atmospheric pressure chemical ionization MS; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; CPBA, 3-chloroperoxybenzoic acid; M, methyl ester; NP-HPLC, normal-phase HPLC; RP-HPLC, reversed-phase HPLC; TMS, trimethylsilyl.

decanoate were purchased from Larodan Fine Chemicals AB (Malmö, Sweden).

Epoxide standards. CLA isomers **1** and **5** were treated with an equivalent amount of CPBA for 0.25 h at room temperature in CH_2Cl_2 as previously described (5) to give the two possible monoepoxide regioisomers. After methylation the monoepoxides were purified by HPLC, as described later, to give methyl 9,10(*Z*)-epoxy-11(*E*)-octadecenoate (**6M**), methyl 11,12(*E*)-epoxy-9(*Z*)-octadecenoate (**7M**), methyl 9,10(*E*)-epoxy-11(*E*)-octadecenoate (**8M**), and methyl 11,12(*E*)-epoxy-9(*E*)-octadecenoate (**9M**).

Enzymatic oxidation. Peroxygenase immobilized on a Fluoropore membrane was prepared as described previously (6). After vacuum infiltration, the membrane was washed with 20 mL CH_2Cl_2, cut into four equal pieces, and placed into a 50-mL glass-stoppered Erlenmeyer flask containing 17.7 μmol CLA substrate **1** in 7.0 ml 50 mM Hepes [*N*(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)], 0.1% (wt/vol) Tween 20, pH 7.5. Reactions were agitated at 20°C for the indicated time. At the start of the reaction and again at 1, 2, and 4 h, 3.38 μmol *t*-butyl hydroperoxide was added, and at 6 h, 20.26 μmol *t*-butyl hydroperoxide was added.

Acidic extraction. The reactions were terminated by adding 20 mL methanol and 10 mL water to each reaction. The pH was lowered to 3.0 with 1.0 N HCl, and the reaction mixture was extracted with two 50-mL portions of diethyl ether. The combined ether fractions were washed with water (2 × 70 mL), dried with anhydrous sodium sulfate, and the ether was removed under a stream of nitrogen. Products were redissolved in 10 mL dichloromethane and methylated with diazomethane.

Solid-phase extraction. A 1 g C-18 Bond Elut column was washed sequentially with 5 mL CH_2Cl_2, acetone, methanol, and H_2O. The reaction solution was added to the column. The column was washed with 5 mL H_2O, and the products were eluted sequentially with 5 mL methanol, acetone, and CH_2Cl_2. The solvent was removed under a stream of nitrogen, and the products were methylated as before.

Cyclic methyl boronic esters and trimethylsilyl (TMS) ethers. Cyclic methyl boronic esters for MS analysis were prepared using methyl boronic acid in dimethoxypropane as previously described (10). TMS derivatives were prepared with BSTFA in pyridine.

Catalytic hydrogenation. Double bonds were hydrogenated by bubbling H_2 gas into an ethanol solution of product containing palladium on activated carbon for 2 h at ambient temperature.

Epoxide hydrolysis products. Methylated epoxide standards were prepared from CLA isomers **1** and **5** using CPBA as already described and were separated using HPLC. These were individually hydrolyzed by placing them in a mixture of acetone, water, and acetic acid (10:2:1, by vol) for 0.25 h and extracting the products with diethyl ether.

Chromatographic and instrumental methods. The purity of the methyl esters of CLA (**1M**) was established by using a gas chromatograph linked to a mass spectrometer (GC–MS); the GC was equippped with a fused-silica capillary column SPTM-2340 (0.25 mm × 60 m) (Supelco, Bellefonte, PA) operated at 130 to 180°C with an increase of 2°C/min using He as the carrier gas. The methyl esters of the diols (**2M**–**4M**) were separated by reversed-phase (RP)-HPLC on two Symmetry 3.5 μm C_{18} reversed-phase columns (150 × 2.1 mm and 50 × 2.1 mm) (Waters, Milford, MA) connected in series. The mobile phase composition and the linear gradient used were 0–5 min H_2O/CH_3CN (40:60, vol/vol); 5–30 min H_2O/CH_3CN (40:60) to CH_3CN (100); 30–54 min CH_3CN (100). The flow rate was 0.25 mL/min. The 1,4-diol isomers (**4M**) were resolved into two isomers by normal-phase (NP)-HPLC on a Dynamax Macro HPLC Si column (250 × 10 mm) (Rainin, Woburn, MA). The mobile phase composition and the gradient used were 0–6 min C_3H_7OH/hexane (5:95, vol/vol); 6–18 min C_3H_7OH/hexane (13:87), 18–35 min C_3H_7OH/hexane (5:95). The flow rate was 2.0 mL/min. Epoxide methyl esters **6M** and **7M** were separated by NP-HPLC on the Dynamax Macro HPLC Si column. The mobile phase composition and the gradient used were 0–30 min C_3H_7OH/hexane (2:98, vol/vol) to C_3H_7OH/hexane (9:91). The flow rate was 1.5 mL/min. Epoxide methyl esters **8M** and **9M** were separated by RP-HPLC using an Adsorbosphere C_{18} 5U column (250 × 10 mm) (Alltech, Deerfield, IL). The mobile phase composition and the gradient used were 0–5 min H_2O/CH_3CN (15:85, vol/vol); 5–30 min H_2O/CH_3CN (15:85) to CH_3CN (100); 30–35 min CH_3CN (100). The flow rate was 1.5 mL/min. Quantification of products was made with a Varex MK III ELSD (Alltech, Deerfield, IL) operated at 55°C using N_2 as the nebulizing gas at a flow rate of 1.5 L/min. Products, after separation by RP-HPLC, were characterized by EI–MS (Thermabeam Mass Detector; Waters) and atmospheric pressure chemical ionization MS (APCI–MS) (Micromass ZMD; Waters). The EI–MS detector was set to scan the mass range *m/z* 55–600 at 1000 amu/s and had an ionization energy of 70 eV. Ionization source, nebulizer, and expansion region temperatures were 200, 64, and 75°C, respectively. When using the APCI–MS, the RP-HPLC gradient contained 0.1% formic acid, and the APCI–MS detector was set to scan the mass range of *m/z* 150–550 at 400 amu/s. The corona, cone, and extractor voltages were 3700, 20, and 5 eV, respectively. The source and APCI heater temperatures were 150 and 400°C, respectively. Proton and carbon NMR spectrometry were obtained as described previously (7).

RESULTS AND DISCUSSION

The purity of the commercial preparation of CLA isomer **1** was determined by GC–MS analysis of its methylated derivative. The commercial preparation contained 96.7% 9(*Z*),11(*E*)-octadecadienoic acid, 2.2% 9(*E*),11(*E*)-octadecadienoic acid, and 1.1% of an unidentified isomer. This preparation of CLA **1** was treated with peroxygenase immobilized on a Fluoropore membrane and *t*-butyl hydroperoxide. Reactions were conducted for 2, 4, 6, and 24 h. After acidification, extraction, and methylation, the products were analyzed by

RP-HPLC by using an ELSD. Appreciable product was formed within 2 h, and by 6 h the reaction was nearly complete (Fig. 1). Two peaks, labeled as **2M** and **3M** (methyl esters of **2** and **3**), accounting for about 40–50% of the product, eluted between 11 and 14 min. An additional peak, labeled as **4M**, eluting at 7–9 min, accounted for the remainder of the product. Mean 24-h yields of four reactions were **2M**, 29%; **3M**, 14%; and **4M**, 45%. Initially, products **2M**–**4M** were thought to be either epoxides or monounsaturated cyclic ethers based on their apparent molecular ions at *m/z* 311 (M + 1) from APCI–MS. However, ^{1}H and ^{13}C NMR analyses showed resonances for protons and carbons adjacent to oxygen that were downfield compared to those published for the monoepoxides of **1M**, which were prepared using *m*-chloroperoxybenzoic acid (5). Furthermore, products **2M**–**4M** showed elution shifts and changes in characteristic ion fragments in MS upon treatment with BSTFA, indicating the presence of a hydroxyl group. Mild acid hydrolysis of the monoepoxides of **1M** prepared according to Reference 5 showed products with elution times on RP-HPLC similar to products **2M**–**4M**, suggesting that the products of peroxygenase treatment of **1** were diols arising from the hydration of initially formed epoxides. Finally, hydrogenated **2M**–**4M** and their bis-TMS ether derivatives gave the expected molecular ion signal for diols.

Structural analysis of 1,2-diol 2M. The FTIR spectrum (film) of compound **2M** showed a broad band at 3630–3090 cm^{-1} (hydroxyl) and a band at 969 cm^{-1} (*E* double bond). The APCI–MS of **2M** gave a base peak at *m/z* 311 ($[M - H_2O(=18) + 1]^+$, 100%), and its bis-TMS ether derivative gave prominent ions at *m/z* 383 ($[M - TMSOH + 1]^+$, 18.4%) and 311 ([M – TMSOTMS + 1], 100%) (Fig. 2). The EI–MS of **2M** gave prominent ions at *m/z* 279 ($[M - OCH_3(=31) - 18]^+$, 3.0%), 187($[CH(OH)(CH_2)_7COOCH_3]^+$, 35.8%), and 155 ($[187 - 32]^+$, 100%) and its bis-TMS ether derivative gave prominent ions at *m/z* 457 ($[M - 15]^+$, 1.1%), 441 ($[M - 31]^+$, 1.0%), 259 ($[TMSOCH(CH_2)_7COOCH_3]^+$, 100%), 213 ($[C_6H_{13}CH{=}CHCHOTMS]^+$, 16.8%), and 155 ($[259 - 73 - 31]^+$, 26.8%). Thus, EI–MS and APCI–MS of **2M** and its bis-TMS ether derivatives gave evidence for alcohols on C-9 and C-10 and unsaturation at C-11 but failed to produce the expected molecular ion peaks. A molecular ion peak also was not observed with the acetylated derivative but could be obtained from the EI–MS of the cyclic methyl boronic acid derivative of **2M**: *m/z* 352 ($[M]^+$, 2.3%), 321 ($[M - 31]^+$, 4.0%), 310 ($[M - CH_3BO]^+$, 19.3%), 292 ($[M - CH_3B(OH)_2]^+$, 10.7%), 279

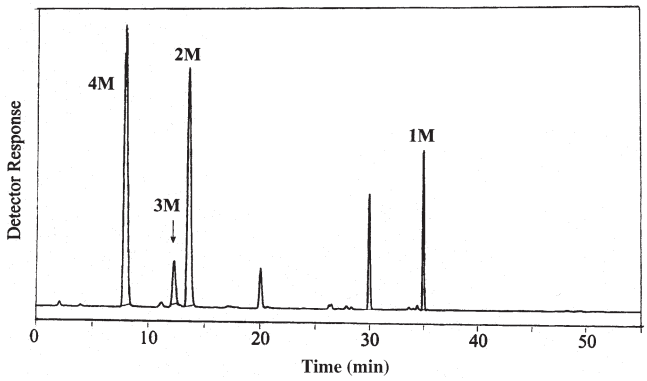

FIG. 1. RP-HPLC analysis of the methylated products formed by the action of immobilized oat seed peroxygenase on CLA **1**. This chromatogram, obtained with ELSD, represents a 6-h reaction; the remaining methylated CLA is indicated as **1M**. The major products are identified as methyl 9,10(*threo*)-dihydroxy-11(*E*)-octadecenoate **2M**, methyl 9,10(*erythro*)-dihydroxy-11(*E*)-octadecenoate **3M**, and methyl 9,12-dihydroxy-10(*E*)-octadecenoate **4M**. Control experiments with no added CLA show that the unmarked peaks at 20 and 30 min are derived from the peroxygenase preparation. From retention times and MS these are tentatively identified as the monoepoxide derivatives of methyl linolenate and methyl oleate.

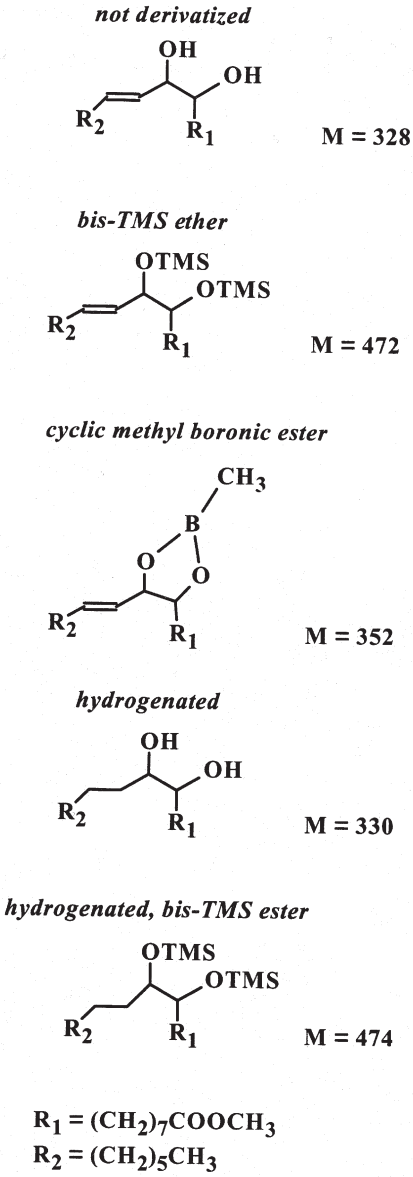

FIG. 2. Methylated allylic 1,2-diols **2M** and **3M** and their derivatives used for mass spectral characterization. Molecular ion peaks were observed with the cyclic methyl boronic ester derivatives, the hydrogenated derivatives, and the hydrogenated, bis-trimethylsilyl (TMS) ester derivatives.

TABLE 1
Proton NMR Data for Methyl 9,10-(*threo*)-dihydroxy-11(*E*)-octadecenoate (2M)[a]

Carbon #	δ (ppm)	Multiplicity	*J* (Hz)
18	1.14	*t*	7.0
3–8, 14–17	1.38–1.82	*m*	
13	2.19	*m*	
2	2.34	*t*	7.4
OCH_3	3.60	*s*	
9	3.61	*m*	
10	3.97	*dd*	6.9, 10.4
11	5.64	*dd*	6.9, 15.4
12	5.86	*td*	6.6, 15.4

[a]Proton NMR spectrum was recorded at 400 MHz in C_6D_6.

($[M - CH_3BO - 31]^+$, 6.4%). Confirmation of the M.W. was obtained from the APCI–MS of a hydrogenated sample of **2M**: *m/z* 331 ($[M + 1]^+$, 100%) and its bis-TMS ether derivative: *m/z* 475 ($[M + 1]^+$, 100%). The EI–MS of hydrogenated **2M** showed the presence of an alcohol group on C-9: *m/z* 187 ($[HOCH(CH_2)_7COOCH_3]^+$, 40.5%), 155 ($[187 - 32]^+$, 100%), and the EI–MS of the bis-TMS ether derivative confirms this assignment: *m/z* 259 ($[TMSOCH(CH_2)_7COOCH_3]^+$, 57.4%), and 215 ($[M - 259]^+$, 66.7%). The ^{13}C NMR (C_6D_6, 100 MHz, δ_C) showed the presence of two hydroxyl groups and one double bond: 51.6 (*C*H_3OC=O), 75.5 (*C*H–OH), 77.3 (*C*H–OH), 131 (*C*H=CH), 134 (CH=*C*H), and 174 (CH_3O*C*=O). The resonance of one hydroxyl group carbon is shifted downfield because it is adjacent to the double bond. The ^1H NMR values, shown in Table 1, also show this, as the C-10 proton is shifted downfield to δ 3.97 compared to the C-9 proton (δ 3.61). The *E* (*trans*) configuration for the double bond is confirmed by the proton coupling constant (J_{11-12}) of the double-bond protons of 15.4 Hz. The two α-hydroxy methine protons in **2M** (δ_H 3.61 and 3.97) are upfield of those in **3M** (δ_H 3.79 and 4.09, Table 2), and by comparison of the shifts of these protons to those in commercial standards of methyl 1,2-dihydroxystearate (*threo*: δ_H 3.49, *erythro*: δ_H 3.61), compound **2M** is assigned as the *threo* isomer. Additional confirmation of this assignment comes from the coupling constants of the α-hydroxy methine protons (J_{9-10}), which are 10.4 Hz. Thus, compound **2M** is identified as methyl 9,10-(*threo*)-dihydroxy-11(*E*)-octadecenoate.

TABLE 2
Proton NMR Data for Methyl 9,10-(*erythro*)-dihydroxy-11(*E*)-octadecenoate (3M)[a]

Carbon #	δ (ppm)	Multiplicity	*J* (Hz)
18	1.14	*t*	7.0
3–8, 14–17	1.38–1.82	*m*	
13	2.20	*m*	
2	2.35	*t*	7.6
OCH_3	3.60	*s*	
9	3.79	*dt*	4.3, 12.4
10	4.09	*dd*	4.3, 7.9
11	5.64	*dd*	7.9, 15.7
12	5.80	*td*	9.6, 15.7

[a]Proton NMR spectrum was recorded at 400 MHz in C_6D_6.

*Structural analysis of 1,2-diol **3M***. The FTIR, APCI–MS, and EI–MS spectra were nearly identical to those for **2M**. The ^{13}C NMR (C_6D_6, 100 MHz, δ_C) spectrum showed the presence of two hydroxyl groups and one double bond: 50.7 (*C*H_3OC=O), 75.0 (*C*H–OH), 76.4 (*C*H-OH), 131 (*C*H=CH), 135 (CH=*C*H), and 174 (CH_3O*C*=O). The resonance of one hydroxyl group carbon is shifted downfield because it is adjacent to the double bond. The ^1H NMR, shown in Table 2, also shows this, as the C-10 proton is shifted downfield to δ 4.09 compared to the C-9 proton (δ 3.79). The *E* (*trans*) configuration for the double bond is confirmed by the proton coupling constant (J_{11-12}) of the double bond protons, which is 15.7 Hz. The two α-hydroxy methine protons in **3M** (δ_H 3.79 and 4.09) are downfield of those in **2M**, and by comparing the shifts of these protons to those in commercial standards of methyl 1,2-dihydroxystearate, compound **3M** is assigned as the *erythro* isomer. Additional confirmation of this assignment comes from the coupling constants of the α-hydroxy methine protons (J_{9-10}), which are 4.3 Hz. Thus, compound **3M** is identified as methyl 9,10-(*erythro*)-dihydroxy-11(*E*)-octadecenoate.

*Structural analysis of 1,4-diols **4aM** and **4bM***. The FTIR spectrum (film) of the mixture of isomers **4M** showed a broad band at 3660–3110 cm (hydroxyl) and at 970 cm^{-1} (*E* double bond). The APCI–MS of **4M** gave a base peak at *m/z* 311 ($[M - 18(H_2O) + 1]^+$, 100%), and its bis-TMS ether derivative gave prominent ions at *m/z* 383 ($[M - TMSOH + 1]^+$, 25.0%) and 311 ([M – TMSOTMS + 1], 100%). The EI–MS of **4M** gave prominent ions at *m/z* 310 ($[M - 18]^+$, 0.6%), 279 ($[M - 31(OCH_3) - 18]^+$, 3.0%), 225 ($[M - C_6H_{13} - 8]^+$, 3.1%), 211 ($M - C_6H_{13} - 32]^+$, 27.4%), 171 ($[C_6H_{13}CH(OH)CH=CHCH(OH)]^+$, 12.9%), 153 ($[171 - 18]^+$, 13.7%); and its bis-TMS ether derivative gave prominent ions at 457 ($[M - 15]^+$, 0.4%), 441 ($[M - 31]^+$, 1.2%), 387 ($[M - C_6H_{13}]^+$, 11.5%), 315 ($[M - C_7H_{14}COOCH_3]^+$, 19.7%), 259 ($[TMSOCH(CH_2)_7COOCH_3]^+$, 10.2%), 225 ($[315 - TMSOH]^+$, 45.4%), 213 ($[M - 259]^+$, 3.1%), 187 ($[C_7H_{14}OTMS]^+$, 16.9%), and 155 ($[259 - 73 - 31]^+$, 19.0%). Thus, EI–MS and APCI–MS spectra of **4M** and its bis-TMS ether derivatives gave evidence for alcohols at C-9 and C-12 and unsaturation at C-10 but failed to produce the expected molecular ion peaks in MS. A molecular ion peak was obtained from the APCI–MS spectrum of a hydrogenated sample of **4M**: *m/z* 331 ($[M + 1]^+$, 100%) and its bis-TMS ether derivative: *m/z* 475 ($[M + 1]^+$, 100%). The EI–MS spectrum of hydrogenated **4M** showed the presence of an alcohol group on C-12: *m/z* 215 ($[M - C_7H_{14}OH]^+$, 18.6%) and 183 ($[215 - 32]^+$, 46.5%), and the EI–MS of the bis-TMS ether derivative confirms this assignment: *m/z* 287 ($[M - C_7H_{14}OTMS]^+$, 50%), and 187 ($[C_7H_{14}OTMS]^+$, 100%).

Product **4M** was separated into two products on NP-HPLC, and these are designated as **4aM**, 55%, and **4bM**, 45%. The ^{13}C NMR (C_6D_6, 100 MHz, δ_C) spectra of the two fractions were nearly identical: **4aM**: 51.6 (*C*H_3OC=O), 72.8 (*C*H–OH), 134 (*C*H=CH), 134 (CH=*C*H), 174 (CH_3O*C*=O); **4bM**: 51.6 (*C*H_3OC=O), 72.9 (*C*H–OH), 134 (*C*H=CH), 135

(CH=*C*H), 174 (CH_3OC=O). The ^{1}H NMR spectra for **4aM** and **4bM** show a multiplet for the double-bond protons (Table 3), and the ^{13}C NMR spectra show identical or closely spaced signals for the double-bond carbons, demonstrating that the protons and the carbons are in identical or nearly identical electronic environments. This condition is satisfied by placing the double bond at C11-C12, directly between the two hydroxyl groups. Products **4aM** and **4bM** exhibited very broad signals in ^{1}H NMR for the protons (9-H and 12-H) adjacent to the alcohols, and difficulty in determining the midpoint of the signal made them useless in assigning the relative conformation of the alcohols. However, the relative conformation could be assigned by comparing proton double-bond resonances to those published for 8,11(*erythro*)-dihydroxy-9(*E*)-octadecenoic acid and 8,11(*threo*)-dihydroxy-9(*E*)-octadecenoic acid: δ_H ($CDCl_3$) 5.68 and 5.63, respectively (11). Table 3 shows chemical shifts obtained in C_6D_6 for **4aM** and **4bM** as δ_H 5.84 and 5.82, respectively. Spectra taken in $CDCl_3$ show δ_H 5.67 and 5.65, respectively, and therefore **4aM** is assigned as methyl 9,12-(*erythro*)dihydroxy-10(*E*)-octadecenoate and **4bM** as methyl 9,12-(*threo*)dihydroxy-10(*E*)-octadecenoate. Interestingly, in the ^{13}C NMR spectra of the reference compounds, the resonance of the carbon atom adjacent to the alcohol in the *threo* isomer (δ_C 72.5) is shifted downfield compared to the *erythro* isomer (δ_C 72.3), and since this resonance in the spectrum of **4bM** is also shifted downfield compared to that in **4aM**, the assignment of **4bM** as the *threo* isomer is confirmed.

Epoxide products. Methylated epoxide standards were prepared as described in the Materials and Methods section with CPBA in CH_2Cl_2. The APCI–MS for methyl 9,10(*Z*)-epoxy-11(*E*)-octadecenoate (**6M**) and methyl 9,10(*E*)-epoxy-11(*E*)-octadecenoate (**8M**) was: *m/z* 311 ($[M + 1]^+$, 100%). EI–MS values for **6M** and **8M** were: *m/z* 310 ($[M]^+$, 3.2%), 279 ($[M - 31]^+$, 7.5%), 185 ($[O=C(CH_2)_7COOCH_3]^+$, 100%), and 125 ($[310 - 185]^+$, 50.5%). The APCI–MS result for methyl 11,12(*E*)-epoxy-9(*Z*)-octadecenoate (**7M**) and methyl 11,12(*E*)-epoxy-9(*E*)-octadecenoate (**9M**) was: *m/z* 311 ($[M + 1]^+$, 100%). The EI–MS values for **7M** and **9M** were: *m/z* 310 ($[M]^+$, 2.1%), 279 ($[M - 31]^+$, 5.4%), 153 ($[M - (CH_2)_7COOCH_3]^+$, 100%), and 113 ($[CH_3(CH_2)_5C=O]^+$, 78.5%). CLA **1** was incubated with peroxygenase and *t*-butyl hydroperoxide as before; reactions were conducted for 2, 4, 6, and 24 h, but the reaction mixture was not acidified, and products were obtained by solid-phase extraction as described in the Materials and Methods section. Analysis of the products by RP-HPLC with ELSD showed that there were new peaks whose retention times corresponded to monoepoxide standards prepared with CPBA. The products were further analyzed by HPLC-EI-MS. From 2-, 4-, and 6-h reactions the major epoxide isomer was thus identified as methyl 9,10(*Z*)-epoxy-11(*E*)-octadecenoate (**6M**), and the minor epoxide isomer as methyl 11,12(*E*)-epoxy-9(*Z*)-octadecenoate (**7M**) (Fig. 3). Incubations conducted for 24 h also showed the presence of a minor amount of methyl 9,10(*E*)-epoxy-11(*E*)-octadecenoate (**8M**).

Reaction mechanism and conclusion. Although epoxide products **6M**, **7M**, and **8M** were identified when products were isolated by solid-phase extraction, epoxide product **6M** was the major product observed. Epoxide **6M** was isolated in pure form by preparative NP-HPLC and then subjected to mild acid hydrolysis. The hydrolysis products, separated using RP-HPLC, consisted of 38% *threo* 9,10-diol (**2M**), 13% *erythro* 9,10-diol (**3M**), and 49% 9,12-diol (**4aM + 4bM**), similar to the distribution of diols obtained from the acidified peroxygenase reaction mixture. A summary of the results is given in Figure 4. The epoxides **7** and **8** are shown in brackets to indicate that they are minor epoxide intermediates. The major epoxide intermediate is **6** in which only the *Z* configuration double bond is acted upon by peroxygenase to form an epoxide, consistent with earlier work that showed that the oat

TABLE 3
Proton NMR Data for *erythro* and *threo* Isomers of Methyl 9,12-dihydroxy-11(*E*)-octadecenoate (4M)[a]

Carbon #	δ (ppm)	Multiplicity	*J* (Hz)
Methyl 9,12-(*erythro*)-dihydroxy-10(*E*)-octadecenoate (**4aM**)			
18	1.14	*t*	6.8
3–8, 13–17	1.40–1.84	*m*	
2	2.35	*t*	7.5
OCH_3	3.61	*s*	
9,12	4.18	*bs*	
10,11	5.84	*m*	
Methyl 9,12-(*threo*)-dihydroxy-10(*E*)-octadecenoate (**4bM**)			
18	1.14	*t*	6.8
3–8, 13–17	1.40–1.84	*m*	
2	2.35	*t*	7.5
OCH_3	3.61	*s*	
9,12	4.16	*bs*	
10,11	5.82	*m*	

[a]Proton NMR spectrum was recorded at 400 MHz in C_6D_6.

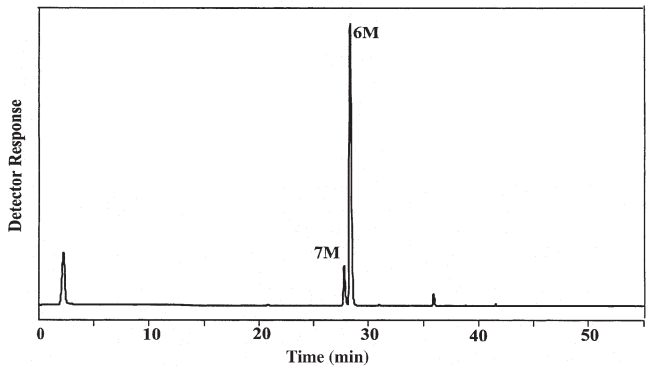

FIG. 3. The reversed-phase (RP)-HPLC chromatogram using ELSD of methylated monoepoxides from the action of peroxygenase on CLA **1**. The epoxides were isolated by solid-phase extraction with no acidification. A chromatogram for a 4-h reaction is shown with the major epoxide products identified as methyl 9,10(*Z*)-epoxy-11(*E*)-octadecenoate (**6M**) and methyl 11,12(*E*)-epoxy-9(*Z*)-octadecenoate (**7M**). Detection was made by comparing the retention times of the products with those of standards prepared with 3-chloroperoxybenzoic acid and by RP-HPLC using EI–MS as the detector. Reactions that were allowed to continue for 24 h also contained a minor amount of an epoxide identified as methyl 9,10(*E*)-epoxy-11(*E*)-octadecenoate (**8M**).

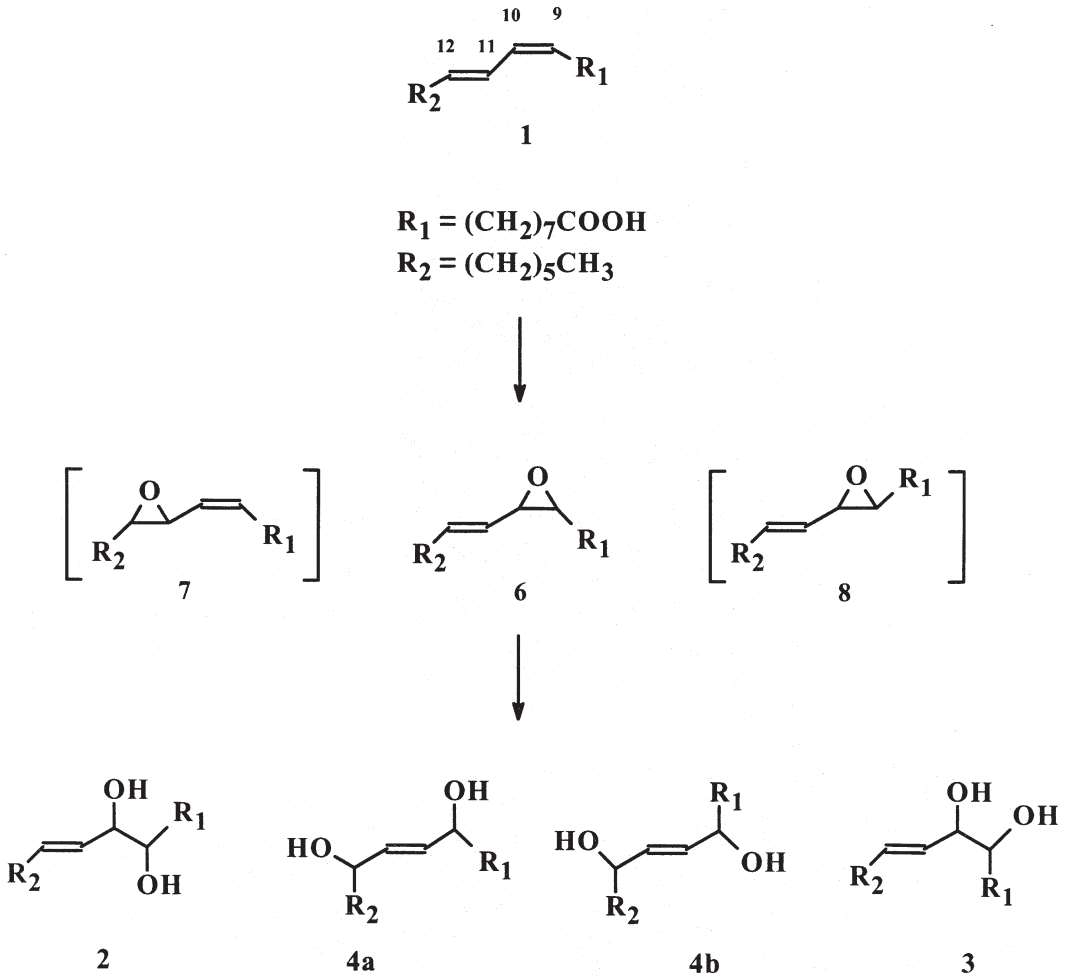

FIG. 4. Proposed reaction sequence for the formation of 1,2- and 1,4-diol products. The diol products (**2**, **3**, **4a**, and **4b**) were obtained from acidified reactions, and the epoxides (**6**, **7**, and **8**) were obtained from reactions that were not acidified. Epoxides **7** and **8** were placed in brackets to indicate that they are minor products. Epoxide **6** was the major product, and its hydrolysis mainly accounts for the observed diol products.

seed peroxygenase exhibits a selectivity for *Z* configuration double bonds (8,9).

Generally, the ring opening of alkyl epoxides has been thought to proceed by a modified S_N2 attack of water, thus giving inversion at one carbon resulting in a *threo* vicinal diol from a *Z* configuration epoxide (12). However, the situation with an allylic epoxide is similar to that of aryl epoxides in which the additional unsaturation can stabilize a carbocation intermediate, resulting in two vicinal diol isomers, an example being the 5,6-diol isomers formed from a 5,6-epoxide intermediate of arachidonic acid metabolism (13). Usually, the *erythro* configuration diol is the predominant isomer from an *E* configuation epoxide, and the *threo* configuration diol is the predominant isomer from a *Z* configuration epoxide. The situation with the CLA epoxides is more complex because both the *Z* and *E* configuration 9,10-epoxides were observed at 24 h. However, at times less that 6 h only the *Z* configuration 9,10-epoxide was observed, and the results of the hydrolysis of the purified **6M** demonstrate that this intermediate is sufficient to account for the vicinal diol isomers. Our results do not exclude the possibility that *E* configuration 9,10-epoxide **8** is the precursor of some of the 9,10-diol, particularly the *erythro* isomer. However, at least at times less than 6 h, epoxide **8** is not a major precursor of this diol.

The lack of substantial production of **7** is responsible for the lack of substantial amounts of 11,12-dihydroxy isomers in the acidified reaction fractions. Additionally, hydrolysis of a purified fraction of methylated isomer **7M** showed that approximately 60% of the product was the 9,12-diol. It is emphasized, however, that traces of the *erythro* and *threo* isomers of methyl 11,12-dihydroxy-9(*Z*)-octadecenoate were detected in acidified and methylated reaction extracts, although the peaks corresponding to these isomers are not visible in Figure 1.

As noted in the introduction, the mode of action of CLA is not well understood, and its metabolism has received only limited attention (1). Although peroxygenase is a plant enzyme, it is functionally related to mammalian "monooxygenase," iron-containing hemoproteins (14). These enzymes play a role in the bioactivation of lipids, resulting in a num-

ber of physiological phenomena (15,16). Evidence for a role for monooxygenase in CLA metabolism is lacking at present, but certainly the results presented here suggest that monooxygenases can convert CLA to oxidized derivatives. These derivatives should be considered as possible contributors to the physiological effects ascribed to CLA.

ACKNOWLEDGMENTS

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Eicosapentaenoic Acid-Induced Apoptosis Depends on Acyl CoA-Synthetase

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ABSTRACT: Marine n-3 FA are known to inhibit proliferation or induce cell death in several cancer cell lines. We have previously reported that EPA promotes apoptosis in the lymphoma cell line Ramos, whereas the U-698 cell line is insensitive to EPA. Furthermore, acyl-CoA synthetase (ACS) is expressed to a higher extent in Ramos cells compared to U-698 cells. To investigate the importance of ACS in EPA-induced apoptosis, we incubated Ramos cells with triacsin C, an inhibitor of ACS. This caused a 70% reduction in the amount of cell-associated EPA and diminished activation of EPA. In addition, triacsin C caused a 90% reduction in EPA-induced apoptosis. Several different approaches were tried to overexpress ACS4 in EPA-insensitive lymphoma cell lines, but we did not obtain viable cells with high expression of acyl-CoA activation. However, we show that overexpression of ACS4 in the more robust COS-1 cells caused up to a fivefold increase in activation of EPA and a 67% increase in the amount of cell-associated radiolabeled EPA. Furthermore, we observed 28% elevated cellular level of TAG in EPA-incubated COS-1 cells overexpressing ACS4. The present study provides new information about ACS as an important enzyme for EPA-induced apoptosis in Ramos cells. Our data offer a potential mechanism that may explain the effect of dietary marine n-3 PUFA on growth of certain malignant cells.

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Cancer is a major cause of morbidity and mortality in developed societies, and numerous studies have focused on possible beneficial health effects of PUFA (1–3). Underlying molecular mechanisms by which n-3 PUFA exert their effect as cancer-protective agents have been studied in several cancer cell lines (4–8). Previously, we have investigated 14 different leukemia/lymphoma cell lines for sensitivity to FA based on cell proliferation and cell death. We found that incubation with 30–60 μM EPA, DHA, and arachidonic acid (AA) caused a reduction in cell number in 10 cell lines, whereas 4 cell lines were insensitive to PUFA (9). The sensitivity to FA was specific for PUFA, as none of the cell lines was sensitive to oleic acid (OA) or stearic acid. We also recognized that some of the sensitive cell lines exhibited increased cell death *via* apoptosis (Ramos) or necrosis (Raji) (9,10).

n-3 PUFA are involved in transport and storage of lipids and lipid-soluble vitamins, components of phospholipids, precursors for eicosanoids, and ligands for transcription factors. The diversity of functions of FA implies that specific mechanisms must exist for transport and targeting within cells. Previous investigation of Ramos cells and the PUFA-insensitive U-698 cells revealed similar mRNA levels for the fatty acid-binding proteins cFABP (cytosolic FABP) and fatty acid transport protein 1, whereas plasma membrane FABP and fatty acid translocase (FAT)/CD36 were not detected (11). However, acyl-CoA synthetase (ACS) 3/4 mRNA and ACS activity were elevated in Ramos cells as compared to U-698 cells. Furthermore, the net accumulation of EPA was two- to threefold higher in Ramos cells compared to U-698 cells (11). ACS constitutes a family of proteins, conserved from bacteria to mammals (12), that catalyze the ligation of FA with CoA to produce acyl-CoA. FA activation and the resulting acyl-CoA are crucial for further metabolism of FA, such as β-oxidation or formation of complex lipids. In addition, acyl-CoA and their metabolites may serve as signal molecules. The ACS differ in tissue distribution and substrate preferences, and ACS3 and ACS4 both have high specificity for long-chain PUFA (13–15). Previous observations indicate that ACS may be important for development of apoptosis in Ramos cells (11). Recently, we reported that EPA promotes apoptosis *via* the intrinsic apoptotic pathway in Ramos cells, as shown by activation of caspase-9, but there was no significant detection of caspase-8 activity (16). Thus, EPA has to be present within the cells to initiate apoptosis, rather than to activate the apoptotic process *via* plasma membrane-receptors, as would be expected in the extrinsic pathway. The aims of the present study were to clarify the events preceding activation of the caspases and mechanisms involved in cellular accumulation of EPA. We show that inhibition of ACS by triacsin C reduced net accumulation and activation of EPA in Ramos cells, eventually preventing EPA-induced apoptosis. We were unsuccessful in transient and stable overexpression of ACS4 in the PUFA-insensitive lymphoma cell lines. Therefore, we used the more robust COS-1 cells for overexpression of ACS4. This caused increased net accumulation and activation of EPA in addition to cellular accumulation of TAG. Our findings indicate that activation of EPA is important for net accumulation of the FA.

MATERIALS AND METHODS

Materials. EPA, OA, FA-free BSA, Hoechst 33342 (HO342), RPMI-1640, Dulbecco's modified Eagle's medium (DMEM), L-glutamine, and streptomycin/penicillin were purchased from Sigma Chemical Co. (St. Louis, MO). FCS was obtained from BioWhittaker (Walkersville, MD). [^{14}C]EPA and [^{14}C]-OA were purchased from DuPont, NEN Research Products

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Abbreviations: AA, arachidonic acid; ACS, acyl-CoA synthetase; FABP, fatty acid-binding protein; HO342, Hoechst 33342; OA, oleic acid.

(Boston, MA). Triacsin C was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA).

Cell cultures. Ramos cells were purchased from BioWhittaker. U-698 and U937-1 were kindly provided by Dr. K. Nilsson (University of Uppsala, Sweden). These cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FCS, L-glutamine (2 mM), and streptomycin/penicillin (0.1 mg/mL) and routinely kept in logarithmic growth at $0.2–1.2 \times 10^6$ cells/mL. For experiments, cells were seeded at a density of 0.3×10^6 cells/mL. COS-1 cells, purchased from the American Type Culture Collection (Rockville, MD) were grown in DMEM supplemented with L-glutamine (2 mM), streptomycin/penicillin (0.1 mg/mL), and 10% heat-inactivated FCS. The cell cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Na-salts of the FA were dissolved in H_2O, and then the FA were complexed to BSA at a molar ratio of 2.5:1 prior to cell incubation.

ACS activity assay. Whole cell lysate (15 µg protein/sample) was added to ice-cold reaction mixture (250 µL) containing 150 mM Tris pH 7.4, 6.2 mM $MgCl_2$, 2.5 mM EDTA, 2.3 mM ATP, 0.5 mM CoA, 0.9 mM DTT, and 0.02% Triton X-100. The reaction was initiated by addition of 1.5 mM [^{14}C]EPA (0.25 µCi/mL) at 37°C. Reactions were terminated by addition of isopropanol/heptane/2 M sulfuric acid (40:10:1, by vol) and followed by heptane extraction. A 1-mL sample of the aqueous phase was counted in a WinSpectral 1414 liquid scintillation counter (Wallac, Turku, Finland).

Determination of cell-associated FA. [^{14}C]OA or [^{14}C]EPA (0.25 µCi/mL) was mixed with 60 µM of unlabeled OA or EPA, respectively, before addition to cell cultures. After the incubation period, cells were washed with PBS, then with PBS containing 1% BSA to remove unbound FA from the cell membrane. The cells were then lysed in H_2O and precipitated with equal volumes of ice-cold TCA (10% wt/vol, final concentration). The mixtures were centrifuged at $1570 \times g$ for 10 min at 4°C. The precipitates were then solubilized in 0.5 mL saline with SDS (70 mM) and Triton X-100 (10%), and radioactivity was counted in a WinSpectral 1414 liquid scintillation counter. Cell-associated FA was calculated as nmol/mg protein.

DNA staining. For microscopic analyses of cell viability, 1 mL of Ramos cell suspension was incubated with 10 µL of HO342 (1 mg/mL) in the dark for 20 min. HO342 passes through intact cell membranes, associates with DNA, and emits blue light when exposed to UV light. When HO342 associates with condensed chromatin found in apoptotic cells, the blue color becomes more intense. After staining, the cell pellet was resuspended in 10 µL FCS. Drops of the cell suspension were placed on a microscope slide and air-dried before counting at least 200 cells in a Leitz Ortholux II fluorescence microscope (Leica, Wetzlar, Germany).

Plasmid constructs. For transfections in U-698 and U937-1 cells, the 2.25-kb Pst I/Xba I ratACS4 cDNA-fragment from the SRα-rACS4 plasmid (kindly provided by Dr. Tokuo Yamamoto) was ligated into the pIND-inducible expression vector (Invitrogen Corp., Carlsbad, CA). Alternatively, for transient transfections of COS-1 cells, the 2.25-kb Pst I/Xba I ratACS4 cDNA-fragment was first ligated into pBluescript SK+, then excised with Hind III and Xba I and further ligated into the corresponding restriction sites in the pcDNA3 expression vector (Invitrogen Corp.). Correct inserts were verified by DNA sequencing.

Transfection studies. The U-698 and U937-1 cell lines were transfected by electroporation (180–220 V, 960 µF, 40 µg DNA/50 mill cells). U-698 cells were transiently or stably transfected with ACS4 in pcDNA3. In addition, both U-698 and U937-1 cells were stably transfected with the Ecdysone-inducible expression system (Invitrogen Corp). COS-1 cells were seeded in six-well plates at a density of approximately 60% confluence the day before transfection. The cells were then transiently transfected with ACS4 in pcDNA3 using a standard dextran–chloroquine method (17) with 1 µg DNA/well. Control cells were transfected with pcDNA3. Following transfection, the cells were incubated at standard conditions for 48 h prior to the experiments.

Enzymatic determination of TAG. Cells were harvested in sterile water, sonicated, and the TAG content was measured enzymatically (BioMérieux SA, Lyon, France). The cellular TAG content was calculated as nmol/mg protein.

Statistics. Results are presented as means ± SD. Unless otherwise indicated, Mann-Whitney analysis was used to determine the significance level of differences among sample groups, $P \leq 0.05$. All reported P-values are two-sided.

RESULTS

ACS activity is important for cellular accumulation and activation of EPA. Triacsin C may inhibit ACS activity in cell-free systems (18,19). To investigate whether triacsin C could inhibit ACS in our Ramos cells, we incubated the cells with 4.8 µM triacsin C for 24 h and measured the capacity to convert [^{14}C]EPA to [^{14}C]EPA-CoA (Fig. 1). Triacsin C reduced the formation of [^{14}C]EPA-CoA by 56 ± 6%, indicative of decreased ACS activity. To test whether the reduction of EPA-activation was accompanied by reduced cellular accumulation

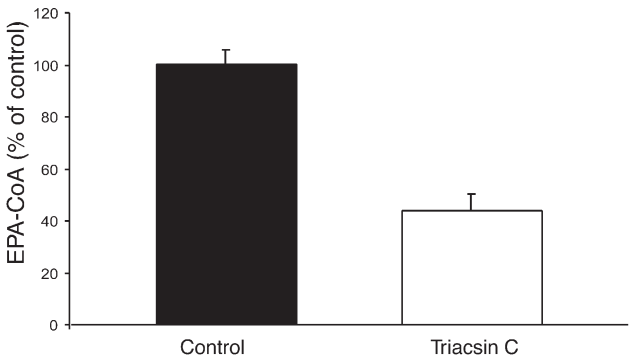

FIG. 1. Acyl-CoA synthetase (ACS) activity in Ramos cells incubated with triacsin C. Ramos cells were incubated with 4.8 µM triacsin C for 24 h, and the capacity to convert [^{14}C]EPA to [^{14}C]EPA-CoA in cell lysates was examined. The means ± SD are presented from three separate experiments performed in triplicate. Absolute values for EPA-CoA (control cells) were 286, 79, and 131 nmol/mg prot in the three separate experiments.

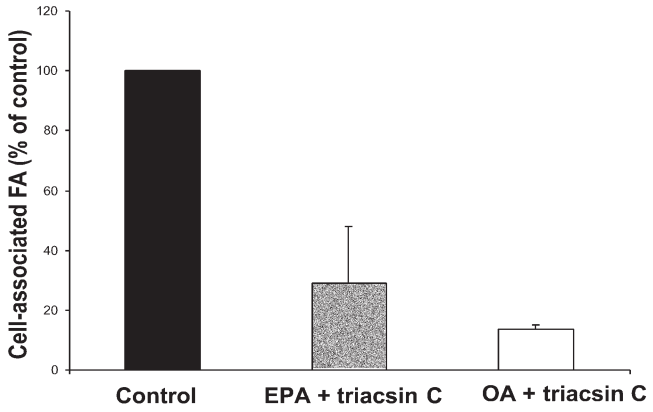

FIG. 2. Cell-associated [^{14}C]EPA and [^{14}C]OA (oleic acid) in Ramos cells incubated with triacsin C. Cell-associated [^{14}C]EPA and [^{14}C]OA were monitored in Ramos cells incubated with 60 μM of the respective FA for 3 h in combination with 4.8 μM triacsin C. The means ± SD are presented from three separate experiments performed in triplicate.

of EPA, we incubated Ramos cells with triacsin C in combination with [^{14}C]EPA and measured cell-associated radiolabeled EPA after 3 h incubation. We found a 71 ± 19% reduction in cell-associated [^{14}C]EPA compared to cells incubated without triacsin C (Fig. 2). In addition, we observed 87 ± 2% reduction in cell-associated [^{14}C]OA, indicating that ACS also is involved in accumulation of monoenes as well as polyenes.

ACS activity is crucial for EPA-induced apoptosis. We hypothesized that EPA-induced apoptosis might be a result of activation and cellular accumulation of EPA. Hence, EPA-induced apoptosis was determined in Ramos cells incubated with triacsin C (Fig. 3). The ACS inhibitor was added in combination with 60 μM EPA, and cells were stained with HO342 after 24 h incubation to assess apoptosis. We found that EPA promoted 45 ± 17% apoptosis, whereas the combination of EPA and triacsin C caused only 5 ± 4% apoptosis, indicating that uptake and activation of EPA are important for induction of apoptosis.

Activation and accumulation of EPA are associated with ACS4. Whereas ACS1 and ACS5 exhibit broad substrate specificity, ACS4 preferentially uses EPA and AA as substrates (14). Based on this observation, along with previous data showing higher expression of ACS3/4 in Ramos cells compared to U-698 cells (11) as well as our present data (Figs. 1–3), we hypothesized that ACS4 may be essential for EPA-induced apoptosis. We assumed that overexpression of ACS4 in the PUFA-insensitive U-698, or in the U937-1 cells (which are less sensitive to PUFA than Ramos cells), would increase uptake and activation of EPA and eventually lead to apoptosis. Thus, we transfected U-698 and U937-1 cells with ACS4 both transiently and stably, using inducible or constitutive expression methods. However, we observed massive cell death when ACS was overexpressed, possibly due to toxic levels of activated PUFA in these cell lines. We then chose to transiently transfect the more robust COS-1 cells with ACS4, and 48 h after transfection we measured the conversion of [^{14}C]EPA to [^{14}C]EPA-CoA in cell lysates. Although ACS4 is known to have higher affinity for EPA than OA, OA was included as a control for ACS4 activity. Overexpression of ACS4 caused a fivefold increase in formation of [^{14}C]EPA-CoA in cell lysates as compared to lysates from cells with the empty expression vector, whereas the level of [^{14}C]OA-CoA was not significantly increased (Fig. 4). Elevated cellular activation of EPA might possibly be detected as an increased level of cell-associated radiolabeled EPA. At time 10–180

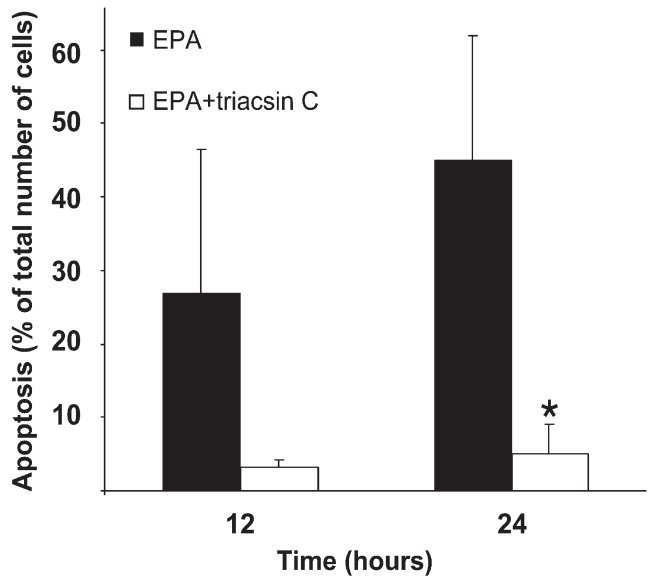

FIG. 3. EPA-induced apoptosis in Ramos cells incubated with triacsin C. Ramos cells were incubated for 12 or 24 h with 60 μM EPA with or without 4.8 μM triacsin C. The number of apoptotic cells was counted after staining with HO342. The means ± SD are presented from two or three separate experiments performed in duplicate. Student's *t*-test was used to determine the significance level of differences between sample groups within each time point. *$P \leq 0.05$.

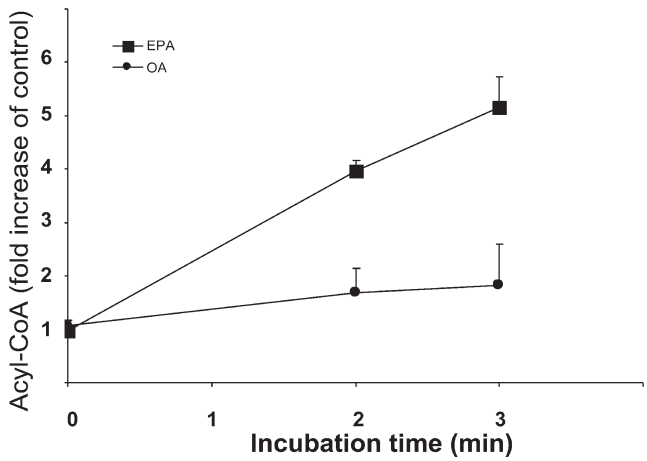

FIG. 4. ACS activity in COS-1 cells overexpressing ACS4. COS-1 cells were transfected with ACS4. Forty-eight hours after transfection, the capacity to activate [^{14}C]EPA and [^{14}C]OA was determined in cell lysates. The means ± SD are presented from three separate experiments performed in triplicate. Absolute values for EPA-CoA (control cells) at zero time were 45, 99, and 52 nmol/mg prot in the three separate experiments. For OA-CoA the values were 34, 26, and 35 nmol/mg prot. For abbreviation see Figure 2.

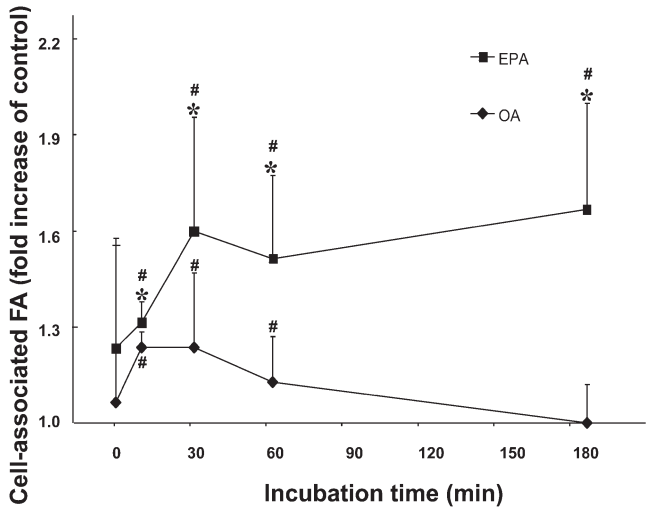

FIG. 5. Cell-associated [^{14}C]EPA and [^{14}C]OA in COS-1 cells overexpressing ACS4. COS-1 cells were transfected with ACS4. Forty-eight hours after transfection, cells were incubated with 60 μM of the indicated FA, then cell-associated [^{14}C]EPA and [^{14}C]OA were monitored at the indicated time. The means ± SD are presented from three separate experiments performed in triplicate. $^{*}P \leq 0.05$: data compared to zero time point. $^{\#}P \leq 0.05$: data compared to cells transfected with empty vector at the indicated time. For abbreviation see Figure 2.

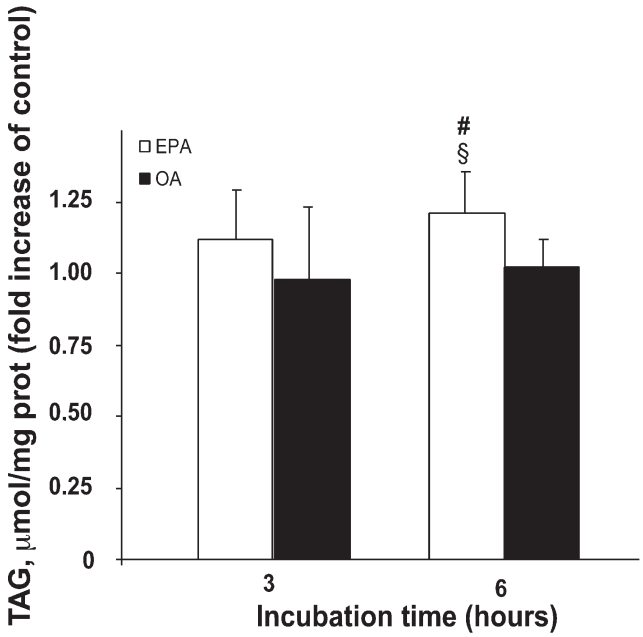

FIG. 6. Amount of TAG in COS-1 cells overexpressing ACS4. Forty-eight hours after transfection with ACS4, COS-1 cells were incubated for 3 and 6 h in the presence of 60 μM EPA or OA, and the cellular content of TAG was measured. The means ± SD are presented from three separate experiments performed in triplicate. $^{\S}P = 0.086$: data compared to zero time. $^{\#}P \leq 0.05$: data compared to cells transfected with empty vector at the indicated time. For abbreviation see Figure 2.

min, the ACS4-transfected COS-1 cells accumulated higher amounts of radiolabeled FA when compared to zero time point and when compared to cells transfected with empty vector (Fig. 5). The level of cell-associated radiolabeled EPA was higher than the level of OA, indicating that activation of EPA by ACS4 is important for cellular accumulation of EPA. To challenge these findings, we measured the content of TAG in ACS4-transfected COS-1 cells. Forty-eight hours after transfection, cells were incubated with either 60 μm EPA or 60 μM OA for 3 or 6 h. A tendency toward increased TAG level in cells incubated with EPA for 6 h was observed when compared to time zero. However, when comparing cells overexpressing ACS4 to cells transfected with empty vector, a significant increase in TAG-level was assessed at 6 h of EPA incubation. There was no difference between control cells and OA-incubated cells (Fig. 6).

DISCUSSION

In the present study we show that inhibition of ACS by triacsin C reduced the level of activated as well as cell-associated radiolabeled EPA when Ramos cells were incubated with [^{14}C]EPA. The reduction in net accumulation and activation of EPA caused by the ACS inhibitor was paralleled by prevention of apoptosis. Furthermore, we showed that overexpression of ACS4 in COS-1 cells increased the ability to activate and accumulate radiolabeled EPA. We also observed that higher cellular capacity for activation and accumulation of EPA was associated with enhanced cellular content of TAG.

Epidemiological studies, animal models, and mechanistic experiments support that n-3 PUFA may inhibit initiation, growth, and metastasis of malignant diseases (20–22). n-3 PUFA may exert their beneficial effects *via* different mechanisms, such as formation of free radicals or lipid peroxides (23,24). Furthermore, n-3 PUFA may induce cell lysis (5), cell cycle arrest, or apoptosis (4,9,10), and thereby reduce cancer cell proliferation. Different antioxidants have been incubated with Ramos cells to study whether EPA initiates apoptosis *via* formation of reactive oxygen species (10). However, the investigated antioxidants did not oppose EPA-induced apoptosis in Ramos cells. Moreover, EPA did not influence cell cycle progression in Ramos cells, and the most likely explanation for the inhibitory effect of EPA on cell proliferation is a direct induction of apoptosis (16). In addition, we have observed that EPA-induced apoptosis *via* the intrinsic pathway by activation of caspase-9 (16). Therefore, we hypothesized that EPA must be present within the cells to initiate apoptosis, rather than to activate the apoptosis *via* receptors on the outer leaflet of the plasma membrane. To challenge this hypothesis, we investigated whether inhibition of cellular accumulation of EPA could influence apoptosis. From previous observations we knew that Ramos cells had elevated levels of cell-associated EPA and expressed ACS3/4 to a greater extent than the PUFA-insensitive U-698 cells (11). Thus, to investigate whether ACS are important for net accumulation/cell-association of EPA, we incubated Ramos cells with triacsin C, known to inhibit ACS4 (25). Despite the observed effect of triacsin C, we cannot rule out that EPA might be attached to the outer leaflet of the plasma membrane when

assessing cell-associated EPA. However, it is most likely that EPA is translocated across the plasma membrane prior to activation as a result of ACS association with several intracellular membranes (18). The intracellular location of EPA is supported by the increased level of TAG following an enhanced level of cell-associated EPA (Fig. 6). Even though ACS4 probably is not involved in direct translocation of EPA across the plasma membrane as a result of its subcellular localization (18), the esterification of FA makes the transport unidirectional and thereby traps the FA within the cell.

Kim and coworkers (25) have suggested that the different ACS play critical roles in partitioning their products toward specific pathways. Moreover, in a previous study we showed that EPA is recovered in different lipid fractions in Ramos cells, with the majority in the TAG fraction (11). It is likely that ACS4 plays an important role for TAG synthesis because ACS4 is mostly located in mitochondria-associated membranes (18). These mitochondria-associated membranes exhibit the highest specific activities for enzymes involved in TAG synthesis (26). Furthermore, the cellular accumulation of EPA in TAG permits continual access of substrate for different pathways. Our present findings indicate that activation of EPA is crucial for induction of apoptosis in Ramos cells, although the molecular links between activation of EPA and induction of the caspase cascade remain unknown.

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Ethyl Arachidonate Is the Predominant Fatty Acid Ethyl Ester in the Brains of Alcohol-Intoxicated Subjects at Autopsy

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ABSTRACT: The role of fatty acid ethyl esters (FAEE), the nonoxidative ethanol metabolites, as mediators of alcohol-induced organ damage is increasingly being recognized. FAEE are detectable in the blood and in liver and adipose tissue after ethanol ingestion, and on that basis, FAEE can be used as markers of ethanol intake. In this study, 10 samples of human brain were collected at autopsy at the Massachusetts Medical Examiner's Office and analyzed for FAEE. FAEE were isolated and quantified as mass per gram of wet weight. The blood ethanol level was also obtained in each case along with the other drugs detected in routine postmortem toxicology screening tests. Ethyl arachidonate was the predominant FAEE species in the brain, representing up to 77.4% of total FAEE in the brain. The percentage of ethyl arachidonate of the total FAEE in the brain was significantly higher than what has been found in all other organs and tissues previously analyzed. Linoleate, the precursor of arachidonate, was a poor substrate for FAEE synthesis, as the percentage of ethyl linoleate of the total FAEE content was extremely low. Thus, this reflects preferred incorporation of arachidonate into newly synthesized FAEE in the brain. Since arachidonate is derived from linoleate, which is depleted in FAEE while arachidonate is enriched, the synthesis of FAEE may be linked to the desaturation and elongation of linoleate to arachidonate.

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Fatty acid ethyl esters (FAEE) are esterification products of ethanol with FA (1–3). FAEE are generated by nonoxidative ethanol metabolism, and many enzymes have been implicated as catalytic for FAEE production (4,5).

FAEE have shown to be toxic mediators of ethanol-induced cell injury in studies with isolated mitochondria, isolated lysosomes, intact HepG2 cells, and *in vivo* in rats infused with FAEE (5,6). FAEE have also been used as markers of ethanol intake (7). FAEE were found in the plasma and serum of individuals as much as 24 h after discontinuing ethanol ingestion (7–9). In addition, FAEE have been reported to be present in both adipose tissue and liver obtained from individuals who had a detectable blood ethanol level at the time of autopsy (10). In this study, three groups of patients—those with a detectable blood ethanol at the time of death, those with a history of chronic alcoholism with no detectable blood ethanol at the time of death, and social drinkers—were tested for FAEE in the liver and in adipose tissue. The FAEE were present in the highest amounts in patients who had died with detectable blood ethanol levels. For this study, in only 10 of the 31 cases was the brain available for testing. The data for brain FAEE were not included in the earlier report (10).

The observations regarding FAEE species in the brain are the basis of this report. The results of this study indicate that the FAEE species distribution in the brain after ethanol intake is markedly different from that found in liver and adipose (10), and from the data in other published studies, it is different from all other organs and tissues analyzed to date (9,11,12). The level of ethyl arachidonate (E20:4) in the brain was extremely high and ethyl linoleate (E18:2) levels were very low in subjects with detectable blood ethanol at the time of death. Since dietary linoleate is converted to arachidonate, the availability of arachidonate for FAEE synthesis in the brain may be linked to the metabolism of linoleate to arachidonate in the brain or other organs.

MATERIALS AND METHODS

The 10 samples used in this study were collected from autopsied cases at the Massachusetts Medical Examiner's Office. Both the Massachusetts Medical Examiner's Office Committee and the Massachusetts General Hospital Pathology Quality Assurance Committee approved the study. The postmortem interval between the estimated time of death and the time when the autopsy was performed ranged from 7 to 15 h, with a mean of 10 h (Table 1). The clinical details were unknown to us at the time of FAEE analysis. Brain samples were collected at the time of autopsy, labeled appropriately by case number, and stored at 4°C for up to 10 h. Immediately on arrival in the laboratory at Massachusetts General Hospital, samples were stored at −80°C until FAEE analysis was performed. After obtaining the FAEE results, relevant information was exchanged about each case. Medical history, history of ethanol ingestion (obtained from the organ bank, treating physician, health insurance records, and/or relatives), and the blood ethanol level at autopsy along with toxicology findings

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Abbreviations: E14:0, ethyl myristate; E16:0, ethyl palmitate; E17:0, ethyl heptadecanoate; E18:0, ethyl stearate; E18:1, ethyl oleate; E18:2, ethyl linoleate; E20:4, ethyl arachidonate; E20:5, ethyl eicosapentaenoate; E22:6, ethyl docosahexaenoate; ETOH, ethanol; FAEE, fatty acid ethyl ester; GC, gas chromatograph.

TABLE 1
Clinical and Biochemical Parameters for 10 Subjects in This Study[a]

Case #	ETOH level at the time of autopsy (mg/L)	Total ethyl arachidonate (pmol/g)	Percentage of ethyl arachidonate of total FAEE	Total FAEE concentration (pmol/g)	Postmortem interval (h)	Relevant clinical toxicology findings
1	960	1,254	31.2	4,020	9	ETOH abuse and CAD
2	1,820	6,143	29.3	20,966	12	Depression/ETOH abuse/suicide
3	1,480	4,895	41.0	11,938	10	ETOH abuse/cirrhosis
4	2,220	18,920	49.4	38,300	10	Heroin use/ETOH abuse
5	1,460	5,409	30.4	17,792	15	ETOH and cocaine abuse
6	1,400	6,818	44.6	15,287	9	Cardiac arrest
7	1,800	16,160	41.3	39,129	10	ETOH and cocaine abuse
8	540	10,214	77.4	13,197	8	ETOH abuse
9	2,260	16,782	34.5	48,643	8	Auto accident
10	2,320	17,864	39.7	44,997	7	Auto accident

[a]ETOH, ethanol; FAEE, fatty acid ethyl esters; CAD, coronary artery disease.

were obtained in each case. Only individuals with detectable blood ethanol levels at the time of autopsy, with or without a history of ethanol abuse, were included in this study. These individuals most likely included chronic alcoholics and/or social drinkers who ingested an excessive amount of ethanol prior to death. Seven cases out of 10 were found to have an ethanol abuse history, 3 of them accompanied by abuse of drugs other than ethanol; 2 cases were involved in an auto accident; 1 case was an apparent sudden cardiac arrest.

FAEE extraction and quantification. A portion of brain tissue was harvested, weighed, and immediately placed on ice, then homogenized (1:10, wt/vol) in protease inhibitor buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid, 20 μg/mL phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 0.01% soybean trypsin inhibitor (pH 7.34) using a Fisher PowerGen 125 Homogenizer (Fisher Scientific, Pittsburgh, PA) equipped with a 10 × 195 mm sawtooth generator. An internal standard of 500 pmol ethyl heptadecanoate (E17:0) (Nu-Chek-Prep, Elysian, MN) was added to 1 mL of the homogenate along with 2 mL of cold acetone. The sample was then vortexed for 1 min and centrifuged for 5 min at 170 × *g* at 4°C, and the supernatant was transferred to a separate tube. Six milliliters of hexane was then added to each sample. The mixture was vortexed for 1 min and centrifuged at 170 × *g* for 5 min at 4°C. The hexane layer was transferred to a separate tube and the aqueous phase re-extracted with an additional 2 mL of hexane. The wash was pooled with the original hexane layer, evaporated to dryness under nitrogen, and resuspended in 200 μL of hexane. FAEE were isolated from the lipid extract using solid-phase extraction. Briefly, aminopropyl columns (Bond-Elut LRC; Varian Diagnostics, Harbor City, CA) were placed on a Vac-Elut vacuum apparatus (Varian Diagnostics) set at 10 kPa. The columns were preconditioned with 4 mL of hexane, followed by 4 mL of dichloromethane. The 200-μL aliquot of lipid extract was then applied, and FAEE were eluted from the column with an additional 4 mL of hexane and 4 mL of dichloromethane. The eluate was next evaporated to a volume of 50 μL, and a 1-μL aliquot was injected into a Hewlett-Packard 5971 gas chromatograph (GC)–mass spectrometer equipped with a Supelcowax 10 capillary column (Supelco, Bellefonte, PA). The injector and detector were maintained at 260 and 280°C, respectively. The oven program was initially maintained at 150°C for 2 min, then ramped to 200°C at 10°C/min for 4 min, ramped again at 5°C/min to 240°C and held for 3 min, and finally ramped to 270°C at 10°C/min and held for 5 min. Carrier gas flow rate was maintained at a constant 0.75 mL/min throughout. Selected ion monitoring was performed, quantifying appropriate base ions for individual FAEE [i.e., ions 67, 88, and 101 for ethyl palmitate (E16:0), ethyl heptadecanoate (E17:0), ethyl stearate (E18:0), ethyl oleate (E18:1), and ethyl linoleate (E18:2); and ions 79, 91 and 117 for E20:4, ethyl eicosapentaenoate (E20:5), and ethyl docosahexaenoate (E22:6)]. FAEE quantification was carried out by interpolation of the slope generated from individually prepared calibration curves, comparing areas of varying concentrations of E14:0–E22:6 to fixed concentrations of the interval standard (E17:0). Mass relationships were obtained for each FAEE by using its individual calibration curve. Total FAEE mass was determined by addition of masses of individual FAEE (E16:0–E22:6). All manipulations were performed under nitrogen to make any losses of FAEE containing PUFA negligible.

Quantitation of blood ethanol from autopsy specimens. Ethanol was assayed in a GC with an FID and an electronic integrator. The serum was diluted with internal standard (an aqueous solution of 1-propanol). The mixture was injected directly into the GC (13).

RESULTS

Table 1 shows the concentration of E20:4 in the brain and other relevant parameters—including the blood ethanol level at the time of autopsy, the percentage of E20:4 of total FAEE, the total FAEE concentration, the postmortem interval, and the clinical and toxicologic findings. In each of the 10 cases, there was a substantial quantity of E20:4 (in pmol/g) in brain tissue. In each of the cases, the blood ethanol levels met the definition for intoxication in most states, with the exception of subject 8, who had a value of 540 mg/L (54 mg/dL). Subject 1 at

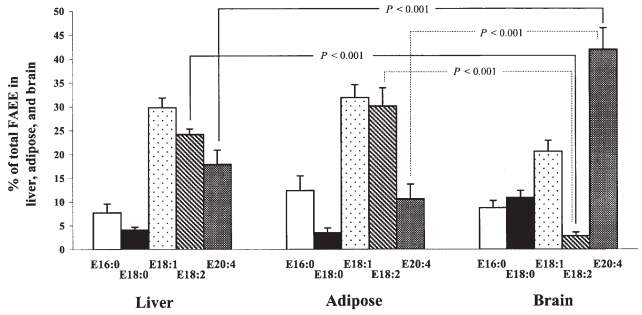

FIG. 1. Fatty acid ethyl ester (FAEE) species in liver, adipose, and brain among 10 individuals with detectable blood ethanol at the time of death. The individual species of FAEE are denoted as E followed by the FA present. Data represent mean ± SEM, $n = 10$.

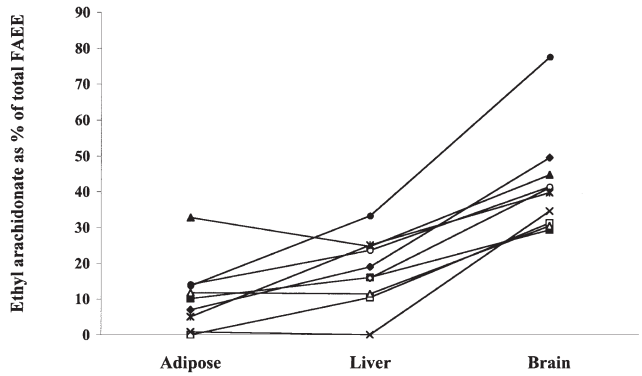

FIG. 2. Results from individual subjects ($n = 10$) for ethyl arachidonate as percentage of total FAEE in adipose, liver, and brain. Each line and symbol type represents a different subject. For abbreviation see Figure 1.

960 mg/L would have been considered intoxicated in states with a legal limit of 800 mg/L. The total FAEE concentration in the brain ranged from 4,020 to 48,643 pmol/g, and the postmortem interval was 7 to 15 h. Seven of the 10 cases represented patients who suffered from ethanol abuse during their lifetime.

Figure 1 shows the mean values ($n = 10$) for the individual FAEE species in the liver, adipose, and brain for the 10 individuals in the study, all of whom had a detectable blood ethanol at the time of death. The brain showed an extremely low level of E18:2 and an extremely high level of E20:4 relative to that found in liver and in adipose. The P values for all the comparisons between brain and liver or brain and adipose were highly statistically significant ($P < 0.01$). There were minimal differences among the three organs/tissues in percentages (relative to total FAEE) of E16:0, E18:0, and E18:1. The average amount of total FAEE in the brain in this study for all 10 subjects was 25.4 ± 5.0 nmol/g wet weight, and this matched with the value of 25 nmol/g in the 1986 study in which FAEE were quantified at autopsy from brain and other organs (5).

Figure 2 shows the data from each individual subject for E20:4 as a percentage of total FAEE. In all 10 cases, the brain had a higher percentage of E20:4 relative to total FAEE than that found in liver and adipose. In general, the liver had slightly higher levels of E20:4 than did adipose tissue, as we showed in an earlier report (10). In one subject, the percentage of total FAEE represented by E20:4 in the brain was extremely high, at 77.4%. However, the amount of E20:4 detected in the brain was not related to the postmortem interval.

To assess both the increase in E20:4 and the decrease in E18:2 at the same time, we calculated the ratio of E20:4 to E18:2 (Table 2) for each of the 10 subjects. The E20:4/E18:2 ratios in the brains of the 10 subjects in this study ranged from 4.07 to infinity. In contrast, the E20:4/E18:2 values in liver and adipose were all less than 1, with the exception of subject 8 for liver, with a value of only 1.33. The statistical comparisons of the mean value for the ethyl ester ratios between brain and liver and brain and adipose were highly statistically significant.

To go beyond the comparisons of FAEE concentration in liver, brain, and adipose as pmol/g of tissue, we also estimated the total organ mass of E20:4 in brain and liver in pmol/organ. The liver had a value of 41,598 ± 7,973 pmol, with a value for brain of 10,446 ± 2,033 pmol. Thus, the liver had a fourfold higher total FAEE content than did the brain. The amount of adipose tissue varies greatly in individuals; therefore, it is impossible even to estimate the total body mass of ethyl esters relative to the amount of adipose tissue. Because the liver is a larger organ than the brain, the largest amount of E20:4 in the body is likely to be in the liver, despite the fact that E20:4 represents a smaller percentage of the total FAEE after ethanol intake than in the brain. This may be relevant if E20:4 is found to be more toxic than other species of FAEE, an issue that remains unresolved. There is substantial evidence in multiple reports that the blood ethanol level correlates with the total plasma FAEE. We found no correlation between the ethanol level in the blood and the percentage of total E20:4 in the brain. The lack of correlation between blood

TABLE 2
Ratio of Ethyl Arachidonate to Ethyl Linoleate (each as a percentage of total FAEE) in Brain, Liver, and Adipose Tissue

	Case #												P value
	1	2	3	4	5	6	7	8	9	10	Mean	SEM	(vs. brain)
Brain	[a]	4.07	20.50	26.00	21.71	12.05	[a]	48.38	5.85	8.63	18.40	5.12	
Liver	0.51	0.59	0.68	0.71	0.52	0.92	0.83	1.33	0.00	0.95	0.71	0.11	<0.0001
Adipose	0.00	0.30	[b]	0.22	0.37	0.82	0.35	0.42	0.13	0.13	0.30	0.08	<0.002

[a]In these cases, the percentage of ethyl linoleate was zero, resulting in an infinite value for the ratio.
[b]No adipose tissue was available for analysis. For abbreviation see Table 1.

ethanol and E20:4 in the brain suggests either that the ethyl esters in the blood do not equilibrate with those in the brain or that there is metabolism of FA or FAEE in the brain to disrupt the linear association between blood ethanol and plasma FAEE levels.

DISCUSSION

This report shows that among FAEE species, the percentage of E20:4 is extremely high and the percentage of E18:2 is extremely low in the brain relative to other organs and tissues. This would suggest that the synthesis of FAEE in the brain may be linked to the desaturation and elongation of linoleate to arachidonate. Despite the high percentage of DHA in the brain, we were not able to detect E22:6, which implies that the brain concentration of a given FA is not necessarily associated with the presence of its corresponding ethyl ester.

There have been a number of reports on the FA composition of the brain from different animals. In some of these studies, the FA composition of individual phospholipids has been determined. In a report by Abedin *et al.* (13), it was shown that there are substantially greater amounts of arachidonate relative to linoleate in brain PE from guinea pigs fed different diets. In another report involving rats, the FA composition of both PC and PE showed a 10- to 20-fold greater amount of arachidonate than linoleate (14). A report by Shetty *et al.* (15) demonstrated a very short half-life for arachidonate in two molecular species of PC—with a fatty composition 16:1–20:4 or 18:2–20:4. The rapid turnover of arachidonate from these molecular species may be related to the increased availability of arachidonate for FAEE synthesis. Finally, there is a report by Fu *et al.* (16) in which guinea pigs received an oral dose of radiolabeled linoleate, and the distribution of its metabolites in various tissue lipids was followed. In these studies, there was much more arachidonate than linoleate in the brain of the guinea pigs (16). Other reports also have indicated that there is a significant preponderance of arachidonate relative to the linoleate in the brain (17,18).

Ethanol consumption has been shown to drastically decrease the percentage of arachidonate in synaptosomal PI while increasing the percentage of linoleate (19). This effect is reversed by dietary linolenic acid. It has also been shown that altering the dietary levels of saturated FA or PUFA can modulate the physiological and neurobehavioral effects of ethanol (20). It is not known whether the neurobehavioral effects of ethanol are actually produced by ethanol's nonoxidative FAEE metabolites, and it is also not known whether FAEE have any impact on FA metabolism in the brain or elsewhere.

There is evidence in the literature for significant conversion of linoleate to arachidonate in the brain. A study by Su *et al.* (21) reported on the kinetics of linoleate and its conversion to arachidonate in the pregnant and fetal baboon. They demonstrated that ^{13}C-linoleate administered to baboons results in the appearance of radiolabeled linoleate in the brain on day 1 after the dose is administered, with no labeled arachidonate detected. By day 2, however, the radiolabeled linoleate had decreased significantly, and arachidonate and other metabolites of linoleate all increased, reaching plateau values by 21 d. These studies show the ability of the brain itself to convert linoleate to arachidonate. Spector (22) indicated in a recent review that linoleate is converted to arachidonate within the brain and in the liver. Thus, brain arachidonate can be derived either from conversion of linoleate within the brain itself or by the uptake from the plasma of arachidonate generated from linoleate in the liver.

Taken together, this reports shows that FAEE in the brain are predominantly represented by E20:4 and that this predominance of arachidonate within FAEE is unique among all organs and tissues analyzed to date.

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Effects of Red Wine Consumption on Kidney FA Composition

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ABSTRACT: We studied the effects of red wine consumption on the FA composition of rat kidney. Four groups of adult male rats were fed a balanced diet for 10 wk. The drinking fluid was water (control), red wine, alcohol-free red wine, or ethanol (12.5%, vol/vol). FA composition, lipid peroxidation, and cytochrome P450 content were determined in the kidney. The antioxidant capacity of plasma was also measured. Ethanol decreased the content of long-chain PUFA, whereas red wine maintained the levels of arachidonic (20:4n-6) and eicosapentaenoic (20:5n-3) acids and alcohol-free red wine significantly increased the levels of 20:4n-6. Lipid peroxidation in the red wine and alcohol-free red wine groups was significantly lower than that of both the control and ethanol groups. The diminished renal lipid peroxidation was associated with an increased antioxidant capacity of plasma. Renal cytochrome P450 was elevated by 50% in the ethanol group and diminished by 20% in the alcohol-free red wine group. These data suggest that moderate red wine consumption could contribute to the preservation of the contents of n-3 and n-6 PUFA, particularly 20:4n-6, in rat kidney. Although ethanol increased the content of cytochrome P450 in the kidney, this effect was eliminated by the nonalcoholic components of red wine.

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PUFA, such as C_{20} and C_{22}, are essential components of all mammalian membrane phospholipids and cholesterol esters. In addition, C_{20} PUFA are precursors of a wide range of metabolically active eicosanoid products (1). Mammals obtain C_{20} and C_{22} PUFA either in the preformed state within animal components of the diet or from endogenous elongation and desaturation of dietary precursors of vegetal origin, such as the EFA linoleic (18:2n-6) and linolenic (18:3n-3) acids. Since no C_{20} or C_{22} PUFA are contained in foods of vegetal origin, they have to be formed by synthesis of C_{18} PUFA, thereby supplying the full mammalian requirements for membrane constituents (2).

In mitochondria, the formation of EPA (20:5n-3) or DHA (22:6n-3) involves the participation of elongases (3). Rat kidney is believed to synthesize enough arachidonic acid (20:4n-6) to satisfy, at least partially, the needs for eicosanoid production (4). On the other hand, PUFA are susceptible to peroxidation by reactive oxygen species (ROS), which alter the FA composition and thus lead to tissue damage (5).

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Abbreviations: FRAP, ferric-reducing ability of plasma; LCPUFA, long-chain PUFA; MDA, malondialdehyde; PLA_2, phospholipase A_2; ROS, reactive oxygen species.

Decreased levels of PUFA in tissue lipids could be caused by an impaired desaturation of EFA precursors (6) or by an enhanced degradation of FA by autoxidation, lipid peroxidation, or increased use of PUFA for eicosanoid synthesis. Another important pathway to reduce the content of 20:4n-6 in the kidney is through the metabolism of 20:4n-6 *via* cytochrome P450 to form hydroxy and epoxy derivatives (7). The high vulnerability of the kidney to lipid peroxidation can be partly attributed to its high content of PUFA, particularly 20:4n-6 and 22:6n-3 (8). Likewise, kidney lipids of rats chronically fed a diet containing ethanol are susceptible to peroxidation (9). On the other hand, administering 20:4n-6 to rats protects the animals against alcoholic liver injury (10).

There is considerable evidence suggesting that ROS are involved in the pathogenesis of ischemic, toxic, or immunologically mediated renal injury. At the cell level, lipid peroxidation causes disruption of the ultrastructural integrity of membranes, thus impairing the cell transport and energy transduction processes. Additionally, ROS promote a reduction of renal blood flow and the glomerular filtration rate, partly due to the liberation of vasoconstrictor mediators involving bioactive lipids (11).

Ethanol behaves as a potent modulator of lipid metabolism, altering the FA profile of several organs (12,13). Alcohol consumption is also known to decrease PUFA content, mainly by inhibiting the elongation and desaturation of n-6 and n-3 FA precursors (14). Although the mechanism by which ethanol decreases the activity of microsomal desaturases of kidney remains to be elucidated, it has been reported that ethanol strongly inhibits the synthesis of PUFA in hepatocytes from spontaneously hypertensive rats. This could explain the deficit of prostaglandin precursors observed in cardiovascular disease linked to ethanol intoxication (15). Ethanol feeding reduces anisotropy of the brush border luminal membrane of the proximal tubule, increasing 18:2n-6 and decreasing 20:4n-6 and 22:6n-3 (16).

The effect of ethanol to diminish PUFA biogenesis could be triggered by its oxidative metabolism, which is known to result in increased ROS production, and it could be counteracted by antioxidants present in various foods, such as fruits, vegetables, tea, or wine. Even though wine polyphenols may enhance the antioxidant status due to their properties to act as ROS scavengers, iron chelators, and modulators of enzymes (17), few attempts have been made to determine their role in the alteration of lipid metabolism in the kidney. This study was undertaken to assess the effect of red wine components on kidney FA composition. The contribution of oxidative

stress in these possible compositional changes was determined through the lipid peroxidation and cytochrome P450 content in the kidney of rats fed water, red wine, ethanol, or alcohol-free red wine.

MATERIALS AND METHODS

Animals and diet. The study protocol was approved by the Comité de Bioética, Facultad de Medicina, Universidad de Chile. Male Wistar rats weighing 200–240 g, from the Departamento de Nutrición, Facultad de Medicina, Universidad de Chile were fed a balanced diet (Table 1) *ad libitum* for 10 wk. The animals were housed individually in wire cages and maintained at 25°C in an animal room with a 12 h light/dark cycle. Management of rats was carried out according to internationally accepted ethical rules.

The animals were divided into four groups, with the type of drinking fluid they were given as the differentiating factor: (i) Group 1, red wine: The animals were given free access to red wine (Cabernet Sauvignon, 1998 harvest, Lomas de Cauquenes, Cauquenes Valley, Chile). The ethanol concentration of wine was 12.5% (vol/vol). (ii) Group 2, alcohol-free red wine: The animals were given the same red wine, but it was previously dealcoholized in a rotary evaporator at 25°C for 4 h (17). (iii) Group 3, ethanol: The animals were given an aqueous ethanol solution having the same alcohol concentration as the red wine used for Group 1 (12.5%, vol/vol). (iv) Group 4, control: The animals were given tap water.

The daily fluid intakes were measured with graduated Richter tubes. Food intake was also estimated by gravimetry to control the calories ingested. Body weight gain was measured daily.

At the end of the treatment period, blood samples from each group were obtained through the carotid artery after intraperitoneal anesthesia with urethane (2 g/kg body weight). The samples were collected in plastic tubes with 5% EDTA and served to measure the antioxidant capacity of plasma, which was assessed by the ferric-reducing ability of plasma (FRAP, expressed in μM) (18). In addition, blood ethanol levels of the red wine and ethanol groups were determined by an enzymatic method, as reported elsewhere (19). The kidneys were perfused with Tris buffer (0.01 M, pH 7.0) prior to homogenization in the same solution.

Preparations of microsomes. Microsomes were prepared by ultracentrifugation as described elsewhere (20), and microsomal protein was measured by the method of Lowry *et al.* (21) using BSA as standard. The total cytochrome P450 content was measured according to the method of Omura and Sato (22).

Analysis of kidney FA. Total kidney lipids were extracted with methanol/chloroform (2:1, vol/vol), according to the method of Bligh and Dyer (23). The chloroform layer was methylated, after being evaporated under nitrogen, to yield FAME. FAME were extracted with hexane prior to capillary GLC analysis. A gas chromatograph (Hewlett-Packard, model 6890), equipped with 50 m × 0.22 mm SGE capillary column (BPX 70.0) was employed to separate FAME. The temperature was programmed from 180 to 230°C at 2°C/min with a final hold, separating 12:0 to 22:6n-3. The temperature of both the detector and injector was 240°C. Hydrogen was used as carrier gas at a flow rate of 1.5 mL/min and a split ratio of 1:80. FAME were identified by comparing their retention times with those of individual purified standards and were quantified using heptadecanoic acid methyl ester as an internal standard with a Hewlett-Packard integrator (HP 3396 Series III).

Lipid peroxidation. The assay for lipid peroxides was performed spectrophotometrically at 532 nm by thiobarbituric acid reaction at pH 3.5, followed by solvent extraction with a mixture of *n*-butanol/pyridine (15:1, vol/vol) (24). Tetramethoxypropane was used as the external standard, and the level of lipid peroxides was expressed as nmol malondialdehyde (MDA)/mg protein.

Statistical analyses. Results are expressed as means ± SD. All statistical analyses of data were computed using the Statistical Analysis System (SAS Institute, Cary, NC). The results for each specific FA were assessed by a one-way ANOVA, and comparisons for individual differences between groups were done using the Scheffé test. The differences were considered statistically significant at $P < 0.05$.

TABLE 1
Diet Composition

Component	g/kg	Component	g/kg
Casein	200	Fiber	40
DL-Methionine	3	Potato starch	75
Cornstarch	297	Mineral mixture[a]	50
Sucrose	185	Water soluble vitamins[b]	30
Canola oil	100	Fat-soluble vitamins[b]	20

[a]Minerals per kg diet: 15.25 g $CaCO_3$; 16.39 g K_2HPO_4; 3.81 g $CaHPO_4 \cdot 2H_2O$; 8.51 g NaCl; 5.18 g $MgSO_4 \cdot 7H_2O$; 510 mg Fe-citrate; 254 mg $MnSO_4$; 38 mg $ZnCl_2$; 51 mg $CuSO_4 \cdot 5H_2O$; 5 mg KI; 1.3 mg $CoCl_2$; 2.5 mg $AlK(SO_4)_2 \cdot 12H_2O$; 0.25 mg $Na_2Se_2O_3$; 5.8 mg NaF.
[b]Vitamins per kg diet: 1.38 mg all-*trans*-retinyl palmitate; 600 mg α-tocopherol (250 UI/g); 25 μg cholecalciferol; 0.75 mg menadione; 5 mg thiamin-HCl; 6 mg riboflavin; 6 mg pyridoxine-HCl, 30 mg nicotinic acid; 15 mg Ca pantothenate; 2 mg folic acid; 2 mg biotin; 0.025 mg cyanocobalamin; 1000 mg choline chloride; 63 mg inositol; 315 mg *p*-aminobenzoic acid.

RESULTS

Analysis of red wine. Total flavonols (free and conjugated myricetin and quercetin) reached 55.2 ± 2.3 mg/L, with a quercetin/myricetin ratio of 0.8. Blood ethanol levels (mg/dL) for the ethanol and red wine groups were 42.1 ± 3.9 ($n = 18$) and 37.8 ± 7.3 ($n = 16$), respectively, showing no significant difference. These blood ethanol concentrations were related to the similar fluid intakes of both groups, expressed as mL/d/100 g body weight, of 7.9 ± 1.3 (ethanol group) vs. 7.2 ± 0.6 (red wine group).

Energy consumption, body weight gain, and plasma antioxidant capacity. Daily energy intake (kcal/d/100 g body weight) during the experimental feeding period (70 d) was similar within all groups (average, 21.0 ± 1.8). Energy intakes

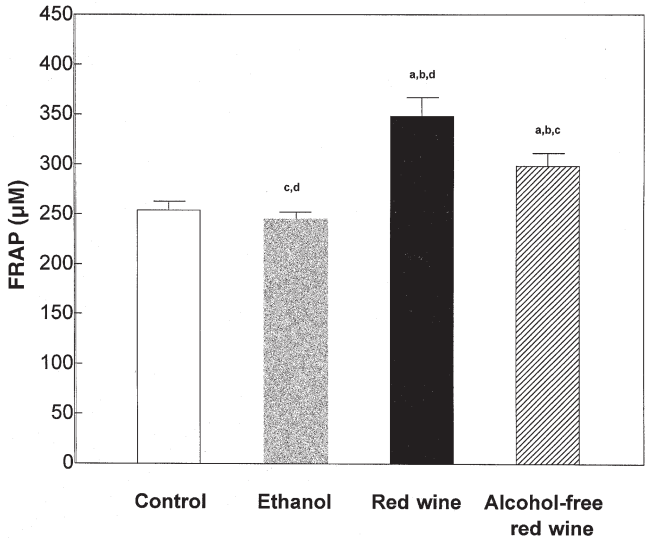

FIG. 1. Antioxidant capacity of plasma, assessed by the ferric-reducing ability of plasma (FRAP). Results are means ± SD (n = 10 for each group). Statistically significant differences, at $P < 0.05$, are indicated by superscript letters: [a]vs. control; [b]vs. ethanol; [c]vs. red wine; [d]vs. alcohol-free red wine, as assessed by a one-way ANOVA followed by the Scheffé test.

due to ethanol consumption were 24 and 28% for the ethanol and wine groups, respectively. Body weight gain (g/d/100 g body weight) was similar between the groups. The total plasma antioxidant capacity, expressed as FRAP (Fig. 1), was significantly increased by alcohol-free red wine and red wine, but ethanol consumption did not influence this parameter. The composition of the experimental diet and the FA composition of this diet are shown in Tables 1 and 2, respectively.

FA composition of kidney lipids. Data on the FA composition of kidney lipids are given in Table 3. Ethanol caused a significant decrease in long-chain PUFA (LCPUFA) (20:4n-6, 20:5n-3, and 22:6n-3). Red wine, as well as ethanol, decreased 22:6n-3, but red wine maintained the levels of 20:4n-6 and 20:5n-3 found in the kidney of the control group. Alcohol-free red wine elevated levels of 20:4n-6 higher than in any other group. The levels of 18:2n-6, 18:3n-3, saturated FA, and monounsaturated FA were not modified by any of the treatments.

Lipid peroxidation. Figure 2 shows the results obtained for kidney lipid peroxidation (nmol MDA/mg protein). In both the red wine and alcohol-free red wine groups lipid peroxidation was diminished by 50 and 35%, respectively, as compared with the control group, whereas no changes were found in the ethanol group.

TABLE 2
FA Composition of Diet

FA	g/kg diet	FA	g/kg diet
14:0	0.07	18:2n-6	25.59
16:0	5.57	18:3n-3	7.10
16:1n-9	0.14	20:0	0.59
18:0	2.00	22:0	0.32
18:1n-9	57.18		

TABLE 3
FA Composition of Kidney Total Lipids[a]

FA (as methyl esters, %)	Control	Ethanol	Red wine	Alcohol-free red wine
SFA total	38.5 ± 1.5	40.0 ± 2.7	36.0 ± 0.3	36.5 ± 0.6
MUFA total	11.7 ± 1.2	10.6 ± 0.4	11.6 ± 0.3	11.7 ± 0.5
PUFA total	43.8 ± 1.4	40.1 ± 0.4[a]	41.8 ± 0.1	43.3 ± 0.5
18:2n-6	2.2 ± 0.4	2.5 ± 0.5	2.2 ± 0.4	2.2 ± 0.5
18:3n-3	0.4 ± 0.2	0.3 ± 0.2	0.4 ± 0.2	0.4 ± 0.2
18:3n-6	12.3 ± 0.9	12.4 ± 1.0[c]	16.3 ± 1.2[a,b]	13.9 ± 0.1[b,c]
20:4n-6	28.6 ± 0.7	21.6 ± 3.1[a,c,d]	28.3 ± 0.7[b]	30.4 ± 0.3[a,b,c]
20:5n-3	2.0 ± 0.7	0.7 ± 0.1[a]	1.6 ± 0.6[b]	1.4 ± 0.1[b]
22:6n-3	3.9 ± 0.6	2.1 ± 0.5[a]	2.9 ± 0.4[a,b]	3.6 ± 0.3[b,c]

[a]Results are means ± SD (n = 10 for each group). Statistically significant differences, at $P < 0.05$, are indicated by superscript letters: [a]vs. control; [b]vs. ethanol; [c]vs. red wine; [d]vs. alcohol-free red wine, as assessed by a one-way ANOVA followed by the Scheffé test. SFA, saturated FA (14:0; 16:0; 18:0; 20:0; 22:0); MUFA, monounsaturated FA (16:1n-7; 18:1n-9); PUFA, polyunsaturated FA (18:2n-6; 18:3n-3; 18:3n-6; 20:4n-6; 20:5n-3; 22:6n-3).

Cytochrome P450 content. As shown in Table 4, the kidney cytochrome P450 content was increased by 50% in the ethanol group and diminished by 20% in the alcohol-free red wine group, as compared with control values. The kidney microsomal protein content was not altered by the treatments.

DISCUSSION

The present study was undertaken to investigate the effects of ethanol, red wine, and alcohol-free red wine intakes on the FA composition, lipid peroxidation, and microsomal cytochrome P450 content in rat kidney. LCPUFA play an important role in the synthesis of eicosanoids that are particularly

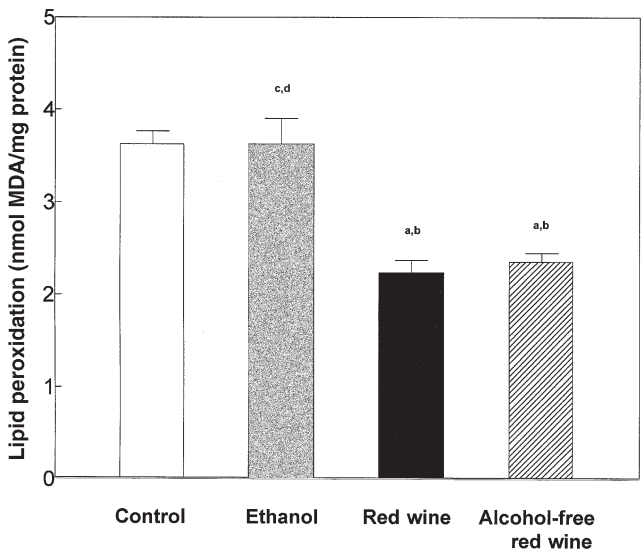

FIG. 2. Lipid peroxidation in whole-kidney homogenates, assessed by malondialdehyde (MDA) production. Results are means ± SD (n = 9 for each group). Statistically significant differences, at $P < 0.05$, are indicated in superscript letters: [a]vs. control; [b]vs. ethanol; [c]vs. red wine; [d]vs. alcohol-free red wine, as assessed by a one-way ANOVA followed by the Scheffé test.

TABLE 4
Rat Kidney Microsomal Protein and Total Cytochrome P450 Content[a]

Group	Microsomal protein (mg/g tissue)	Total cytochrome P450 (mg/mg protein)
Control	9.70 ± 0.65 (10)	0.10 ± 0.03 (8)
Ethanol	9.80 ± 0.47 (10)	0.15 ± 0.04[a,c,d] (8)
Red wine	10.50 ± 0.49 (5)	0.09 ± 0.03 (9)
Alcohol-free red wine	10.70 ± 0.14 (5)	0.08 ± 0.02 (6)

[a]Values are means ± SD. Number of experiments is shown in parenthesis. Statistically significant differences, at $P < 0.05$, are indicated by superscript letters: [a]vs. control; [b]vs. ethanol; [c]vs. red wine; [d]vs. alcohol-free red wine, as assessed by a one-way ANOVA followed by the Scheffé test.

relevant in the regulation of renal physiology (7) and in the maintenance of membrane fluidity.

The present data are in agreement with others reporting that chronic ethanol ingestion alters the profile of FA and decreases the LCPUFA n-3 in heart tissue of rats (25). Chronic administration of ethanol, in a micropig model, caused reduced anisotropy in kidney brush border membranes, which correlated inversely with the levels of 18:2n-6 and directly with the levels of 20:4n-6 and 22:6n-3. On the other hand, the wine polyphenols could have inhibited the activity of cyclooxygenase and lipoxygenase, as reported for closely related flavonoids (26), thereby avoiding the use of 20:4n-6. Hence, the strong antioxidant effect of red wine polyphenols could have contributed to the increased levels of 20:4n-6. Notably, the abundance of polyphenols found in the red wine used in the present study was higher than that reported for samples from numerous geographical regions (27), which supports an antioxidant effect in the kidney as well as in the levels of antioxidant capacity of plasma. Arachidonic acid is known to be liberated from mammalian membranes by phospholipase A_2 (PLA_2). This enzyme is activated by ethanol exposure in mouse brain membrane, exhibiting a preference for 20:4n-6 (28). These results suggest that the properties of ethanol-activated PLA_2 found in mouse brain membrane could also be found in a variety of cell types, including rat kidney. This could account for the low levels of 20:4n-6 found in the latter in the present study. A tremendous decrease of 20:4n-6 biosynthesis was evidenced in alcohol-intoxicated hepatocytes; this effect was dose-dependent and reinforced when ethanol concentration was high, mainly for Δ5-desaturase (15).

The activity of Δ6-desaturase increased by more than twofold when the content of vitamin E was increased in brain microsomal membrane suspension (29). This raises the question of the role of antioxidants, such as vitamin E or polyphenols (30), in membrane LCPUFA content. This effect could be due to an increased synthesis of these compounds and/or to a protection against their peroxidation. This view is consistent with the finding in the present paper showing decreased lipid peroxidation in kidney (Fig. 2) and increased FRAP levels in plasma (Fig. 1) in red wine and alcohol-free red wine groups. Furthermore, the increased total cytochrome P450 content found in the ethanol group could account for the consumption of 20:4n-6 and the generation of hydroxy and epoxy derivatives in the kidney, as reported previously (7).

Another important pathway of 20:4n-6 utilization is its metabolism *via* cyclooxygenase- and lipoxygenase-catalyzed reactions. Tea polyphenols were found to inhibit cyclooxygenase-dependent arachidonic metabolism in microsomes of normal colon mucosa (30). Accordingly, the increased levels of 20:4n-6 found in the kidney of the alcohol-free red wine group (Table 3) are consistent with a decreased level of cyclooxygenase activity, an effect that might be caused by wine polyphenols. However, measurements of this enzyme activity should be carried out to demonstrate this hypothesis.

In summary, the results reported here suggest that moderate red wine consumption could contribute to the preservation of n-3 and n-6 PUFA, particularly 20:4n-6. It could be hypothesized that this effect might be due to the ability of the nonalcoholic components of red wine to decrease lipid peroxidation and inhibit the activity of the enzymes involved in eicosanoid synthesis. However, further studies on the pharmacological properties of these compounds that might elucidate their effects *in vivo* are still lacking.

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Functionalization of Unactivated Carbons in 3α,6- and 3α,24-Dihydroxy-5β-cholane Derivatives by Dimethyldioxirane

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ABSTRACT: Direct remote functionalization of unactivated carbons by dimethyldioxirane (DMDO) was examined for 3α,6- and 3α,24-dihydroxy-5β-cholane derivatives. DMDO oxidation of stereoisomeric methyl 3α,6-diacetoxy-5β-cholanoates caused the direct, unexpected 14α- and 17α-hydroxylations, in analogy with that of the 5α-H analogs, regardless of the differences in stereochemical configuration of the A/B-ring junction and of the acetoxyl groups at C-3 and C-6. On the other hand, the ester derivatives of 3α,24-dihydroxy-5β-cholane with DMDO were transformed into the corresponding 5β-, 14α-, and 17α-hydroxy compounds, whereas the ether derivatives yielded the 5β-hydroxy, 3-oxo, and C-24 oxidized products, accompanied by their dehydrated ones.

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In analogy with enzyme-controlled reactions *in vivo,* such as a cytochrome P-450 oxidase-dependent system, remote functionalization of unactivated carbon atoms in steroids is a key reaction in an efficient and short-step synthesis of biologically and physiologically active compounds, starting from abundantly available steroids such as bile acids and sterols. Hence, various methods have been developed for the remote functionalization reactions in steroids, as has recently been reviewed by Reese (1). Of various specific remote-oxidizing reagents, dimethyldioxirane (DMDO) (2,3) and an oxygen-transfer system (e.g., iodosylbenzene and heteroaromatic *N*-oxides) catalyzed by a metalloporphyrin (4–7) are attractive oxidants, because they cause a direct O-insertion on unactivated methine and methylene carbons in substrates to produce hydroxylation and/or ketonization products. Particularly noteworthy is the simplicity in the preparation and handling of the DMDO reagent, as well as in the experimental and workup procedures.

In a previous paper, we reported a facile and stereoselective oxyfunctionalization of unactivated methine carbons in the 5β-cholane (A/B-*cis* junction; 5β-H) and 5α-cholestane (A/B-*trans* junction; 5α-H) series of steroids by DMDO, which provides a variety of mono- and dioxygenated compounds in one step (3). By utilizing the DMDO procedure, we also successfully attained for the first time the synthesis of so-called avicholic acid, or 3α,7α,16α-trihydroxy-5β-cholanoic acid (8), a novel primary bile acid in the Shoebill stork (*Balaeniceps rex*) and herons (9). In a continuation of a program on the remote functionalization reaction of steroids, we report here DMDO oxidation of 3,6- and 3,24-dihydroxycholane derivatives, most of which are of the 5β-series.

EXPERIMENTAL PROCEDURES

Melting points (mp) were determined on a microstage apparatus and are uncorrected. IR spectra were obtained on a Bio-Rad FTS-7 FTIR spectrometer as KBr discs. ^{1}H and ^{13}C NMR spectra were obtained on a JEOL JNM-EX 270 FT NMR instrument at 270 and 68.80 MHz, respectively, with $CDCl_3$ unless otherwise noted; chemical shifts were expressed as δ ppm relative to tetramethylsilane. Electron ionization (EI) low-resolution mass spectra (LR-MS) were obtained on a JEOL JMS-Automass 150 gas chromatograph–mass spectrometer at 70 eV. High-resolution mass spectra (HR-MS) were performed using a JEOL-LCmate double-focusing magnetic mass spectrometer equipped with an electrospray ionization (ESI) or an atmospheric-pressure chemical ionization (APCI) probe under positive ion mode (PIM) or negative ion mode (NIM). HR-MS were also obtained on a JEOL JMS-AX500 mass spectrometer with an EI under the PIM. A Shimazdu GC-17A gas chromatograph equipped with a FID was used isothermally at 300°C: It was fitted with a chemically bonded fused-silica capillary column (25QC3/BPX5; 25 m × 0.32 mm i.d.; film thickness, 0.25 μm; SGE Japan, Yokohama, Japan). The apparatus used for medium-pressure liquid chromatography (MPLC) consisted of a Shimamura YRD-880 refractive index detector (Tokyo, Japan) and a uf-3040S chromatographic pump (Shimamura Tech., Tokyo, Japan) using silica gel 60 (230–400 mesh, Nacarai Tesque, Kyoto, Japan) as the normal-phase (NP) adsorbent or ODS-AM 120-S50 as the reversed-phase (RP) adsorbent (YMC Co. Ltd., Kyoto, Japan). TLC was performed on precoated silica gel plates (0.25 mm layer thickness; E. Merck, Darmstadt, Germany) using hexane/EtOAc/acetic acid mixtures (80:20:1–20:80:1, by vol) as the developing solvent.

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Abbreviations: APCI, atmospheric-pressure chemical ionization; DMDO, dimethyldioxirane; EI, electron ionization; ESI, electrospray ionization; HR-MS, high-resolution mass spectra; LR-MS, low-resolution mass spectra; MPLC, medium-pressure liquid chromatography; NIM, negative ion mode; NP, normal phase; PIM, positive ion mode; RP, reversed phase; S.C., side chain; TBDMS, *tert*-butyldimethylsilyl; TES, triethylsilyl.

A solution (0.33 mol/L) of DMDO in $CHCl_3$ was prepared according to a literature method using oxone®, $NaHCO_3$, and acetone (10). Substrates **1**–**6** used in this study were from our laboratory collection. The triethylsilyl (TES) ether derivative (**7**) of 3α,24-dihydroxy-5β-cholane was prepared by a literature method (11,12).

General procedure for the oxidation of steroids by DMDO. To a solution of a steroid (1 mmol) in CH_2Cl_2 (6 mL) was added a freshly prepared solution of DMDO (2 mmol; 6 mL) in $CHCl_3$. The mixture was left at room temperature for 12 h, and excess DMDO and solvent were evaporated off under reduced pressure. The above procedure was repeated for one to three runs (36 h) with monitoring by TLC. After the reaction, the product was purified by passage through an open column of silica gel (70–230 mesh), eluting with benzene/EtOAc (98:2–2:8, vol/vol) mixtures and then by NP-MPLC on silica gel (230–400 mesh), eluting with hexane/benzene/EtOAc (5:3:2, by vol) or benzene/EtOAc (8:2, vol/vol), or by RP-MPLC on C_{18} bonded silica gel, eluting with methanol/water (9:1–6.5:3.5, vol/vol).

*Oxidation products of methyl 3α,6α-diacetoxy-5β-cholanoate (**1**)*. The crude DMDO oxidation product of **1** was subjected to an open column of silica gel (70–230 mesh, 15 g), eluting with benzene/EtOAc (95:5, vol/vol), followed by NP-MPLC on silica gel (20 g) eluting with hexane/benzene/EtOAc (5:3:2, by vol).

(i) Methyl 3α,6α-diacetoxy-14α-hydroxy-5β-cholanoate (***8***). Isolated as colorless needles [Fraction (Fr.) 1] recrystallized from aqueous methanol: mp, 163–165°C. IR, λ_{max} (cm^{-1}): 1726 (ester C=O), 3557 (OH). ^{1}H NMR, δ: 0.78 (*s,* 3H, 18-CH_3), 0.89 (*d,* 3H, *J* = 6.2 Hz, 21-CH_3), 0.98 (*s,* 3H, 19-CH_3), 2.01 and 2.03 (*s,* each 3H, 3α- and 6α-$OCOCH_3$), 3.66 (*s,* 3H, $COOCH_3$), 4.71 (*br m,* 1H, 3β-H), 5.17 (*br m,* 1H, 6β-H). LR-MS, *m/z*: 506 (<1%, M), 428 (3%, M – AcOH – H_2O), 368 (8%, M – 2AcOH – H_2O), 313 (13%), 281 (8%), 253 [100%, M – 2AcOH – H_2O – side chain (S.C.)], 211 (26%, M – 2AcOH – H_2O – S.C. – ring D). HR-MS (ESI-PIM), calcd. for $C_{29}H_{46}O_7Na$ $[M + Na]^+$: 529.3142. Found: *m/z*, 529.3154.

(ii) Methyl 3α,6α-diacetoxy-17α-hydroxy-5β-cholanoate (***9***). Isolated as a noncrystalline substance (Fr. 2). IR, λ_{max} (cm^{-1}): 1727 (ester C=O), 3556 (OH). ^{1}H NMR, δ: 0.73 (*s,* 3H, 18-CH_3), 0.90 (*d,* 3H, *J* = 7.0 Hz, 21-CH_3), 0.98 (*s,* 3H, 19-CH_3), 2.02 and 2.04 (*s,* each 3H, 3α- and 6α-$OCOCH_3$), 3.67 (*s,* 3H, $COOCH_3$), 4.70 (*br m,* 1H, 3β-H), 5.15 (*br m,* 1H, 6β-H). LR-MS, *m/z*: 506 (<1%, M), 373 (4% M – 2AcOH – CH_3), 353 (9%, M – 2AcOH – CH_3 – H_2O), 313 (18%), 281 (30%), 253 (100%, M – 2AcOH – H_2O – S.C.), 228 (49%, M – 2AcOH – H_2O – S.C. – part of ring D), 214 (37%, M – 2AcOH – H_2O – S.C. – ring D). HR-MS (ESI-PIM), calcd. for $C_{29}H_{46}O_7Na$ $[M + Na]^+$: 529.3142. Found: *m/z*, 529.3158.

*Oxidation products of methyl 3α,6β-diacetoxy-5β-cholanoate (**2**)*. The crude DMDO oxidation product was subjected to an open column of silica gel (15 g) eluting with benzene/EtOAc (90:10, vol/vol), followed by NP-MPLC on silica gel (20 g) eluting with hexane/benzene/EtOAc (5:3:2, by vol).

(i) Methyl 3α,6β-diacetoxy-14α-hydroxy-5β-cholanoate (***10***). Isolated as a noncrystalline substance (Fr. 1). IR, λ_{max} (cm^{-1}): 1727 (ester C=O), 3559 (OH). ^{1}H NMR, δ: 0.83 (*s,* 3H, 18-CH_3), 0.90 (*d,* 3H, *J* = 6.2 Hz, 21-CH_3), 1.03 (*s,* 3H, 19-CH_3), 2.01 and 2.04 (*s,* each 3H, 3α- and 6β-$OCOCH_3$), 3.67 (*s,* 3H, $COOCH_3$), 4.68 (*br m,* 1H, 3β-H), 4.77 (*m,* 1H, 6α-H). LR-MS, *m/z*: 506 (<1%, M), 428 (8%, M – AcOH – H_2O), 353 (17%, M – 2AcOH – H_2O – CH_3), 314 (17%), 281 (13%), 253 (100%, M – 2AcOH – H_2O – S.C.), 207 (32%). HR-MS (ESI-PIM), calcd. for $C_{29}H_{46}O_7Na$ $[M + Na]^+$: 529.3142. Found: *m/z*, 529.3100.

(ii) Methyl 3α,6β-diacetoxy-17α-hydroxy-5β-cholanoate (***11***). Isolated as a noncrystalline substance (Fr. 2). IR, λ_{max} (cm^{-1}): 1730 (ester C=O), 3559 (OH). ^{1}H NMR, δ: 0.77 (*s,* 3H, 18-CH_3), 0.91 (*d,* 3H, *J* = 6.5 Hz, 21-CH_3), 1.02 (*s,* 3H, 19-CH_3), 2.02 and 2.04 (*s,* each 3H, 3α- and 6β-$OCOCH_3$), 3.67 (*s,* 3H, $COOCH_3$), 4.68 (*br m,* 1H, 3β-H), 4.71 (*m,* 1H, 6α-H). LR-MS, *m/z*: 506 (<1%, M), 489 (13%, M – H_2O), 429 (19%, M – H_2O – AcOH), 354 (13%, M – 2AcOH – $2CH_3$), 337 (30%, M – 2AcOH – $2CH_3$ – H_2O), 315 (30%), 281 (18%), 253 (60%, M – 2AcOH – H_2O – S.C.), 228 (47%, M – 2AcOH – H_2O – S.C. – part of ring D), 213, (100%, M – 2AcOH – H_2O – S.C. – ring D). HR-MS (ESI-PIM), calcd. for $C_{29}H_{46}O_7Na$ $[M + Na]^+$: 529.3142. Found: *m/z*, 529.3098.

*Oxidation products of methyl 3α,6α-diacetoxy-5α-cholanoate (**3**)*. The crude DMDO oxidation product of **3** (500 mg) was subjected to an open column of silica gel (15 g) eluting with benzene/EtOAc (9:1, vol/vol), followed by NP-MPLC on silica gel (20 g) eluting with hexane/benzene/EtOAc (55:30:15, by vol).

(i) Methyl 3α,6α-diacetoxy-14α-hydroxy-5α-cholanoate (***12***). Isolated as a colorless amorphous solid (Fr. 1) recrystallized from aqueous acetone: mp, 157–159°C. IR, λ_{max} (cm^{-1}): 1728 (ester C=O), 3558 (OH). ^{1}H NMR, δ: 0.79 (*s,* 3H, 18-CH_3), 0.87 (*s,* 3H, 19-CH_3), 0.88 (*d,* 3H, *J* = 7.2 Hz, 21-CH_3), 2.02 and 2.06 (*s,* each 3H, 3α- and 6α-$OCOCH_3$), 3.66 (*s,* 3H, $COOCH_3$), 4.68 (*m,* 1H, 6β-H), 5.16 (*br s,* 1H, 3β-H). LR-MS, *m/z*: 506 (<1%, M), 446 (8%, M – AcOH), 429 (7%, M – AcOH – H_2O), 368 (9%, M – 2AcOH – H_2O), 313 (15%), 281 (15%), 253 (100%, M – 2AcOH – H_2O – S.C.), 208 (66%). HR-MS (ESI-PIM), calcd. for $C_{29}H_{46}O_7Na$ $[M + Na]^+$: 529.3142. Found: *m/z*, 529.3135.

(ii) Methyl 3α,6α-diacetoxy-17α-hydroxy-5α-cholanoate (***13***). Isolated as a noncrystalline substance (Fr. 2). IR, λ_{max} (cm^{-1}): 1735 (ester C=O), 3562 (OH). ^{1}H NMR, δ: 0.73 (*s,* 3H, 18-CH_3), 0.86 (*s,* 3H, 19-CH_3), 0.90 (*d,* 3H, *J* = 7.5 Hz, 21-CH_3), 2.02 and 2.06 (*s,* each 3H, 3α- and 6α-$OCOCH_3$), 3.67 (*s,* 3H, $COOCH_3$), 4.65 (*m,* 1H, 6β-H), 5.06 (*br s,* 1H, 3β-H). LR-MS, *m/z*: 506 (<1%, M), 415 (5%, M – AcOH – $2CH_3$), 354 (9%, M – 2AcOH – H_2O – CH_3), 339 (8%, M – 2AcOH – H_2O – $2CH_3$), 313 (11%), 288 (14%), 253 (63%, M – 2AcOH – H_2O – S.C.), 215 (100%, M – 2AcOH – H_2O – S.C. – ring D). HR-MS (ESI-PIM), calcd. for $C_{29}H_{46}O_7Na$ $[M + Na]^+$: 529.3142. Found: *m/z*, 529.3160.

*Oxidation products of 3α,24-diacetoxy-5β-cholane (**4**)*. The crude DMDO oxidation product of **4** (400 mg) was sub-

jected to an open column of silica gel (30 g) eluting with benzene/EtOAc (9:1–7:3, vol/vol), followed by NP-MPLC on silica gel (20 g) eluting with benzene/EtOAc (8:2, vol/vol).

(i) 3α,24-Diacetoxy-5β-hydroxycholane (***14***). Isolated as a noncrystalline substance (Fr. 1). IR, λ_{max} (cm^{-1}): 1736 (acetyl C=O), 3497 (OH). ^{1}H NMR, δ: 0.64 (*s,* 3H, 18-CH_3), 0.90 (*s,* 3H, 19-CH_3), 0.92 (*d,* 3H, *J* = 6.2 Hz, 21-CH_3), 2.02 and 2.04 (*s,* each 3H, 3α- and 24-$OCOCH_3$), 4.02 (*m,* 2H, 24-CH_2OAc), 5.15 (*br m,* 1H, 3β-H). LR-MS, *m/z*: 462 (1%, M), 444 (2%, M – H_2O), 402 (3%, M – AcOH), 384 (13%, M – AcOH – H_2O), 348 (15%), 255 (28%, M – AcOH – H_2O – S.C.), 213 (100%, M – AcOH – H_2O – S.C. – ring D). HR-MS (ESI-PIM), calcd. for $C_{28}H_{46}O_5Na$ $[M + Na]^+$: 485.3243. Found: *m/z*, 485.3287.

(ii) 3α,24-Diacetoxy-5β,17α-dihydroxycholane (***15***). Isolated as colorless needles (Fr. 2) recrystallized from aqueous acetone: mp, 164–167°C. IR, λ_{max} (cm^{-1}): 1739 (acetyl C=O), 3466, 3529 (OH). ^{1}H NMR, δ: 0.74 (*s,* 3H, 18-CH_3), 0.91 (*s,* 3H, 19-CH_3), 0.91(*d,* 3H, *J* = 4.6 Hz, 21-CH_3), 2.02 and 2.04 (*s,* each 3H, 3α- and 24-$OCOCH_3$), 4.05 (*m,* 2H, 24-CH_2OAc), 5.09 (*br m,* 1H, 3β-H). LR-MS, *m/z*: 478 (<1%, M), 427 (2%, M – $2H_2O$ – CH_3), 400 (13%, M – AcOH – H_2O), 313 (13%, M – 2AcOH – $3CH_3$), 281 (19%), 253 (100%, M – 2AcOH – $2H_2O$ – S.C.), 228 (94%, M – 2AcOH – $2H_2O$ – S.C. – part of ring D). HR-MS (ESI-PIM), calcd. for $C_{28}H_{46}O_6Na$ $[M + Na]^+$: 501.3192. Found: *m/z*, 501.3189.

*(iii) 3α,24-Diacetoxy-5β,14α-dihydroxycholane (**16**).* Isolated as a noncrystalline substance (Fr. 3). IR, λ_{max} (cm^{-1}): 1736 (acetyl C=O), 3528 (OH). ^{1}H NMR, δ: 0.78 (*s,* 3H, 18-CH_3), 0.93 (*s,* 3H, 19-CH_3), 0.92 (*d,* 3H, *J* = 6.5 Hz, 21-CH_3), 2.02 and 2.04 (*s,* each 3H, 3α- and 24-$OCOCH_3$), 4.03 (*m,* 2H, 24-CH_2OAc), 5.08 (*br m,* 1H, 3β-H). LR-MS, *m/z*: 478 (<1%, M), 442 (1%, M – $2H_2O$), 400 (7%, M – AcOH – H_2O), 382 (11%, M – $2H_2O$ – AcOH), 346 (6%), 313 (11%, M – 2AcOH – $3CH_3$), 281 (19%), 253 (100%, M – 2AcOH – $2H_2O$ – S.C.), 228 (94%, M – 2AcOH – $2H_2O$ – S.C. – part of ring D). HR-MS (ESI-PIM), calcd. for $C_{28}H_{46}O_6Na$ $[M + Na]^+$: 501.3192. Found: *m/z*, 501.3223.

*Oxidation products of 3α,24-diformyloxy-5β-cholane (**5**).* The crude DMDO reaction product of **5** (400 mg) dissolved in benzene (5 mL) was poured onto an open column of neutral alumina (activity II, 60 g) and left in contact with it overnight. Elution with benzene/EtOAc (8:2, vol/vol) gave a mixture of three components, which was then subjected to RP-MPLC on C_{18} silica gel (20 g), eluting with methanol/water (9:1–65:35, vol/vol).

(i) 3α,24-Dihydroxy-5β-chol-14-ene (***19***). Isolated as colorless needles (Fr. 1) recrystallized from aqueous methanol: mp, 168–171°C. IR, λ_{max} (cm^{-1}): 3252 (OH). ^{1}H NMR, δ: 0.89 (*s,* 3H, 18-CH_3), 0.92 (*s,* 3H, 19-CH_3), 0.93 (*d,* 3H, *J* = 5.6 Hz, 21-CH_3), 3.62 (*br m,* 1H, 3β-H), 3.63 (*t,* 2H, *J* = 6.4 Hz, 24-CH_2OH), 5.14 (*br s,* 1H, 15-H). LR-MS, *m/z*: 360 (<1%, M), 342 (2%, M – H_2O), 327 (2%, M – H_2O – CH_3), 275 (50%, M – S.C.), 255 (100%, M – H_2O – S.C.). HR-MS (EI-PIM), calcd. for $C_{24}H_{40}O_2$ $[M]^+$: 360.3028. Found: *m/z*, 360.3011.

(ii) 3α,5β,24-Trihydroxycholane (***17***). Isolated as colorless thin plates (Fr. 2) recrystallized from aqueous methanol: mp, 208–211°C. IR, λ_{max} (cm^{-1}): 3389 (OH). ^{1}H NMR (CD_3CD), δ: 0.64 (*s,* 3H, 18-CH_3), 0.89 (*s,* 3H, 19-CH_3), 0.93 (*d,* 3H, *J* = 6.5 Hz, 21-CH_3), 3.64 (*m,* 2H, 24-CH_2OH), 4.03 (*br m,* 1H, 3β-H). LR-MS, *m/z*: 378 (<1%, M), 360 (4%, M – H_2O), 342 (28%, M – $2H_2O$), 306 (100%), 281 (34%), 255 (56%, M – $2H_2O$ – S.C.), 213 (91%, M – $2H_2O$ – S.C. – ring D). HR-MS (ESI-NIM), calcd. for $C_{26}H_{45}O_5$ $[M + AcO]^-$: 437.3267. Found: *m/z*, 437.3272.

(iii) 3α,5β,17α,24-Tetrahydroxycholane (***18***). Isolated as colorless needles (Fr. 3) recrystallized from aqueous methanol: mp, 200–203°C. IR, λ_{max} (cm^{-1}): 3334 (OH). ^{1}H NMR, δ: 0.74 (*s,* 3H, 18-CH_3), 0.89 (*s,* 3H, 19-CH_3), 0.91 (*d,* 3H, *J* = 6.5 Hz, 21-CH_3), 3.62 (*t,* 2H, *J* = 6.2 Hz, 24-CH_2OH), 4.00 (*br m,* 1H, 3β-H). LR-MS, *m/z*: 376 (<1%, M – H_2O), 358 (2%, M – $2H_2O$), 340 (9%, M – $3H_2O$), 325 (16%, M – $3H_2O$ – CH_3), 281 (24%), 253 (100%, M – $3H_2O$ – S.C.), 215 (74%). HR-MS (APCI–NIM), calcd. for $C_{24}H_{41}O_4$ $[M - H]^-$: 393.3005. Found: *m/z*, 393.2999.

*Oxidation products of 3α,24-di(*tert*-butyldimethylsilyloxy)-5β-cholane (**6**).* To the crude DMDO oxidation product of **6** (400 mg) dissolved in methanol (15 mL) was added five drops of conc. HCl, and the mixture was left to stand overnight at room temperature. Most of the solvent was evaporated, and the residue was extracted with EtOAc. The EtOAc layer was washed with saturated brine, dried with Drierite®, and evaporated. The residue was separated on an open column of silica gel (20 g), eluting with benzene/EtOAc (98:2, vol/vol), followed by NP-MPLC on silica gel (10 g), eluting with hexane/benzene/EtOAc (5:4:1, by vol).

*(i) Methyl 3-oxo-5β-cholanoate (**21**).* Isolated as a colorless amorphous solid (Fr. 1) recrystallized from aqueous acetone: mp, 118–120°C [lit. (13), mp, 119–120 °C]. IR, λ_{max} (cm^{-1}): 1712 (ketonic C=O), 1735 (ester C=O). ^{1}H NMR, δ: 0.68 (*s,* 3H, 18-CH_3), 0.92 (*d,* 3H, *J* = 6.2 Hz, 21-CH_3), 1.01 (*s,* 3H, 19-CH_3), 3.66 (*s,* 3H, $COOCH_3$). LR-MS, *m/z*: 388 (14%, M), 373 (4%, M – CH_3), 355 (4%, M – H_2O – CH_3), 318 (31%), 273 (100%, M – S.C.), 231 (96%, M – S.C. – ring D).

*(ii) Methyl 3-oxo-4-cholenoate (**22**).* Isolated as colorless thin plates (Fr. 2) recrystallized from aqueous acetone: mp, 123–126°C [lit. (14), mp, 125–126°C]. IR, λ_{max} (cm^{-1}): 1678 (ketonic C=O), 1735 (ester C=O). ^{1}H NMR, δ: 0.71 (*s,* 3H, 18-CH_3), 0.92 (*d,* 3H, *J* = 6.2 Hz, 21-CH_3), 1.18 (*s,* 3H, 19-CH_3), 3.66 (*s,* 3H, $COOCH_3$), 5.72 (*s,* 1H, 4-H). LR-MS, *m/z*: 386 (12%, M), 371 (M – CH_3), 355 (3%, M – OCH_3), 344 (4%, M – CH_2CO), 329 (5%, M – CH_3 – CH_2CO), 271 (13%, M – S.C.), 263 (29%), 229 (100%, M – S.C. – CH_2CO).

*(iii) Methyl 3α-hydroxy-5β-cholanoate (**23**).* Isolated as colorless needles (Fr. 3) recrystallized from methanol and identified as the title compound (methyl lithocholate) by comparison with an authentic reference compound.

(iv) Methyl 3α,5β-dihydroxycholanoate (***20***). Isolated as colorless thin plates (Fr. 4) crystallized from aqueous methanol: mp, 171–174°C. IR, λ_{max} (cm^{-1}): 1736 (ester

C=O), 3336 (OH). ^{1}H NMR, δ: 0.64 (*s,* 3H, 18-CH_3), 0.88 (*s,* 3H, 19-CH_3), 0.91 (*d,* 3H, *J* = 6.7 Hz, 21-CH_3), 3.66 (*s,* 3H, $COOCH_3$), 4.01 (*br m,* 1H, 3β-H). LR-MS, *m/z*: 406 (1%, M), 388 (6%, M – H_2O), 370 (20%, M – $2H_2O$), 334 (29%), 255 (76%, M – $2H_2O$ – S.C.), 213 (100%). HR-MS (ESI-NIM), calcd. for $C_{27}H_{45}O_6$ [M + AcO]$^-$: 465.3216. Found: *m/z*, 465.3212.

Oxidation products of 3α,24-di(triethylsilyloxy)-5β-cholane (7). The crude DMDO oxidation product of **7** (600 mg) was treated with conc. HCl and processed as described for **6**. The reaction product was chromatographed on a column of silica gel (30 g). Elution with benzene/EtOAc (9:1–2:8, vol/vol) afforded four well-resolved homogeneous fractions. Each of the these compounds was identified as follows: methyl 3-oxo-5β-cholanoate (**21**, Fr. 1), methyl 3α-hydroxy-5β-cholanoate (**23**, Fr. 2), methyl 3α,5β-dihydroxycholanoate (**20**, Fr. 3), and 3α,5β,24-trihydroxycholane (**17**, Fr. 4), as evidenced by their chromatographic and spectral data.

RESULTS AND DISCUSSION

Substrates examined in this study were the ester (acetate and formate) or ether [*tert*-butyldimethylsilyl (TBDMS) and triethylsilyl (TES)] derivatives of stereoisomeric methyl 3α,6-dihydroxycholanoates (**1**–**3**) and of 3α,24-dihydroxy-5β-cholanes (**4**–**7**). Such derivatizations were made to prevent the simultaneous oxidation of the hydroxy groups to the carbonyl with DMDO and also to investigate the effect of the hydroxy-protecting groups on the remote functionalization products by nucleophilic DMDO: Esters are classified into an electron-withdrawing group and ethers into an electron-donating group.

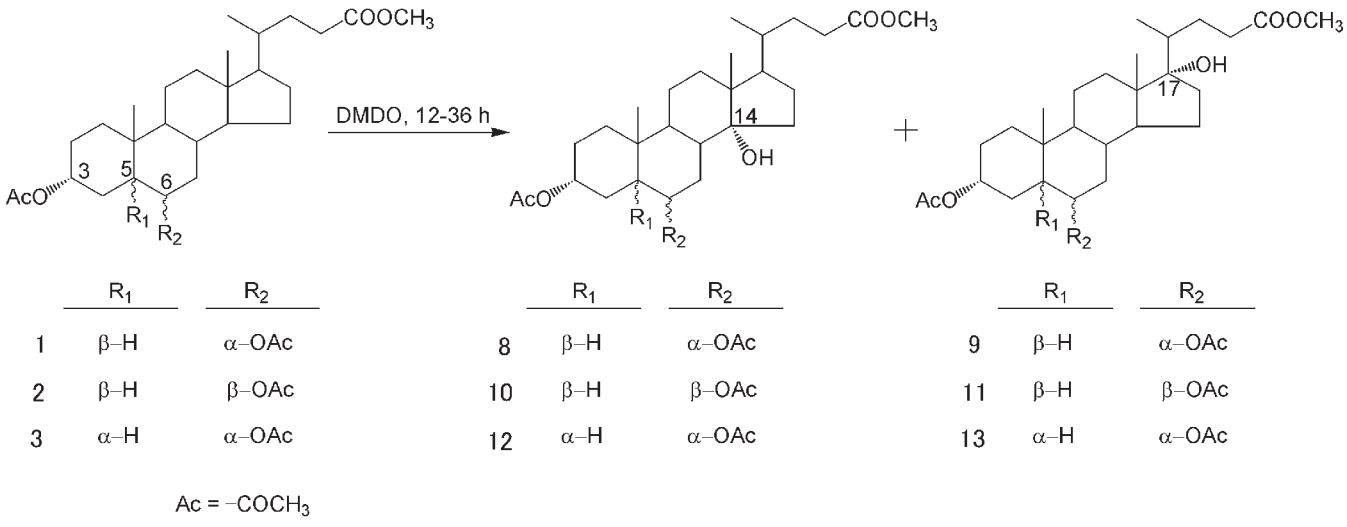
Essentially identical results were obtained when the three variants of methyl 3α,6-diacetoxycholanoates (**1**–**3**) were subjected to the DMDO reaction, regardless of the differences in the stereochemistry of the A/B-ring junction (*cis* 5β-H or *trans* 5α-H) and of the acetoxyl group (axial or equatorial) at the C-3 and the C-6 positions. As shown in Scheme 1 and Table 1, the respective oxidation products of **1**–**3** with DMDO, which consisted of a mixture of two major components, were characterized as the corresponding 14α- and 17α-hydroxy compounds (**8**, **10**, **12**, and **9**, **11**, **13**, respectively), although the ratio of the resulting 14α- vs. 17α-ols depended on the stereochemical nature of the substrates: *ca*. 1:1 in **1**, 2:1 in **2**, and 1:2 in **3**. The structures of the *tert*-hydroxylated compounds, particularly for the position and stereochemical configuration of the hydroxyls, were exclusively determined on the basis of the ^{1}H and ^{13}C NMR (Table 2) and GC–MS data, in comparison with those of analogous hydroxy steroids (2,3).

TABLE 1
Oxidation Products of 3,6- and 3,24-Dihydroxycholane Derivatives with DMDO

Substrate	Reaction time (h)	Starting compound recovered (%)	Products (yield, %)[a]
1	36	(43)	**8** (13), **9** (13)
2	12	(47)	**10** (24), **11** (15)
3	36	(53)	**12** (9), **13** (21)
4	36	(17)	**14** (30), **15** (17), **16** (13)
5	12	(20)	**17** (40), **18** (10), **19** (26)
6	36	(0)	**20** (27), **21** (30), **22** (16), **23** (25)
7	36	(7)	**17** (17), **20** (20), **21** (35), **23** (22)

[a]Determined by capillary GC; for conditions, see Experimental Procedures section. DMDO, dimethyldioxirane. For chemical structures of compounds in this table see Schemes 1 and 2.

The result for the DMDO oxidation of **1** and **2** is very similar to that reported previously for 5α-steroids (15), but much differs from that observed for 5β-steroids (10,16): Expected 5β-hydroxylation did not occur at all under the experimental conditions used. Since 5β-hydroxylation is usual for 5β-steroids by DMDO oxidation, the above finding suggests that the 6α- and 6β-acetoxyl groups in **1** and **2**, both of which have gauche conformation with respect to the adjacent 5β-H, com-



SCHEME 1

TABLE 2
^{13}C NMR Chemical Shifts for Oxyfunctionalization Products

Carbon no.	8	9	10	11	12	13	14	15	16	17[c]	18	19	20	21	22
1	35.1[a]	35.0	35.2	35.0	27.2	27.2[a]	29.3	29.3	29.4	30.7	29.9	35.0	29.9	37.1	35.6
2	26.1	26.1	26.1	26.0	25.6	25.6	26.1	26.0	26.0	29.2[a]	28.1	30.7	28.6	36.9	33.9
3	73.6	73.5	74.9	74.4	68.9	68.9	71.3	71.3	71.3	68.4	67.7	71.8	68.0	213.5	199.6
4	26.5	32.4	31.1	32.4	32.9	32.9	38.1	37.9[a]	37.9	42.5	41.8	36.3	41.8	42.3	123.7
5	45.4	45.3	44.9	44.8	46.0	44.0	75.3	75.2	75.0	76.2	75.4	41.9	75.7	44.2	171.5
6	71.0	70.8	73.2	73.0	72.7	72.6	36.8	36.7	36.4	37.1	36.7	26.8	36.9	25.7	32.9
7	26.3	26.3	26.6	26.0	31.8	37.5	28.6	28.5	23.2	30.7	29.6	24.1	29.7	26.5	31.9
8	37.6	34.8	34.4	31.4	36.8	34.2	34.9	35.0	37.8	36.2	35.1	35.2	34.9	35.4	35.5
9	32.2	39.1	32.8	39.0	43.6	53.0	43.1	42.7	35.1[a]	44.5	39.5	40.1	43.1	40.6	53.7
10	35.9	35.9	35.2	34.0	36.7	36.7	39.6	39.5	39.6	40.6	39.6	34.6	39.6	34.8	38.5
11	19.6	20.4	19.4	20.2	19.6	20.4	21.0	20.8	20.2	22.1	20.8	21.7	21.0	21.1	20.9
12	32.1	32.1	32.1	32.0	32.5[a]	32.2[b]	39.8	32.4	32.0	41.2	32.3	42.5	39.8	40.0	39.5
13	46.7	47.8	46.7	47.5	46.6	47.6	42.4	47.4	46.4	43.5	47.3	47.1	42.4	42.7	42.4
14	85.3	50.6	85.3	50.5	85.3	50.6	56.5	50.9	85.4	57.9	50.8	155.4	56.4	56.3	55.8
15	32.9[a]	23.3	32.8	23.3	32.6[a]	23.3	24.1	23.4	32.8	25.2	23.4	116.8	24.1	24.1	24.1
16	26.9	38.0	26.9	37.8	26.8	37.8	28.1	38.0[a]	26.9	29.7[a]	37.9	35.5	28.0	28.1	28.0
17	50.6	86.1	50.7	86.0	50.6	86.1	55.9	86.2	50.6	57.4	86.1	58.7	55.7	55.9	55.7
18	15.7	14.4	15.7	14.5	15.6	14.4	11.9	13.7	15.6	12.3	14.3	16.8	11.9	12.0	11.9
19	22.9	23.2	24.6	24.7	12.2	12.4	16.2	16.2	15.8	16.9	16.2	23.0	16.2	22.6	17.3
20	35.1	39.4	34.4	39.8	35.1	39.1	35.3	39.4	35.5[a]	36.8	42.6	33.7	35.3	35.3	35.2
21	18.1	13.5	18.1	13.4	18.1	13.5	18.4	14.4	18.3	19.1	13.8	18.8	18.2	18.2	18.2
22	31.0	27.4	31.4	27.3	31.0	27.4[a]	25.1	26.8	25.3	30.1	28.5	29.4	30.9	30.9	30.9
23	31.0	31.2	31.4	31.3	31.0	32.1[b]	31.8	28.3	31.8	33.0	31.0	31.7	31.0	30.9	31.0
24	174.6	174.3	174.6	174.3	174.6	174.4	65.0	64.7	64.9	63.5	63.0	63.6	174.7	174.7	174.6
$COO\underline{C}H_3$	51.5	51.5	51.5	51.3	51.4	51.5							51.4	51.4	51.4
$O\underline{C}OCH_3$	170.5	170.5	170.2	170.1	170.5	170.5	170.4	170.5	170.5						
	170.5	170.5	170.4	170.3	170.9	170.9	171.2	171.1	171.1						
$OCO\underline{C}H_3$	21.3	21.3	21.3	21.2	21.2	21.2	21.0	20.9	20.9						
	21.4	21.4	21.6	21.4	21.5	21.5	21.4	21.4	21.3						

[a,b]Assignments down a vertical column may be interchanged.
[c]Measured in CD_3OD.

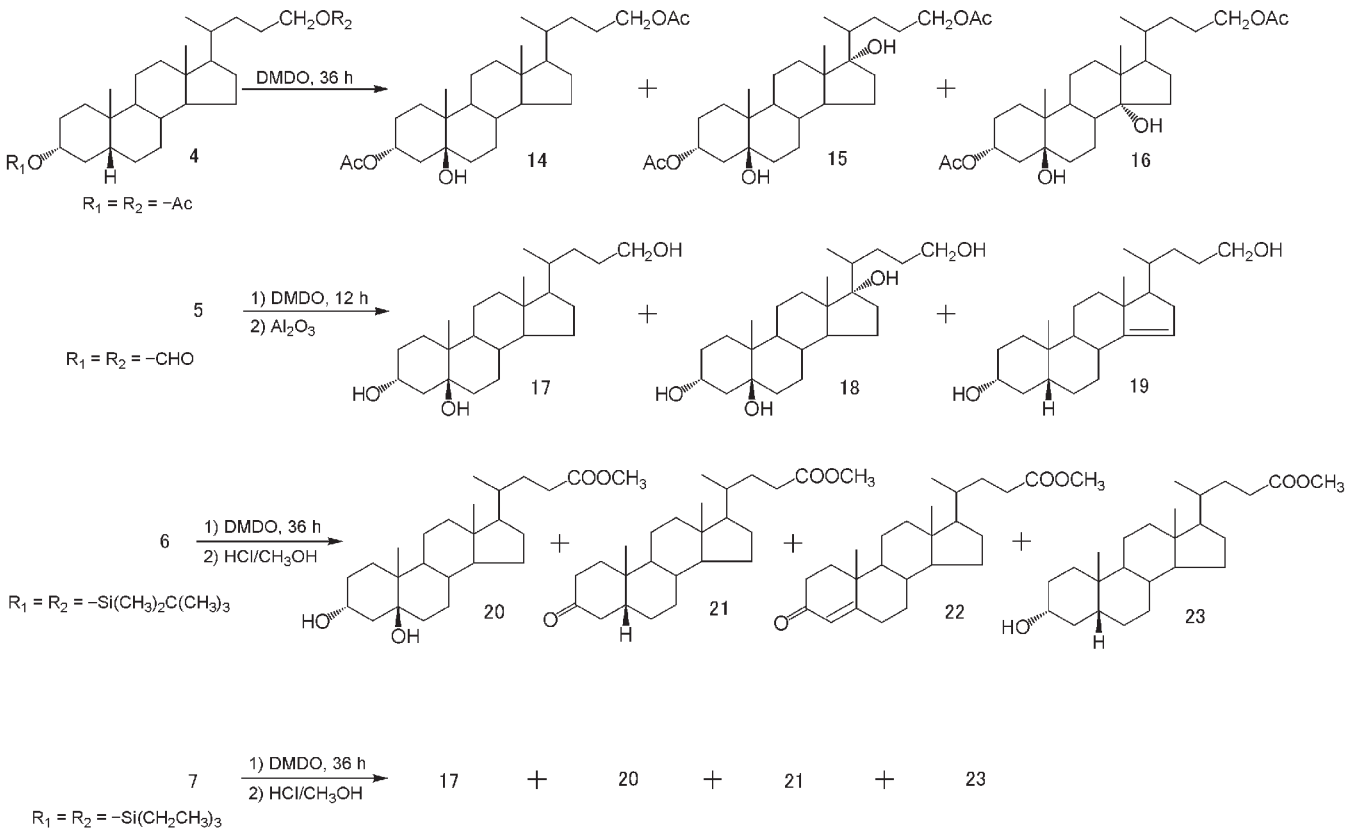
pletely shield the access of a DMDO molecule to the 5β-H by steric hindrance. As a result, oxidation of methine carbon protons at 14α-H and 17α-H, instead of 5β-H, in the 5β-steroids was accelerated efficiently, in analogy with 5α-steroids.

Meanwhile, oxidation of the ester or ether derivatives of 3α,24-dihydroxy-5β-cholanes (**4**–**7**) with DMDO was significantly influenced by the chemical nature of the hydroxy-protecting groups and produced a wide variety of oxidized products (Scheme 2). DMDO oxidation of 3α,24-diacetoxy-5β-cholane (**4**) gave a mixture of three oxyfunctionalization products, which were identified as the expected 5β-hydroxy compound (**14**, 30%) as the major component, along with the two minor double-oxyfunctionalized 5β,14α- and 5β,17α-dihydroxy compounds (**16**, 13%, and **15**, 17%, respectively).

A less bulky, analogous ester derivative, 3α,24-diformyloxy-5β-cholane (**5**), was then treated with DMDO. After DMDO oxidation of **5**, when the resulting product was poured onto a column of neutral alumina (activity II) and allowed to stand overnight, hydrolysis of the formyl groups took place (17). Elution with benzene/EtOAc (8:2–2:8, vol/vol) afforded the crude hydrolysis product, which in turn was purified by RP-MPLC on C_{18} bonded silica gel, eluting with methanol/water (9:1–6.5:3.5, vol/vol) to give the unprotected free 3α,5β,24-triol (**17**, 40%) and 3α,5β,17α,24-tetrol (**18**, 10%), along with an appreciable amount of 5β-chol-14-en-3α,24-diol (**19**, 26%). The unexpected **19** may be formed by elimination of the 14α-hydroxy group *via* a possible 3α,24-diformyloxy-14α-hydroxy intermediate during the hydrolytic step on a column of activated alumina.

On the other hand, when 3α,24-di(*tert*-butyldimethylsilyloxy)-5β-cholane (**6**) was subjected to DMDO oxidation, followed by the cleavage of the TBDMS ether linkage of the oxidation product with conc. HCl, four components were isolated after chromatographic separation. Those were characterized as methyl 3α,5β-dihydroxycholanoate (**20**, 27%), methyl 3-oxo-5β-cholanoate (**21**, 30%), methyl 3-oxo-4-cholenoate (**22**, 16%), and methyl 3α-hydroxy-5β-cholanoate (methyl lithocholate; **23**, 25%). Our previous paper pointed out that the TBDMS ether derivative of bile acids is stable within a few hours on exposure to a DMDO solution (18). However, the formation of an appreciable amount of **21**–**23** may imply that by prolonged reaction, partial (at C-3) and/or complete (at C-24) elimination of the TBDMS ether linkage gradually occurs prior to remote functionalization of unactivated carbons in the substrate to give the methyl ester of 3-oxo and 3-hydroxy derivatives. In addition, the route to the enone **22** probably involves dehydration of the 5β-hydroxy group, *via* intermediary methyl 3-oxo-5β-hydroxycholanoate (16), by conc. HCl treatment, in analogy with **19** mentioned above.

A similar oxidation was also observed for the DMDO



SCHEME 2

reaction of sterically less hindered 3α,24-di(triethylsilyloxy)-5β-cholane (**7**), which, on treatment with DMDO and subsequent cleavage of the TES ether linkage with conc. HCl, yielded **17**, **20**, **21**, and **23**. The formation of appreciable amounts of **20**–**23** apparently suggests that silyloxy groups as hydroxyl-protecting groups, particularly at the C-24 position, are prone to elimination by DMDO treatment, compared with the acyl groups.

Thus, oxidation of the ester and ether derivatives (**4**–**7**) of 3α,24-dihydroxy-5β-cholane with DMDO afforded a number of unique 5β-, 14α-, and 17α-*tert*-hydroxy compounds and/or their dehydrated analogs, along with C-3 and C-24 oxidized products, depending on the chemical nature of the hydroxy-protecting groups in the substrates. The results strongly suggest that the site-selectivity of the DMDO oxyfunctionalization is significantly influenced by the presence of an electron-withdrawing group (acetyl and formyl) and/or of an electron-donating group (TBDMS and TES), as well as their bulkiness and stability.

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An *in vitro* Method for Studying the Proliferation and Differentiation of Atlantic Salmon Preadipocytes

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ABSTRACT: The aim of the present study was to develop a cell culture system for studying the proliferation and differentiation of preadipocytes isolated from Atlantic salmon adipose tissue. The expression of proliferating cell nuclear antigen (PCNA) was used as a marker for cell proliferation. The cells started to proliferate within 48 h after seeding and continued to proliferate throughout the culture period of 2 wk. Undifferentiated preadipocytes showed a fibroblast-like morphology with a homogeneous cytoplasm devoid of lipid droplets. At confluence, an exogenous lipid mixture was added to the cell cultures. The preadipocytes became larger and rounder during the subsequent days, and the cytoplasm gradually filled with lipid-rich droplets. These droplets were revealed by oil red O staining. Immunocytochemical staining showed that differentiated adipocytes expressed detectable levels of the three regulatory proteins associated with adipocyte differentiation: peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding protein α (C/EBPα), and leptin. The cells also showed activity of glycerol-3-phosphate dehydrogenase (GPDH) (EC 1.1.1.8), a biochemical marker of adipocyte differentiation. The morphological and biochemical data presented here show that fish preadipocytes have properties that are similar to those of preadipocytes in mammals. We conclude therefore that salmon adipose tissue contains a sizable population of preadipocytes. Exogenous lipids promote the activation of adipose-related genes and induce the differentiation of fish preadipocytes *in vitro*.

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Fat levels in the diets of farmed salmon have increased significantly in recent years, and at present about 50% of dietary energy comes from fat. In spite of the high levels of fat in salmon diets, it is generally not well understood how various dietary FA influence the development and distribution of adipose tissue, lipid accumulation, and consequently salmon quality. White adipose tissue develops in a process that continues throughout life in which preadipocytes differentiate to adipocytes. However, the details of the molecular processes that control adipocyte differentiation in fish are not known. In mammals, on the other hand, knowledge about this process has improved significantly during the last decade. Cell culture studies of primary preadipocytes (1–4) and preadipocyte cell lines (5–8) have provided a great deal of information. An important advantage of primary cells, in comparison to cell lines, is that they more closely resemble preadipocytes that are present *in vivo,* since there have been no genomic modifications of these cells.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily of ligand-activated transcription factors. This receptor is a key regulator of adipocyte differentiation in mammals, and high levels are expressed in adipose tissue (9). We have previously shown that PPARγ m-RNA is expressed in Atlantic salmon liver and in adipose tissue, and that PPARγ is involved in the regulation of FA metabolism in the salmon liver (10). One of the aims of the present study was to investigate whether PPARγ also is directly involved in the regulation of adipocyte differentiation in Atlantic salmon, as shown for mammals. Mammalian studies have further shown that PPARγ cooperates with other families of transcription factors, including CCAAT/enhancer binding proteins (C/EBP), to regulate adipocyte differentiation. C/EBPα regulates terminal adipocyte differentiation by turning on fat-specific genes that are required for the synthesis, uptake, and storage of long-chain FA (reviewed in Ref. 11).

Leptin is an adipostatic circulating hormone that is secreted from adipocytes (12). Studies in mammals have demonstrated that this hormone regulates food intake and body weight by interacting with the hypothalamus to decrease food intake (reviewed in Ref. 13). Leptin also acts locally at the tissue site from which it originates to suppress adipocyte differentiation (5). Whether these adipogenic factors are expressed during salmon preadipocyte differentiation *in vitro* has not been previously studied.

The overall aim of the present study was to develop a cell culture system for studying the proliferation and differentiation of fish preadipocytes. A second aim of the study was to use this system to gain knowledge about the molecular events that control the growth of adipose tissue in Atlantic salmon.

EXPERIMENTAL PROCEDURES

Materials. Atlantic salmon (*Salmo salar*) fry were obtained from Aqua Gen (Sunndalsøra, Norway) and raised at a local

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Abbreviations: AEC, 3-amino-9-ethylcarbazole; AP, alkaline phosphatase; BCIP/NBT, 5-bromo-4-chloro-3 indolyl phosphate *p*-toluidine salt/nitro blue tetrazolium chloride; C/EBPα, CAAT enhancer binding protein α; GPDH, glycerol-3-phosphate dehydrogenase; HRP, horseradish peroxidase; PBS-T, PBS with 0.05% Tween; PCNA, proliferating cell nuclear antigen; PPARγ, peroxisome proliferator-activated receptor γ; PVDF, polyvinylidene difluoride.

aquarium at the Agricultural University of Norway. DMEM, FBS, antibiotics, HEPES, L-glutamine, lipid mixture [containing 4.5 g/L cholesterol, 10 g/L cod liver oil FA (methyl esters), 25 g/L polyoxyethylenesorbitan monooleate, and 2.0 g/L D-α-tocopherol acetate], HBSS, collagenase, laminin, Thermanox cover slips, oil red O, and metacain (MS-222) were all obtained from Sigma-Aldrich (St. Louis, MO). Tissue culture plasticware was obtained from NalgeNunc International (Naperville, IL). Paraformaldehyde and formalin were obtained from Electron Microscopy Sciences (Fort Washington, PA). Polyvinylidene difluoride (PVDF) transfer membranes were from Amersham Pharmacia Biotech (Buckinghamshire, England), Mini Trans-Blot apparatus from Bio-Rad (Hercules, CA) and secondary horseradish peroxidase (HRP)-conjugated antibodies used in Western blotting were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Goat anti-human leptin, normal goat IgG, normal rabbit IgG, blocking serum, HRP-3-amino-9-ethylcarbazole (AEC), and alkaline phosphatase (AP)-5-bromo-4-chloro-3 indolyl phosphate *p*-toluidine salt/nitro blue tetrazolium chloride (BCIP/NBT) immunodetection kits were provided by R&D Systems, Ltd. (Abingdon, United Kingdom). Rabbit anti-rat C/EBPα (14AA) and rabbit anti-human PPARγ (H-100) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA). All antibodies were polyclonal.

Proliferating cell nuclear antigen (PCNA) immunodetection kit, Histomount, Clearmount, and Mayer's hematoxylin were supplied by Zymed Laboratories Inc. (South San Francisco, CA). Cells in culture were observed using a Diaphot inverted light microscope (Nikon, Japan). A Leitz Laborlux S light microscope (Leica, Germany) was used to view all stained cells. A Leica DC100 camera integrated with the Laborlux S microscope was used to capture digitized images. All image acquisitions were controlled by Image Pro Plus 4.0 software from Media Cybernetics (Silver Spring, MD).

Cell culture conditions. Atlantic salmon were reared on a commercial diet to an average weight of 500 g. They were anesthetized with metacain. The arch bows of the gills were cut and after bleeding for a couple of minutes, the fish were killed by a blow to the head. The abdominal cavity was exposed and visible white adipose tissue surrounding the intestinal tract (visceral adipose tissue) was dissected from five or six fish and placed into sterile HBSS. The tissue was cut into small pieces and washed twice with HBSS. After centrifugation at 300 × *g* for 5 min, the tissue was suspended in 0.2% collagenase in HBSS (1 g tissue/5 mL). Collagenase digestion was allowed to proceed with gentle shaking for 1 h at 13°C.

The digested tissue was filtered through 250 and 100 μm nylon filters to remove large particulate material. The resulting cell suspension was then centrifuged at 700 × *g* for 10 min. The fat layer and adipocytes on top of the centrifuge tube and the digestion medium were removed by aspiration. After two washing steps, the sedimented cells were resuspended in growth medium (DMEM containing 10% FBS, 2 mM L-glutamine, 10 mM HEPES, and antibiotics). Cells were plated (day 0) at a density of approximately 1×10^4 cells/cm^2 in laminin-precoated 24-well plates equipped with Thermanox cover slips. The cells were incubated at 13°C with 3% CO_2. Most cells were attached on the next day, and they were extensively washed with medium. The cells were maintained in growth medium until reaching confluence, which occurred after 1 wk.

Confluent preadipocyte cultures were differentiated in a differentiation medium composed of growth medium supplemented with lipid mixture (10 μL/mL; corresponding to 45 μg/mL cholesterol, 100 μg/mL cod liver oil FA (methyl esters), 250 μg/mL polyoxyethylenesorbitan monooleate, and 20 μg/mL D-α-tocopherol acetate). Cells in differentiation medium were grown in parallel with cells in growth medium for 1 wk postconfluence. The media were changed every 2–3 d. Cultures for morphological studies (oil red O staining) and proliferation assessment (PCNA) were fixed every day throughout the culture period. Cultures for immunocytochemical studies were fixed on day 11. The cells were examined using light microscopy in order to determine whether they had differentiated, based on their morphology. Cells that contained lipid droplets that absorbed oil red O stain were considered to be in a differentiated stage. Cells filled with cytosolic lipid were considered to be fully differentiated.

Assessment of cell proliferation. Cell proliferation was assessed by the immunocytochemical detection of PCNA. Cells were washed in PBS and fixed in 70% ethanol for 30 min at 4°C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. The cells were washed three times in PBS, then incubated with a mouse anti-PCNA monoclonal antibody (clone PC10) using a PCNA immunodetection kit, following the manufacturer's instructions. The cells were counterstained with Mayer's hematoxylin for 2 min, washed in water, dehydrated in a graded series of alcohol solutions, cleared with xylene, and mounted with Histomount. PCNA-containing nuclei were stained dark brown. Two hundred cells were observed, and the percentage of proliferating cells was calculated.

Oil red O staining. Cells were stained with oil red O according to Ramírez-Zacarías *et al.* (14), to visualize accumulated TAG. Briefly, medium was aspirated from three culture wells and the cover slips were washed twice with PBS before fixation in cold 10% neutral buffered formalin for 30 min. The cells were rinsed in water and stained for 2 h with filtered oil red O in isopropanol, then exhaustively rinsed with water. The nuclei were counterstained with Mayer's hematoxylin for 2 min. After one further rinse in water, cover slips with cells were mounted on slides with one drop of Clearmount.

PAGE and Western blot analysis. After 4 d in differentiation medium, cells were washed in PBS, harvested in heated lysis buffer [2% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.06 M Tris-HCl, pH 6.8], and frozen at –80°C until analysis. Approximately 50 μg of denaturated protein was loaded to each well on a 10% SDS-polyacrylamide gel under nonreducing conditions. The gel electrophoresis was performed on ice at 100 V for about 2 h, and the polypeptides were transferred

onto moist PVDF transfer membranes by electroblotting. The membranes were prewetted in methanol and rinsed in distilled water before blotting overnight using a Mini Trans-Blot apparatus at 4°C. Protein blots were treated with blocking solution (PBS containing 5% dry milk and 0.1% Tween 20) for 2 h at room temperature to block nonspecific binding. The blots were then incubated with one of the primary antibodies (PPARγ, C/EBPα, leptin), diluted 1:500 in PBS with 0.05% Tween (PBS-T) for 4 h at 4°C. After rinse in PBS-T for 3 × 15 min, the blots were incubated with secondary HRP-conjugated antibodies (diluted 1:100,000 in PBS-T) for 2 h at 4°C. Binding was detected by autoradiography using Agfa X-ray film HC-SL and SuperSignal ULTRA.

Immunocytochemistry. Cells for immunocytochemical staining were washed in PBS and fixed in freshly prepared 4% paraformaldehyde in PBS, pH 7.4, for 30 min at 4°C. The cells were treated with 0.1% Triton X-100 in PBS for 15 min to ensure better immunoglobulin penetration. After rinsing in PBS, the cells were incubated with blocking serum and then incubated with primary antibody. PPARγ and C/EBPα antibodies were diluted 1:50 and leptin antibody 1:20 in dilution buffer (1.5% FBS in PBS-T). PPARγ and leptin were visualized using HRP-AEC (red color) detection kits and C/EBPα were visualized using an AP-BCIP/NBT (blue color) detection kit, following the manufacturer's instructions. Rabbit primary antibodies were replaced by normal rabbit IgG, and goat primary antibody was replaced by normal goat IgG in the negative controls. As a second negative control, no antibodies were used, only the dilution buffer.

Glycerol-3-phosphate dehydrogenase (*GPDH) activity and protein determination.* The activity of GPDH (EC 1.1.1.8) was used as a marker of adipocyte differentiation. This activity was determined as described by Wise and Green (15), with the exception that the assay was run at 12°C. Cells that had been cultivated for 4 d postconfluence either in growth medium or in differentiation medium were harvested in PBS and sonicated. The cells were centrifuged at 300 × *g* for 10 min, and then the pellet was frozen until it was analyzed. Protein concentration was determined by the method of Lowry *et al*. (16).

RESULTS

Proliferation. Proliferation and differentiation were studied in preadipocytes grown in primary culture over a period of 2 wk. After 24 h, the isolated preadipocytes were small and PCNA-negative (Fig. 1A). PCNA staining, showing that proliferative activity had started, was detected 48 h after seeding. Growth continued during the subsequent days with the cells becoming more elongated and developing connections with neighboring cells. Approximately 15% of the nuclei were labeled with PCNA after 3 d (Fig. 1B). After 5 d in culture the cells exhibited a very extended cytoplasm, and approximately 40% of them showed proliferative activity (Fig. 1C). The cells proliferated at an increasing rate until reaching confluence after 1 wk. Approximately 50% of the cells showed proliferative activity at this stage. Cells continued to proliferate after confluence, in the presence of both growth medium and differentiation medium, and the cell density continued to increase. We observed a high replicative potential throughout the 2-wk culture period. After 4 d in differentiation medium (day 11), most cells were PCNA-positive and contained large lipid inclusions (Fig. 1D).

Preadipocyte morphology and differentiation. The morphology of undifferentiated preadipocytes was similar to that of fibroblasts, with a cytoplasm devoid of lipid droplets. We observed none or occasionally a few very tiny lipid inclusions in the cytoplasm of preadipocytes prior to confluence (Fig. 2A). At confluence, the cells showed an extensive and a relatively homogeneous cytoplasm, devoid of lipid droplets (Fig. 2B). The cell cultures were supplemented with a differentiation medium containing lipid mixture at this stage in order to test the ability of the cells to differentiate. The cells responded rapidly. Preadipocytes showed many small lipid droplets in the cytoplasm 24 h after replacing the growth medium with differentiation medium. More lipid droplets with different sizes appeared continuously in the cytoplasm of cells treated with differentiation medium during the subsequent days. The lipid droplets were easily detectable in the microscope as light-refringent spheres in the cytoplasm (Fig. 1D). The lipid nature of these inclusions was confirmed by oil red O staining (Fig. 2D, 2E). After 3 d in differentiation medium, cells contained many large lipid droplets. The phenotype of almost all cells was that of differentiated adipocytes at this stage (Fig. 2D). In comparison, cells incubated in growth medium in the same period showed almost no differentiation (Fig. 2C). However, the degree of differentiation reached by the preadipocytes was heterogeneous; some cells possessed only small lipid droplets, and we believe that these cells differentiate more slowly. Other cells were almost filled with cytosolic lipid (Fig. 2E). Cells in differentiation medium gradually became more spherical, and the lipid vacuoles became larger. This latter effect was probably due to the fusion of small droplets. Continuous exposure of the cells to differentiation medium for 1 wk postconfluence resulted in a remarkable increase in the number of round cells with a cytoplasm that was almost completely filled with lipid. We considered these cells to be fully differentiated. The cells easily became detached from the culture support and floated in the medium. In contrast, no (or a few small) intracellular lipid droplets appeared in preadipocytes maintained in growth medium for 1 wk of culture after confluence.

Expression of adipogenic transcription factors and leptin. We performed immunocytochemical studies on salmon adipocytes differentiated *in vitro* in order to examine the expression of PPARγ, C/EBPα, and leptin. Immunoreactivity for both transcription factors was detected in cells after they had been incubated in differentiation medium for 4 d. An intense immunoreaction for PPARγ (Fig. 3A) and C/EBPα (Fig. 3C) was detected in the nuclei. Adipocytes observed 4 d postconfluence typically showed a positive immunoreaction for leptin that was intense in a concentrated perinuclear region of the cytoplasm

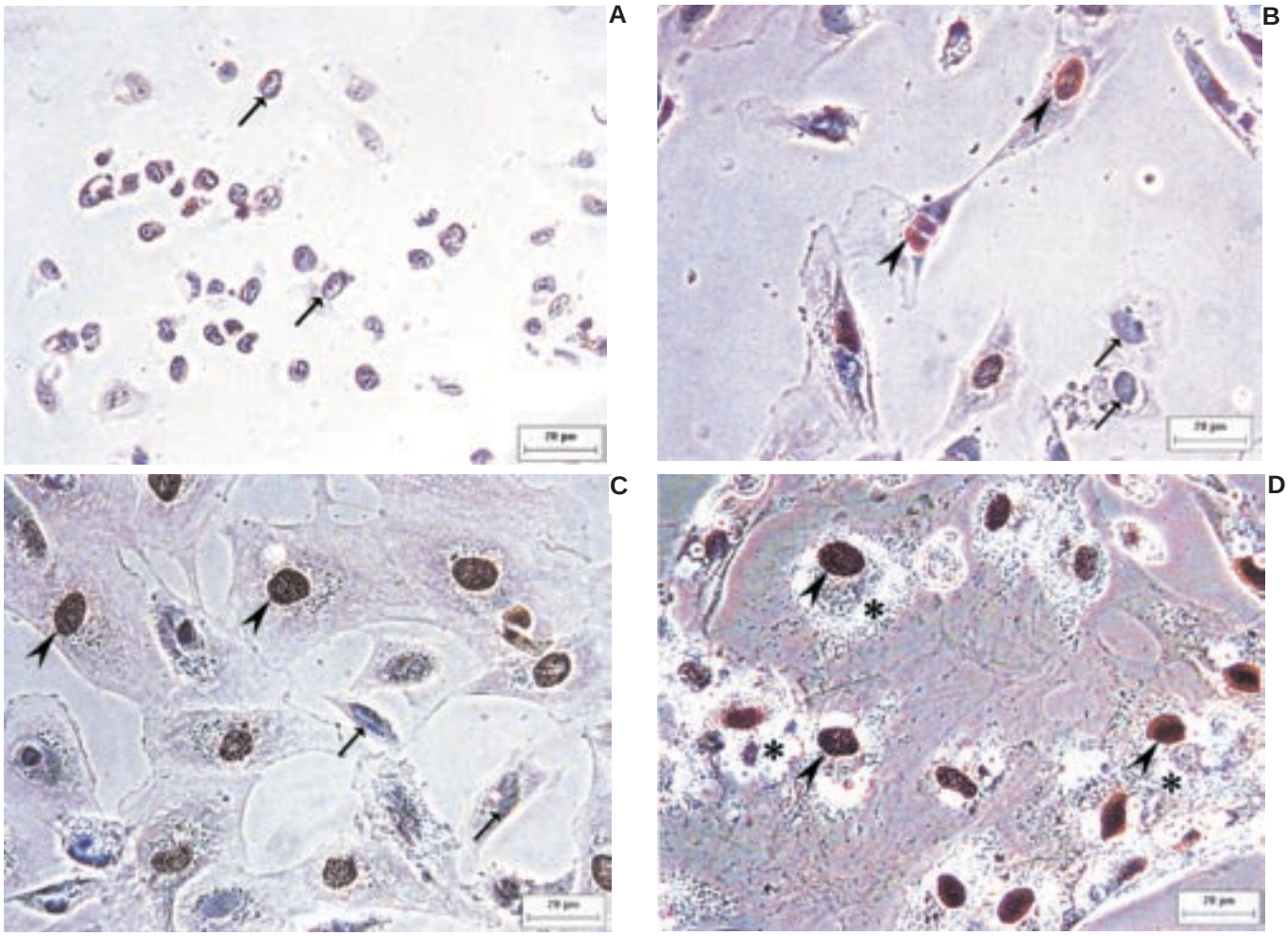

FIG. 1. Light micrographs of salmon preadipocytes and adipocytes in primary culture. Cell proliferation was assessed by immunocytochemical detection of proliferating cell nuclear antigen (PCNA). PCNA-containing nuclei are stained dark brown (arrowheads). Counterstaining with Mayer's hematoxylin gives PCNA-negative nuclei a blue color (arrows). Twenty-four hours after seeding, the cells were small and round and did not show PCNA-staining (A). Three days after seeding, approximately 15% of the cells were PCNA-positive (B). Five days after seeding, approximately 40% of the cells were PCNA-positive (C). The preadipocytes reached confluence after 7 d in culture. At this stage the preadipocytes were incubated in differentiation medium containing FBS and a lipid mixture. On day 11 most cells were PCNA-positive (D), and the cells showed a differentiated phenotype with large, light-refringent lipid inclusions surrounding the nuclei (*). Bars = 20 μm.

(Fig. 3E). Occasionally we observed cells with a more diffuse leptin staining distributed throughout the cytoplasm. Controls for PPARγ (Fig. 3B), C/EBPα (Fig. 3D), and leptin (Fig. 3F) showed little or no staining. Each of the three antibodies labeled one specific protein band on the Western blots (Fig. 4). The M.W. of these bands were approximately 47, 43, and 15 kDa for PPARγ, C/EBPα, and leptin, respectively.

GPDH activity. Cells exposed to differentiation medium at confluence showed a GPDH activity of 6.5 nmol min^{-1} mg^{-1} protein 4 d later. Preadipocytes cultivated in growth medium showed no GPDH activity.

DISCUSSION

We have shown for the first time in a fish species that preadipocytes can be isolated from adipose tissue and be caused to differentiate to mature adipocytes in culture. Isolation of preadipocytes from the visceral region resulted in very clean cultures with a high proportion of cells differentiating to adipocytes when exposed to differentiation medium. Adipose tissues from different locations differ in their replicative and adipogenic potential (17), and we therefore routinely isolated preadipocytes from the visceral region.

There was a gradual increase in the proportion of proliferating cells until reaching confluence. The cells were exposed at confluence to a differentiation medium containing FBS and a lipid mixture. This medium promoted the development of an adipocyte phenotype and permitted continued proliferative activity. The cells also possessed a high mitotic capacity after reaching the adipocyte phenotype, as evidenced by an accumulation of lipid droplets and the positive immunostaining of PCNA, PPARγ, C/EBPα, and leptin. A study on 3T3-L1 cells showed that cell cycle arrest is as important as the expression of adipogenic genes (PPARγ and C/EBPα) in promoting cell

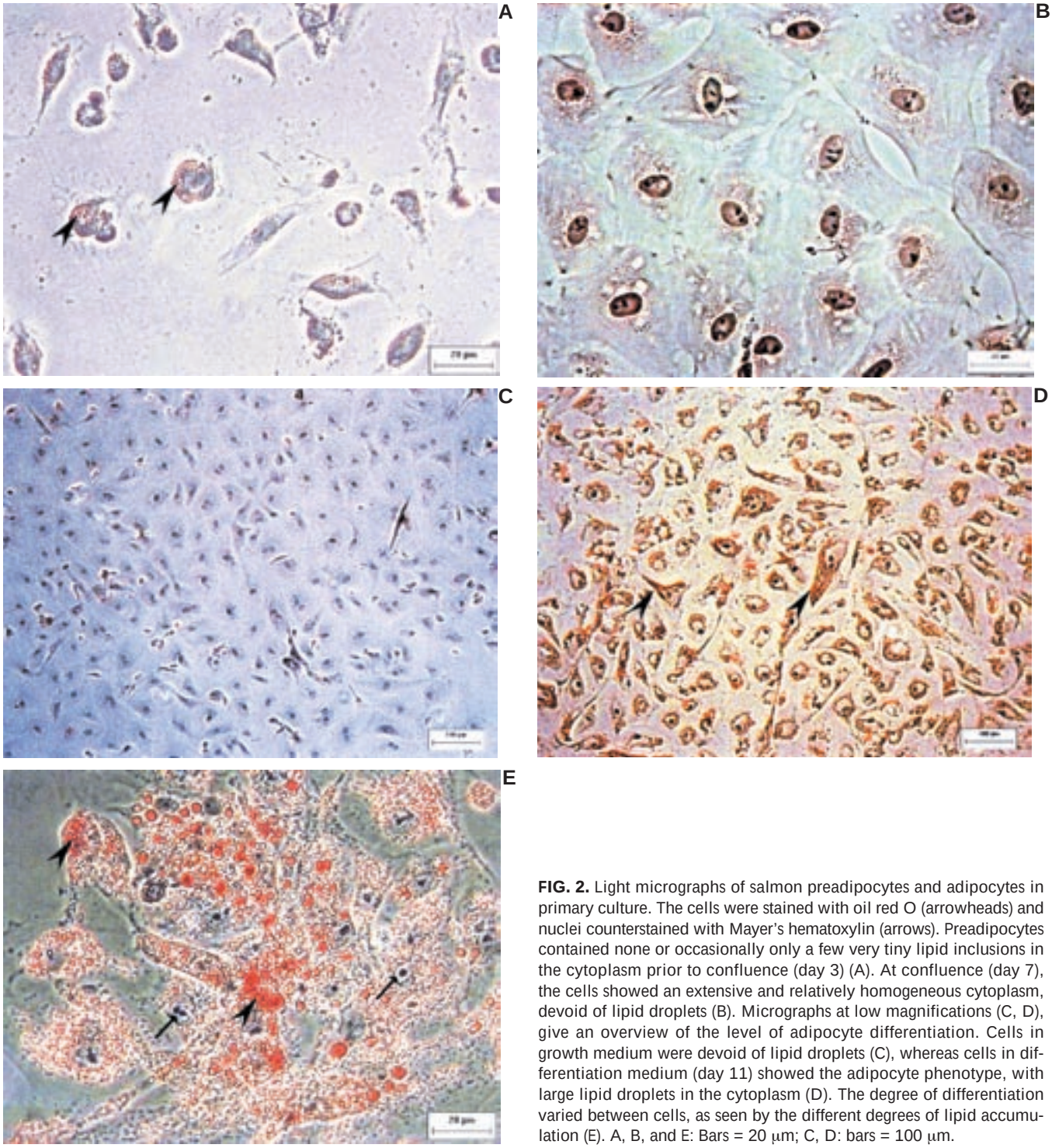

FIG. 2. Light micrographs of salmon preadipocytes and adipocytes in primary culture. The cells were stained with oil red O (arrowheads) and nuclei counterstained with Mayer's hematoxylin (arrows). Preadipocytes contained none or occasionally only a few very tiny lipid inclusions in the cytoplasm prior to confluence (day 3) (A). At confluence (day 7), the cells showed an extensive and relatively homogeneous cytoplasm, devoid of lipid droplets (B). Micrographs at low magnifications (C, D), give an overview of the level of adipocyte differentiation. Cells in growth medium were devoid of lipid droplets (C), whereas cells in differentiation medium (day 11) showed the adipocyte phenotype, with large lipid droplets in the cytoplasm (D). The degree of differentiation varied between cells, as seen by the different degrees of lipid accumulation (E). A, B, and E: Bars = 20 μm; C, D: bars = 100 μm.

differentiation (6). C/EBPα has a role in growth-arrest, and this transcription factor is expressed relatively late during adipogenesis (reviewed in Ref. 11). We did not see any evidence that C/EBPα has an antimitogenic nature in salmon preadipocytes.

We have shown that salmon preadipocytes proliferate and differentiate in the presence of FBS. Serum has a strong growth-promoting activity, and it contains many other poorly defined factors that may positively and/or negatively interfere with differentiation (18). However, the presence of serum often results in a decreased frequency of adipocyte differentiation (19). Incubation with medium that contains FBS does not inhibit the differentiation of salmon preadipocytes in culture. One of the future challenges will be to develop culture conditions without serum supplementation in order to study the differentiation process under fully defined conditions. Differentiation medium

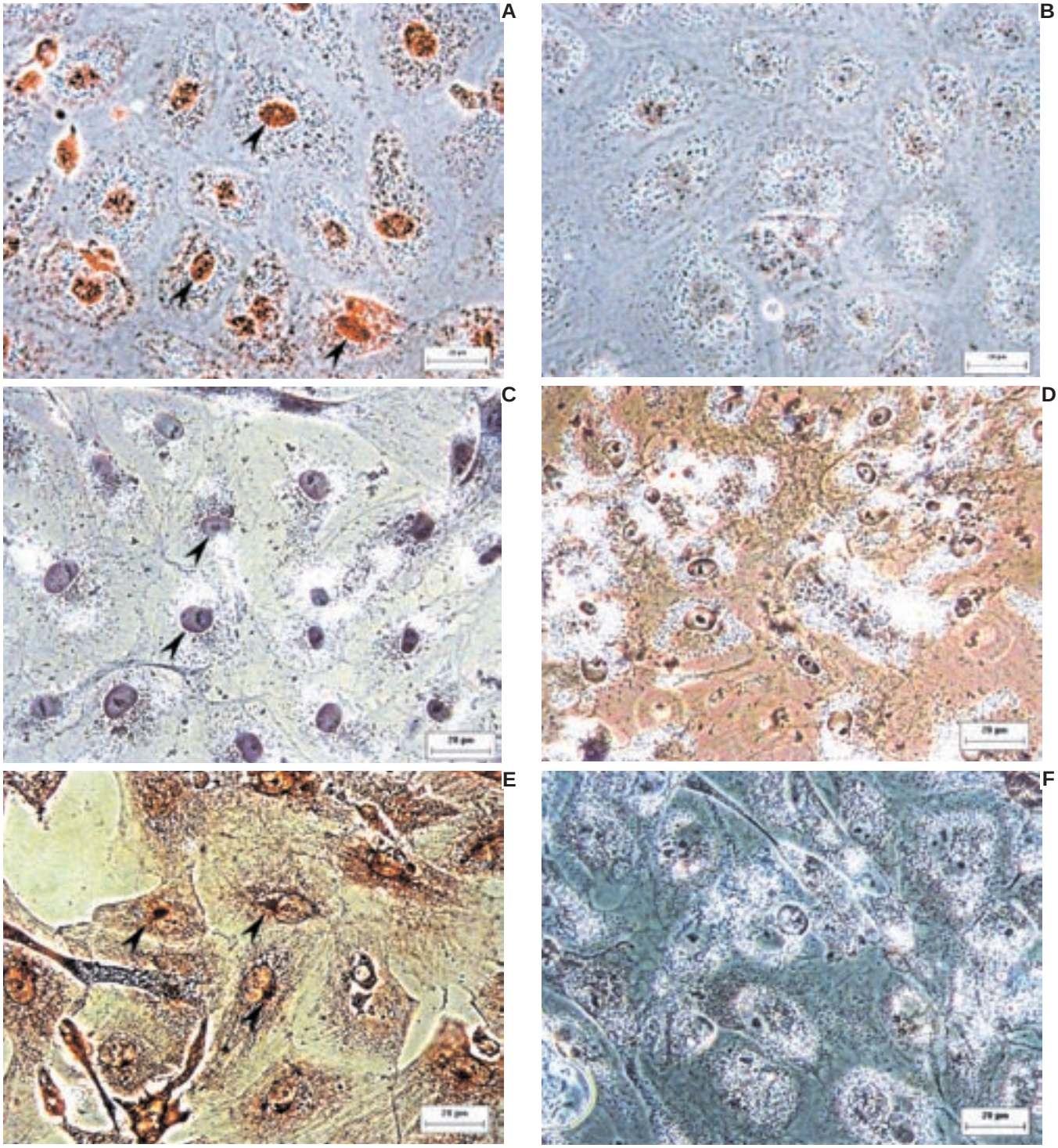

FIG. 3. Light micrographs showing immunocytochemistry of differentiated salmon adipocytes in primary culture. Cells were incubated in differentiation medium from confluence until day 11 and then immunostained (arrowheads) for peroxisome proliferator-activated receptor γ (PPARγ) (A), CCAAT enhancer binding protein α (C/EBPα) (C), and leptin (E). PPARγ (red) and C/EBPα (blue) were expressed in the nuclei. Leptin reactivity (red) was typically strong in a concentrated spot of cytoplasm, close to the nucleus. Negative controls for PPARγ (B), C/EBPα (D), and leptin (F) were without immunostaining. Bars = 20 μm.

containing lipid mixture promoted a rapid and extensive differentiation of salmon preadipocytes. The first evidence of the adipogenic process was the accumulation of lipid droplets. One or more components of the lipid mixture act as an adipogenic factor, and the mixture also provides cholesterol and lipids for the fat droplets that are increasing in number and size.

Previous studies on Ob 1771 cells (7) and C2C12 myoblasts (20) have shown that FFA have an adipogenic effect

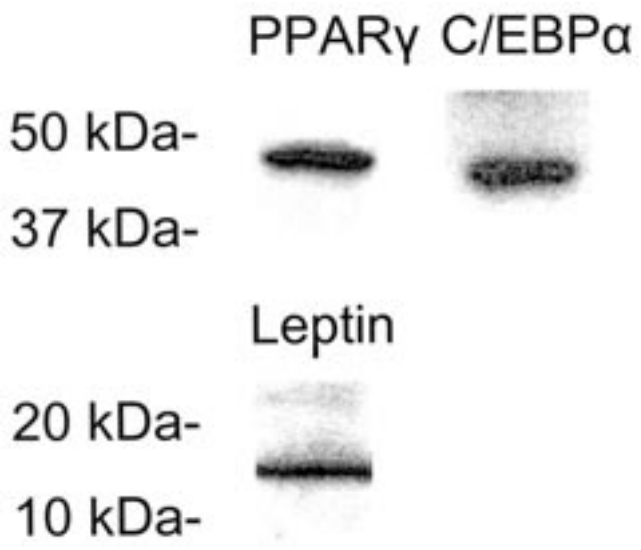

FIG. 4. Western blots demonstrating a single protein band present for each antibody tested. For abbreviations see Figure 3

on the differentiation. Several *in vitro* studies on mammalian adipocytes have shown that PUFA can activate PPARγ (21). Following the activation, PPARγ recognizes and binds to specific responsive elements in the promoter regions of genes that encode enzymes and other proteins involved in lipid metabolism, thereby regulating their activities. We performed immunocytochemical labeling of salmon adipocytes differentiated *in vitro* in order to study the expression of PPARγ, C/EBPα, and leptin. We found that PPARγ was expressed in the nuclei. The role of PPARγ in the initiation of differentiation is well documented in mammals. PPARγ is expressed early in the differentiation of preadipocytes and it is absolutely required for adipogenesis (8).

Studies on mammals have shown that PPARγ cooperates with C/EBPα to regulate adipocyte differentiation. We found that C/EBPα was expressed in salmon adipocytes, indicating a possible adipogenic function of this factor in fish. C/EBPα has been found to be expressed in tissues with high lipogenic capacity, including adipose tissue, liver, intestine, and lung in mammals (22). Transgenic mice unable to express this transcription factor lack adipose tissue (23). Although expression of C/EBPα is required for adipocyte differentiation, it is not sufficient to induce differentiation. Several studies have demonstrated that PPARγ is required for preadipocyte differentiation *in vivo* and *in vitro* in addition to members of the C/EBP family (24).

We here describe for the first time in a fish species the immunolocalization of leptin in adipocytes differentiated *in vitro*. The staining was most intense in a small area close to the nucleus, which we assume to be the Golgi region. This agrees with the observation that newly synthesized leptin is rapidly secreted from the cell (25). Johnson *et al*. (26) presented the first evidence that leptin is expressed in fish. They found leptin in blood, brain, heart, and liver. Cultured human adipocytes produce and release high levels of leptin (3). Leptin in differentiating human adipocytes is mainly attached to cellular membranes and in small vesicle-like structures in the cytoplasm. This led Bornstein *et al*. (4) to suggest that leptin is secreted by a regulated exocytotic mechanism.

GPDH often has been used as a sensitive marker for determining the extent of adipocyte differentiation. We detected GPDH activity in differentiated lipid-containing cells after incubation with lipid mixture for 4 d postconfluence. We did not observe any GPDH activity at an equivalent stage in the growth of undifferentiated cells cultured in growth medium.

In summary, we have developed a method for studying the proliferation and differentiation of fish preadipocytes in culture. The method is a useful new tool for studying fish adipocyte development and metabolism *in vitro*. We have shown that salmon adipose tissue contains a sizable population of preadipocytes that are prone to differentiate into mature adipocytes when exposed to a differentiation medium supplemented with lipids. The level of GPDH, an enzymatic marker of adipogenesis, increased during the differentiation process. Leptin and the adipogenic transcription factors PPARγ and C/EBPα were expressed in differentiated adipocytes. Our results show that fish preadipocytes have properties that are similar to preadipocytes in mammals.

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A History of Fats and Oils in Canada

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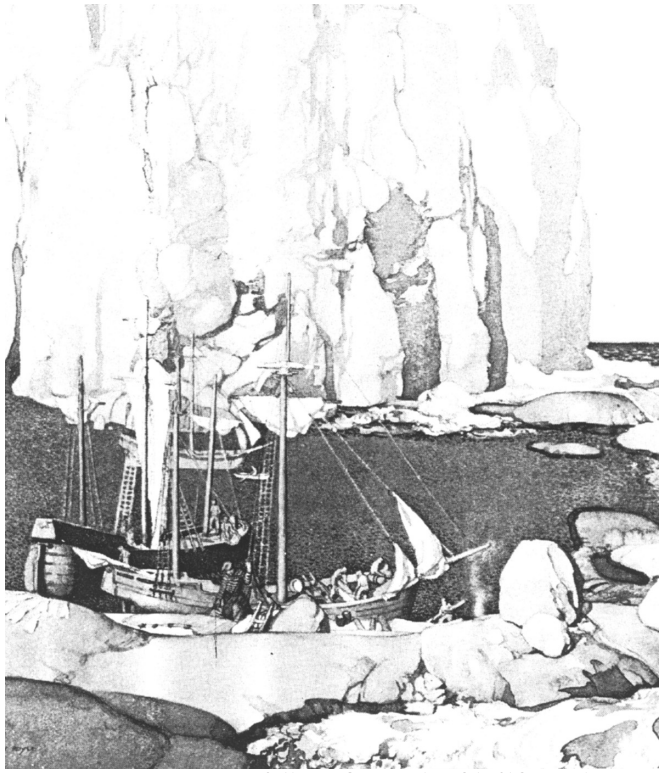
The first "fats" industry in Canada was for marine oils. Before Christopher Columbus crossed the Atlantic Ocean, Basque whalers had established themselves seasonally at Red Bay in Canada. Located above the narrow extension of northern Newfoundland on the southern shore of Labrador, this activity was unknown until two decades ago when archaeologists discovered papers such as insurance records in San Sebastian that suggested where these hardy European mariners might have gone. The location is in fact a natural funnel and ideal as a place in which to capture and process migrating whales for blubber. Current technology in Europe did not then permit large-scale production of fish oils, but whale oil rendering was a simple process that lasted unchanged into the 19th century.

Commercially valuable secrets leak out eventually, and a little later, legitimate European explorers such as John Cabot (1488) and Jacques Cartier (1543) followed the Basques and commented on the remarkable stocks of codfish off Newfoundland. International fishing fleets soon followed. Figure 1 is an imaginative view of the seasonal activity of visiting Europeans of many nations in Labrador and Newfoundland in the 16th century. At first glance, the row of objects on the rocks at the lower left are large enough to be mistaken for sealskins. I am confident that they are large cod fillets, salted and laid out to dry. Even in the 1950s, it was not uncommon to see codfish a meter in length landed in Halifax, and selective fishing with large hooks, "jigging," could handle very large fish. Drying salt cod on wooden "flakes" was widespread in Atlantic Canada, and salt fish remained an export product of Newfoundland and Labrador into the 1970s. This surge of fishing activity, which was later referred to as "The Cod Rush," led to semipermanent and then permanent settlements in Newfoundland, and the development of the "cod oil" industry. Notice that the word "liver" is left out, even though the product came from the livers of codfish. These contained 30–50% oil. In small outports, they were tossed into a barrel on the end of the wharf, as far as possible removed from houses, and left for the sun and natural enzymes to break down the proteins. The oil could then be skimmed off and collected for larger centers to ship to Great Britain in wooden barrels. This was done, from experience, as deck cargo. If the oil still contained some protein, it settled along with water to the bottom as "foots." Bacteria then generated gases, and the barrel would sometimes explode in a very messy accident. This oil was liquid, cheap, and useful for paints at a time when large-scale vegetable oil production did not exist, except for olive oil. Cod oil had a high FFA content (5–25%). It could be sulfonated to make it emulsify better. By coincidence, it was used in a tannery where I had my first summer laboratory job. In one section of the plant, girls rubbed the fine-quality calfskins with this oil to make them soft and flexible; even today, in a fine leather goods store, I sniff appreciatively and nostalgically. To give you an idea of the later



STANLEY ROYLE, R.B.A., R.C.A., SACKVILLE, N.B., 1941

FIG. 1. A view of how the early temporary codfish processing sites for Europeans in Newfoundland and Labrador might have looked.

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(~1950) scale of the cod oil industry, small tankers carried 500 tonnes at a time from Newfoundland to the Boston area for the leather industry there.

High-quality cod liver oil for pharmaceutical (vitamin) use was eventually produced, and after completing my B.Sc., my first laboratory job was to measure the vitamins A and D in these oils. Sadly, both cod liver industries were wiped out a decade ago when the fishery for cod collapsed.

Before leaving the Atlantic coast, it is interesting to note that Newfoundland once had a law requiring that lighthouses burn seal oil. This oil was produced by a fishery conducted basically for seal skins, until this process was suppressed in large part to cater to urban activists in the United States with no knowledge of local Newfoundland economics or the appetite of seals for eating fish, especially the bellies of codfish containing the livers as the softest part of that fish.

It would be remiss not to mention that these salt-water activities described for Newfoundland were also actively taking place on the coasts of the three Maritime provinces of Canada, and of Québec. However the latter area had rich agricultural land along the St. Lawrence river, and today the Québec dairy industry produces a variety of very fine cheeses as a unique contribution to the edible fats and oils industry of Canada.

Surprisingly, centuries ago, fats made an important contribution to the explorations of western Canada conducted from Québec. Beyond the forests of Ontario, the French found the prairie "First Nations" people. Their contribution, aside from providing horses, guides, and wives, was based on "pemmican," an "iron ration" developed long ago for winter survival as well as travel. Lean meat was thoroughly dried, pulverized, and mixed with buffalo or other animal fats, often with dried berries mixed in as well if they were available. Buffalo (bison) meat was especially suitable. A few French place names linger in western Canada to mark where the early explorers established trading posts as far as the eastern side of the Rocky Mountains. After the British arrived in Canada, the fur trade continued; the hardy Scots continued this exploration, and curiously, fish oils helped point their way through the Rocky Mountains.

On the Pacific coast, other First Nations people discovered that the small, oily, but edible fish called the oolacon, or eulachon, were not only edible but useful in other ways. Fish could be tossed into a clay-lined hole in the ground and after a while an oil floated to the surface. The whole fish and the oil had two unique and distinct virtues. One was that, if properly dried, a whole fish could be stuck into a cleft stick, or the ground, and lit as a candle, hence the English name of candlefish applied by traders and settlers. Long before that, the First Nations of the plains discovered that the oil made a fine base for decorative body and facial paints, and so "grease trails" through the Rocky Mountains were developed to trade the oil to the interior, another exploitation of human vanity that led to explorations and trade as in many other historical societies. One of the famous Scottish explorers, Alexander MacKenzie, followed such a trail and when he saw the Pacific Ocean at the mouth of the Dean River, wrote on a large rock his name, followed by "from Canada, by land, 22nd July 1793. Latitude 52%20.48/N." This was the subject of a painting at one time reproduced in all Canadian schoolbooks until history was abolished in favor of social studies. Because it was a long way to carry paint from Europe or Québec, he is said to have painted it in a mix of "grease" (probably bear fat, often carried because it was thought to have medicinal properties) and local "vermillion," as the red pigment. In 1968 I discovered that the secret of the eulachon oil was that the fat contained ~14% squalene, explaining most of the distinctive properties of this fish and its oil.

The fabulous river runs of the Pacific salmon eventually led to canneries and oil production from fish waste. Herring were also plentiful and a source of marine oil, and eventually a small shark, the dogfish, supported a thriving vitamin A and D industry until synthetics displaced this liver oil source by the 1950s. Much of this was faithfully recorded by scientists of the Vancouver laboratory of the FRBC (Fisheries Research Board of Canada) in hardcover books, notably Bulletin 89 published in 1952. Their documentation of the composition and physical properties of Canadian marine oils is useful down to this day. Not far from Vancouver, Seattle had a comparable fisheries laboratory that benefited from Saltenstall-Kennedy funding in the early days of GLC and filled in many of the missing details for Pacific marine oils as recorded by M.E. Stansby and colleagues. I was fortunate enough to bring a knowledge of GLC from the United Kingdom to Canada in 1956 and introduced this technology to the Halifax laboratory of the FRBC to my great advantage, and that of Atlantic fish oils and lipids.

Historically, rapeseed or "colza" oil was not as popular for lamp use in Canada as in Europe. By 1870, France alone devoted 400,000 acres to growing of this "brassica" or "kale" for oil use, and at that time, India shipped 150,000 tonnes/annum of seed to Europe for this use. Ingenious lamps, for example, the "Argand" or the "student" lamps, fed the viscous vegetable oils by gravity or pressure to the wick. On the other hand, kerosene could be fed from below by capillary action in the wick, and the proximity of the early Pennsylvania oil fields to Canada facilitated its rapid adoption when introduced in the mid-1800s. It rapidly displaced candles and other oils in most lighting using fats. The self-sustaining farm base of the population of eastern Canada used lard, tallow, butter, and similar fats as lubricants, but eventually farm and household use of petroleum products spread, although many still used lard that came in 5-lb metal pails with handles that were very handy for subsequent uses.

Indirectly, the present Canada oilseed industry came about from the building of the Canadian Pacific Railway. By the 1870s, the 24,000 European and Canadian miners, lumberjacks, and settlers living in what is now British Columbia demanded a rail link to eastern Canada, and one of our most famous prime ministers, Sir John A. MacDonald, obliged. He promoted and floated, in public and private ways, including various exchanges of a lot of money, the development of this railway. Part of this deal gave the railway 25,000,000 acres of

undeveloped land along the right of way in the western prairie provinces. The railway advertised this free farmland in Europe, hoping to expand traffic and trade along its right of way. Very hard-working people from eastern Europe, in particular, emigrated to settle in western Canada, adding generously to our ethnic mixture of peoples.

By 1936, "Polish" or "turnip" rapeseed, *Brassica campestris*, was known to be grown privately in Saskatchewan and soon several oilseed varieties were being tested by the Canadian Department of Agriculture. Argentina supplied another national name applied to the variety *Brassica napus* introduced from there. World War II created a demand for rapeseed oil as a marine steam engine lubricant, and by 1943, oil production began in earnest, with the *B. napus* oil preferred for this use, after minor refining.

Remarkable advances in breeding, agronomy, and potential food use for the oil took place starting about 1950, and the NRC (National Research Council) Prairie Regional Laboratory became an active participant, along with regional universities, in the development of this oil for food use. Interestingly, free exchange with European scientists and input from a large commercial operation, Canada Packers Ltd., assisted in their projects, and Figure 2 is a roll of honor for some of the participants in this work, most of whom appeared at various times as members of, or meeting attendees at, the NRC "Canadian Committee on Fats and Oils." There, the free exchange of information prevailed. The use of rapeseed oil in liquid salad or frying oils, shortenings, and margarines was eventually (~1960) blessed, albeit feebly, by the Canadian Department of National Health and Welfare.

Rapseseed/Canola Oil Pioneers

J. Gordon Ross	H.J. Lips	K.K. Carroll
H.K. Sallans	A. Zuckerman	J. Beare-Rogers
B.M. Craig	J.M. Bell	J.A. Campbell
L.R. Wetter	J.B. O'Neil	B.R. Stefansson
C.G. Youngs	R.A. Burt	R.K. Downey
N.H. Grace	B.F. Teasdale	G. Rakow
J. Ward	J.R. Reynolds	D.R. Clandinen

FIG. 2. An honor roll of some of the many pioneers in the development of the rapeseed industry, later the canola industry, in Canada.

The same year saw the start of the triumph of Canadian rapeseed plant breeding. A low-erucic acid (22:1) plant gene that had been recognized elsewhere was transferred to the principal rapeseed varieties by 1964.

In 1970, questions about the safety of erucic acid consumption in human health arose and were aired in a celebrated international rapeseed meeting at St. Adèle, Québec. Luckily, new varieties of LEAR (low-erucic acid rapeseed) were already in development and could be rapidly multiplied by having them grown in Chile during the Canadian winter; by 1973, this crisis was under control. Eventually, the name "canola" became common for the genetically improved rapeseed oils. Western farmers finally had an attractive crop alternative to the traditional growing of wheat. The facility with which rapeseed oil FA could be manipulated genetically was

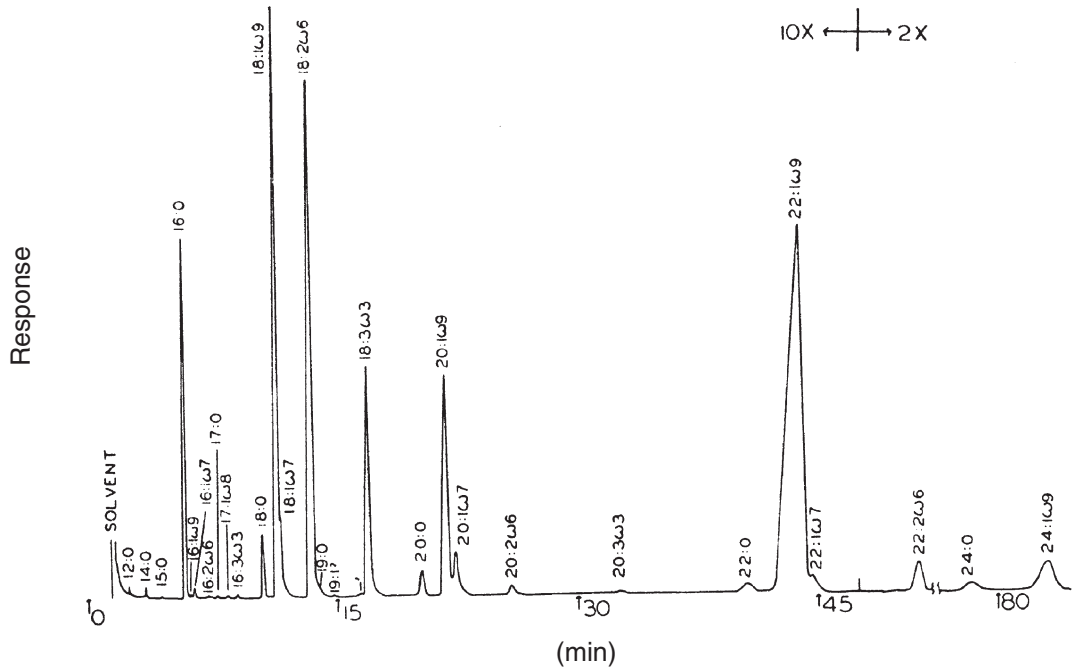

FIG. 3. A 1966 gas–liquid chromatogram of the methyl esters of rapeseed oil. Note the large 22:1ω9 peak and attenuations at the top. The BDS liquid phase was butanediol succinate, often called the Craig polyester. It was developed by B.M. Craig of the Prairie Regional Laboratory of the National Research Council of Canada. He also pioneered removal of the oil part of industrial seeds for FA analysis and then raising any promising varieties from the rest of the seed.

always recognized and is the subject of a recent review published from France (1); however, the definitive work done in Canada had been recorded earlier by G.S. Boulter (2).

My role in the development of canola was based principally on the use of open-tubular (capillary) GLC, and Figure 3 shows the original rapeseed oil GLC profile that I published in the *Journal of the American Oil Chemists' Society* in 1966, based on that technology. As an active member of the Canadian Committee on Fats and Oils, I was also eventually useful as an intermediary between scientists of the Canadian Department of Agriculture and scientists of the Department of National Health and Welfare. Ministerial rulings that they could not directly exchange scientific information on parallel research into the effects of erucic acid in animals did not include Halifax. Scheduling of hot-line calls to my laboratory soon kept those informed who most needed to know how animal feeding trials with LEAR oils were progressing in Ottawa laboratories actually only a few miles apart.

Not much could be done to enable continued food use of hydrogenated marine oils, many of which basically contained a 22:1n-11 isomer (cetoleic acid) and little 22:1n-9 erucic acid. In effect, use of these oils in margarine and shortening was eliminated when a 5% limit on total 22:1 content was introduced. Unlike U.S. menhaden oil, north Atlantic and Pacific fish oils have always contained 5–30% 22:1n-11. Curiously, although world production of industrial fish oils from inedible or underutilized species continues to be nearly 1.5 million tonnes per year, and although Canada is a major player in the salmon aquaculture industry and therefore a major user of fish oils, Canada now produces very little raw fish oil.

It has been my good fortune to be a participant in fats and oils research from the era when fish oils had little direct human use except as sources of vitamins A and D, to the revolutionary and advanced thinking that the term "essential fatty acids" could well mean only dihomo-γ-linolenic, arachidonic, eicosapentaenoic, and docosahexanoic acids, and not be used for linoleic and α-linolenic acids. I am happy to see that my early work in fish oils and their analysis, including n-3 FA, is now widely useful internationally and a Canadian contribution to the work of many scientists active in this still developing clinical nutritional field.

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The Potential Role for Arachidonic and Docosahexaenoic Acids in Protection Against Some Central Nervous System Injuries in Preterm Infants

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ABSTRACT: The risk of central nervous, visual, and auditory damage increases from 2/1000 live births in the normal birthweight to >200/1000 as birthweight falls below 1500 g. Such babies are most likely to be born preterm. Advances in infant care have led to increasing numbers of very-low-birthweight, preterm infants surviving to school age with moderate to severe brain damage. Steroids are one of the current treatments, but they cause significant, long-term problems. The evidence reported here suggests an additional approach to protecting the very preterm infant by supporting neurovascular membrane integrity. The complications of preterm, very-low-birthweight babies include bronchopulmonary dysplasia, retinopathy of prematurity, intraventricular hemorrhage, periventricular leukomalacia, and necrotizing enterocolitis, all of which have a vascular component. Arachidonic acid (AA) and DHA are essential, structural, and functional constituents of cell membranes. They are especially required for the growth and function of the brain and vascular systems, which are the primary biofocus of human fetal growth. Molecular dynamics and experimental evidence suggest that DHA could be the ligand for the retinoid X receptor (RXR) in neural tissue. RXR activation is an obligatory step in signaling to the nucleus and in the regulation of gene expression. Very preterm babies are born with minimal fat stores and suboptimal circulating levels of these nutrients. Postnatally, they lose the biomagnification of the proportions of AA and DHA by the placenta for the fetus. No current nutritional management repairs these deficits. The placental biomagnification profile highlights AA rather than DHA. The resultant fetal FA profile closely resembles that of the vascular endothelium and not the brain. Without this nourishment, cell membrane abnormalities would be predicted. We present a scientific rationale for a common pathogenic process in the complications of prematurity.

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Abbreviations: AA, arachidonic acid; ACh, acetyl choline; BPD, bronchopulmonary dysplasia; CI, confidence interval; CPG, choline phosphoglycerides; EPG, ethanolamine phosphoglycerides; IL, interleukin; IVH, intraventricular hemorrhage; LA, linoleic acid; MRI, magnetic resonance imaging; NEC, necrotizing enterocolitis; OR, odds ratio; RXR, retinoid X receptor; ROP, retinopathy of prematurity; PVL, periventricular leukomalacia.

Biomembranes are pivotal interfaces between different physicochemical phases of living systems. They also support the structural and functional aspects of cellular organization and function. One-third of all proteins known to date exist in biomembranes and depend for their function on the local domains in which they sit as well as the ability of the lipid bilayer of the membrane to optimize movement and separate two different aqueous phases. This separation enables ionic concentrations to exist with consequent electrochemical gradients across the membrane. These gradients are pivotal in signaling processes, which are particularly specialized in the brain. The lipids of the membrane are important determinants of the membrane's physical properties. These properties in turn, depend on the type of lipids involved; the major variables are the types of FA and the cholesterol/phosphoglyceride/sphingolipid balance. The polyenoic FA component of the membrane provides motional freedom and contributes to special properties of protein/lipid domains. They also provide signaling regulators of inflammation, vascular tone, adhesion, aggregation, and gene expression. Disturbance of membrane lipid composition results in functional abnormalities that carry many hallmarks of the vascular disorders associated with the complications of prematurity

CENTRAL NERVOUS SYSTEM MORBIDITY IN PRETERM INFANTS

Advances in obstetrics and pediatrics have led to increased survival from shorter gestational ages. Morbidity has not declined similarly (1–4), and increases with the degree of prematurity. Of the complications of prematurity, "Brain damage is the most feared consequence" (5). Although severe central nervous system damage occurs in 2 of 1000 live births with normal gestations, it rises to >200 of 1000 in babies born below 1500 g and 30 wk gestation (6). There is no need to detail the morbidity implications of very preterm delivery whose costs are disproportionately high because of the lifelong effect. However, in those very preterm infants who escape severe disorder, long-term follow-up reveals significant cognitive and visual impairments (7–9). Researchers have

studied the volumes of cortical subdivisions, ventricular system, cerebellum, basal ganglia, corpus callosum, amygdala, and hippocampus, derived from structural magnetic resonance imaging (MRI) scans. They compared preterm and term children and found correlations of regional brain volumes with cognitive measures (at age 8 yr) and perinatal variables among preterm children (10). Their data indicate that preterm birth is associated with regionally specific, long-term reductions in brain volume and that morphological abnormalities are, in turn, associated with poorer cognitive outcome.

RETINOPATHY

Detachment of the retina can lead to blindness unless diagnosed early and treated with cryotherapy. Although the retinal blood vessels are implicated in the pathogenesis of retinopathy of prematurity (ROP), it has recently been suggested that the photoreceptor rods are also involved (11). The lipid bilayers of the rods are made up of phosphoglycerides with >50% DHA. That the rods seem also to be involved could be coincidental. The coincidence could be related to a lack of the DHA-rich membrane lipids consistent with the same process in the retinal blood vessels. Postnatal depletion of these lipids in healthy preterm infants reduces rod function (12,13). It is not unusual for several of these complications to coexist in one individual. The need for increased oxygen to ensure neuronal survival often has to be balanced against the retinal toxicity it causes.

NECROTIZING ENTEROCOLITIS (NEC)

The protective effect of breast milk for NEC has been known for some time. Breast milk contains arachidonic acid (AA) and DHA but only one study has been done to test the possibility that AA and DHA might be protective as well. Many studies (14) have been done on AA and DHA in visual and cognitive development. It was found that infants fed formula with added AA and DHA developed significantly less stage II and III NEC than infants fed the control formula (2.9 vs. 17.6%, $P < 0.05$), but had similar rates of bronchopulmonary dysplasia (23.4 vs. 23.5%), septicemia (26 vs. 31%), and ROP (38 vs. 40%) (15). Compared with the control formula, the experimental formula provided sevenfold more esterified choline, AA (0.4% of total FA), and DHA (0.13%). Phospholipids are constituents of mucosal membranes and intestinal surfactant, and their components, AA and choline, are substrates for the production of intestinal vasodilatory and cytoprotective eicosanoids (AA) and the vasodilatory neurotransmitter, acetylcholine (choline), respectively.

The research referred to above used phospholipids to supply the AA and DHA, which could be envisaged as benefiting the intestine directly *via* the oral route, even at relatively low concentrations. Such low concentrations when diluted in the circulation may be less effective for the brain or lungs. The researchers were mimicking human milk content, which is physiologically designed for term delivery and not as a replacement for nutrition *via* the placenta. Our hypothesis is that AA and DHA levels approaching the intrauterine experience would be required to test for the prevention of ROP, bronchopulmonary dysplasia (BPD), and periventricular leukomalacia (PVL).

PRENATAL STEROID THERAPY

Steroids are given routinely to women who are expected to have a very preterm delivery because of their beneficial effects when given antenatally on BPD and the delay in delivery (16,17). Steroid use has become widespread because of their lung maturing effects. However, there has been concern regarding the gains and losses of steroid treatment (18). In a review of the data (19), it was concluded that antenatal steroid treatment was associated with >50% reduction in the incidence of PVL in preterm neonates. In experiments on rats, however, prenatal exposure to high levels of glucocorticoids increased the susceptibility of cerebellar granule cells to oxidative stress-induced cell death (20). In another study, human infants in the dexamethasone group were less likely than those in the placebo group to be receiving oxygen supplementation 28 d after birth ($P = 0.004$) or open-label dexamethasone ($P = 0.01$), but were more likely to have hypertension ($P < 0.001$), and were more likely to be receiving insulin treatment for hyperglycemia ($P = 0.02$) (21). During the first 14 d, spontaneous gastrointestinal perforation occurred in a larger proportion of infants in the dexamethasone group (13 vs. 4% in the placebo group; $P = 0.02$) although it appeared to be due to an unfavorable additive effect of dexamethasone and indomethacin. The dexamethasone-treated infants had a lower weight ($P = 0.02$) and a smaller head circumference ($P = 0.04$) at 36 wk postmenstrual age. In a systematic review, it was concluded that "Postnatal pharmacologic steroid treatment for prevention or treatment of bronchopulmonary dysplasia is associated with dramatic increases in neuro-developmental impairment. As there is no clear evidence in the literature of long-term benefit, their use for this indication should be abandoned" (22). This conclusion would, of course, be possible only in the postsurfactant era.

THE PRETERM INFANT—STEROID TREATMENT AND A DIFFERENT APPROACH

Chronic lung disease and BPD are associated with a significant inflammatory response of the airways and the interstitium of the lungs. In addition to inflammatory cells, various cytokines, lipid mediators, proteolytic enzymes, and toxic oxygen radicals may play an essential role in the pathogenesis of this disease. Intrauterine exposure to chorioamnionitis or proinflammatory cytokines has been shown to induce a pulmonary and systemic inflammatory response in the fetus (23).

When given in addition to preformed surfactant, one aim of steroid therapy is to reduce the inflammatory complications in both the mother and the newborn and reduce the effect of

BPD. Although steroids are generally effective for inflammatory conditions, it is of interest that steroid treatment has been associated with increased risk of morbidity. Shinwell *et al.* (24) compared dexamethasone-treated preterm infants (n = 132) with a saline placebo group (n = 116). The dexamethasone-treated group had a significantly higher incidence of cerebral palsy than those receiving placebo [39/80 (49%) vs. 12/79 (15%), respectively; odds ratio (OR) 4.62, 95% confidence interval (95% CI) 2.38 to 8.98]. The most common form of cerebral palsy was spastic diplegia. Developmental delay was also significantly more common in the dexamethasone-treated group (44/80 (55%) than in the placebo-treated group (23/79 (29%); OR 2. 87, 95% CI 1.53 to 5.38). The authors concluded that a 3-d course of dexamethasone administered shortly after birth in preterm infants with respiratory distress syndrome was associated with a significantly increased incidence of cerebral palsy and developmental delay.

The downregulation of inflammatory processes by steroids may also allow reassembly of damaged tissue. However, in a fetus, cell growth is more rapid than at any other time. Seventy percent of the energy delivered to the fetus is devoted to brain growth. This demands a simultaneous development and growth of a competent vascular system to deliver the energy and nutrients. The very preterm infant is still physiologically a fetus. Steroid therapy may suppress the signs of damage and thus even help deliver nutrients but it does nothing for the high fetal demand for nutrient supply *per se*. Steroids do not replace the structural nutrients if they are in inadequate supply. The evidence shows that in some systems, EPA and DHA are anti-inflammatory and may protect the brain from ischemic stress and also provide for cell structural growth (25–27). Therefore, a complementary approach to steroid therapy of very preterm infants would be to target cell strutural requirements as supplied by the placenta, which would simultaneously be anti-inflammatory.

BRAIN MEMBRANE LIPIDS AND THE INDEPENDENCE OF THE LONG-CHAIN, POLYENOIC FA

The composition of the brain is dominated by AA and DHA (see Fig. 1). The brain does not use the polyenoic FA precursors, linoleic and α-linolenic acids in its cell membranes. Discussions on the possibility that α-linolenic acid would be converted directly to DHA frequently recur. However, the evidence accumulated by 1978 had already demonstrated that it was not to an appreciable extent (28). AA and DHA are preferentially taken up by the developing brain from the earliest phases of its growth with adult levels reached soon after birth (29). Feeding either linoleic acid (LA) or α-linolenic acid to rat pups during brain development demonstrated a highly selective uptake of the preformed molecules (30) with only 1 in 10–30 molecules of LA and α-linolenic acids becoming incorporated as brain AA and DHA acids, respectively (31). Approximately 50–60% of ^{14}C-labeled LA and α-linolenic acids are oxidized in the first 24 h after administration (32).

Moreover, ^{13}C studies have shown that of the oral α-linolenic acid that reaches the brain, there is 30–50 times as much of the isotope recovered from palmitic acid and cholesterol over incorporation into DHA (33). Indeed, it may well be that the high oxidation rate of α-linolenic acid provides it with a rather special role for brain cholesterol and myelin synthesis without which the brain cannot form. The brain is totally dependent on its own endogenous synthesis of cholesterol, which is a major building block of its cell membranes. Quite precise ^{13}C studies have now been done in adult humans confirming the poor conversion of α-linolenic acid to EPA and DHA, which may be as low as only 5% (34).

The isotope studies and what we know about function indicate that the preformed AA and DHA acids are independently important in their own right. This independence is strikingly visualized by the biomagnification process of the

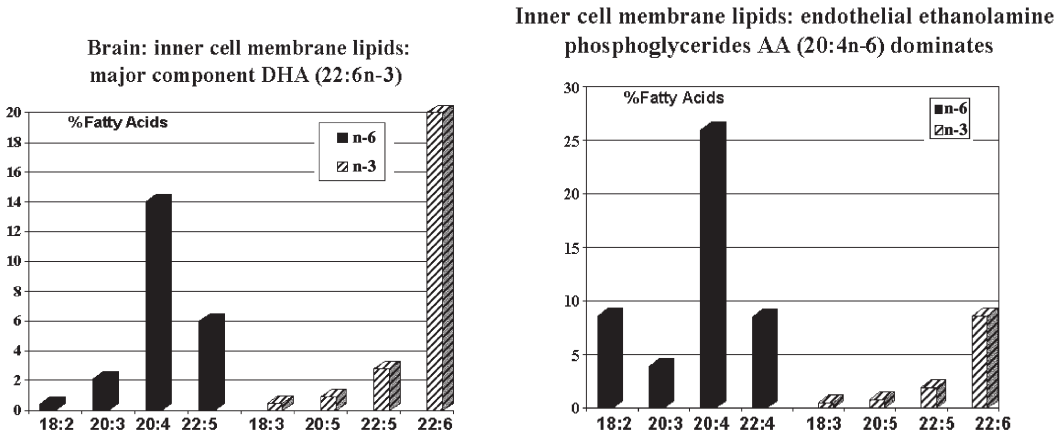

FIG. 1. Brain and vascular endothelium, inner cell membrane composition. In all mammals studied to date, the compositional data are very similar despite large differences in food selection practices, liver, and muscle membrane composition (84). The variation among species is in relative brain size, not composition. This compositional conservation against extremes of nonneural composition (e.g., liver ethanolamine phosphoglycerides from 1–42%, DHA from 0.5–22%) is strong evidence for arachidonic acid (AA) and DHA as determinants of brain growth and evolution (85).

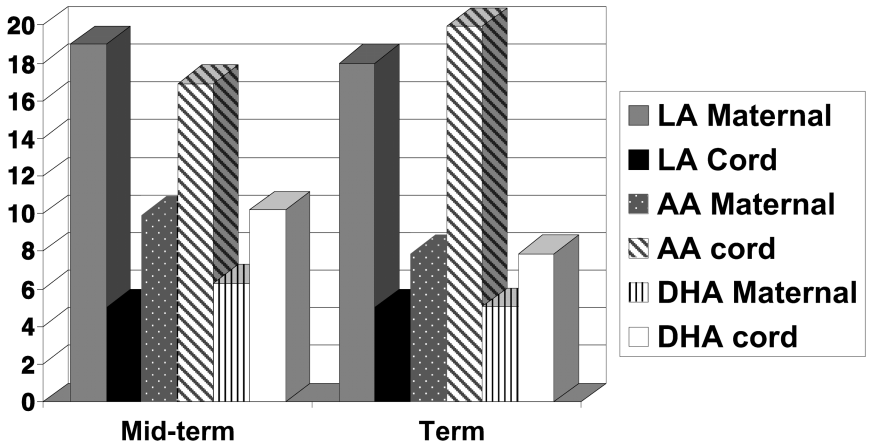

FIG. 2. Biomagnification across the placenta emphasizes fetal arachidonic acid (AA) and restricts linoleic acid (LA). Mid-term data are derived from mid-term elective abortions; term data from normal deliveries 39–42 wk gestation and birthweights >3.0 kg. Note that the proportions of maternal AA and DHA are less at term than mid-term in both mother and fetus (cord blood). By contrast, the fetal AA is greater at term than mid-term. The striking biomagification of AA can be appreciated from calculating the ratio of LA/AA in the mother (0.25) compared with the fetus (2.7), which means that the change in the ratio at term is >10-fold.

placenta. The proportions in the phosphoglycerides on the fetal side of the placenta are greatly enhanced compared with the maternal side. In Figure 2 it can be seen that this biomagnification process is especially striking for AA. Not only is its proportion more than doubled, but that of LA, its precursor, is more than halved by the time they reach the fetal circulation. It has been shown that this process is due to *selection* and *not metabolic conversion* by the placenta (35). Thus, the biomagnification by the placenta is taking preformed AA and DHA from the mother to nourish the fetus. In Table 1, we

TABLE 1
Key Polyenoic FA Differences Between Pregnant and Nonpregnant Korean Women: Data from Plasma Choline Phosphoglycerides (CPG) and TG[a]

Normal pregnancies[b] FA, % of total	Pregnant *n* = 40	Nonpregnant *n* = 40	*P*-value
Healthy birthweights 3.0–4.5 kg			
Plasma CPG			
Linoleic acid	20.1 ± 4.1	21.2 ± 2.51	NS
Arachidonic acid	7.98 ± 2.2	9.2 ± 1.4	0.005
DPA n-6	0.73 ± 0.41	0.18 ± 0.07	<0.0001
DHA	5.43 ± 0.98	7.48 ± 1.1	<0.0001
∑ n-3 FA	7.02 ± 1.23	11.0 ± 2.20	<0.0001
Supplemented with DHA 3–9 mon	Postnatal month 3 (*n* = 28)	Postnatal month 9 (*n* = 28)	
Low birthweight <2.5 kg			
Plasma CPG			
DHA	2.45 ± 0.8	3.85 ± 0.68	<0.0000
TG			
DHA	0.84 ± 0.37	0.95 ± 0.23	NS

[a]TG contain little DHA and arachidonic acid (AA) as these are preferentially incorporated into the CPG. Whereas the CPG responded to the DHA supplement, the TG did not, so it is important to measure separated phosphoglyceride rather than TG or total FA in plasma or adipose tissue. In the dolphin liver and muscle phosphoglycerides, we found high proportions of AA, which might be expected to be limiting for marine mammals. The same FA was barely detectable in the adipose TG, which contained an abundance of EPA and DHA. The opposite was the case in zebras, a grass-eating (n-3) species whose adipose fat was rich in α-linolenic acid with small quantities of linoleic acid (Ref. 86). Liver and muscle lipids, however, were rich in linoleic acid. The speculation derived from such data is that a mechanism must exist to preferentially satisfy membrane requirements, whereas adipose tissue acts as a store of the residue. In both situations, neither adipose fat nor phosphoglyceride composition is a true reflection of diet. The phosphoglycerides are clearly a better reflection of "status," but only assessment of both together provides a reflection of the diet.
[b]Women were matched for age and ethnicity. Supplementation was with 300 mg DHA/d. NS, not significant.

compare pregnant and nonpregnant women from Seoul, Korea, matched for age and ethnicity. The data in this table show that maternal LA is not different, but AA and DHA are lower, in the pregnant woman. This difference suggests that pregnancy selectively depletes maternal stores of these long-chain polyenoic FA. The stability of LA implies that any conversion to AA is not keeping pace with the placental/fetal demand.

PRENATAL CONTRIBUTIONS TO THE COMPLICATIONS OF PREMATURITY

We propose that replicating the placental input to the fetus of the AA and DHA needed for cell membranes (see Fig. 2) could downregulate inflammatory processes and simultaneously provide the lipids nutrients for membrane growth, thus providing an optimum environment in which to prevent or treat the complications of prematurity. We have shown that the preterm infant is born with reduced and greatly variable levels of AA and DHA (36–38). These FA are essential cell membrane constituents of particular importance for the vascular endothelium, the brain, and the visual system. At birth, a large part of the variation for AA is explained by birthweight and of DHA by gestational age, with both related to head circumference (39). These variations are likely to be prenatal in origin.

Infection has been claimed to be one contributor to the development of cerebral palsy among preterm babies (40). Conversion of the T-cell surface molecule CD45 from naïve $CD45RA^+$ to $CD45RO^+$ occurs in response to antigen. The T lymphocytes are predominantly naïve in healthy newborn infants. Infection has been shown to upregulate CD45RO on fetal T cells, which is consistent with a fetal inflammatory response and activation of immunological memory *in utero*. The authors claim that the concomitant increase in the proportion of $CD45RO^+$ suggests that the activation resulted from exposure to antigen rather than brain injury, hypoxia, or parturition. Conversion of naïve $CD45RA^+$ T cells to $CD45RO^+$ takes up to 10 d *in vitro*. Therefore, it is likely that an inflammatory process or some tissue damage has occurred possibly as much as 2 wk before delivery in some babies. This time course is consistent with the MRI study of the brains of these infants because some images clearly show longstanding lesions.

There is some evidence that the cytokines interleukin (IL)-6 and IL-8 are associated with preterm delivery (41). Chorioamnionitis is considered to be a leading cause of preterm birth and subsequent neonatal complications. Even in the absence of a proven infection, fetuses and neonates can present with a systemic inflammatory response, which can be identified by radiological and morphological examination of the thymus. The frequent occurrence of brain injury in neonates with chorioamnionitis is probably linked to systemic mechanisms that have not yet been completely clarified (42). A source of antigen stimulating a fetal immune response could be uteroplacental infection, which is common before preterm delivery. As Duggan *et al*. (43) commented, there is no known mechanism whereby common infections such as urinary tract and intravaginal infections would damage the brain.

On the other hand, some consider the possibility that it is the infant's own cytokines that damage the brain (44). Yet the tight cell junctions in its specialized endothelium selectively and specifically protect the brain. Penetration of activated cells or cytokines would be expected to require a failure in this protective system. There is an allergic encephalomyelitis model of multiple sclerosis that is created by injection of brain protein into the foot pad of rats. The result is lymphocyte penetration into the brain and extensive demyelination. The original model was developed in the 1960s by making rats deficient in EFA to diminish the blood brain barrier function. To avoid the tedious nutritional deprivation, genetically susceptible rats were selected. It has now been shown that these genetically susceptible rats can be protected by feeding certain EFA (45), thus proving the importance of the membrane lipids to the protection of the brain.

In preterm infants, the brain damage seen before birth is likely to involve intraventricular hemorrhage, which does not resemble experimental allergic encephalomyelitis. A common view on the cause of intraventricular hemorrhage is that it results from the rupture of fragile blood vessels that are poorly supported during very early brain development. This idea is a statement of fact rather than an explanation. Why are the blood vessels fragile? Why do they rupture? The actual rupture of the blood vessels can be explained by a surge of blood pressure or defective hemostasis, including platelet-vascular interactions. The mechanisms at work in the premature brain are as yet unknown, but data we obtained during a study of preterm infants may provide a possible explanation. Studying the membrane lipids in the vascular endothelium of umbilical arteries of babies of different birth weights and gestational ages at birth, we found striking correlations of AA and DHA status with birthweight and gestational age, respectively. Of particular interest was the fact that the correlations were the strongest ($r > 0.8$; $P < 0.001$) with the tetraene/triene ratio as a measure of AA status and the n-6 DPA/docosatetraenoic acid ratio as an index of DHA status, rather than the values of the individual FA themselves (46). Although there were only 14 infants in this study, a subsequent study of 63 preterm infants' plasma choline phosphoglycerides (CPG) produced similar results, namely, that AA status correlated with birthweight and DHA with gestational age (47,48). Moreover, of special interest to us was the astonishingly high proportions of Mead acid (20:3n-9) in the vascular endothelium. Similar data were first reported by Hornstra's group (49). This FA is the eicosatrienoic desaturation and elongation product of oleic acid, which is seen in significant amounts in EFA deficiency only when there is reduced membrane AA. In the preterm endothelium, the Mead acid was present at 1.8–6.4% of the total FA. Moreover the Mead elongation product (22:3n-9) was also present. The total of the

TABLE 2
Ranges of Individual FA Composition of Umbilical Artery, Endothelial CPG and Ethanolamine Phosphoglycerides (EPG), Birthweights, and Head Circumferences[a]

	Birthweight (g)	Head circumference (cm)									
Lowest	820	25.5									
Highest	4310	36.5									
FA		n-6					n-3				n-9	
% of total		18:2	20:3	20:4	22:4	22:5	18:3	20:5	22:5	22:6	20:3	22:3
EPG												
Lowest		0.45	0.37	10.8	2.29	2.91	Trace	Trace	Trace	7.36	1.85	1.0
Highest		3.1	3.60	26.1	7.24	7.62	0.25	1.1	0.97	18.0	6.40	3.6
CPG												
Lowest		Trace	1.26	5.63	1.17	1.66	Trace	Trace	Trace	0.10	1.74	0.6
Highest		3.1	2.90	10.1	3.03	3.80	0.36	0.23	Trace	5.20	4.46	2.1

[a]Data are from the study described in Reference 44. Note that the regression coefficients for the various markers of status were substantially stronger than the coefficients for the individual FA. For example, head circumference vs. 22:6n-3 gave $r = +0.49$, $T = 1.78$ and was not significant. However, the head circumference vs. 22:5n-6/22:4n-6 ratio, which is a sensitive marker for DHA status, gave for the CPG head circumference $r = -0.46$, $T = 2.42$, and $P < 0.05$. However the ethanolamine phosphoglycerides (EPG) gave $r = -0.83$, $T = 5.09$, and $P < 0.001$. It is possible that the EPG, which is largely an inner membrane lipid, contains greater historical information than the CPG, which, in the outer leaflet, will have more exchange with the day-to-day information in the circulation. Independently, Mead acid proved to be a better marker of birthweight and head circumference than any of the other individual FA. It is noteworthy that even in the infants with the highest levels of Mead acid, it was not present in such large amounts as in the umbilical artery. The plasma data are presented in Reference 44. For abbreviation see Table 1.

two reached a maximum of 10%. In the corresponding plasmas, the proportions were <1%.

Fetal plasma composition is a reflection of constant perfusion by the placenta and the metabolic demands of fetal growth. The vascular system, and the umbilical artery in particular, is growing rapidly. Some cells will be newly formed, but others will have developed some time ago. Thus, the tissue may be a better reflection of the dynamic history of the intrauterine experience than the plasma. Consistent with this idea is the fact that the ethanolamine phosphoglycerides (EPG) expressed the greatest biochemical signs of EFA deficiency. For head circumference the triene/tetraene ratio, EPG correlation coefficient was –0.83 ($P < 0.001$) compared with –0.63 ($P < 0.01$) for the CPG (Tables 2, 3, and 4). The EPG fraction is largely on the inner side of the membrane and therefore less in contact with the circulating lipids and, we suggest, of greater historical significance.

In babies born at term, Mead acid was present in the vascular endothelium at levels >1% which, among tissues, is highly unusual and significant. Fetal endothelial cell membranes are growing very rapidly. The presence of Mead acid suggests to us that the system is at the limits of the supply of their polyenoic FA component. If the deficit is severe enough, the inadequate supply of AA and DHA could result in functionally abnormal cell membranes with consequent loss of integrity and increased risk of rupture. Rupture would release intracellular proteins, and free AA for peroxidation to vasoconstrictor, thrombogenic, and inflammatory responses.

Support for a biochemical defect with membrane instability comes from a genomic investigation of the endothelium of preterm births. This study indicated that there is a molecular pathologic condition of fetal membranes regardless of any inflammatory status (50).

TABLE 3
Regression of Birthweight (BW) and Head Circumference (HC) with Individual FA in Endothelium of Cord Arteries: EPG[a]

		Pearson's coefficient	*t*	*n*	*P*-value
Anthropometry	FA				
BW vs.	20:4n-6	+0.53	2.98	14	<0.02
HC vs.	20:4n-6	+0.44	2.34	12	<0.05
BW vs.	22:6n-3	+0.54	2.19	14	<0.05
HC vs.	22:6n-3	+0.49	1.78	12	—
BW vs.	16:0	–0.52	1.93	13	—
HC vs.	16:0	–0.45	1.49	11	—
BW vs.	18:0	–0.52	2.11	14	—
HC vs.	18:0	–0.48	1.68	12	—
Mead acid					
BW vs.	20:3n-9	–0.61	3.43	14	<0.01
HC vs.	20:3n-9	–0.75	4.00	12	<0.01
Choline phosphoglycerides					
BW vs.	20:4n-6	+0.35	1.28	14	—
HC vs.	20:4n-6	+0.37	2.08	12	—
Mead acid					
BW vs.	20:3n-9	–0.50	2.84	14	<0.02
HC vs.	20:3n-9	–0.42	2.27	12	<0.05

[a]See footnote *a*, Table 2.

PRETERM INFANTS AT GREATEST RISK

If the limiting conditions on polyenoic FA supply comprise a correct interpretation of the data on preterm infants, then its application to the blood vessels in the brain could provide an

TABLE 4
Regression of Birthweight (BW) and Head Circumference (HC) with Indices of EFA Status in Endothelium of Cord Arteries: EPG[a]

		Pearson's coefficient	*t*	*n*	*P*-value
Triene/tetraene ratio (EFA deficiency)					
BW vs.	20:3n-9/20:4n-6	–0.87	6.46	14	<0.001
HC vs.	20:3n-9/20:4n-6	–0.83	5.14	12	<0.001
Pentaene/tetraene ratio (n-3 deficiency)					
BW vs.	22:5n-6/22:4n-6	–0.79	5.07	14	<0.001
HC vs.	22:5n-6/22:4n-6	–0.83	5.09	12	<0.001
Choline phosphoglycerides					
Triene/tetraene ratio (EFA deficiency)					
BW vs.	20:3n-9/20:4n-6	–0.68	3.86	14	<0.01
HC vs.	20:3n-9/20:4n-6	–0.63	3.20	12	<0.01
Pentaene/tetraene ratio (n-3 deficiency)					
BW vs.	22:5n-6/22:4n-6	–0.66	3.74	14	<0.01
HC vs.	22:5n-6/22:4n-6	–0.46	2.42	12	<0.05

[a]See footnote *a*, Table 2.

explanation for fragility and rupture. The greatest risk of brain damage is in those babies born at the lowest birthweights and the lowest gestational ages. Our data indicate that the babies with the lowest birthweights have the lowest AA, and those born the earliest have the lowest DHA levels in their plasma CPG and indeed in both CPG and EPG of the umbilical endothelium. It is unlikely that this expression of physiologic growth stress does not also apply in some measure to the blood vessels in the brain.

The message from the placental biomagnification profile is that it is serving vascular growth. Vascular growth is a prerequisite for brain growth and arguably has to precede brain growth to meet the phenomenal demand, which can be as high as 70% of the total fetal demand for energy in the last trimester. Given the FA data, we have a potential explanation for the vascular damage in the brain and possibly also the vascular involvement in ROP, BPD, and NEC. A relative deficiency of both AA and DHA during a period of rapid demand would be expected to lead to fragile, leaking vessels, rupture, and even the obliteration seen in ROP.

Any prenatal polyenoic FA deficiency in the very preterm infant will be exacerbated after birth. The proportion of AA the placenta delivers ranges from 14 to >20%. The proportion provided by the present formulae sold for preterm infants is ~0.4%. Similarly, for DHA, the placental delivery is >6% of the FA, whereas the formulae designed for preterm infants is ~0.3%. That is a 50-fold reduction in AA and a >10-fold reduction in DHA compared with the physiologic expectation at this time. When the plasma phosphoglyceride levels are followed from birth, it is evident that the levels of AA plummet to a third of what they would have been had the baby remained as a fetus. DHA status is similarly damaged. Moreover, the proportions of LA rise from 4–8% to as much as 30%, effectively replacing AA in the β-position of the phosphoglycerides.

AA has not been incorporated into preterm formulae to any significant degree. The reasons given vary, but the main excuse is that LA is converted to AA and α-linolenic acid is converted to DHA. The single fact that LA rises steeply and AA levels fall sharply after birth indicates that the claim that LA is adequately converted to AA is incorrect.

DHA IN GENE EXPRESSION

There is good reason to consider that DHA is not simply a structural feature of the signaling process but is also a potential ligand for nuclear receptors. Molecular dynamic studies show that the structure of vitamin A is similar in three dimensions to that of DHA in their minimized energy configurations. The steric energy for the lowest energy conformation of vitamin A (calculated) was 29.5 kcal/mol; for DHA, the corresponding steric energy was 35.6 kcal/mol. This steric energy term is most important relative to any higher energy conformations that can occur in the molecule.

In the case of vitamin A, the molecule is innately quite rigid and flat. The only real question about the conformation of vitamin A is that the six-member ring actually has a mirror image conformation. There is a dimethyl group on the ring and the CH_2 group adjacent to this ring can kind of pucker toward either one of the methyl groups. It is, however, in a portion of the molecule that may have a minimal effect on its binding properties. Retinoic acid has a carbonyl in place of a CH_2 group; thus, its overall length is very close to that of vitamin A. The retinoids have 11–12 planar bond lengths. For the same distance, DHA similarly has equivalent length divided among 12–14 planar bond lengths. The reason more bond lengths fit over the same distance is that in DHA, each double bond is attached to a CH_2 group in which the bonds are tetrahedral, not planar, i.e., more bonds fit in precisely the same planar projected distance.

Retinaldehyde, all-*trans* retinoic acid, and 13- and 9-*cis*-retinoic acid are all to a lesser or greater degree ligands for the retinoid receptors that activate gene expression. The natural coexistence of vitamin A and DHA is of some interest. The plant precursors occur together in the same foods as do the preformed molecules themselves (e.g., green leafy plants and sea foods, respectively). Both DHA and vitamin A are functionally critical for the photoreceptor. The retinoids are the principal ligands for nuclear receptors, which include the retinoid X receptors (RXR) and the nuclear peroxisome proliferator-activated receptors (51,52). Because the 3-D structure of DHA is similar to half of the retinoid structure, DHA might well be expected to compete with retinoid ligands for the RXR. Such ligand activity for DHA has been reported to be the case for brain RXR (53).

In the liver, the quantity of retinoids is large. However, there is very little retinoid in neural cells in which the quantity of DHA is large. It is plausible that DHA is operating as a ligand for RXR in the developing brain. Control may be exercised by variation in the content of the DHA in the membrane where stoichiometric release would provide the nutrition-dependent regulation. Brain DHA levels are difficult to alter.

If DHA was indeed a regulator of neural gene expression, its compositional stability would offer the advantage of the characteristic consistency and remodeling, typical of brain physiology, that operates without continuous cell renewal. Nonetheless, long exposure to n-3–deficient diets or high levels of n-6 could reduce the DHA content and the gene expression responses. Indeed, it is being suggested that such dietary alterations, which are of recent origin, could contribute to the rapid rise in mental ill health. The latter seems to be inversely proportional to sea food intake, the major source of dietary DHA (54). Ligand activation of RXR is an obligatory step in the process of signaling to the nucleus. Our structural calculations imply that DHA will fit into the active pocket in RXR. That could mean that DHA is a natural ligand for RXR in the brain.

As described above, there is increasing evidence that, although the majority of preterm babies may escape severe brain damage, there are many apparently healthy babies who are poor achievers at school, and who may have an attention deficit disorder or other behavioral problems. One of the most interesting developments in our understanding of the role of DHA in biomembranes is its function in stimulating neural gene expression (55,56). With DHA involved as a nerve growth factor in the expression of genes controlling synaptic plasticity, dendrite formation, neural outgrowth, and those concerned with cell growth, cytoskeleton, signal transduction, ion channel formation, energy metabolism, and regulatory proteins, it is not difficult to understand how a relative deficiency could result in impoverished neural development.

There is general agreement that supplementing preterm infants with AA and DHA improves visual and mental development (57). The converse is being claimed from the inverse correlation between seafood intake, depression, and violence (58,59). The results in term infants have not been so clear-cut. A low-dose study showed no effect. However, at doses that correspond more to the expectation in human milk, significant effects were seen (60,61). For any such effects to be long-lasting would require a role for these FA not simply in immediate regulation of gene expression but also in programming.

PEROXIDATION

Saugstad proposed that some of the damage associated with very preterm delivery of infants is due to peroxidation. Antioxidant defenses are compromised when an infant is born preterm against a physiologic expectation of the reduced oxygen tension of the intrauterine environment. Saugstad also suggested that the complications of prematurity are "facets of one disease" (62). His suggestion is consistent with our own notion of a common vascular etiology. We find that preterm infants are born with only half the red cell membrane activity for Cu/Zn superoxide dismutase (63), which does not catch up with babies born at term. That is, their cellular antioxidant defenses are compromised by being born preterm. They are born at a time when their physiology expects the low, intrauterine oxygen tension. They are then exposed to a high oxygen tension, often exacerbated by the use of oxygen respiration needed to avoid overt neuronal death.

Saugstad's concept is also consistent with a common biomembrane disorder underpinning the complications of prematurity. One of the major attacks of peroxidation at the membrane would be the highly susceptible, unsaturated FA AA and DHA. Hence loss of AA and DHA due to the absence of the placental input for an infant born very preterm would be exacerbated by peroxidation. Peroxidation, as Saugstad points out, would make its own contribution to cell damage. However, attempts to protect against peroxidation *per se* have had equivocal results (64). It is our view that both antioxidant defenses and cell membrane structures must be supported simultaneously as would occur in nature.

Animal studies on peroxidative damage to the brain have demonstrated, unsurprisingly, that a lack of cell membrane integrity and peroxidation go together (65,66). These experimental studies, which involve massive cerebellar damage, raise three interesting points. First, only the cerebellum is affected. Why not the cerebrum? The answer is most likely that in the postnatal chicken, it is the cerebellum that is growing rapidly and acquiring its unsaturated FA. Second, observable neurologic damage will occur only if there is a deficiency of both the antioxidant and n-3 FA. Third, the damage can be arrested by supplementing with n-3 FA (67). The message is that protection against peroxidation damage would have to be accompanied by simultaneous provision of the substrates needed for cell membrane growth.

BIOCHEMICAL PATHOGENESIS: INTRAVENTRICULAR HEMORRHAGE (IVH) AND A POTENTIAL ROLE FOR AA IN THE ENDOTHELIUM

Reduced membrane AA and or DHA, or increased peroxidation will predictably lead to leaking cell membranes as in the

BPD, obliteration of blood vessels as in ROP, and hemorrhage as in IVH. Even raised blood pressure can be attributed to inadequate vascular function. Stiffening of the vascular system and depressed elasticity with relaxation dysfunction would be a precursor for raised blood pressure. Reduced AA could also impair the balance of pro- and antithrombotic, vasoaortic eicosanoids produced by the endothelium and platelets (68).

In experimental studies aimed at testing the role of the high-fat diet of Western countries on vascular development, we found that feeding a high-fat diet to pregnant rats resulted in vascular contractile and relaxation dysfunction in the arteries of newborns. This dysfunction appeared to be permanent and was associated with an inhibition of the transfer of polyenoic FA from the mother to the fetus with resultant deficits on the cell membrane AA and DHA (69). We therefore studied the effect of preincubating AA, EPA, and DHA to test for their individual effects on vascular function.

We found that preincubation of AA but not DHA (Figs. 3, 4) significantly improved vascular function. Although EPA counteracted the effect of a thromboxane mimetic, it did not improve vascular relaxation. It seems from these data that AA is operating as an endothelium-derived hyperpolarization factor.

The compositional data of the endothelium combined with these effects of AA on vascular relaxation provide a rationale for the selective and striking enrichment of the fetal circulation with AA by the placenta. With the contributions of long-chain polyenoic n-6, n-3, and antioxidant deficiency to blood vessel fragility in the brain and given that the lowest weight and earliest deliveries are those at greatest risk for brain damage (70) and those same babies have the lowest AA and DHA status, a role for deficiency of AA and DHA in the pathogenesis of IVH is plausible.

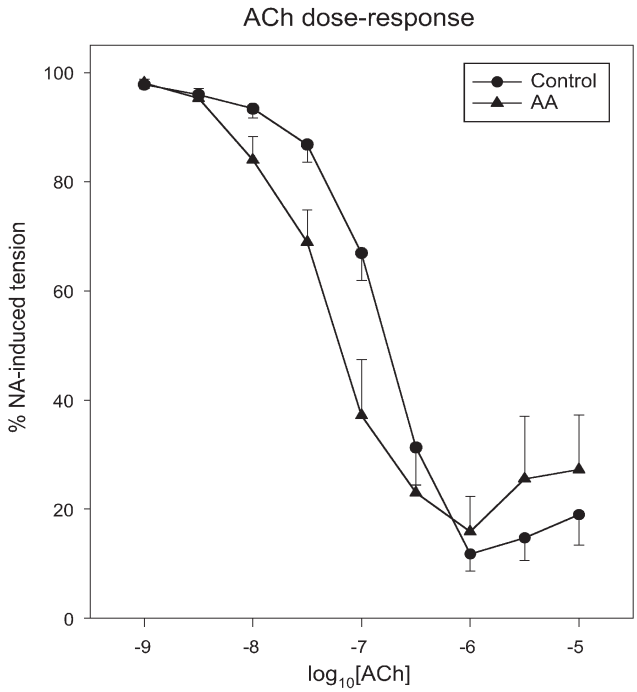

FIG. 3. Concentration response to acetylcholine (ACh) in rat small mesenteric arteries: effect of preincubation with AA. The myograph technique allows segments of small arteries to be mounted as ring preparations. This technique is characterized by being relatively atraumatic and provides measurements of highly isometric responses. The contractile response of mounted rat mesenteric small arteries is >250 mN/mm^2 of media. If allowance is made for the histologically determined smooth muscle fraction of the media (~70%) (86), this corresponds to an active stress of >350 mN/mm^2. After contracting the arteries with noradrenaline (NA), we added progressive amounts of ACh from 10^{-9} to 10^{-5} mol/L. Compared with controls, there was a progressive relaxation of the treated arteries starting very early (10^{-9}) and continuing until (10^{-6}). At this point, the lines cross, and treated arteries start to contract more than the controls and both curves separate again. It is possible to see that the AA curve relaxes first, at a low concentration of ACh and its pEC_{50} changes significantly, demonstrating increased sensitivity to ACh (pEC_{50} 7.32 ± 0.13 treated vs. 6.82 ± 0.05 control, $n = 10$, $P < 0.01$). Later increases in contractility with the highest doses of ACh between controls and treated groups were not significant (methods after Ref. 86). For other abbreviation see Figure 1.

BIOCHEMICAL PATHOGENESIS: PVL AND CEREBRAL PALSY

The pathophysiology of PVL involves ischemia. An ischemic lesion can resolve without leaving a scar. If the lesion is in the brain and becomes cystic, it will, with certainty, lead to a permanent functional defect. Because the newborn baby has little precise motor control, a cystic lesion can pass unnoticed for some time. It is only when motor milestones fail to be reached that the alarm bells are rung. In fact, pediatric neurologists now usually consider that it is not possible to diagnose cerebral palsy with certainty until 2 yr of age.

The failure of the placenta to transfer the appropriate quantities of AA and DHA, whether because of maternal insufficiency or placental dysfunction, could equally contribute to the etiology of cystic PVL. The consequences of cell membrane breakdown are now well understood. Normal blood flow is favored by cyclooxidation of dihomo-γ-linolenic acid and AA in the endothelium to vasodilatory and antiaggregatory eicosanoids, which act as local hormones. They act synergistically with nitric oxide. DHA is not without its contribution either. Recent data suggest that DHA increases NO production by potentiating inducible NO synthase expression induced by IL-1β through a mechanism involving a p44/42 mitogen-activated protein kinase signaling cascade in rat vascular smooth muscle cells (71). Indeed, DHA has been found to protect against oxidative stress in the fetus (72). If the cell membrane is deficient in its polyenoic FA component, it is more likely to rupture. The consequences are lysosomal activation and AA release from the membrane phosphoglycerides. If the platelets are activated by exposure to collagen, they will aggregate and adhere to the broken cells. In this context, they will be stimulated by the free AA, which they will convert to thromboxane A_2.

The classical response to injury will then follow. Eicosanoid and cytokine activity will favor vasoconstriction and ischemia. Under normal conditions, NO is formed by the endothelium and is washed away in the circulation. NO and prostacyclin act on the underlying smooth muscle cells,

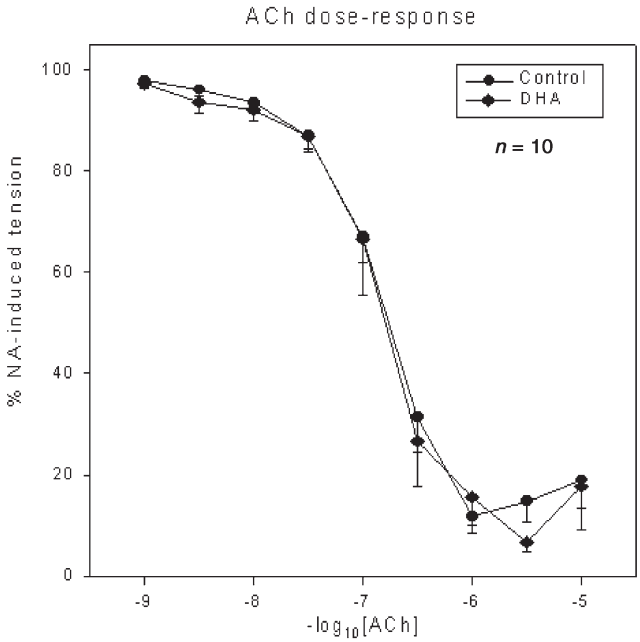

FIG. 4. Concentration response to ACh in rat small mesenteric arteries: effect of preincubation with DHA. The DHA dose-response curve to agonist ACh, NA, spermine NONOate, or U46619 did not differ from control arteries. The arteries preincubated with EPA did not relax in response to ACh. There were no differences between treated and control arteries in contraction to NA or the relaxation response to spermine NONOate. On the other hand, when the concentration response to the thromboxane mimetic, U46619, was tested, there was a progressive shift of the curve to the left, suggesting an increase in the contractility of EPA-treated arteries (87). For abbreviations see Figure 3.

causing vasodilation, thus lowering blood pressure and providing a valuable local vascular tone function. During ischemia, there is synaptic discharge with glutamate release, which opens a gate for calcium to enter the cell and activate NO synthase. On reperfusion, the release of NO will cause large amounts of peroxynitrite to form (73,74). Peroxynitrite is a highly potent lipid peroxidizing agent. It is not difficult to see how this situation in the brain, which is rich in very unsaturated FA that are susceptible to peroxidation, would lead to cell death and be a potential mechanism leading to cystic PVL (75–77).

This paper has developed the hypothesis that deficits of AA and DHA may contribute to the complications of prematurity. The mechanism would simply be reduced vascular or endothelial integrity with the predictable signaling consequences in hemorrhage or ischemia. This hypothesis suggests the possibility of supplementation of the mother before birth and the infant afterwards, with AA and DHA. There is recent evidence that fish oil (rich in DHA and EPA) will reduce arterial plaque (78) and ameliorate hypertension-induced vascular damage (79). Combined with the evidence presented above on AA as an endothelial relaxation factor as well as its dominant role in the in endothelial membrane lipids (Fig. 1), we suggest it would be important to test the effect of both AA and DHA.

CONCLUSION AND HYPOTHESIS

We suggest that an important component of the complications of prematurity is loss of vascular integrity (80). BPD commences with the leaking of fibrin through the pulmonary blood vessel wall; retinopathy is described in the pathology text books as starting with obliteration of the retinal blood vessels, whereas IVH and PVL have self-evident blood vessel involvement.

The evidence on placental biomagnification for the fetus of AA and DHA implies that the placenta is a super pump for AA (see Fig. 2). The inner cell membrane lipid of the endothelium is especially rich in AA (Fig. 1), which provides for membrane properties, signaling, and protein kinase C activation (81), and acts as a precursor for a range of small molecules that play a pivotal role in cell trafficking, communication, and vasoregulation. Indeed, the use of steroids in some situations could be counterproductive due to reduced phospholipase A_2 activity and the suppression of the beneficial products of AA and DHA.

This discussion leads us to conclude that the placenta is acting primarily to serve blood vessel growth as a prerequisite to supply the extraordinary and unique demands of human fetal brain growth (82). Just as preterm infants have been found to require intakes of protein, energy, and minerals that match the placental output, so also it is likely that they will require the placental output of lipids, i.e., AA and DHA. These are currently being denied. In the context of this denial, it must be remembered that this is a time when 70% of the energy for growth is focused on the brain and the brain is 60% lipid. It is thus not surprising that membrane-rich systems fail under the stress of rapid growth and a dearth of the very substances needed for their growth and integrity.

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Diets Could Prevent Many Diseases

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ABSTRACT: The 2002 ISSFAL Meeting arranged a special evening discussion with professional dietitians about diet-tissue-disease relationships involving essential fatty acids and eicosanoids. The balance of eicosanoid precursors in human tissues differs widely, reflecting voluntary dietary choices among different groups worldwide. An empirical quantitative diet-tissue relationship fits these diverse values as well as other research reports on essential fatty acid metabolism. Information for dietitians and nutritionists about essential fatty acids and eicosanoids is also given in two distance learning web sites, http://ods.od.nih.gov/eicosanoids/ and http://efaeducation.nih.gov/, which facilitate dietitian education and diet counseling. These sites also have an innovative, interactive diet planning software program with the empirical equation embedded in it to help evaluate personal food choices in the context of the diet-tissue-disease relationship and other widely recommended dietary advice.

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BENEFITS FROM DECREASING EXCESSIVE AUTACOID SIGNALING

Non-steroidal anti-inflammatory drugs (NSAID) are some of the most widely used medications worldwide. The >70–100 million NSAID prescriptions written annually offer a huge profitability potential with annual sales of $13 billion. The 24.5 million Celebrex® prescriptions in 2001 in the United States were closely matched by 23.7 million prescriptions for Vioxx®; these two alone had worldwide sales of $5.7 billion. The drugs are taken to decrease an overactive self-healing response by the body's autacoids (auto = self, akos = healing), the n-6 eicosanoids derived from essential fatty acids obtained from the diet (1). This review examines ways in which less costly dietary approaches might also beneficially decrease those overactive responses. The NSAID produce their beneficial anti-inflammatory, analgesic, antipyretic, antithrombotic effects by inhibiting the cyclooxygenase enzyme that converts highly unsaturated fatty acid (HUFA) precursors into active eicosanoids. The worldwide success of NSAID provides ample "proof of principle" for the desirability of preventing excessive n-6 eicosanoid formation in many disorders such as thrombosis, the arrhythmia of heart attacks, and inflammatory/immune problems in atherosclerosis, arthritis, asthma, and tumor angiogenesis. Moderating those excesses brings an improved quality of life to millions of people. Unfortunately, nearly all of the drugs have some undesired side effects or limitations that are much discussed in assessing benefits and risks of medical treatments.

Ironically, the self-healing eicosanoids that are so important in the body's normal adaptive processes are sometimes overly mobilized, causing people to seek various self-administered medication to decrease the "friendly fire" of those overresponses. The n-6 eicosanoid is formed more rapidly in the case of prostaglandin derivatives (2,3), and acts more intensely in receptor signaling (4) compared with its competing n-3 eicosanoid analog (esp. leukotriene B chemotaxis). These differences produce less intense actions when the mixture of released HUFA precursors has a lower proportion of the n-6 analog to compete for the eicosanoid-forming enzymes. Physiologic benefits depend on moderate transient actions of these autacoids formed from local precursors present in nearly all tissues of the body (1). Once formed, eicosanoids are rapidly inactivated so that active autacoid usually appears only in small amounts at nearby tissue receptors and travels little distance from its site of synthesis. As a result, a small increase or decrease in the rate of synthesis causes a very important difference in the intensity of an autacoid's action at local receptors (1). Inhibitory drugs are voluntarily used by people to moderate an overresponse when eicosanoid action is more intense or for a longer time than desired. However, the difference in potency between the n-3 and n-6 eicosanoids gives another opportunity to decrease chronic excessive formation and action of n-6 eicosanoids by increased dietary intakes of naturally occurring competitive n-3 analogs rather than with pharmaceutical antagonists.

PROPORTIONS OF PRECURSORS RELATE TO INDIVIDUAL HUFA AND TO CORONARY HEART DISEASE (CHD) DEATH RATES

Dietary intake is the only route of entry for eicosanoid precursors, and food choices have an important effect on tissue HUFA levels and tissue autacoid responses. Dietary 18-carbon essen-

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Abbreviations: AA, arachidonic acid; CHD, coronary heart disease; DGLA, di-homo-γ-linolenic acid; HUFA, highly unsaturated fatty acids; MRFIT, multiple risk factor intervention trial; NSAID, non-steroidal anti-inflammatory drugs.

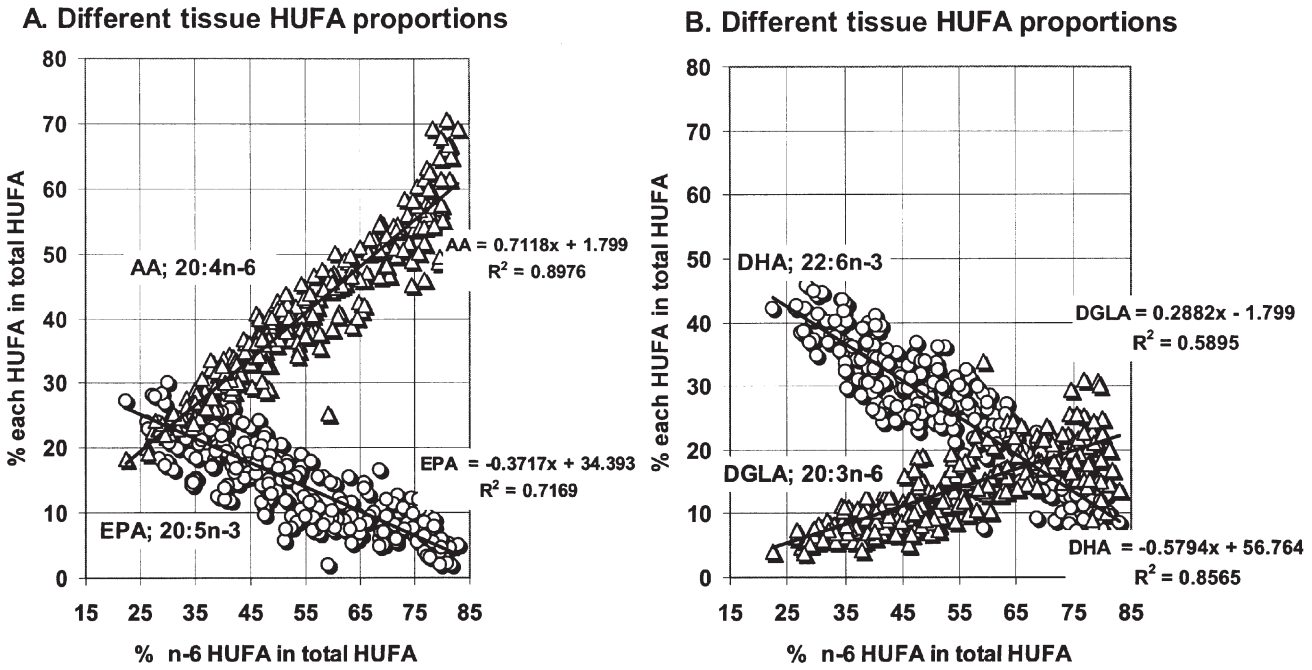

FIG. 1. Different tissue highly unsaturated fatty acid (HUFA) proportions among humans. The phospholipid fatty acids from 380 different plasma samples described in other studies from the United States [n = 293 (8)] and Japan [n = 87 (9)] were analyzed by gas chromatography. The proportions of individual n-3 HUFA decrease (A: EPA, 20:5n-3, ○; B: DHA, 22:6n-3, ○) as the n-6 HUFA increase [A: arachidonic acid (AA), 20:4n-6, △; B: di-homo-γ-linolenic acid (DGLA), 20:3n-6, △].

tial fatty acids are metabolized to different chain lengths [such as the 20-carbon arachidonic acid, AA (20:4n-6), and EPA (20:5n-3)] and stored as HUFA precursors esterified to tissue phospholipids from which they are mobilized by phospholipase-catalyzed hydrolysis (5). The closely related n-3 and n-6 essential fatty acids compete with each other for accumulation in tissue phospholipids, a process long recognized since the description of competitive hyperbolic interactions for these two types of nutrient by Mohrhauer and Holman (6,7). The voluntary food choices that people make day by day provide diverse proportions of n-6 and n-3 essential fatty acids associated with very different proportions of n-6 HUFA among the total HUFA of plasma phospholipids, which vary from 25 to 85% (Fig. 1). Competition between the n-6 and n-3 fatty acids is clearly evident in the decreased proportions of n-3 HUFA (DHA and EPA) associated with increased proportions of n-6 HUFA [AA and di-homo-γ-linolenic acid (DGLA)] that are stored in the tissue phospholipid HUFA.

High proportions of the n-6 precursor in the tissue HUFA that will be released during a stimulus will give high rates of formation of n-6 eicosanoids, whereas low proportions will give low rates of formation. In this way, the balance of n-3 and n-6 acids in the diet influences the balance of n-3 and n-6 HUFA in tissues and therefore the eventual balance of n-3 and n-6 eicosanoid actions in self-healing processes. CHD involves excessive n-6 eicosanoid actions in chronic and acute inflammatory processes in vascular walls that predispose people to fatal heart attacks as well as in the thrombosis and arrhythmia of the acute event. Because CHD is a major cause of death, many drug treatments are marketed vigorously to meet the need to treat people and reduce an imminent risk. Figure 2 shows that the age-adjusted risk of CHD mortality is less when the proportion of n-6 eicosanoid precursors in people's tissue HUFA is lower. The wide diversity in abscissa values for Figures 1 and 2 raises the question, "What proportion of n-6 eicosanoid precursor is stored on the shelves of your body's medicine chest?" It also prompts a closer, more quantitative look at the association between tissue HUFA and CHD mortality.

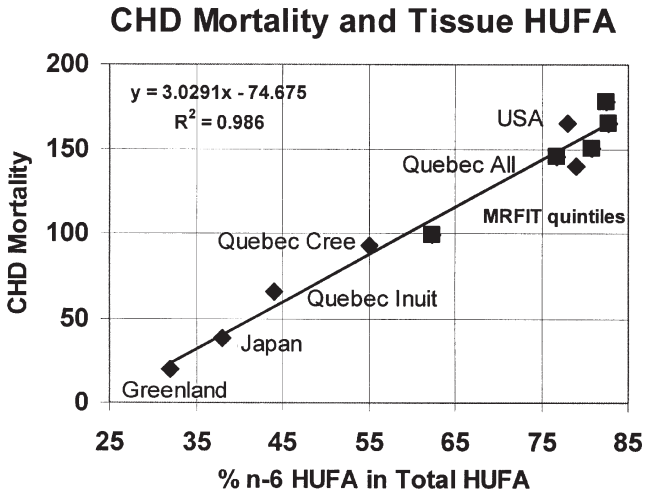

FIG. 2. Coronary heart disease (CHD) mortality rates associated with tissue HUFA proportions. Results from the United States, Japan, and Greenland were discussed earlier (10,11,12) as were quintile results from the Multiple Risk Factor Intervention Trial (MRFIT) study [■ (13)] and those from Quebec Inuit (14), Quebec Cree (15), and Quebec overall (16). For abbreviation see Figure 1.

The Multiple Risk Factor Intervention Trial (MRFIT) was a multimillion-dollar clinical trial in which the "Usual Care" cohort provided prospective longitudinal evidence that people consuming greater levels of n-3 HUFA had a lower relative risk of CHD than those eating less (13). The voluntary intake of n-3 HUFA for 6258 men (expressed as percentage of food energy, en%) had quintile means of 0.001, 0.004, 0.019, 0.063, and 0.272 en%. These relatively low intakes corresponded to proportions of 83, 82, 81, 77, and 62% n-6 HUFA in overall tissue HUFA, respectively. The 1251 men with the most n-3 HUFA intake had a significantly lower relative risk of CHD, 0.6 compared with 1.0, 1.08, 0.91, 0.88. However, Table 1 shows that the limited diversity among Americans in voluntary intake of n-3 HUFA causes this significant effect to be less apparent when all five quintiles are combined together. Predicting the likely proportion of n-6 HUFA in tissue HUFA for each of the quintiles (in column 6) and combining them with the known fraction of deaths in each quintile (column 7) predicts values of 78% for cases and 75% for surviving controls (column 8). Direct gas chromatographic analysis of the HUFA in plasma phospholipids gave good agreement with the values predicted from the dietary data, with an average of 79% n-6 HUFA in tissue HUFA for 94 cases and 76% for 94 controls (17). The clear benefit seen for one quintile and emphasized by Dolecek and Grandits (13) seems less visible when combining all quintiles together as reported by Simon *et al*. (17). Nevertheless, the latter authors concluded that a 1 SD increase in n-6 DGLA (20:3n-6) was associated with an increase in CHD risk of 40%, whereas a 1 SD increase in DHA (22:6n-3) was associated with a decrease in CHD risk of 33% (17). They concluded that the decreased CHD incidence may reflect decreased platelet aggregability after increased dietary intake of n-3 polyunsaturated fatty acids.

Recent results from Quebec (see Fig. 2) fit closely to the relationship observed for the MRFIT cohort as well as for the United States, Japan, and Greenland populations:

$$\text{CHD mortality} = 3 \times (\%\ \text{n-6 HUFA in tissue HUFA}) - 75 \quad [1]$$

Although cross-national analyses have been regarded with caution, the Quebec population of groups from the same province of the same country (14–16) follows the same trend for tissue HUFA influence on the likelihood of fatal CHD events. Knowledge of the molecular mechanisms for n-6 eicosanoid actions in the disease processes of inflammation, thrombosis, and arrhythmia and the success of many NSAID in moderating those processes leads inevitably to considering dietary steps to prevent excessive n-6 eicosanoid actions before they occur rather than relying only on treatment after disease processes become evident. The strong correlation coefficient of 0.99 ($r^2 = 0.986$) for the results in Figure 2 suggests that making dietary choices that decrease the proportion of tissue HUFA that is n-6 HUFA can be an effective primary prevention strategy to decrease the risk of fatal CHD events in a population. Figure 1 shows that many people already do. There are undoubtedly other eicosanoid-mediated diseases for which better nutrition may prevent what must otherwise be treated with drugs.

FOOD CHOICES AFFECT PROPORTIONS OF PRECURSORS IN TISSUE HUFA

Diet choices influence the composition of HUFA in tissue phospholipids, whose composition affects the likelihood of phospholipase releasing n-6 eicosanoid precursors; that in turn affects the probable intensity of n-6 eicosanoid synthesis and action in tissues. In maintaining health and preventing chronic diseases, the tissue is the issue. Extending the work of Mohrhauer and Holman, we examined quantitative metabolic relationships between dietary supply and the proportions of n-6 and n-3 eicosanoid precursors stored in the HUFA of tissue phospholipids. The proportion of n-6 in tissue HUFA can be estimated by an empirical relationship (12) for the quantitative metabolic competitions among four separate types of essential fatty acids in the diet:

1. 18-carbon n-3 PUFA; 18:3n-3
2. 18-carbon n-6 PUFA; 18:2n-6
3. 20- and 22-carbon n-3 HUFA; 20:5; 22:5; 22:6n-3
4. 20- and 22-carbon n-6 HUFA; 20:3; 20:4; 22:4; 22:5n-6

Each of the four types contributes differently to the overall tissue balance. Discussing the four types combined into a single

TABLE 1
Combined Quintile Results from the MRFIT Study[a,b]

1 Quintile of n-3 HUFA	2 *n*	3 Deaths	4 RR	5 Dietary en%	6 Estimated % n-6 HUFA	7 3 × 6 Cases	8 (2–3) × 6 Controls
MRFIT #5	1251	24	0.6	0.272	62.3	1495	76,434
MRFIT #4	1252	35	0.88	0.063	76.8	2687	93,428
MRFIT #3	1251	35	0.91	0.019	80.9	2830	98,335
MRFIT #2	1197	39	1.08	0.004	82.4	3215	95,447
MRFIT #1	1307	42	1	0.001	82.8	3476	104,692
Total	6258	175			Dolecek data predict	78.3 Cases	74.8 Controls
					Simon data observed	78.7	76.5

[a]Results in the first five columns are from Dolecek and Grandits (13), and the proportion of n-6 HUFA in total HUFA was estimated by an empirical quantitative metabolic relationship (12). Column 7 combines columns 3 and 6 to obtain the average estimated value for coronary heart disease cases, whereas column 8 gives the average estimated value for survivors. Analytical results of Simon *et al.* (17) are also in columns 7 and 8.
[b]Abbreviations: MRFIT, Multiple Risk Factor Intervention Trial; HUFA, highly unsaturated fatty acids; RR, relative risk; en%, percentage of food energy.

ratio of total dietary n-6/total dietary n-3, the ratio has no clear meaning because tissue proportions of n-6 HUFA in total HUFA result from the four separate contributions expressed as a percentage of total food energy (en%). To make estimates with all four variables, a simple calculator is accessible at http://efaeducation.nih.gov/sig/dietbalance.html to help design general features of dietary intervention protocols.

To facilitate estimates of how different combinations of specific foods can give very different proportions in body tissues, we developed an interactive personalized computer program that manages U.S. Department of Agriculture information about the essential fatty acids in 9214 different servings of food. The software combines information concerning a person's daily food choices and predicts the resulting tissue HUFA proportions, which are biomarkers for essential fatty acid intake (as noted in Ref. 12) as well as surrogate clinical markers (as seen in Fig. 2) that are also metabolically related to the probable intensity of an eicosanoid response as noted above. It can be downloaded without cost from http://ods.od.nih.gov/eicosanoids/. Some ethnic food choices achieve healthy tissue proportions and moderate eicosanoid responses by containing large amounts of n-3 HUFA (Japanese or Greenlanders), whereas some populations eat lower amounts of the n-6 18-carbon fatty acids (Mediterranean people). Although intake of linoleate is similar in the United States and Japan (quintile means from 3 to 9 en%), the mean intake for the lowest quintile of dietary n-3 HUFA in Japan (0.31, 0.46, 0.56, 0.71, 0.92 en%) is greater than the highest for the United States (0.001, 0.003, 0.017, 0.057, 0.249), making tissue eicosanoid responses appreciably different for the two populations.

To help clinical investigators design diets that produce the desired tissue HUFA balance, a simple spreadsheet was arranged to combine the four separate dietary influences with the empirical relationship described earlier (12) to predict the likely long-term outcome. Illustrations of three typical ethnic dietary combinations of essential fatty acid intakes (expressed as en%) are in the columns at the right of Table 2. In a 2400 kcal/d diet, 266 mg of fat is 0.10 en%, and a standard 1-g fish oil supplement is equivalent to 180 mg 22:6 + 120 mg 20:5, i.e., ~0.11 en% n-3 HUFA per capsule.

Dietitians who know the importance of voluntary food choices can use their familiarity with nutrient tables and the new interactive software on essential fatty acids to help people choose healthy combinations of palatable foods that will fit their personal tastes and meet their personal target for the balance of tissue HUFA. The interactive software combines the selected foods into daily meal plans, tracking the calories and estimating the probable proportions of n-6 HUFA in the total tissue HUFA, which can vary from 25 to 85%. The American Heart Association has recommended at least two meals per week of fatty fish to help provide better tissue balance. Careful attention to raising the relative amount of n-3 to n-6 contents of salad and cooking oils is another easy step in shifting tissue HUFA to a healthier balance. Also, discovering and eating the vegetables and legumes with relatively higher proportions of n-3 acids is made easier with a computerized "sort" command. All of the above aspects open the way for dietitians to lead in developing primary prevention strategies that decrease the frequency and severity of eicosanoid-mediated disorders for the whole population.

TABLE 2
Daily Dietary Intakes Affect Tissue HUFA[a]

Type of essential fatty acid in the diet	Trial diet	Typical diets		
		U.S.	Mediterranean	Japan
1 en% 18:3n-3	1.00	0.85	0.50	0.83
2 en% 18:2n-6	6.00	6.82	2.30	5.41
3 en% n-3 HUFA	0.10	0.03	0.09	0.71
4 en% n-6 HUFA	0.10	0.08	0.08	0.09
% n-6 in total HUFA	75	80	63	48

[a]For abbreviations see Table 1.10

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Fatty Acids, the Immune Response, and Autoimmunity: A Question of n-6 Essentiality and the Balance Between n-6 and n-3

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ABSTRACT: The essentiality of n-6 polyunsaturated fatty acids (PUFA) is described in relation to a thymus/thymocyte accretion of arachidonic acid (20:4n-6, AA) in early development, and the high requirement of lymphoid and other cells of the immune system for AA and linoleic acid (18:2n-6, LA) for membrane phospholipids. Low n-6 PUFA intakes enhance whereas high intakes decrease certain immune functions. Evidence from *in vitro* and *in vivo* studies for a role of AA metabolites in immune cell development and functions shows that they can limit or regulate cellular immune reactions and can induce deviation toward a T helper (Th)2-like immune response. In contrast to the effects of the oxidative metabolites of AA, the longer-chain n-6 PUFA produced by γ-linolenic acid (18:3n-6, GLA) feeding decreases the Th2 cytokine and immunoglobulin (Ig)G1 antibody response. The n-6 PUFA, GLA, dihomo-γ-linolenic acid (20:3n-6, DHLA) and AA, and certain oxidative metabolites of AA can also induce T-regulatory cell activity, e.g., transforming growth factor (TGF)-β-producing T cells; GLA feeding studies also demonstrate reduced proinflammatory interleukin (IL)-1 and tumor necrosis factor (TNF)-α production. Low intakes of long-chain n-3 fatty acids (fish oils) enhance certain immune functions, whereas high intakes are inhibitory on a wide range of functions, e.g., antigen presentation, adhesion molecule expression, Th1 and Th2 responses, proinflammatory cytokine and eicosanoid production, and they induce lymphocyte apoptosis. Vitamin E has a demonstrable critical role in long-chain n-3 PUFA interactions with immune functions, often reversing the effects of fish oil. The effect of dietary fatty acids on animal autoimmune disease models depends on both the autoimmune model and the amount and type of fatty acids fed. Diets low in fat, essential fatty acid deficient (EFAD), or high in long-chain n-3 PUFA from fish oils increase survival and reduce disease severity in spontaneous autoantibody-mediated disease, whereas high-fat LA-rich diets increase disease severity. In experimentally induced T cell-mediated autoimmune disease, EFAD diets or diets supplemented with long-chain n-3 PUFA augment disease, whereas n-6 PUFA prevent or reduce the severity. In contrast, in both T cell- and antibody-mediated autoimmune disease, the desaturated/elongated metabolites of LA are protective. PUFA of both the n-6 and n-3 families are clinically useful in human autoimmune-inflammatory disorders, but the precise mechanisms by which these fatty acids exert their clinical effects are not well understood. Finally, the view that all n-6 PUFA are proinflammatory requires revision, in part, and their essential regulatory and developmental role in the immune system warrants appreciation.

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The purpose of this overview and analysis was to bring together the immunologic, biochemical, metabolic, and nutritional aspects relevant to the role and significance of n-6 and n-3 polyunsaturated fatty acids (PUFA) in the immune system and autoimmune disease. A general overview of the immune system, particularly in relation to those areas that are most relevant to fatty acids and related lipids, is included for readers who are generally unfamiliar with the field and for those who simply require updating. It also provides the basis for integrating the nutritional and biochemical aspects of fatty acids and related lipids with the immunology.

OVERVIEW OF THE IMMUNE SYSTEM

The immune system is a complex set of interactive cells and molecules that has evolved to maintain the internal environment, particularly against invading microbes. Innate or nonadaptive immunity includes the antimicrobial components of body fluids, e.g., the bactericidal action of lysozyme, physical barriers such as skin, complement proteins, and internal defensive cells, which include mononuclear (e.g., macrophages), polymorphonuclear (e.g., neutrophils) phagocytes, and natural killer (NK) cells. Phagocytic cells mediate the killing of micro-organisms through engulfment, oxygen-

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; APC, antigen-presenting cells; CREAE, chronic relapsing EAE; DHA, docosahexaenoic acid; DHLA, dihomo-γ-linolenic acid; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis; EFAD, essential fatty acid deficiency; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; IL, interleukin; INF, interferon; LA, linoleic acid; LFA, leucocyte function-associated antigen; LT, leukotriene; MHC, major histocompatibility complex; NK, natural killer; PG, prostaglandin; PHA, phytohemagglutinin; RA, rheumatoid arthritis; ROI, reactive oxygen intermediates; SLE, systemic lupus erythematosus; TcR, T cell antigen receptor; TGF, transforming growth factor; Th, T helper; TNF, tumor necrosis factor.

dependent killing, and digestion. They are made more efficient in host defense through the sequential activation of a cascade-like event of serum proteins known as the complement system. Components of the complement system assist in the clearance of antigens by direct killing (lysis), attraction of, and recognition by phagocytic cells (chemotaxis and opsonization). The proinflammatory cytokines, e.g., tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) produced by mononuclear phagocytes and other cell types in response to injury and infection, which can also be produced by immune-mediated activation, induce the acute phase response and immune cell trafficking to the site of injury (1). Specific (or acquired) adaptive immune responses are characterized by specificity of immunoglobulins (antibodies) and lymphocytes as well as memory. Immunoglobulins also interact with the innate system by coating the surfaces of microorganisms so that specific cells can recognize and eliminate them. Central to adaptive immune responses is the activation of clones of lymphocytes that specifically recognize and bind antigen through receptors, thus providing the basis for the molecular shape identification of antigens. Lymphocytes can be grouped into T- and B-lymphocyte populations. Membrane immunoglobulin is the B-lymphocyte (B cell) antigen receptor, and these cells are able to secrete immunoglobulins (antibodies) with identical antigen specificity to that of its surface receptor. Immunoglobulins can be divided into various classes and subclasses, e.g., in humans, IgM, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, and IgE. Individual B cells can change the class of antibody they produce and may secrete more than one class at the same time. Class switching of immunoglobulins can also occur during the development of an immune response. Some B cells are able to recognize antigen in its native form (T-independent) but most require T-lymphocyte (T cell) help (T-dependent).

The antigen receptor on T cells (TcR) is related to but distinct from immunoglobulin and consists of two different polymorphic chains that are associated with the cell surface molecule CD3 (a complex of polypeptides involved in cell activation), which forms the TcR/CD3 complex (2). After primary exposure to antigen, a specific antibody or T-cell response develops; on secondary exposure to the same antigen, marked enhancement of the specific response is observed. In contrast to B cells, T cells recognize antigen only when it is presented in association with molecules known as the major histocompatibility complex (MHC). T cells can be divided into $CD4^+$ T cells and $CD8^+$ T cells. $CD8^+$ T cells recognize antigen in association with MHC class I molecules, which are found on all nucleated cells; they are primarily responsible for cytotoxic killing of virally infected cells as well as transplant rejection and can mediate suppression of immune responses. $CD4^+$ T cells recognize antigen in association with class II MHC molecules; they generally provide T-cell help to immune responses and are central to immune regulation (2). Some $CD4^+$ T cells are important in cell-mediated reactions for the destruction of microbial and nonmicrobial antigens and are known as T delayed-type hypersensitivity (DTH) cells. Antigen presentation to $CD4^+$ T cells in an appropriate form is carried out by cells, termed antigen-presenting cells (APC), which process and express fragments (peptides) of antigen at the membrane with MHC class II molecules (2). In addition to correctly presented antigens and costimulatory molecule interaction (e.g., CD28/CD80), T cells require signalling from cytokines, a heterogenous group of proteins that mediate intercelluar signaling, which induce proliferation and differentiation (2).

In recent years, important new doctrines have emerged in T-cell biology which, as we shall see, are are significant to fatty acid biochemistry, metabolism, and nutrition in relation to the immune response. Mosman and Coffman (3) have characterized two different patterns of cytokine secretion by T cells, which give rise to different functional responses. T helper-1 (Th1) cells produce interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor-β (TNF-β), which are not synthesized by Th2 cells. In contrast, Th2 cells (but not Th1) produce IL-4, IL-5, IL-6, IL-10, and IL-13. Th1 cells enhance cell-mediated inflammatory activity and induce B-cell antibody-class switching to and production of immunoglobulin IgG2a, whereas Th2 cells synthesize cytokines that help B cells develop into IgG1 and IgE antibody-producing cells (4). Th2 cytokines can downregulate production of Th1 cytokines and vice versa (4). There are also T cells able to produce both Th1 and Th2 cytokines referred to as Th0 cells (4). Weiner (5) has further characterized a Th3 T cell subset that primarily produces transforming growth factor (TGF)-β, provides help for IgA production, and has potent immune and inflammatory-suppressive properties. Importantly, the overall balance of cytokine production by Th1, Th2, and Th3 cells affects the type of immune response generated (4). The Th cytokine pattern also depends on the dose and properties of antigens, site of exposure, and the environment in which the T cells are found, e.g., production of antigen-presenting cell-derived IL-12 can direct Th1 responses *via* induction of IFN-γ (4,6–8). The dietary fatty acid effect on the nutritional microenvironment of the cell should now also be included with the foregoing factors that influence Th cells and their cytokine pattern (9–11).

ESSENTIALITY OF n-6 PUFA FOR CELLS AND TISSUES OF THE IMMUNE SYSTEM

Essentiality of n-6 PUFA for immune cell functions. The late John Rivers discussed the many problems regarding the nutrient essentiality concept (12). Essential fatty acid (EFA) deficiency in relation to the immune system illustrates many of the fundamental problems in attributing the term essential to a nutrient. For example, on the one hand, we may observe a decrease in a particular immune function, whereas on the other, we may observe an increase in another immune function under exactly the same conditions of essential fatty acid deficiency (EFAD). Although this appears problematic from a nutritional perspective, it is fundamentally inappropriate to consider a particular immunologic function measured in iso-

lation as a proxy for the whole of the immune response. This can be illustrated by the difference in response to the same antigen depending on the immunologic compartment challenged, and probably accounts for the different functional data, compared with others, observed by Boissonneault and Johnston (13), for route of antigen challenge under conditions of EFAD. Furthermore, Jeffery *et al.* (14) have demonstrated differences in dietary fatty acid effects on immune functions depending on the tissue origin of the lymphocytes. Moreover, the immune system displays immune deviation (15) whereby there is preferential selection of a particular type of response, e.g., Th1 or Th2 (2,8). Therefore, a decrease in a particular response may not necessarily demonstrate immune deficiency or suppression but deviation. Lymphoid organs and tissues have (like the brain) a relatively fast and high degree of growth, expansion, and development both pre- and early postnatally (16,17). Growth and development of lymphoid tissues, the structural and in some cases functional integrity of lymphoid cells (T and B cells) and tissues are severely affected under conditions of EFAD (13,18–20). These effects can be attributed specifically to the n-6 fatty acids linoleic acid (18:2n-6, LA) and its longer-chain derivative arachidonic acid (20:4n-6, AA) because of the associated decrease in these tissue n-6 fatty acids without any change in n-3. Consistent with this view, AA, and to a lesser extent LA, are (unlike the n-3 fatty acids) major fatty acid components of lymphocyte membrane phospholipids (21–25). Moreover, $CD4^+$ T cells, which play a central and critical role in immunoregulation, have a high proportion of membrane AA (~25% of the total fatty acids) with, by contrast, very little (~4%) docosahexaenoic acid (DHA; 22:6n-3) (26). Other cells of the immune system (e.g., monocytes and macrophages) also have a high membrane phospholipid AA composition, ~20–25% of total fatty acids (22,27). Similarly, EFAD also gives rise to loss of functional integrity in these cells and in neutrophils, particularly chemotaxis and eicosanoid production (28–30).

There would be a high requirement for PUFA during the generation of an immune response in lymph nodes due to increased cell division and proliferation. This is in agreement with the finding that lymphocytes preferentially incorporate n-6 fatty acids (LA and AA) during growth and proliferation *in vitro* (24,31). On the other hand, the feeding of a n-3 fish oil-rich diet leads to enrichment of lymphocyte membrane phospholipids with long-chain n-3 fatty acids at the expense of n-6, particularly AA (32). The high macrophage membrane AA is linked in part to its prostaglandin production capacity (27); however, the role of AA in monocyte/macrophage and lymphocyte membranes, particularly $CD4^+$ T cells, is not fully understood. T cells do not appear to be able to produce prostaglandins and thromboxanes (33) to any great degree; thus, the high membrane AA composition may fulfill other essential functions. Interaction between the TcR and MHC class II molecules on antigen-presenting cells [the so-called "immunological synapse"; Dustin *et al.* (34)] may require a critical level of membrane AA for optimal function (perhaps analogous to DHA in the "neuronal synapse"). Arachidonate appears to affect lymphocyte membrane microviscosity (24,35), which could affect the affinity/number of TcR-MHC complexes, glycolipid-enriched membrane rafts, and/or TcR signal transduction, all of which can determine the nature and magnitude of the T-cell response (36,37). Support for an involvement of fatty acids in the TcR-MHC interaction comes from n-3 fatty acid fish-oil feeding studies in which Fujikawa *et al.* (38) demonstrated a functional decrease in antigen presentation. Importantly, much *in vitro* evidence exists for a regulatory role of AA oxidative metabolites on immune cell development and functions, including thymocyte and monocyte growth and differentiation (39–41), T cell proliferation and migration (33,42,43), lymph-node shutdown (44), Th1 and Th2 cytokine regulation (45–48), antigen-presenting cell functions (49,50), macrophage TNF-α, IL-1 and IL-12 regulation (51–54), and induction of T suppressor cells (55–60). Observations *in vivo* appear to corroborate some but not all of the reported *in vitro* effects of AA metabolites mentioned above (39,44,61–67). The overall impression from the *in vitro* and *in vivo* research is that oxidative metabolites of AA at concentrations in the lower physiologic range appear to augment or have no effect on immune functions, but at higher concentrations, they are inhibitory, particularly on lymphocyte functions. Furthermore, at the higher concentrations, there appears to be a shift in the Th cytokine balance toward a Th2-like profile. These studies suggest therefore that oxidative AA metabolites can serve to limit cellular immunological reactions and may act to deviate the immune response.

Thymus development and programming in relation to n-6 PUFA. The thymus occupies a central role in T-cell biology; it is the main source of all T cells and it is where T cells diversify and are programmed into an effective primary T-cell repertoire. During T-cell development in the thymus, inappropriate ligation (high affinity binding) of the antigen receptors (TcR-CD3 complex) on immature thymocytes ($CD4^+CD8^+$), i.e., by autoantigen, leads to cell death, whereas ligation of the TcR on mature T cells (plus costimulatory signals) results in proliferation (68). Similar mechanisms operate to eliminate immature autoreactive B cells in the bone marrow. There is a striking accretion of AA in the mouse thymus during early growth and development (Fig. 1). This finding is consistent with the developing thymus compositional data from both control rats and rats fed an EFA-rich milk diet (20), although this aspect was not the focus of the investigation and not recognized in the conclusions by the authors (20). This accretion of AA in the thymus (thymocytes) would also be consistent with Crawford's findings of placental AA enrichment of the fetus and the supply of AA *via* maternal milk (69–71). Lymphocyte phospholipid fatty acid studies in breast-fed human infants also appear to corroborate the foregoing view (72). Additionally, results presented by Catherine Field (ISSFAL 2002 Congress) show differences in $CD3^+$ (T cells) counts and IFN-γ production between infant formula without AA and DHA, compared with formula milk with AA

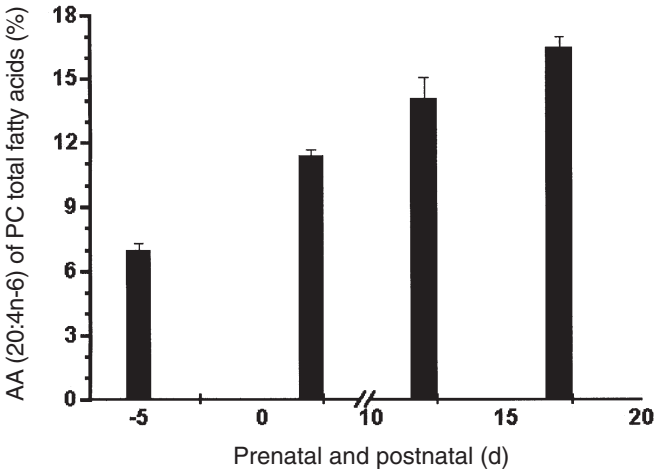

FIG. 1. Arachidonic acid (AA) accretion in the developing mouse thymus. Thymi were obtained from fetal mice at days 14–15 of pregnancy (prenatal; $n = 3$), and from neonate mice at days 2, 12, and 17–18 after birth (postnatal; $n = 3$–4/time point). The prenatal value was significantly different from the postnatal day 2 value ($P < 0.01$) and the postnatal day 2 value was significantly different from the postnatal day 18 value ($P < 0.01$). Values are means ± SD. Maternal dietary intake consisted of a standard rodent breeding diet. Thymus choline phosphoglyceride (PC) fatty acid composition was analyzed by previously described analytical methods (66,99). The accretion of linoleic acid (18:2n-6) in the developing mouse thymus was also found to follow a pattern similar to that observed for AA (Harbige, L.S., unpublished data).

and DHA, and also breast-fed babies. This could, in part, be consistent with the previously mentioned thymus/thymocyte AA accretion because breast milk contains AA (also DHA). However, caution must be exercised because other immunologically important factors such as growth factors and immune cells are found in breast milk, which could also account for the differences, and there would be differences in the antigenic nature of the different milks. Investigation of lymphocyte membrane AA in preterm babies deserves special attention in order to establish any link with functional immunological changes, because we and others have reported deficits in AA and other PUFA-related nutrients (73–75).

The full significance of the thymus accretion of AA is unclear, but there is *in vivo* and *in vitro* evidence to show that oxidative AA metabolites play an important role in thymocyte growth, differentiation, and apoptosis (41,76–78). It is possible therefore that AA accretion, in addition to growth requirements, is linked to thymic programming (education) in relation to autoreactive T cells and tolerogenic mechanisms and the subsequent development of autoimmune and allergic disorders (11,79). In summary, there is therefore much evidence for an essential role for n-6 fatty acids and their oxygenated metabolites in the lymphoid system but not for n-3 fatty acids. Further research is still required to characterize the precise effects of suboptimal and deficient n-6 fatty acid states on, for example, lymphocyte subpopulations and their cytokines and to establish clearly whether n-3 fatty acids might have an essential role.

AN EVOLUTIONARY PERSPECTIVE OF n-6 PUFA AND THE IMMUNE SYSTEM

As previously mentioned, the functions of the lymphocyte-based response are inhibited or limited by high AA oxidative metabolite concentrations. In contrast, however, they are potent mediators of the early (acute) inflammatory response, e.g., vasodilatation, chemotaxis, neutrophil responses, and platelet aggregation (80–82). In this way, n-6 fatty acids appear to have dual proinflammatory and immunosuppressive actions; this appears contradictory, but could explain much of the conflicting data in the literature (to be described later). We can perhaps better understand this from an evolutionary perspective (83). One of the early primordial roles for n-6 fatty acids (particularly oxidative metabolites of AA) at a metazoan level of organization would have been in the defensive response against injury (83). This would have involved signaling certain primitive but specialized cells to aggregate, and primitive phagocytic cells to move to the site of injury, with the subsequent plugging and sealing of the damage as well as phagocytosis of pathogens (if infection occurred) and repair. However, with the evolution of complex lymphocyte-based immune systems and adaptive immune responses to defend against, e.g., certain viruses, intracellular pathogens, and perhaps cancers (84), there is the problem of regulatory control of such systems. Early in lymphocyte-based evolution, the use of the already existent oxidative AA metabolite system (used in early simple inflammatory reactions) for the resolution of the lymphocyte response could have fulfilled such a function (with AA metabolites being further increased by activation of APC, which are more advanced phagocytic cells). This would be consistent with Morley's (85) original negative-feedback homeostatic mechanism, which proposed E-type prostaglandins (PGE) in the regulatory need to control lymphocyte reactivity. Current views on the physiological role of both PGE_2 and TGF-β (certain cells constitutively release these factors) as potent natural endogenous immune suppressor factors are also consistent with such an evolutionary view (TGF-β is also an evolutionary primordial molecule).

Interestingly, some cancer cells have utilized (evolved) this system to suppress local immune reactions, which might otherwise compromise their survival (86–88) and which further suggests that this form of regulation is both highly effective and has a long evolutionary history. Increased comparative research in acoelomate and primitive coelomate animals is required to better understand the early evolutionary history of fatty acids and other lipids in these early defense systems (83). From what has been described previously in this review, it is apparent that n-6 fatty acids and their oxidative metabolites play an important role in the immunoregulatory control of advanced immune systems. Evolutionary divergence and adaptation to a particular dietary pattern (e.g., herbivores and carnivores) may explain some of the differences between species in functional immunologic responses and differences in response induced by a change in dietary fatty acids. Fur-

thermore, in Eutheria mammals, the evolution of the placenta and mammary nutrition in relation to the delivery of specific fatty acids, as well as other factors for growth and development of the fetal and neonatal lymphoid system and transfer of protective immunity, is a fundamental evolutionary advance.

AUTOIMMUNITY, IMMUNE FUNCTIONS, AND n-6 PUFA

Effects of n-6 PUFA on immune cell functions. Addition of different n-6 fatty acids to lymphocyte cell cultures and their effects on T and B cell mitogen-induced lymphoproliferation demonstrate that low concentrations enhance whereas high concentrations inhibit these *in vitro* functions (9,89). The above inhibitory effects of n-6 fatty acids at high concentrations are independent of eicosanoids (42,90), while *in vitro* data indicate that antioxidants (including vitamin E) can (91) or cannot (92) reverse this effect. Moreover, some antioxidants are able to directly suppress T-cell proliferation *in vitro,* and certain n-6 fatty acids induce specific cytokine gene suppression, e.g., IL-2, or induction, e.g., TGF-β, which can inhibit T-cell proliferation (25,66,90,93). In animal feeding studies, differences between investigations such as mode and length of feeding, species differences, vitamin E stripped oil, tissue source of lymphocytes, and different cell culture conditions make it difficult to compare studies of the effects of LA intake on *ex vivo* lymphoproliferation (10,94). In most reports, however, high LA intakes suppress *ex vivo* lymphoproliferative responses in animal studies (19,95–98), whereas low intakes (above the minimal standard growth requirements for LA in laboratory animals) appear to enhance the response (19,99). Enhancement of the *ex vivo* lymphoproliferative response by low LA intakes (but higher than controls) probably represents enriched cellular supply of this fatty acid for membrane synthesis over and above that of control cultures where it may be in more limited supply. One exception to the above is found in rabbits, where there is no effect of high LA intake on lymphoproliferation (100); however, it is a species adapted to a herbivorous diet, rich in α-linolenic acid (18:3n-3, ALA), and therefore lymphocytes may have different requirements in this species. The latter is supported by the findings of Kelly *et al.* (101) showing that feeding of ALA-rich linseed oil enhanced the lymphoproliferative response in rabbits. Studies *in vivo* also support the inhibitory effects of a high dietary intake of LA on T-cell functions, as measured by the DTH test, which is an *in vivo* measure of T-cell function (102,103).

There are few reports on the effects of n-6 fatty acids on antibody production; nevertheless, two studies examining the effects of diets high in LA showed reductions in IgM and IgG (19,102). In *ex vivo* culture studies by Yamada *et al.* (104), high concentrations of LA and AA inhibited IgM, IgG, and IgA and increased IgE (Th2-like) synthesis and/or secretion. This would agree in part with other observations of the *in vitro* effects of PGE (derived from the latter precursor fatty acids) on Th1 and Th2 cytokine production (45,48). Our *in vivo* findings, however, appear to conflict with those of Yamada *et al.* (104), in that we found no effect on total IgG and a decrease in antigen-specific IgG1 antibody (but not IgG2a) production in mice, a Th2-like response (11). This apparent dichotomy probably reflects the level, metabolic, and functional differences of *in vitro* LA and AA vs. dietary GLA on antibody production, and the use of antigen as well as the complex differences between generating an immune response *in vivo* compared with *in vitro*. Therefore, more research is warranted to clarify the effects of n-6 PUFA on antibody production, particularly in relation to Th2 responses. Because antibody production in autoimmune disease is complicated by the underlying aberrant immune state, the effects of dietary n-6 fatty acids are discussed separately in this review.

In relation to allograft survival, results of experiments carried out by Peter Medawar, Jürgen Mertin, and Ruth Hunt (105) showing prolongation of allograft transplantion by LA administration appear inconsistent with those reported by Otto *et al.* (106). Differences in the allograft organ used and in background diet compositions between these studies may be significant. Moreover, the data of Otto *et al.* (106) indicate that prolonged allograft survival is related to the balance between n-6 and n-3 PUFA. Rossetti *et al.* (107) reported a reduction in T-cell proliferation with γ-linolenic acid (18:3n-6, GLA)-rich borage oil supplementation in humans. Supplementation with GLA-rich borage oil increases the proportions of the PGE_1 and PGE_2 precursor fatty acids dihomo-γ-linolenic acid (20:3n-6, DHLA) and AA, respectively, in human mononuclear cell membrane phospholipids (25). We found no significant effect on lymphoproliferative response to phytohemagglutinin (PHA) and anti-CD3, but supplementation did increase the production of PHA-stimulated peripheral blood mononuclear cell TGF-β_1 and decreased the production of the Th2 cytokines IL-4 and IL-10 (25,108). These findings concur with our additional findings on the effects of PGE_1 and PGE_2 on IL-4 and IL-10 secretion in human T-cell clones and our *in vivo* findings of decreased antigen-specific IgG1 antibody production in mice (11,109). Others have shown decreased Th1 cytokines with PGE_2 using murine and human T-cell clones (45,110); however, there are few *in vivo* data to support these *in vitro* findings at present (66). The *in vitro* differences may reflect the phenotype differences of the T-cell clones used, methods of activation, and the eicosanoid concentrations adopted. Reduction *ex vivo* in the secretion of IL-1 and TNF-α has been observed with oral administration of GLA-rich oil to volunteers and by the addition of GLA to *in vitro* cell cultures (111). In agreement with these findings, we have also found marked reductions in both of these proinflammatory cytokines with oral administration of GLA-rich oil in human studies (Harbige, L.S., Hollifield, R.D., Varney, E., and Sharief, M.K., unpublished data). In relation to NK cells, it appears that n-6 PUFA have little effect on their functional activity, which is in direct contrast to the effects of n-3 PUFA (9). In summary, low intakes of n-6 PUFA enhance, whereas high intakes can suppress, immune functions such as lymphoproliferation and DTH. GLA, DHLA, and AA can act to suppress Th2-like responses and can reduce

the production of the proinflammatory cytokines IL-1 and TNF-α. Figure 2 illustrates schematically the effects of n-6 PUFA and their eicosanoids on immune functions.

Autoimmune disease and n-6 PUFA. NZB × NZW F1 and MRL/lpr mice are useful spontaneous animal models of the archetype human autoimmune disease systemic lupus erythematosus (SLE) in which autoantibody mediates much of the pathology. The Th2 cytokines IL-4, IL-6, and IL-10 are proinflammatory in NZB × NZW F_1 mice, involving an antibody-mediated pathology (112). In contrast, in experimental autoimmune encephalomyelitis (EAE), a $CD4^+$ Th_1 cell-mediated autoimmune disease, IL-4 and IL-10 are associated with remission (113–115). In NZB × NZW F_1 autoimmune mice, high-fat LA-rich diets exacerbate the disease (116,117) or have no effect (118). Similarly, in MLR/lpr autoimmune-prone mice, a LA-rich diet also appears to enhance the disease or to have no effect (119–121). Lin *et al.* (117,122) found that a high-fat diet (equal amounts of lard and soybean oil, i.e., high-LA diet) induced a more severe disease and shortened the life span in NZB × NZW F_1 mice, which was associated with decreased Th1 and increased Th2 cytokine and autoantibody responses. These findings therefore indicate that a high LA intake may induce Th2-like responses, which are proinflammatory in these spontaneous autoimmune diseases. Conversely, in MLR/lpr autoimmune-prone mice, Godfrey *et al.* (121) observed a greater survival rate for mice supplemented with evening primrose oil (72% LA and 9% GLA). The above findings suggest that LA, the parent n-6 fatty acid, is unable to ameliorate antibody-mediated spontaneous autoimmune disease, whereas its desaturated n-6 fatty acid metabolites can. We found that administration of botanical sources of GLA was completely protective, depending on the dose, in the $CD4^+$ Th1 (IFN-γ, IL-2) cell-mediated diseases: experimental autoimmune encephalomyelitis (EAE), chronic relapsing EAE (CREAE), and experimental autoimmune uveitis (EAU) (66,99,123; Fig. 3). Natural recovery in EAE is mediated by expansion of suppressor (regulatory) lymphoid cells (124), some of which have been characterized as TGF-

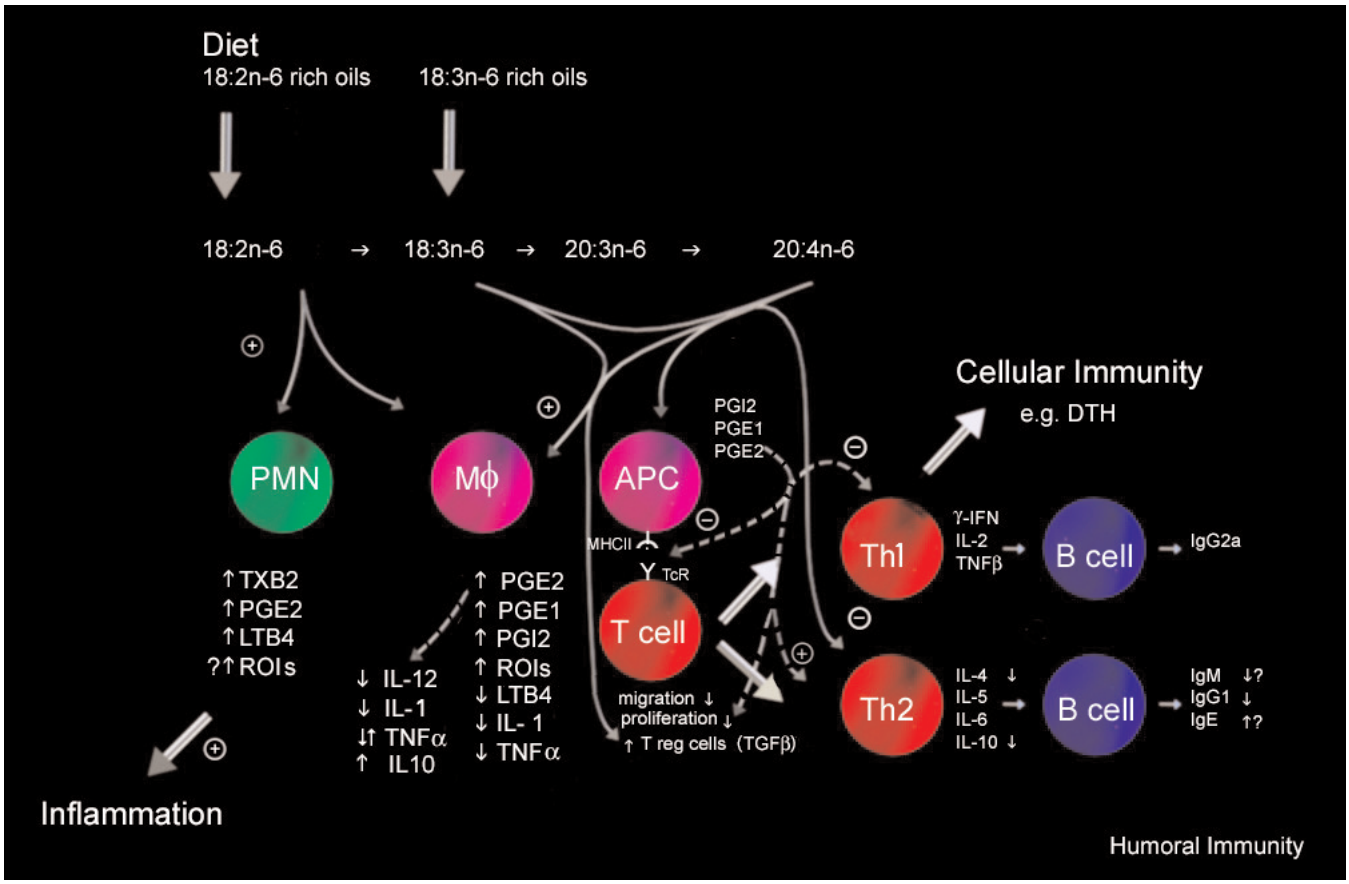

FIG. 2. The effects of n-6 polyunsaturated fatty acids and their eicosanoid metabolites on immune functions. Key: Polymorphonuclear cell (PMN), macrophage (Mϕ), antigen-presenting cell (APC), T helper-1 (Th1), T helper-2 (Th2). Major histocompatibility complex class II (MHC II), T-cell receptor (TcR), delayed-type-hypersensitivity (DTH), T-regulatory cells (T reg). Interleukin-1 (IL-1), -2, -4, -5, -6, -8, -10, and -12; tumor necrosis factor-α (TNF-α), tumor necrosis factor-β (TNF-β), interferon-γ (IFN-γ); transforming growth factor-β (TGF-β), prostaglandin E_1 (PGE_1), prostaglandin E_2 (PGE_2); prostacyclin (PGI_2), thromboxane A_2 (stable derivative TXB_2), leukotriene B_4 (LTB_4), leukotriene B_5 (LTB_5), reactive oxygen intermediates (ROI). Solid lines represent effects of fatty acids *in vivo* or *ex vivo,* and dashed lines represent the effects of eicosanoids established from *in vitro* studies. Importantly, some effects of fatty acids appear to be independent of those observed for eicosanoids studied *in vitro,* e.g,, *in vivo* and *ex vivo* studies showed that the longer-chain n-6 fatty acids decrease Th2 responses, whereas PGE_2 enhances Th2 cytokines and decreases Th1 cytokines *in vitro.* See text for details.

β–producing $CD4^+$ T cells by Karpus and Swanborg (125). Consistent with these findings, the protective effect of GLA-rich oil in EAE is linked to increased T-cell TGF-β transcription and production (66) (Fig. 4). In addition, PGE_2-producing monocytes (suppressor/regulatory monocytes) also appear to be involved (66), which is in keeping with the inhibitory effect of oxidative AA metabolites on lymphocyte functions mentioned previously.

In EAU, analysis of serum antibody to the autoantigen (S-antigen) showed that GLA treatment of animals resulted in significantly more IgG1 anti-S-antibody and less IgG2a anti-S-antibody than controls (total anti-S-antigen IgG levels were the same for both groups), indicating immune deviation toward a Th2-like response in this model (123). This Th2 deviating effect in EAU may be related to the high oil dose given in these particular experiments and the fact that the GLA-rich borage oil used contained a high level of LA as well as GLA. Antigen-specific lymphoproliferative responses were unaffected by n-6 fatty acid feeding in all of the above EAE and EAU studies, demonstrating that antigen presentation was not affected. Furthermore, the EAE and EAU findings clearly show overall that the protective effects of GLA, which bypasses the normally slow LA conversion rate and results in increased long-chain n-6 PUFA *in vivo*, are not due to gross immunosuppression. This is in contrast to long-chain n-3 fatty acid-rich fish oil feeding, which decreases MHC class II expression on dendritic and other antigen-presenting cells and produces a functional decrease in antigen presentation and T-cell proliferation (11,38,126,127). GLA feeding studies in both animals and humans have shown a decrease in the production of monocyte PGE_2 and leukotriene B_4 (LTB_4) and an increase in PGE_1 (128–130). The reduction of LTB_4 would be in keeping with an anti-inflammatory action, whereas increased production of PGE_1 and/or PGE_2, according to many of the studies cited earlier, has an inhibitory effect on lymphocyte functions. Calculation of phospholipid DHLA/AA ratios from data published by Fan and Chapkin (129) and our own data (66) reveals that it is this ratio that determines whether there is an overall greater production of PGE_1 or PGE_2. Thus, the lower ratio in our study predicts the greater level of PGE_2 production observed. The beneficial effects of GLA-rich borage oil on nonimmune neutrophil/monocyte-mediated inflammatory models linked with a concomitantly decreased LTB_4 (130) and the beneficial findings in T cell-mediated autoimmune disease linked with increased PGE_2 (66) are not, therefore, paradoxical findings. Similarly, under EFAD, T cell-mediated autoimmune disease is potentiated (131), whereas in NZB × NZW F_1 mice, it increases the survival rate (118). These findings probably reflect the loss of oxidative AA metabolites under EFAD (29,30). This would potentiate T cell-mediated autoimmunity, for which they would have inhibitory actions, but diminish neutrophil

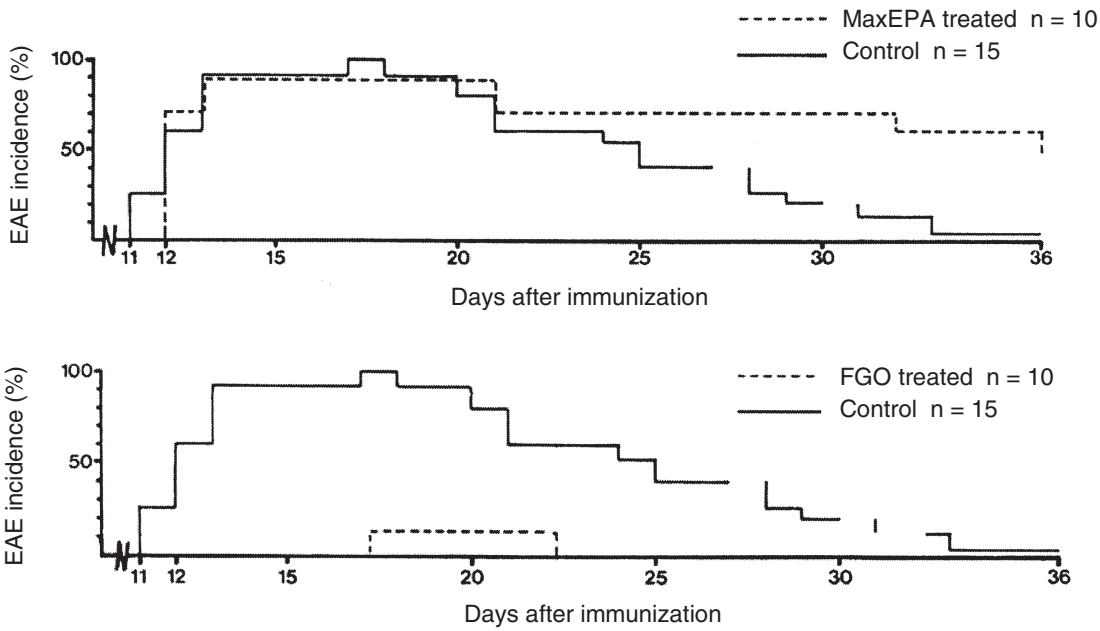

FIG. 3. Effect of orally feeding fish oil (MaxEPA) and fungal oil (FGO) on the clinical incidence of experimental autoimmune encephalomyelitis (EAE) in Lewis rats. Rats were sensitized (immunized) with neuroantigen (spinal cord homogenate) on day 0 and treated 7 d latter (until day 21) with EPA-rich fish oil (MaxEPA) at 500 mg/kg EPA, or γ-linolenic (GLA)-rich fungal oil (FGO) at 500 mg/kg GLA. There were significant differences in the course of the disease compared with control EAE values at day 28 ($P < 0.05$) and day 36 ($P < 0.025$) in the fish-oil-treated group, i.e., fish oil prolonged the disease course. Fungal oil markedly reduced the incidence of EAE compared with controls, i.e., full protection against clinical disease. Linoleic acid-rich safflower oil at 500 mg linoleic acid/kg body weight was without effect and importantly does not contain GLA. At higher doses, safflower oil reduced the clinical severity of EAE but not the incidence. There was also no difference between controls and linseed oil at 500 mg α-linolenic acid/kg. (See Refs. 99 and 175 for methodological details and additional data.)

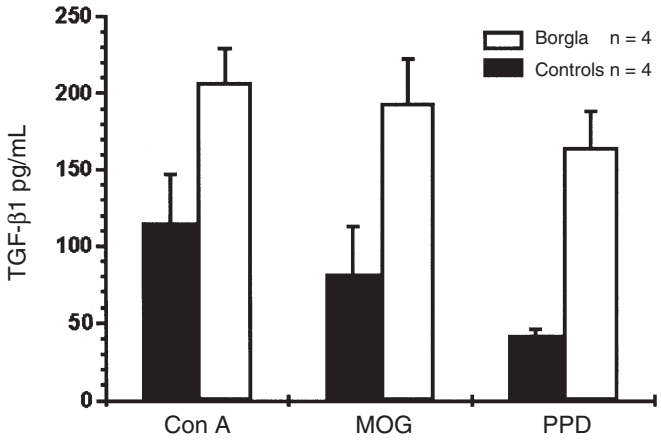

FIG. 4. Spleen mononuclear cell TGF-β1 production *ex vivo* in SJL mice fed borage oil (GLA-rich) and control mice with myelin oligodendrocyte glycoprotein (MOG)-induced EAE. Mice were gavaged daily with 250 µL of ultrarefined borage oil or saline fed from day 7 onward after induction of EAE. Cells were isolated from the spleens of borage oil (BORGLA)-treated and control EAE mice (n = 4/group) 21 d after disease induction. Borage oil-treated mice displayed no clinical signs of EAE. Cells were stimulated *ex vivo* with 1 µg/mL concanavalin A (Con A), 5 µg/mL of the encephalitogenic peptide (aa 92–106) of MOG and 5 µL/mL protein purified derivative (PPD) for 24 h. Culture supernatants were assayed for cytokine by enzyme-linked immunosorbent assay. Values are means ± SD. Significance of difference of borage oil-treated EAE compared with control EAE: for Con A, $P < 0.05$; MOG, $P < 0.05$; and PPD, $P < 0.05$. There was no detectable cytokine in response to a nonencephalitogenic MOG peptide (aa 50–64). For abbreviations see Figures 2 and 3. Adapted from Reference 66.

inflammatory responses in spontaneous autoantibody-mediated disease, in which these cells are important in the type II immune complex-mediated pathology (132).

Consistent with the foregoing explanations and coupled with a LA-induced Th2-like response under autoimmune (autoantibody) conditions, as mentioned earlier, high LA-rich diets accelerate the disease course in NZB × NZW F_1 mice; conversely, there is a protective effect of LA on T-cell autoimmune disease. This would explain many of the paradoxical observations of dietary n-6 fatty acid effects on the different autoimmune disease models. High LA intake therefore appears to have a proinflammatory role under certain autoimmune circumstances, whereas the longer-chain derivatives do not. In addition, there is evidence that LA can increase the level of reactive oxygen intermediates (ROI) (133) and that LA or its hydroperoxides can enhance the production of matrix metalloproteinases (134) and activate endothelial cell NF-κB (135). However, these effects are (135), or are likely to be, dependent on the vitamin E level. It is possible that LA may act in a proinflammatory capacity when it is in excess of requirements as a membrane structural fatty acid and there is an associated vitamin E insufficiency (135– 138). Several human disorders involving an autoimmune-inflammatory pathogenesis appear to be clinically responsive to certain n-6 fatty acids. Beneficial clinical effects of LA supplementation have been reported in multiple sclerosis (139) and in atopic eczema patients (140). In addition, GLA supplementation has been shown to have a degree of clinical effectiveness in rheumatoid arthritis (141–143), atopic eczema (140), and possibly Sjogren's syndrome (141). On the basis of the animal model evidence described previously, it would be interesting to investigate the effects of GLA supplementation in human SLE. Mechanisms by which n-6 fatty acids exert clinical effects in these disorders are not well understood and could include fundamental links between immunological dysregulation and a disturbed n-6 metabolism (144,145).

In summary, LA has been shown to be protective in experimental T cell-mediated disease, whereas high-fat LA-rich diets increase disease severity in experimental autoantibody-mediated disease. However, the desaturated/elongated metabolites of LA are protective in both T cell- and antibody-mediated experimental autoimmune disease. A Th2 deviating effect may be behind the augmentation of autoantibody-mediated disease by high LA, whereas expansion of regulatory T-cell and TGF-β production in addition to PGE-mediated effects appears important in the protective effects of GLA, DHLA, and AA. Although limited data on the effects of n-6 PUFA in human autoimmune disease exist at present, there is evidence of clinical benefit in multiple sclerosis and rheumatoid arthritis.

The proinflammatory n-6 PUFA paradox. It has generally been thought that n-6 fatty acids are proinflammatory based on several lines of evidence. These include (i) the enhancement of certain autoimmune diseases, as mentioned previously; (ii) the association of plasma n-6 fatty acid levels with certain inflammatory diseases; and (iii) the classic role of membrane phospholipid-liberated AA as a precursor to cyclooxygenase and lipoxygenase products. These oxygenated products are important because of their potent vasoactive and chemotaxic properties. Paradoxically, despite this suggested proinflammatory background, the n-6 fatty acids have been reported to have protective effects in *both* immune-mediated and nonimmune-mediated inflammatory diseases as described in this review (99,121,130,146). Furthermore, studies that purport to show simply increased AA levels in plasma in certain inflammatory disorders and therefore, by this definition alone, a proinflammatory state, cannot be taken as unequivocal proof that n-6 fatty acids are proinflammatory. It is evident that plasma AA levels (both esterified and nonesterified) in several inflammatory disease states show far from consistent results and can demonstrate either high or low or no change in AA (147–151). Moreover, the concentration of plasma phospholipid AA can be inversely associated with clinical evidence of inflammatory activity (150). It is clear from the examples described previously and others (99, 152,153) that GLA, DHLA, and AA do not reflect the functions of LA and vice versa. Some of the pharmacological mediators derived from free AA can clearly be classified as proinflammatory, but the view that all n-6 fatty acids are therefore proinflammatory is inappropriate and our views have to be reconciled with the evidence outlined in this overview and analysis.

AUTOIMMUNITY, IMMUNE FUNCTIONS, AND n-3 PUFA

Effects of n-3 PUFA on immune cell functions. The addition of different n-3 fatty acids to lymphocyte cell cultures and their effects on T- and B-cell mitogen-induced lymphoproliferation indicate that low concentrations enhance, whereas high concentrations inhibit, these *in vitro* functions independently of eicosanoids (9,42,89). Diets high in long-chain n-3 fatty acids from fish oil suppress *ex vivo* lymphoproliferative responses in both animal and human studies [see Calder (9)]. Compared with high dietary intakes, low fish oil intake was reported by Hinds and Sanders (154) to increase cell-mediated immunity in mice, as measured *in vivo* (host vs. graft response). In addition, a low intake of fish oil plus additional vitamin E increased the *ex vivo* lymphoproliferative response in rats (155). Furthermore, Wu *et al.* (156) observed increased peripheral-blood lymphocyte responses to mitogens in primates fed eicosapentaenoic acid (EPA; 20:5n-3) and DHA-rich diets, without any change in the level of circulating vitamin E, suggesting that in the presence of adequate vitamin E, lymphoproliferative responses can be enhanced. Because supplementation with vitamin E has a stimulatory effect on the $CD4^+$ T cell count and the lymphoproliferative response (157), it is difficult to differentiate effects of vitamin E from the effects of long-chain n-3 fatty acids in the above studies. In rats fed a high fish oil diet, Yaqoob *et al.* (98) found no difference in the proportion of $CD4^+$ and $CD8^+$ T cells. In humans, however, fish oil supplementation decreased the relative percentage of peripheral blood $CD4^+$ T cells and decreased the DTH response, i.e., an *in vivo* measure of T-cell function (158). Similar findings have been reported by Wu *et al.* (156) in monkeys and by Bell *et al.* (159) in HIV-infected patients. In agreement with the previous observations, Robert Chapkin (ISSFAL 2002 Congress) reported that $CD4^+$ T cells are susceptible to long-chain n-3-induced apoptosis. Conversely, we found no effects of LA- or GLA-rich plant oils on $CD4^+$ and $CD8^+$ T cells in experimental animal and human studies (25,99,108). There are few definitive studies of the effects of n-3 fatty acids on antibody production. One study showed increased IgG and IgE to ovalbumin in rats fed a high fish oil diet (160); conversely, we found a marked decrease in antigen-specific IgG1 (Th2-like), and to a lesser extent Ig2a (Th1-like), in mice fed a high fish oil diet (11). The latter finding would be consistent with the effects of n-3 fatty acid-rich fish oils on autoantibody in spontaneous autoimmune animal models, but will be discussed later in this review because the preexisting immunologic disorder makes comparisons difficult. It is also conceivable that there are other long-chain n-3 fatty acid effects on B cells, in addition to changes in cytokine-induced effects, such as increased apoptosis (11).

The expression of several adhesion molecules has been investigated in both fish oil feeding studies and in *in vitro* culture experiments with EPA and DHA (89,126). In general, stimulated and unstimulated lymphocytes and/or monocytes from feeding studies display reduced expression of CD54 (intercellular adhesion molecule-1), CD58 (leucocyte function-associated antigen-3, or LFA-3), CD62L (L-selectin), CD2, with inconsistent information regarding CD11a (LFA-1) (89,126,161,162). Pretreatment of endothelial cells with DHA decreased the functional adhesion of lymphocytes to stimulated and unstimulated endothelial cells *in vitro* (162). Despite doubts over the importance of small changes in the expression of some of these surface molecules induced by long-chain n-3 fatty acids (162), it is likely that lymphocyte and monocyte trafficking will be altered by feeding these fatty acids under both normal and inflammatory conditions. Some of these molecules are also involved in costimulatory functions; therefore, aspects other than adhesion, such as the immune synapse, may also be affected, and there is *in vitro* evidence that DHA and EPA can inhibit the CD28-lymphocyte activation pathway (163). As mentioned previously, fish oil feeding can reduce MHC class II expression on dendritic and other APC and decrease functional antigen presentation. The effects of fish oil supplementation have been studied extensively on *ex vivo* human peripheral blood mononuclear cell cytokine production, particularly IL-1α, IL-1β, IL-2, IL-6, IL-8, and TNF-α, showing decreased production of these proinflammatory cytokines [see Calder (89)]. There are, however, conflicting studies in animals in which production of TNF-α and IL-1α was increased with fish oil feeding [see Blok *et al.* (164)] and studies in humans in which no effects on these cytokines were observed with fish oil feeding [see Calder (89)]. These findings may reflect the site of cell origin, species differences, mode of elicitation/activation, cytokine gene polymorphisms, or diet component differences, e.g., vitamin E. Furthermore, the University of Southampton group (ISSFAL 2002 Congress) reported that combining dietary antioxidants (vitamins A, E, C, and selenium) with fish oil reversesd the effects of a fish-oil-only-induced decrease in IFN-γ and lymphoproliferation. This may be an effect mediated by an inhibition of long-chain n-3 fatty acid peroxidation. However, vitamin E can stimulate IFN-γ production (157); thus, like the lymphoproliferative response mentioned earlier, vitamin E can have effects that are independent from long-chain n-3 fatty acids. This further highlights the complex interactions among the long-chain n-3 PUFA, vitamin E, and immune functions.

Some but not all studies [see Calder (89)] have shown that fish oil feeding can induce an appreciable increase in the stimulated production of ROI, e.g., superoxide and nitric oxide (NO) from mononuclear phagocytes (133). Furthermore, both the proliferation of Th1 cells and their production of IL-2 and IFN-γ are inhibited by NO, whereas Th2 cells are unaffected (165). This may be one explanation for the apparent reversal of effects with antioxidants reported by the University of Southampton group and may explain some of the reported effects of long-chain n-3 fatty acid-rich fish oil on Th1 and Th2 responses. Several studies in rodents have noted that the feeding of linseed oil-rich in ALA and fish oil-rich diets suppresses NK cell activity (9). Whether this has an effect on host resistance to certain viral infections or tumor cell growth is unclear at

present, but may be relevant to several reports of increased susceptibility to certain infections with fish oil feeding, which will be described later in this review. In this section, it is clear that high intakes of long-chain n-3 PUFA, particularly in the form of fish oils, suppress a wide range of immune variables such as lymphoproliferation, $CD4^+$ cells, antigen presentation, adhesion molecule expression, Th1 and Th2 responses, and proinflammatory cytokine and eicosanoid production. Some of these effects with high n-3 PUFA appear to be reversed by vitamin E, whereas limited evidence indicates that low intakes of n-3 PUFA can enhance certain immune functions. Figure 5 illustrates schematically the effects of high intakes of n-3 PUFA on immune functions.

n-3 PUFA, autoimmune disease, and infection. Fish oils rich in long-chain n-3 fatty acids increase longevity and delay the onset of clinical manifestations of autoantibody-mediated disease in NZB × NZW F_1 and MRL/lpr mice (119,121,166). Compared with corn oil feeding, fish oil delays the onset of autoimmune disease in NZB × NZW F_1 mice; this is associated with elevated IL-2 and IL-4, increased TGF-β_1, and lower c-myc and c-ras in the spleen as well as reduced autoantibody production (167). The same authors also reported high kidney TGF-β_1 mRNA for corn oil-fed and low kidney TGF-β_1 mRNA expression for fish oil-fed NZB × NZW F_1 mice, the reverse of their findings in the spleen (167). This finding may be important for the renal disease normally observed in these mice and also demonstrates that dietary fatty acids can regulate gene expression in an organ-specific manner (167,168), which would be relevant to the observations of site-specific differences in lymphocyte functions induced by dietary fatty acids mentioned earlier. Fernandes *et al.* (169) also showed that dietary fish oil feeding in NZB × NZW F_1 mice increases programmed cell death of lymphocytes, which may therefore prevent the accumulation of self-reactive immune cells in lymphoid organs. This increased apoptosis of lymphocytes (169) in fish oil-fed mice appears to be the result of increased oxidative stress induced by highly peroxidizable long-chain n-3 fatty acids (170). In a fish oil study in MRL/lpr mice, fish oil plus vitamin E was found to have a synergistic effect in delaying the onset of disease (171). This could indicate that peroxide-mediated mechanisms are not directly involved in the protective effects observed with fish oil and that vitamin E directly or indirectly alters the effect of fish oil on the

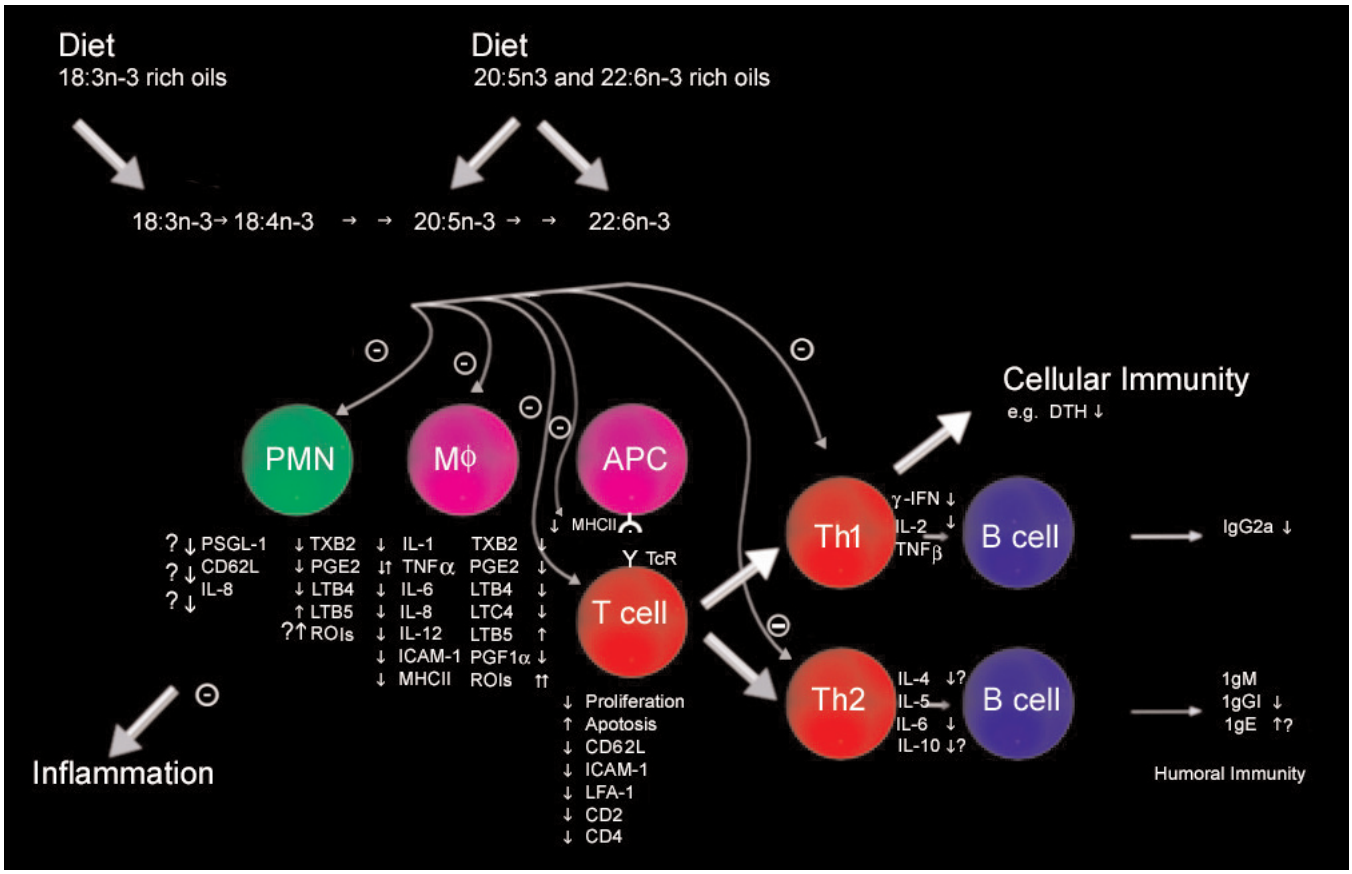

FIG. 5. The effects of high intakes of n-3 polyunsaturated fatty acids on immune functions. Key: Polymorphonuclear cell (PMN), macrophage (Mϕ), antigen-presenting cell (APC), T-helper1 (Th1),Th2. Major histocompatibility complex class II (MHC II), T-cell receptor (TcR), delayed-type-hypersensitivity (DTH), T-regulatory cells (T reg). Clusters of differentiation (CD markers), CD2, CD4, CD62L (L-selectin), leucocyte function antigen-1 (LFA-1), intercellular adhesion molecule-1 (ICAM-1), PSGL-1 (a mucin-like adhesion molecule). Interleukin-1 (IL-1), -2, -4, -5, -6, -8, -10, and -12; tumor necrosis factor-α (TNF-α), TNF-β, interferon-γ (IFN-γ); prostaglandin E_1 (PGE_1), PGE_2; prostacyclin (PGI_2) and its stable product $PGF_{1\alpha}$, thromboxane A_2 (stable derivative TXB_2), leukotriene B_4 (LTB_4), LTB_5, LTC_4, reactive oxygen intermediates (ROI). Arrows depicting an increase or decrease in relation to cell surface molecules indicate expression; for cytokines and eicosanoids, they indicate production/secretion. See text for details.

immune response. Interestingly, fish oil plus vitamin E further reduces the decreased production of proinflammatory LTB_4 and thromboxane B_2 from polymorphonuclear leucocytes induced by feeding fish oil alone (172). This demonstrates the importance of the interaction between vitamin E and long-chain n-3 fatty acids and inflammatory functions.

The effects of n-3 fish oil fatty acids in experimentally induced T cell-mediated models of autoimmune arthritis appear to conflict. In mice, Leslie *et al.* (173) found that fish oil protected against experimental collagen-induced autoimmune arthritis, whereas in rats, fish oil was reported to augment the disease (174). We found that fish oil feeding augmented EAE in rats (175) (Fig. 3). However, suppression of EAE using lower doses of fish oil has been reported by Mertin (176), although the animals developed more severe clinical disease after discontinuation of treatment. The mechanisms involved in fish oil-induced augmentation of experimental T cell-mediated autoimmune disease are not well understood. Increased severity and delayed recovery induced by fish oil feeding in EAE (175; Fig. 3) may be the consequences of inhibition of the proliferative expansion of regulatory T cells, which, as previously explained, normally mediate recovery in EAE and/or a decrease in PGE_2 from regulatory monocytes (11). All of the above findings demonstrate a wide range of fish oil-induced immune inhibition, which is consistent with their effects in prolonging graft survival (177) and delaying the onset of autoantibody-mediated autoimmune disease (119,121,166,167). These could, however, also be undesirable long-term effects of high-dose fish oil, compromising host immunity.

The generalized immunosuppressive effects of high fish oil intake are also further evidenced by *in vivo* reports of impaired host resistance to a range of bacterial, viral, and fungal infections (178–182). Fritsche *et al.* (183) showed that fish oil reduces IL-12 and IFN-γ production during the early phase of *Listeria monocytogens* infection in mice, factors known to be important in host defense against *Listeria*. Conversely, there are studies showing protective effects of fish oil feeding in certain bacterial (184,185), viral (186), and parasitic infections, particularly malaria (187,188), and to endotoxin (189). However, in some of these studies, the protective effects of fish oil are observed only in the presence of a vitamin E-deficient diet (188). Again, this demonstrates the importance of vitamin E in relation to the interaction of long-chain n-3 fatty acids and immune functions. Further work is required on the effects of fish oil feeding in viral infections in which $CD8^+$ T cells, neutralizing antibodies, and expression and function of MHC class I molecules are all important immunologic factors involved in host resistance and clearance of infection. Future research must clearly link immunologic mechanisms with fish oil-induced resistance or susceptibility to infection, and the role of vitamin E in such interactions. Fish oil feeding increases the leukocyte phospholipid EPA/AA ratio, which correlates with a decreased production in the proinflammatory lipid mediator LTB_4 and an increase in the less proinflammatory lipid mediator LTB_5 in animal and human studies (190–193). This latter finding may account for much of the clinical effectiveness of fish oil in rheumatoid arthritis (RA) because fish oil reduces the production of LTB_4 from neutrophils, and the number of tender joints correlates with decreased neutrophil LTB_4 production (194–197). This suggests that it is the secondary inflammatory consequences of immune activation that are modulated by fish oil in RA, whereas the underlying immune dysregulation may not be affected, as evidenced by no effect on immunologic parameters such as rheumatoid factor (194,196). Beneficial clinical effects of fish oil supplementation in Crohn's disease (198), IgA nephropathy (199), and inflammatory skin diseases (200,201) have all been reported. In addition, although some fish oil supplementation studies have been equivocal in SLE, a study by Walton *et al.* (202) found clinically significant improvements in SLE patients following a low-fat diet plus fish oil. Despite clinically important observations, the mechanisms by which fish oils exert their effects in such studies are not well characterized but are likely to include effects on autoantibody production, T-cell proliferation, apoptosis of autoreactive lymphocytes, and reduced immune-mediated proinflammatory cytokine (e.g., TNF-α, IL-1) and leukotriene (e.g., LTB_4) production.

In summarizing this section on n-3 PUFA, autoimmune disease, and infection it is clear that many of the observations made on the effects of these fatty acids on immune functions in health are relevant to the effects of these fatty acids observed in infection and autoimmunity. Much evidence exists, consistent with the generalized immunosuppressive effects of high fish oil intakes, demonstrating impaired resistance to certain experimental infections. However, there is also a good deal of evidence for a protective effect of fish oils in other experimental infections. The immunological mechanisms behind this apparent paradox are unclear, but vitamin E in some cases is important in this interaction. Consistent with their immunosuppressive and anti-inflammatory effects, long-chain n-3 PUFA from fish oils increase the survival and reduce the severity of spontaneous autoantibody-mediated disease in animal models. However, n-3 PUFA can exacerbate experimental T cell-mediated autoimmune disease, although the mechanisms are not well understood. There is good evidence for clinically beneficial effects of long-chain n-3 PUFA in human autoimmune/inflammatory diseases such as RA, SLE, and Crohn's disease, although the primary mechanisms of action and the long-term effects of high-dose fish oil warrant further investigation.

FATTY ACIDS, AUTOIMMUNITY, EPIDEMIOLOGY, AND AGING

The striking effect on susceptibility and resistance to autoimmune disease in animal models and the clinically beneficial reports of n-6 or n-3 fatty acids in established human autoimmune/inflammatory disease raise the possibility of what the effects of dietary fatty acids might be on these disorders in different human populations. For example, what effect might there be on specific autoimmune diseases after migration from an area of low-fat consumption to an area of high-

saturated LA-rich fat consumption? In addition to new viral exposure and immunogenetic considerations, the relatively low incidence of SLE in West Africans (203) but more common occurrence in black Americans descended from West Africans, compared with white Americans in the United States (204), may be linked to a high-fat LA-rich diet. There is convincing epidemiological evidence for a protective role of n-6 and n-3 PUFA in multiple sclerosis (205,206), which is considered an inflammatory/autoimmune disease of the central nervous system, and dietary fatty acids and antioxidants in RA (207,208). In addition, there is a low prevalence of autoimmune/inflammatory diseases recorded in Greenland Eskimos (209) consuming a high-seafood diet containing oil rich in long-chain n-3 PUFA. Interestingly, there is an early immunologic, possibly autoimmune, involvement in atherosclerosis (210), a disease in which the effect of dietary fats is well documented. Clearly more epidemiological research is required to clarify further the effects of dietary fats, particularly n-6 and n-3 PUFA, on human autoimmune/inflammatory disease.

There is an age-related effect on the immune system, e.g., loss of immune function, and the onset of many autoimmune phenomena, e.g., autoantibodies, is linked to increasing age (211–217). Laganiere and Fernandes (22) reported that the age-related decrease in mitogenic proliferative responses of spleen lymphoid cells is associated with increased long-chain PUFA and decreased LA in rats. Interestingly, the same fatty acid changes were observed for bone marrow cells (B cells) but not for thymocytes (T cells), which had the reverse pattern (22). Similar age-related increases in the long-chain n-3 and n-6 PUFA composition of lymphocyte membranes have been reported in human studies (218), and it has been demonstrated that the decline of mitogen responsiveness during human aging is correlated with dysregulated lymphocyte membrane lipid and fatty acid metabolism (219). The precise role of n-6 and n-3 PUFA in these age-related immune changes is unclear, but they may also be linked directly or indirectly to loss of immunoregulation and increased autoantibody production by B cells.

CONCLUSIONS AND PERSPECTIVES

Clearly, it is important not to compromise the essential developmental and immunoregulatory role of n-6 fatty acids, particularly AA for the immune system during early development. It may also be important to ensure that under certain autoimmune disease states, in which the tissue levels of n-6 fatty acids are reduced, they are maintained to provide for normal physiological immunoregulatory functions. High intakes of long-chain n-3 fatty acids (fish oils) in experimental animals are inhibitory on a wide range of immune functions and should therefore be classed as immunosuppressive, although there are still limited human data in this area at present. The effect of dietary fatty acids on animal autoimmune disease models depends on both the animal autoimmune model (T cell-mediated vs. autoantibody-mediated) and the amount and type of fatty acids fed. Diets low in fat, deficient in EFA, or high in n-3 fatty acids from fish oils increase survival and reduce disease severity in spontaneous autoantibody-mediated disease, whereas high-fat LA-rich diets increase disease severity. In experimentally induced T cell-mediated autoimmune disease, EFAD diets, or diets supplemented with fish oil n-3 fatty acids augment disease, whereas n-6 fatty acids prevent or reduce the severity of the disease. In contrast, in both T cell- and antibody-mediated autoimmune disease, the desaturated/elongated metabolites of LA (normally a slow conversion rate) are protective. Suppression of autoantibody and T-lymphocyte proliferation, apoptosis of autoreactive lymphocytes, and reduced proinflammatory cytokine and leukotriene production by high-dose fish oils are all likely mechanisms by which long-chain n-3 fatty acids ameliorate certain autoimmune diseases. The ability of long-chain n-3 fatty acids to control proinflammatory eicosanoids and cytokines does appear useful clinically in certain human autoimmune/inflammatory disorders. However, high fish oil intake may not be beneficial long term, i.e., it may compromise host immunity and may address only the secondary consequences of immune activation in some clinical conditions. Mechanisms by which various n-6 fatty acids are protective in T cell-mediated autoimmune disease include GLA-, DHLA-, and AA-sensitive immunoregulatory circuits involving PGE_1-, PGE_2-, and TGF-β_1-mediated effects, and possible Th2 inhibition in autoantibody-mediated disease. High LA intakes may act, under certain autoimmune conditions, *via* immune deviation toward a Th2-like humoral response and enhancement of neutrophil-mediated inflammation (as part of an immune complex-mediated pathology), thus potentiating autoantibody disease. Importantly, more rigorous nutritional and immunological studies are required to further test some of the above conclusions.

Moderate intake of n-6 PUFA (with LA and AA as the important n-6 in most human diets) and an appropriate ratio between n-6 and n-3 PUFA are therefore important in relation to the physiologic functioning of the immune system, whereas manipulation of the n-6 to n-3 ratio can be beneficial in autoimmune/inflammatory disease. A key question for the future will be: What is the optimal balance between n-6 and n-3 fatty acids that can ensure both an effective immunologic response against pathogens and satisfy tolerogenic responses? This balance may also be different during different periods of the life cycle, e.g., in early human development and in aging. A second question is: What is the optimal therapeutic n-6/n-3 balance for the treatment of autoimmune/inflammatory disorders with different underlying immune-mediated pathologies, e.g., in favor of n-3 PUFA in predominately autoantibody-mediated diseases such as SLE? It is likely that the best way to control the immune response is by manipulation of the molecules derived from the immune system itself, which act through natural pathways of immune inhibition. This principle should be a useful guide for further research in relation to fatty acid manipulation of the immune response. When investigating dietary fatty acid effects on immune functions, the interaction of fatty acids with other associated nutrients such

as vitamin E, in addition to the interactions of these nutrients themselves with the immune system, must be taken into account. Finally, fatty acids and eicosanoids are important factors in the evolution of both primitive and advanced defense systems; thus, further comparative research should give more insight into the evolutionary history of the immune system. From this overview and analysis, it is clear that future research should be fruitful in relation to fatty acids, the immune response, and autoimmune/inflammatory disease from basic biology to application in clinical medicine.

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n-3 Polyunsaturated Fatty Acids and Inflammation: From Molecular Biology to the Clinic

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ABSTRACT: The immune system is involved in host defense against infectious agents, tumor cells, and environmental insults. Inflammation is an important component of the early immunologic response. Inappropriate or dysfunctional immune responses underlie acute and chronic inflammatory diseases. The n-6 PUFA arachidonic acid (AA) is the precursor of prostaglandins, leukotrienes, and related compounds that have important roles in inflammation and in the regulation of immunity. Feeding fish oil results in partial replacement of AA in cell membranes by EPA. This leads to decreased production of AA-derived mediators, through several mechanisms, including decreased availability of AA, competition for cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, and decreased expression of COX-2 and 5-LOX. This alone is a potentially beneficial anti-inflammatory effect of n-3 FA. However, n-3 FA have a number of other effects that might occur downstream of altered eicosanoid production or might be independent of this effect. For example, dietary fish oil results in suppressed production of proinflammatory cytokines and can modulate adhesion molecule expression. These effects occur at the level of altered gene expression. Fish oil feeding has been shown to ameliorate the symptoms of some animal models of autoimmune disease and to protect against the effects of endotoxin. Clinical studies have reported that oral fish oil supplementation has beneficial effects in rheumatoid arthritis and among some asthmatics, supporting the idea that the n-3 FA in fish oil are anti-inflammatory. There are indications that the inclusion of fish oil in enteral and parenteral formulae is beneficial to patients.

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This article will briefly describe the nature of the inflammatory process, the role of n-6 PUFA- derived eicosanoids as mediators and regulators of inflammation, the effects of n-3 PUFA on eicosanoid and inflammatory cytokine production and on adhesion molecule expression, recent developments regarding n-3 PUFA and the expression of inflammatory genes, and the evidence that supports the use of n-3 PUFA in a range of inflammatory conditions.

INFLAMMATION IN HEALTH AND DISEASE

Inflammation is the body's immediate response to infection or injury. Its role is to begin the immunological process of elimination of invading pathogens and toxins and to repair damaged tissue. These responses must be ordered and controlled. The movement of cells into the inflammatory/infected site is induced by the upregulation of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selection on the surface of endothelial cells, allowing leukocyte binding and subsequent diapedesis. The activity of leukocytes is induced by certain triggers. One important exogenous trigger is bacterial endotoxin (also known as lipopolysaccharide or LPS), a component of the cell wall of gram-negative bacteria. LPS can directly activate monocyte/macrophages, inducing them to form cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and IL-8; eicosanoids, such as prostaglandin (PG)E_2; nitric oxide; matrix metalloproteinases (MMP); and other mediators. LPS also induces adhesion molecule expression on the surface of endothelial cells and leukocytes. The cytokines produced by monocyte/macrophages also serve to regulate the whole-body response to infection and injury (see Fig. 3 of Ref. 1). Thus, inflammation and the inflammatory response are part of the normal, innate immune response; inflammatory mediators also provide a link between the innate and acquired immune responses (see Fig. 3 of Ref. 1). However, when inflammation occurs in an uncontrolled manner, disease ensues. High levels of TNF-α, IL-1β, and IL-6 are particularly destructive. Chronic overproduction of TNF-α and IL-1 may cause muscle wasting and loss of bone mass. TNF-α, IL-1, and IL-6 are implicated in causing some of the pathologic responses that occur in endotoxic shock, in adult respiratory distress syndrome, and in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease.

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Abbreviations used: AA, arachidonic acid; COX, cyclooxygenase; FLAP, 5-lipoxygenase activating protein; GM-CSF, granulocyte macrophage colony stimulating factor; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; IκB, inhibitory subunit of NFκB; IκK, IκB kinase; IL, interleukin; LOX, lipoxygenase; LPS, lipopolysaccharide; LT, leukotriene; MMP, matrix metalloproteinase; NFκB, nuclear factor κB; PG, prostaglandin; PPAR, peroxisome proliferator-activated receptor; SIRS, systemic inflammatory response syndrome; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor; TX, thromboxane; VCAM-1, vascular cell adhesion molecule-1.

FA AND EICOSANOIDS

The FA composition of inflammatory cells. The link between FA and inflammation relates to the composition of inflammatory cell membrane phospholipids. This is because the FA composition of membrane phospholipids can influence various membrane activities, which can, in turn, influence cellular responses (Fig. 1). For example, the physical nature of the membrane, often referred to as fluidity, is regulated in part by the FA composition of its constituent phospholipids (2). Membrane fluidity affects the activity of membrane-bound proteins including receptors, transporters, and enzymes (3); thus, it can alter the responsiveness of inflammatory cells to a stimulus (for a review, see Ref. 4). Intracellular signals, such as DAG, inositol phosphates, and ceramide, are produced from membrane phospholipids in response to a suitable cell stimulus, and there is evidence that the ability of the phospholipase enzymes to generate these signaling molecules can be altered by the FA composition of the substrate phospholipids (for references, see Ref. 5). Another group of mediators, the eicosanoids, are generated from FA liberated from membrane phospholipids; the ability to produce these mediators is therefore strongly influenced by the FA composition of membrane phospholipids (i.e., substrate availability) (see below). Thus, the FA composition of inflammatory cells is important in terms of regulating the functional responses of those cells.

Inflammatory cells typically contain a high proportion of the n-6 PUFA arachidonic acid (AA; 20:4n-6) and low proportions of n-3 PUFA, especially EPA (20:5n-3). The exact proportion of AA in human inflammatory cells varies according to cell type and the lipid fraction examined (see, for example, Refs. 6,7). Increased consumption of fish oil, which is rich in long-chain n-3 PUFA such as EPA and DHA (22:6n-3), results in increased proportions of those FA in inflammatory cell phospholipids, partly at the expense of AA (see, for example, Refs. 6,7).

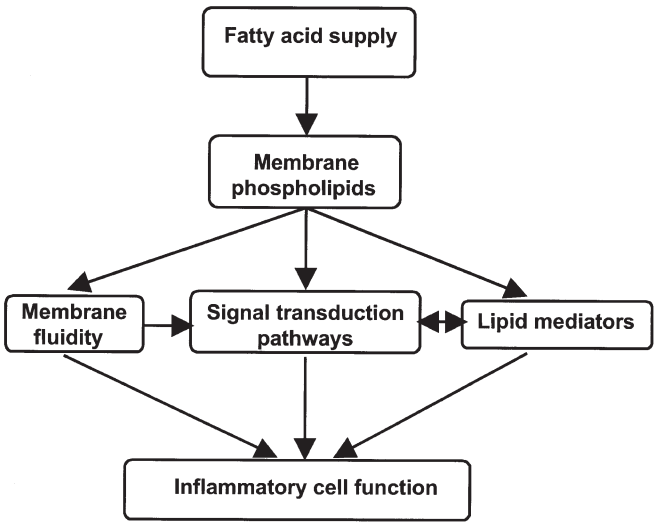

FIG. 1. Mechanisms by which altered FA supply could affect inflammatory cell function.

AA as an eicosanoid precursor. The principal functional role for AA is as a substrate for synthesis of the eicosanoid family of bioactive mediators [e.g., PG, thromboxanes (TX), leukotrienes (LT), or hydroxyeicosatetraenoic acids (HETE); see Fig. 4 of Ref. 1]. AA in cell membranes can be mobilized by various phospholipase enzymes, especially phospholipase A_2, and the free AA can subsequently act as a substrate for the enzymes that synthesize eicosanoids (Fig. 2). Metabolism of AA by cyclooxygenase enzymes (COX) gives rise to the 2-series PG and TX (Fig. 2). There are two isoforms of COX: COX-1 is a constitutive enzyme and COX-2 is induced in inflammatory cells as a result of stimulation and is responsible for the markedly elevated production of PG that occurs upon cellular activation. PG are formed in a cell-specific manner. For example, upon activation monocytes and macrophages produce large amounts of PGE_2 and $PGF_{2\alpha}$, neutrophils produce moderate amounts of PGE_2, and mast cells produce PGD_2. Metabolism of AA by the 5-lipoxygenase (5-LOX) pathway gives rise to hydroxy and hydroperoxy derivatives [5-HETE and 5-hydroperoxyeicosatetraenoic acid (5-HPETE), respectively], and the 4-series LT, LTA_4, B_4, C_4, D_4, and E_4 (Fig. 2). 5-LOX is found in mast cells, monocytes, macrophages, and granulocytes.

AA-derived eicosanoids and inflammation. Eicosanoids are involved in modulating the intensity and duration of inflammatory responses (for reviews, see Refs. 8–10). For example, PGE_2 has a number of proinflammatory effects including inducing fever, increasing vascular permeability and vasodilation, and enhancing pain and edema caused by other agents such as bradykinin and histamine. PGE_2 also promotes IgE production by B lymphocytes; IgE is a mediator of allergic inflammation. PGE_2 suppresses production of TNF-α, IL-1, and IL-6, which, in these respects, is an anti-inflammatory action. LTB_4 increases vascular permeability, is a vasoconstrictor, enhances local blood flow, is a potent chemotactic agent for leukocytes, induces release of lysosomal enzymes, enhances the generation of reactive oxygen species, and enhances production of TNF-α, IL-1, and IL-6. Thus, LTB_4 is proinflammatory in nature. In inflammatory conditions, increased rates of production of AA-derived eicosanoids occur, and elevated levels of these eicosanoids are observed in blood and tissues from patients with trauma, burns, and a variety of inflammatory disorders (8,10). Interestingly, recent studies have shown that PGE_2 inhibits 5-LOX, thereby preventing the generation of the inflammatory 4-series LT (11). Recent studies have also reported novel anti-inflammatory effects of certain lipoxins generated from AA by 15-LOX (12,13). PGE_2 was found to induce generation of one of these, lipoxin A_4 (11), indicating that PGE_2 is involved in mediating the resolution of inflammation through effects on the generation of other eicosanoids.

n-3 PUFA and eicosanoid production. Because significantly increased consumption of long-chain n-3 PUFA results in a decrease in the amount of AA in the membranes of inflammatory cells, there will be less substrate available for synthesis of eicosanoids from AA. However, it is now apparent that the ability of long-chain n-3 PUFA to influence pro-

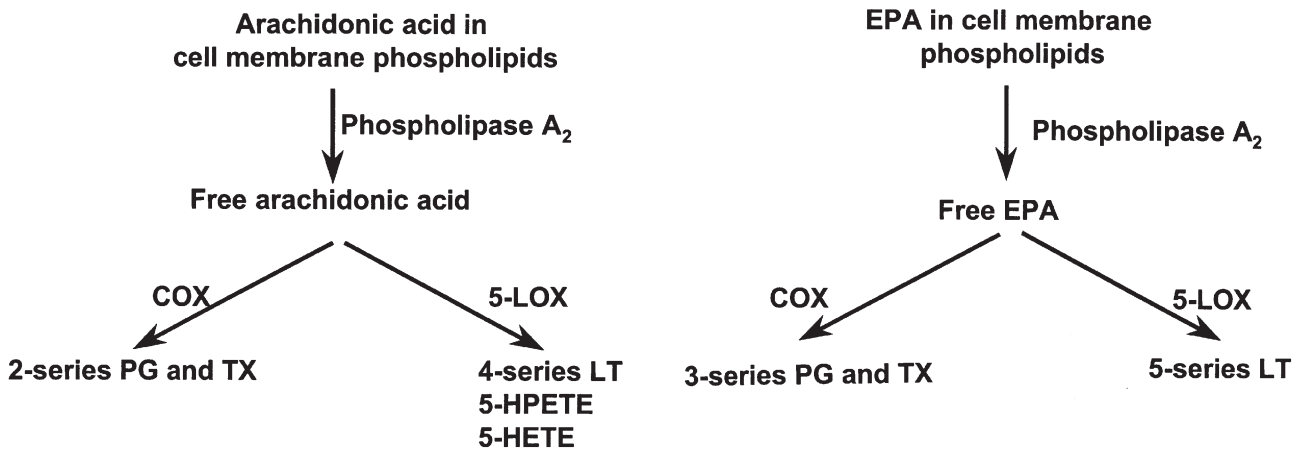

FIG. 2. Synthesis of eicosanoids from arachidonic acid and EPA. COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin; TX, thromboxane.

duction of eicosanoids extends beyond simply decreasing substrate availability. For example, EPA competitively inhibits the oxygenation of AA by COX (14). Recent cell culture studies have demonstrated that n-3 PUFA suppress cytokine-induction of COX-2 and 5-LOX gene expression (15,16). Owing to these various actions, fish oil feeding results in a decreased capacity of inflammatory cells to synthesize COX- and 5-LOX-derived eicosanoids from AA (see, for example, Refs. 7,17–20). The reduction in the generation of AA-derived mediators that accompanies fish oil consumption has led to the idea that fish oil is anti-inflammatory.

In addition to inhibiting the metabolism of AA, EPA is able to act as a substrate for both COX and 5-LOX (Fig. 2), giving rise to derivatives that differ in structure from those produced from AA (i.e., 3-series PG and TX, and 5-series LT). Thus, the EPA-induced suppression in the production of AA-derived eicosanoids may be accompanied by an elevation in the production of EPA-derived eicosanoids. This is most evident for the 5-LOX products of EPA metabolism (7,20). The eicosanoids produced from EPA are considered to be less biologically potent than the analogs synthesized from AA, although the full range of biological activities of these compounds has not been investigated. Additionally, n-3 PUFA have been shown to give rise to novel anti-inflammatory eicosanoids generated *via* COX-2 (21).

n-3 PUFA AND INFLAMMATORY CYTOKINE PRODUCTION

EPA and DHA can inhibit the production of IL-1β and TNF-α by cultured monocytes (see Ref. 22), and the production of IL-6 and IL-8 by cultured venous endothelial cells (23,24). More recent studies have extended such findings to include suppression of tissue factor production by n-3 PUFA (25). Consistent with cell culture studies involving n-3 PUFA, fish oil feeding decreased *ex vivo* production of TNF-α, IL-1β, and IL-6 by rodent macrophages (26–28) and decreased circulating TNF-α, IL-1β, and IL-6 concentrations in mice injected with LPS (29). Supplementation of the diet of healthy human volunteers with fish oil providing more than 2.4 g/d EPA plus DHA decreased production of TNF-α, IL-1, and IL-6 by mononuclear cells (17,19,30,31). These inhibitory effects of n-3 PUFA on inflammatory cytokine production are not those that would be predicted on the basis of antagonism of PGE_2 production, suggesting some other mechanism of action. This might be *via* effects on production of eicosanoids other than PGE_2 or *via* eicosanoid-independent effects.

n-3 PUFA AND ADHESION MOLECULE EXPRESSION

Culture of murine macrophages with n-3 PUFA decreased their ability to bind to various surfaces (32); how this effect occurred was not investigated. Later studies showed that culture of human venous endothelial cells with EPA or DHA decreased cytokine- or LPS-induced surface expression of E-selectin, ICAM-1, and VCAM-1 (23,33), and diminished the adhesion of ligand-bearing monocytes (33,34). In another cell culture study, EPA decreased surface expression of ICAM-1 on monocytes stimulated with interferon (IFN)-γ (35). Dietary fish oil decreased expression of ICAM-1 on the surface of murine macrophages (36). Supplementing the diet of healthy humans with fish oil providing ~1.5 g/d EPA plus DHA resulted in a lower level of expression of ICAM-1 on the surface of blood monocytes stimulated *ex vivo* with IFN-γ (37). More recently, dietary fish was found to decrease circulating levels of soluble VCAM-1 in elderly subjects (38), but it is not clear whether this represents decreased surface expression of VCAM-1. If so, then this effect might be indicative of decreased endothelial inflammation *in vivo*.

n-3 PUFA AND INFLAMMATORY GENE EXPRESSION

de Caterina *et al.* (23) demonstrated that the downregulation of VCAM-1 expression on the surface of endothelial cells caused by DHA was exerted at the level of VCAM-1 gene expression, and that this effect was independent of any effects on eicosanoid production and on antioxidant status. This was among the first demonstrations of an effect of n-3 PUFA on the expression of inflammatory genes. More recently, it was

shown that culturing bovine chondrocytes with α-linolenic acid, EPA, or DHA dramatically decreased cytokine-mediated induction of expression of the COX-2 (but not COX-1), TNF-α, IL-1α, and aggrecanase-1 and -2 genes (15). Recently, this study was extended by a study using cultured explants of human osteoarthritic cartilage (16). Including α-linolenic acid, EPA, or DHA in the culture medium markedly decreased the cytokine-induced upregulation of expression of the COX-2, IL-1α, IL-1β, TNF-α, 5-LOX, 5-LOX activating protein (FLAP), MMP-3, MMP-13, and aggrecanase-1 genes in these cells. The n-3 PUFA did not affect expression of the COX-1, 12-LOX, or 15-LOX genes, which were not induced by cytokines (16). Also, there was little effect of n-3 PUFA on the expression of genes for the tissue inhibitor of metalloproteinase (TIMP)-1, -2, or -3, which again were not cytokine inducible (16). These studies indicate a marked capacity of n-3 PUFA to suppress the expression of inflammatory genes, with little effect on the expression of housekeeping (e.g., COX-1) or anti-inflammatory (TIMP) genes. They also indicate that an important, hitherto unrecognized contributor to the reduction in the generation of AA-derived eicosanoids after fish oil feeding may be decreased expression of the enzymes and proteins responsible (e.g., COX-2, 5-LOX, FLAP). These observations provide exciting new understanding of the anti-inflammatory effects of n-3 PUFA.

A limited number of feeding studies have demonstrated an effect of dietary fish oil on inflammatory gene expression. Dietary fish oil completely abolished mRNA for TNF-α, IL-1β, and IL-6 in the kidneys of autoimmune disease-prone mice (39). Feeding mice a fish oil-rich diet significantly decreased the level of IL-1β mRNA in LPS-stimulated spleen lymphocytes (40). This study further demonstrated that the lower IL-1β mRNA level was not due to accelerated mRNA degradation but to impaired mRNA synthesis (40). Fish oil lowered LPS-stimulated TNF-α mRNA levels in murine peritoneal macrophages (27). ICAM-1 mRNA levels were lower in peritoneal macrophages from mice fed fish oil (36). Thus, significant evidence is emerging of an effect of dietary fish oil on inflammatory gene expression.

Because eicosanoids derived from AA regulate inflammatory gene expression, the effects of n-3 PUFA might come about through antagonism of the effects of AA-derived mediators. However, at least some of these effects have been demonstrated to occur in an eicosanoid-independent manner (see, for example Ref. 23). Recent studies indicate that n-3 PUFA exert some effects through direct actions on the intracellular signaling pathways that lead to activation of one or more transcription factors.

Nuclear factor κB (NFκB) is a transcription factor involved in the induction of numerous inflammatory genes including COX-2, ICAM-1, VCAM-1, E-selectin, TNF-α, IL-1β, IL-6, nitric oxide synthase, acute phase proteins, and MMP in response to inflammatory stimuli (41–43) (Fig. 3). NFκB exists as an inactive heterotrimer in the cytosol of resting inflammatory cells; one of the subunits is called the inhibitory subunit of NFκB (IκB). Upon stimulation, a signal-

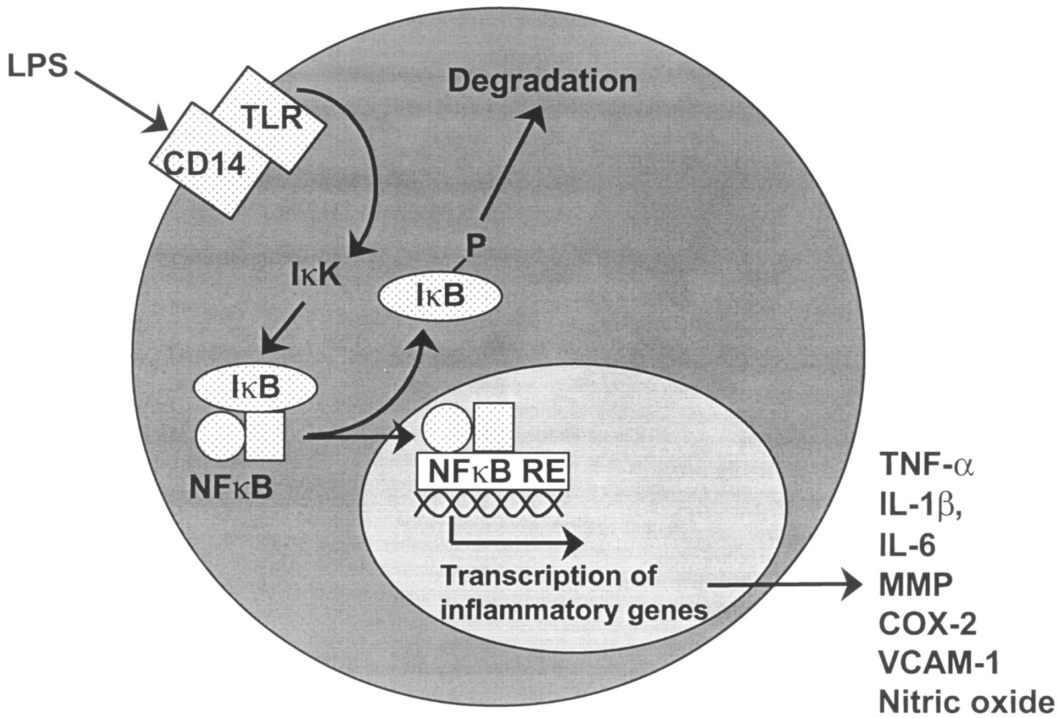

FIG. 3. Outline of the pathway of upregulation of inflammatory gene expression *via* nuclear factor κB. CD14, cluster of differentiation 14 (the LPS receptor); COX, cyclooxygenase; IκB, inhibitory subunit of NFκB; IκK, IκB kinase; IL, interleukin; LPS, lipopolysaccharide; MMP, matrix metalloproteinases; NFκB, nuclear factor κB; RE, response element; TLR, toll-like receptor; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule. Figure previously published in Reference 43.

ing cascade activates a protein complex known as IκB kinase (IκK). Activated IκK phosphorylates IκB, causing its dissociation from the rest of the inactive NFκB trimer (44,45). The phosphorylated IκB is degraded. The remaining NFκB heterodimer is rapidly translocated to the nucleus where it binds to response elements in target genes, thus regulating their transcription. Recent studies suggest that one aspect of the anti-inflammatory action of fish oil is decreased activation of NFκB. For example, dietary fish oil resulted in a lower level of NFκB in the nucleus (i.e., activated NFκB) of LPS-stimulated murine spleen lymphocytes compared with feeding corn oil (46). How n-3 PUFA decreases the activation of NFκB is not clear. However, incubating human monocytes with EPA decreased LPS-induced activation of NFκB, and this was associated with decreased phosphorylation of IκB (47). This suggests an effect of n-3 PUFA on the signaling process leading to activation of IκK. Incubation of a pancreatic cell line with TNF-α markedly upregulated degradation of IκB, and this could be totally abolished by prior incubation of the cells with EPA, but not with AA (48). This effect could be due to inhibition of phosphorylation of IκB, thereby preventing it from being targeted for degradation, or to inhibition of the degradation process itself.

A second group of transcription factors currently eliciting much interest because of their potential role in inflammation is the peroxisome proliferator-activated receptors (PPAR). Although PPARα and -γ play important roles in liver and adipose tissue, respectively (49), they are also found in inflammatory cells (50,51). PPAR act through dimerization with the retinoid-X-receptor and subsequent regulation of gene expression. PPAR can bind, and appear to be regulated by PUFA and eicosanoids (52,53). Mice deficient in PPARα have a prolonged response to inflammatory stimuli (53), leading to the suggestion that PPARα activation might be "anti-inflammatory." Activators of both PPARα and -γ have been shown to inhibit the induction of a range of inflammatory genes including TNF-α, IL-1β, IL-6, IL-8, COX-2, VCAM-1, nitric oxide synthase, MMP, and acute phase proteins (51,54–60). Two mechanisms for the anti-inflammatory actions of PPAR have been proposed (for reviews, see Refs. 61,62). The first is that PPAR stimulate the breakdown of inflammatory eicosanoids through induction of peroxisomal β-oxidation. The second is that PPAR might interfere with or antagonize the activation of other transcription factors, including NFκB. Expression of PPARα and -γ in liver and adipose tissue, respectively, is increased by feeding mice fish oil (63). The effect of fish oil on PPAR expression in inflammatory cells has not been reported. However, it is possible that n-3 PUFA might act by increasing the level of these anti-inflammatory transcription factors in such cells.

A number of other transcription factors are activated by inflammatory signals and play a role in the expression of inflammatory genes (for a review, see Ref. 64). Possibly n-3 PUFA might affect the activation of these factors, but this has not been studied in detail. However, effects of n-3 PUFA on signaling processes that lead to activation of various transcription factors have been reported. For example, incubation of murine macrophages with EPA was found to decrease LPS-induced phosphorylation and activation of mitogen-activated protein kinase (65).

CLINICAL APPLICATIONS OF THE ANTI-INFLAMMATORY EFFECTS OF n-3 PUFA

Chronic inflammatory diseases. Chronic inflammatory diseases are characterized by a dysregulated T-cell response that drives an ongoing immune response to normally benign, often host, antigens. There is a genetic predisposition to such diseases. The response has a strong inflammatory component, and inflammatory mediators are responsible for damage to host tissues and for the metabolic changes seen at the whole-body level. Rheumatoid arthritis is an example of such a disease. It is characterized by a dysregulated T helper 1-type response that promotes the production of inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-8 (66,67). High levels of TNF-α, IL-1β, IL-6, IL-8, and granulocyte/macrophage-colony stimulating factor (GM-CSF) are present in synovial biopsies from patients with rheumatoid arthritis (68). Furthermore, cultured synovial cells produce TNF-α, IL-1β, IL-6, IL-8, and GM-CSF without any additional stimulus (68), suggesting chronic stimulation. COX-2 expression is increased in the synovium of rheumatoid arthritis patients, and in the joint tissues in rat models of arthritis (69). PGE_2, LTB_4, 5-HETE, and platelet activating factor are found in the synovial fluid of patients with active rheumatoid arthritis (70). The efficacy of nonsteroidal anti-inflammatory drugs in rheumatoid arthritis indicates the importance of proinflammatory COX pathway products in the pathophysiology of the disease. Increased expression of E-selection, VCAM-1, and ICAM-1 is found in patients with arthritis, and blocking ICAM-1 or VCAM-1 with antibodies reduces leukocyte infiltration into the synovium and synovial inflammation in animal models of the disease (for references, see Ref. 71).

The effects of fish oil on inflammatory eicosanoid and cytokine production and on adhesion molecule expression suggest that it might have a role in prevention and therapy of rheumatoid arthritis (and other chronic inflammatory diseases). Certainly, dietary fish oil has been shown to have beneficial effects in animal models of arthritis. For example, dietary fish oil delayed the onset and reduced the incidence and severity of type II collagen-induced arthritis in mice (72). It was recently reported that both EPA and DHA suppress streptococcal cell wall-induced arthritis in rats, but that EPA was more effective (73).

Numerous (at least 14) randomized, placebo-controlled, double-blind studies of fish oil in rheumatoid arthritis have been reported. These trials were reviewed in some detail elsewhere (74,75) and are summarized briefly here. The trials used between 1 and 7.1 g/d EPA plus DHA (average dose was 3.3 g/d) with a duration of 12–52 wk. Various improvements in clinical outcomes were reported. These included reduced duration of morning stiffness, reduced number of tender or swollen joints, reduced joint pain, reduced time to fatigue, increased grip strength, and decreased use of nonsteroidal anti-inflammatory drugs. In

an editorial commentary discussing the use of fish oil in rheumatoid arthritis, it was concluded that "the findings of benefit from fish oil in rheumatoid arthritis are robust," "that dietary fish oil supplements in rheumatoid arthritis have treatment efficacy," and that "dietary fish oil supplements should now be regarded as part of the standard therapy for rheumatoid arthritis" (76).

The efficacy of dietary fish oil has been examined in other chronic inflammatory diseases including ulcerative colitis, Crohn's disease, lupus, and multiple sclerosis (reviewed in Ref. 22). Although there are examples of trials reporting clinical improvement in each of these conditions, in general, these studies show little benefit (22). This may be because the dose of n-3 PUFA used was too low, the duration of the studies was too short, the studies were insufficiently powered to detect a significant effect, or a number of other reasons.

Atopic disease. There is currently considerable interest in the relative effects of n-3 and n-6 PUFA in asthma (and other atopic diseases). The discussions center on the roles of various eicosanoids produced from AA in mediating allergic inflammation and in programming T lymphocytes to a phenotype that predisposes to such inflammation. AA-derived eicosanoids such as PGD_2, LTC_4, D_4, and E_4 are produced by the cells that mediate pulmonary inflammation in asthma (mast cells) and are believed to be the major mediators of asthmatic bronchoconstriction. Thus, provision of n-3 PUFA to asthmatics might be beneficial because of the resulting decrease in production of 4-series LT (7,20) and other AA-derived mediators. However, the situation is complicated by the fact that different eicosanoids have different effects, some antagonizing others. For example, the observations that PGE_2 inhibits 5-LOX (11) and promotes the generation of lipoxins that act as inflammation "stop signals" (11–13) indicate that PGE_2 could, in fact, be protective in active asthma. Thus, interventions that aim to suppress PGE_2 production could be counterproductive, at least in some asthmatics. Nevertheless, numerous trials of fish oil in asthma and related atopic diseases have been performed (for reviews, see Refs. 77,78). Most of these studies reveal limited clinical effect, despite significant biochemical changes (e.g., reduced 4-series LT production), although some have shown some significant clinical improvements at least in some patient groups (78). However, a study performed by Broughton *et al.* (79) suggests that fish oil should be used cautiously in asthmatics. These researchers found that although n-3 PUFA ingestion resulted in markedly improved lung function in >40% of adult asthmatic subjects, some patients did not respond favorably to the high n-3 PUFA intake (79). This study suggests that there are patients who respond positively to fish oil intervention and patients who are nonresponders.

Another area of current interest relates to the putative predisposing role of PGE_2 toward atopic disease, particularly in childhood. There has been a rapid increase in the prevalence of childhood atopic disease in developed countries over the last 30 years (80,81), and this coincides with the period of increase in the intake of n-6 PUFA. Because PGE_2 regulates T-lymphocyte differentiation, promoting the development of the T helper 2-type phenotype that underlies sensitization to environmental allergens (82), it is suggested that the pattern of change in dietary FA intake over the last 40 yr is responsible for the increase in childhood atopic disease (83–85). Certainly there are biochemical measurements suggesting an inverse relationship between n-3 FA status and atopic disease. The proportions of EPA, docosapentaenoic acid, and DHA were higher in umbilical cord serum phospholipids from nonallergic compared with allergic mothers (86). Higher n-3 PUFA in breast milk were associated with a decreased likelihood of atopy in the infants (87). The proportions of DHA and of total n-3 PUFA were higher in the serum phospholipids from 12- to 15-yr-old nonatopic controls than in those from children with asthma and/or atopic dermatitis (88). There is also epidemiologic evidence to support a protective role of long-chain n-3 PUFA in atopic disease. Data from the first and second National Health and Nutrition Surveys in the United States found that dietary fish intake was positively associated with lung function (89) and protected against wheezing (90), respectively. Australian schoolchildren who included oily fish in their diet had a much lower likelihood of having asthma than children who did not consume oily fish (91). Schoolchildren who went on to develop atopy had previously consumed more margarine and less butter (and thus more n-6 PUFA) than those who did not develop atopy (92). Despite a biologically plausible mechanism and the supportive biochemical and epidemiologic data, the key to demonstrating a protective effect of increased long-chain n-3 PUFA consumption toward atopic disease must come from well-designed, placebo-controlled intervention studies. It is now recognized that sensitization to allergens occurs early in life (93); thus, the characteristics of the maternal diet may be very important in determining predisposition to atopy, and studies addressing this question should be performed in pregnant women. Several such studies are currently under way, and their findings are eagerly anticipated.

Systemic inflammatory response to surgery and injury. It is now recognized that a hyperinflammatory response, characterized by overproduction of TNF-α, IL-1β, IL-6, and IL-8, is important in the progression of very ill patients toward sepsis, i.e., markedly elevated circulating concentrations of these mediators are seen in sepsis (see, for example, Refs. 94,95); Vervloet *et al.* (96) stated that "these mediators are largely, if not completely, responsible for the clinical signs and symptoms of the septic response to a bacterial infection." Enhanced production of AA-derived eicosanoids such as PGE_2 is also associated with such a pathophysiology (97,98). The inflammatory effects of infection can be mimicked by administration of LPS, which causes an elevation of circulating concentrations of inflammatory cytokines and an upregulation of adhesion molecule expression (for references, see Ref. 43). Laboratory animals can be protected against bacterial- and LPS-induced shock by neutralizing these cytokines or by blocking or gene deletion of VCAM-1 or ICAM-1 (for references, see Ref. 43). The ability of n-3 PUFA to decrease production of inflammatory cytokines and eicosanoids and to decrease adhesion molecule expression suggests that fish oil

might be a useful agent to aid the control of endotoxemia and the so-called systemic inflammatory response syndrome (SIRS). Fish oil feeding or infusions enhanced the survival of guinea pigs after LPS challenge and decreased the accompanying metabolic perturbations in guinea pigs and rats (for references, see Ref. 43). Mice fed fish oil and then injected with LPS had lower plasma TNF-α, IL-1β, and IL-6 concentrations than mice fed safflower oil (29), whereas fish oil-containing parenteral nutrition decreased serum TNF-α, IL-6, and IL-8 concentrations in burned rats (99,100). Postsurgery patients administered parenteral fish oil after major abdominal surgery had lower serum concentrations of TNF-α and IL-6 than those given a standard lipid mix (101). In another study in postsurgical patients, parenteral fish oil decreased TNF-α production by LPS-stimulated whole blood and decreased serum IL-6 concentration compared with the control group that was given standard parenteral nutrition (102). Postoperative stay in the intensive care unit and in hospital tended to be shorter in the fish oil group (102). These studies indicate the potential for significant modification of the inflammatory changes induced by major surgery by infusion of n-3 PUFA in the form of fish oil (101,102). However, larger studies are required to evaluate the effects on complication rates, hospital stay, and mortality.

Numerous clinical trials (at least 20) have been performed in intensive care or surgical patients using enteral formulae containing n-3 PUFA. The majority of these trials used formulae that also contained other nutrients proposed to be beneficial such as arginine and nucleotides. Many of these trials report beneficial outcomes, including decreased numbers of infections and infectious or wound complications, decreased severity of infection, decreased need for mechanical ventilation, decreased progression to SIRS, and decreased length of intensive care unit and/or total hospital stay (for a meta-analysis, see Ref. 103). A number of these studies also measured circulating inflammatory cytokine levels or *ex vivo* cytokine production. These studies reported lower plasma concentrations of inflammatory cytokines (especially IL-6) in patients given n-3 PUFA-containing enteral formula pre- or postsurgery than in those administered standard enteral nutrition (104–107). Although this is in agreement with the effects of n-3 PUFA reported in other settings, and could be used as evidence of their efficacy in the trauma and postsurgery settings, the complex nature of the formulae prevents such a clear interpretation. The effects could be due to any one of the specified nutrients (i.e., arginine, nucleotides, n-3 PUFA) or to the combination of these nutrients.

A trial performed in patients with moderate and severe acute respiratory distress syndrome used an enteral formula claimed to differ only in lipid source from the control (32% canola oil + 25% medium-chain TAG + 20% borage oil + 20% fish oil + 3% soy lecithin vs. 97% corn oil + 3% soy lecithin) (108). However, in addition to the difference in FA composition between the formulae, the n-3 PUFA-rich formula contained more vitamin C and E than the control and contained β-carotene, taurine, and carnitine, whereas the control did not. The study included a number of patients with surgical trauma, sepsis, and pneumonia; all patients had respiratory failure and about one third had failure of at least one other organ system. Patients were given ~7 g EPA, 3 g DHA, 6 g γ-linolenic acid, 1.1 g vitamin C, 400 IU vitamin E, and 6.6 mg β-carotene daily for up to 7 d. By day 4, the numbers of leukocytes and neutrophils in the alveolar fluid had significantly declined in the treatment group and were lower than in the control group. Furthermore, arterial oxygenation and gas exchange were improved in the treatment group. Patients in the treatment group had decreased requirement for supplemental oxygen, reduced time on ventilation support, and shorter length of intensive care unit stay. Total length of hospital stay also tended to be shorter. Fewer patients in the treatment group developed new organ failure. Mortality was 19% in the control group and 12% in the treatment group, but this was not a significant difference. Nevertheless, this study suggests the efficacy of n-3 PUFA (in combination with γ-linolenic acid, medium-chain TAG, antioxidant vitamins, taurine, and carnitine) in this group of patients.

CONCLUDING STATEMENT

Inflammation is a component of a range of acute and chronic human diseases, and is characterized by the production of inflammatory cytokines, AA-derived eicosanoids, other inflammatory mediators (e.g., platelet-activating factor), and adhesion molecules. The n-3 PUFA decrease the production of inflammatory mediators and the expression of adhesion molecules. They act both directly (e.g., by replacing AA as an eicosanoid substrate and inhibiting AA metabolism) and indirectly (e.g., by altering the expression of inflammatory genes through effects on transcription factor activation). Thus, n-3 PUFA are potentially potent anti-inflammatory agents. As such, they may be of therapeutic use in a variety of acute and chronic inflammatory settings. Evidence of their clinical efficacy is stronger in some settings (e.g., in rheumatoid arthritis) than others (e.g., in asthma, in trauma patients).

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n-3 Long-Chain FA Decrease Serum Levels of TG and Remnant-Like Particle-Cholesterol in Humans

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ABSTRACT: A large number of papers have reported that administration of n-3 FA reduced serum TG concentrations in hypertriglyceridemic patients. However, few studies have examined the effect of n-3 FA on serum concentrations of remnant-like particle (RLP) cholesterol. Volunteers (n = 41) whose serum TG concentrations were 100–300 mg/dL were recruited and randomly assigned to either an n-3 FA group or a control group with stratification by sex, age, and serum TG level in a double-blind manner. The subjects in the n-3 FA group were administered 125 mL of fermented soybean milk with fish oil containing 600 mg of EPA and 260 mg of DHA/d for 12 wk. The controls consumed control soybean milk with olive oil. Fasting blood samples were obtained before the start of administration and at 4, 8, and 12 wk. EPA concentrations in red blood cells increased significantly in all but one subject in the n-3 FA group, with no significant changes in the control group. TG levels decreased more in the n-3 FA group than in the control group at weeks 4 ($P < 0.05$), 8 ($P < 0.01$), and 12 ($P < 0.05$) with their baseline as covariate. RLP cholesterol levels decreased more in the n-3 FA group than in the control at weeks 8 ($P < 0.01$) and 12 ($P < 0.05$) with their baseline as covariate. The groups did not differ in the other lipid levels. It is likely that n-3 long-chain FA may exert anti-atherosclerotic effects by lowering serum TG and RLP-cholesterol levels even at the dose of 860 mg/d.

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The consumption of fish, which is a good source of n-3 long-chain PUFA (LC-PUFA) such as EPA and DHA, is now widely recommended for preventing heart disease (1). To date, three large-scale intervention studies have been performed using n-3 LC-PUFA for the secondary prevention of coronary heart disease, and all of them have been successful in terms of prevention of cardiac death (2–4). A meta-analysis of randomized control trials with coronary heart disease and n-3 FA suggested that intake of n-3 FA reduced overall mortality, mortality due to myocardial infarction, and sudden death (5). In the GISSI-Prevenzione Study (4), the risk of sudden death was reduced by 45% in patients who took ethyl esters of EPA and DHA (~0.87 g/d) for 3.5 yr. A recent observational study (6) also indicated that compared with men whose blood levels of n-3 LC-PUFA were in the lowest quartile, the adjusted relative risk of sudden death in those whose blood levels were in the highest quartile was reduced by 80%. The beneficial effects of n-3 LC-PUFA on sudden cardiac death could be due to antiarrhythmic effects, as reported from experimental models (7). However, studies on the mechanisms of anti-sudden death effects of n-3 FA are still far from conclusive.

Nakamura *et al.* (8) reported that, in 10 patients with type 2 diabetes mellitus, the administration of 0.9–1.8 g of EPA ethyl ester for 3 mon significantly reduced serum levels of remnant-like particle (RLP)-cholesterol from 14.5 to 3.3 mg/dL. They also found a similar reduction of RLP-cholesterol (from 12.7 to 2.5 mg/dL) with EPA ethyl ester administration for 3 mon in 14 hyperlipidemic patients who were treated with statins (9). These beneficial effects of EPA on RLP-cholesterol may be related to the anti-sudden death effects of n-3 LC-PUFA. Actually, Takeichi *et al.* (10) showed that postmortem RLP-cholesterol was the best predictor for coronary atherosclerosis among the lipids and lipoproteins and that there was a marked difference in RLP-cholesterol levels between victims of sudden cardiac death (12.4 mg/dL) and those of sudden death from non-cardiac causes (5.4 mg/dL). [Serum levels of total cholesterol, lipoproteins and apolipoproteins were reported to be stable for at least 24 h after death (11,12).]

Although Nakamura *et al.* (8,9) showed an effect of n-3 LC-PUFA on RLP-cholesterol for the first time, their studies were open trials and not placebo controlled. In the present study, we used a dose of n-3 LC-PUFA very similar to that in the GISSI-Prevenzione Study and measured serum lipids,

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Abbreviations: apo, apolipoprotein; LC-PUFA, long-chain PUFA; RBC, red blood cells; RLP, remnant-like particle; SRL, Special Reference Laboratories.

including RLP-cholesterol, in a placebo-controlled, double-blind style.

SUBJECTS AND METHODS

Subjects. Normolipidemic and hypertriglyceridemic subjects (n = 70) were recruited from students and employees of Toyama Medical and Pharmaceutical University and local companies, and their fasting serum TG were screened. Of those with TG levels of 100–300 mg/dL, 41 were asked to participate in the present study. They had not taken any lipid-lowering medications or hormone replacement (including estrogen and birth control pills) during the previous 3 mon. They were all considered healthy by routine health checkups 3–4 mon previously.

Study protocol. The present study was a randomized, placebo-controlled, double-blind trial of 12 wk. Subjects (n = 41) were randomly assigned to two groups (n-3 FA and control groups). Subjects in the n-3 FA group (n = 20) consumed one pack (125 mL) of fermented soybean milk containing 600 mg of EPA and 260 mg of DHA for 12 wk. The other subjects (the control group, n = 21) consumed the same amount of control fermented soybean milk containing olive oil instead of fish oil. The two kinds of soybean milk were prepared by Nippon Suisan Kaisha (Tokyo, Japan). The ingredients in the fermented milk are listed in Table 1. Subjects in both groups were told that the fermented milk could be consumed at any time during the day. They were also asked to maintain their body weights and physical activity levels and to consume their habitual diets during the study. Fasting blood samples were taken at weeks 0 and 4, 8, and 12 after soybean milk supplementation. At weeks 0, 4, 8, and 12, the subjects were asked to complete a food-frequency questionnaire (13) for the previous 4 wk. At the end of the study, they were asked to complete another questionnaire about any adverse effects of soybean milk, and to guess the content of their soybean milk by choosing one of the following three: control milk, "hard to tell," or EPA milk. The study was approved by the ethics committee of Toyama Medical and Pharmaceutical University, and written informed consent was obtained from each participant.

TABLE 1
Composition of the Two Kinds of Fermented Soybean Milk[a]

	Group	
	Control	n-3 FA
Energy (kcal/pack)	73	74
Protein (g/pack)	2.1	2.1
Carbohydrate (g/pack)	6.9	6.9
Fat (g/pack)	4.1	4.2
Oleic acid (g/pack)	2.0 (49%)	0.54 (13%)
Linoleic acid (g/pack)	0.85 (21%)	0.72 (17%)
EPA (g/pack)	ND	0.60 (14%)
DHA (g/pack)	ND	0.26 (6%)

[a]One pack contained 125 mL of fermented soybean milk; subjects consumed one pack of fermented soybean milk/d for 12 wk. ND, not detected.

Measurements. Red blood cells (RBC) separated from EDTA-containing blood samples were washed with saline three times and frozen at 30°C. They were sent to Special Reference Laboratories (SRL) Tokyo for FA analysis of the total FA fraction. The FA composition of the total lipid fraction of RBC was analyzed as described previously (14) with slight modifications. Briefly, the fraction was extracted by the method of Folch *et al.* (15); FA of the extract were transmethylated with BF_3 and analyzed by a gas chromatograph (GC17A Shimadzu, Kyoto) with a capillary column (Omegawax 250, 0.25-mm i.d. × 30 m; Supelco, Bellefonte, PA). Serum samples were also sent to SRL for enzymatic measurement of TG (16), total cholesterol, LDL-cholesterol, and HDL-cholesterol (17). RLP-cholesterol was measured at SRL as follows: RLP were separated from serum by immunoaffinity chromatography with gel equipped with an anti-apolipoprotein (apo) A-1 monoclonal antibody (H-12 antibody) and a specific apoB-100 monoclonal antibody (JI-H antibody) (Japan Immunoresearch Laboratories, Takasaki, Japan) that did not recognize apoB-48 (18). These antibodies eliminate HDL, newly synthesized chylomicrons containing apoA1, and all apoB-100-containing lipoproteins except for certain apoB-100-containing lipoproteins enriched in apoE [a large amount of apoE is known to hinder the binding somehow of JI-H antibody and apoB-100 (19)]. Consequently, the separated RLP were a mixture of remnant-like VLDL and chylomicron remnants. The intra- and interassay variances of RLP-cholesterol were <10%.

Statistical analysis. Results are expressed as means ± SD. FA composition was analyzed parametrically (t-tests and ANOVA). Because some of the TG and RLP-cholesterol values were not normally distributed, those were analyzed nonparametrically (Wilcoxon's test and Mann–Whitney's U test). The χ^2 test was used for the comparison of subjects' guesses about the randomization status between the two groups. Correlation was analyzed by the least squares method. $P < 0.05$ was considered to be significant. StatView (ver. 5.0) was used for statistical analysis.

RESULTS

Three subjects of the control group complained of minor adverse effects (mild digestive tract disorder), as did one of the n-3 FA group (mild digestive tract disorder). One new case of mild hypertension occurred in the n-3 FA group. No subjects discontinued the study or complained about the taste of the milk. At the end of the study, the subjects guessed the content of their soybean milk. The guesses in the control and n-3 FA groups were similar and were almost equally distributed among the control, "hard to tell," and EPA milk choices, namely, in the n-3 FA group, the distribution was 7, 8, and 5, respectively; in the control group, it was 5, 11, and 5, respectively.

Baseline characteristics assessed at week 0 indicated that there were no significant differences between the two groups (Table 2). The average changes in body mass index over the

TABLE 2
Baseline Characteristics of Randomized Subjects[a]

Characteristic	Group	
	Control	n-3 FA
n	21	20
Men/women (*n*)	12/9	13/7
Smoking (*n*)	9	9
Total cholesterol (mg/dL)	216 ± 28	206 ± 29
TG (mg/dL)	146 ± 44	162 ± 54
RLP-cholesterol (mg/dL)	5.1 ± 1.6	5.6 ± 2.3
HDL-cholesterol (mg/dL)	56 ± 11	51 ± 9
LDL-cholesterol (mg/dL)	131 ± 23	123 ± 29
Age (yr)	48 ± 11	44 ± 11
BMI (kg/m^2)	24 ± 3	25 ± 3
Blood pressure		
Systolic (mmHg)	128 ± 15	124 ± 26
Diastolic (mmHg)	83 ± 8	82 ± 11

[a]The baseline values are given as means ± SD. The groups did not differ. RLP, remnant-like particle; BMI, body mass index.

TABLE 3
Change in the Total FA Composition in Red Blood Cells[a,b]

FA	Group			
	Control (*n* = 21)		n-3 FA (*n* = 20)	
	Week 0	Week 12	Week 0	Week 12
	(%)			
16:0	22.9 ± 0.7	24.2 ± 1.0	22.9 ± 0.8	24.7 ± 0.9
18:0	14.3 ± 0.6	14.1 ± 0.6	14.6 ± 0.6	14.4 ± 0.4
18:1n-9	12.3 ± 0.8	11.9 ± 0.7	12.6 ± 0.7	12.0 ± 0.8
18:2n-6	8.6 ± 1.1	8.9 ± 1.1	8.3 ± 0.7	8.4 ± 0.9
20:4n-6	10.5 ± 0.7	9.7 ± 0.8	10.7 ± 1.3	9.3 ± 1.1*
20:5n-3	1.8 ± 0.6	1.8 ± 0.7	1.6 ± 0.6	2.4 ± 0.7**
22:5n-3	2.5 ± 0.3	2.5 ± 0.3	2.4 ± 0.4	3.0 ± 0.4**
22:6n-3	8.3 ± 0.9	8.4 ± 1.0	7.9 ± 1.1	8.0 ± 1.0

[a]Values are means ± SD. Baseline values between the groups did not differ. Asterisks indicate difference from baseline,*$P < 0.003$; **$P < 0.0001$ (ANOVA).
[b]Subjects in the n-3 FA group were administered 125 mL of fermented soybean milk with fish oil containing 600 mg of EPA and 260 mg of DHA/d for 12 wk. Controls consumed fermented soybean milk with olive oil.

study period of 12 wk were minimal (0.2 ± 0.5 and –0.2 ± 0.9 kg/m^2 in the control and n-3 FA groups, respectively). EPA concentrations in RBC were significantly increased in the n-3 FA group (58.5 ± 41.1%) during the study period, with no significant changes in the control group (–3.4 ± 13.6%). Changes in the FA composition of RBC are shown in Table 3. EPA levels in RBC were significantly increased as early as week 4 (Table 3). In the n-3 FA group, docosapentaenoic acid was slightly but significantly increased but not DHA; on the contrary, arachidonic acid was significantly decreased at the end of the study (Table 3). Food analyses showed that there were no significant differences in the average intakes of macronutrients, n-3 LC-PUFA (500 ± 310 mg EPA and 830 ± 490 mg DHA/d and 400 ± 180 mg EPA and 670 ± 290 mg DHA/d in the control and n-3 FA groups, respectively; $P > 0.2$ for both) or other FA between the two groups (data not shown).

Total cholesterol, HDL-cholesterol, and LDL-cholesterol in the n-3 FA group did not change over the study period compared with the control group (Table 4). However, TG levels were significantly decreased in the n-3 FA group compared with the control at wk 4, 8, and 12 (Table 4), and RLP-cholesterol levels were significantly decreased in the n-3 FA group compared with the control group at weeks 8 and 12 (Table 4). Figure 1 shows a significant positive correlation between changes over 8 wk in serum levels of TG and RLP-cholesterol. There was also a similar correlation between those two values at weeks 4 ($r = 0.82$) and 12 ($r = 0.86$).

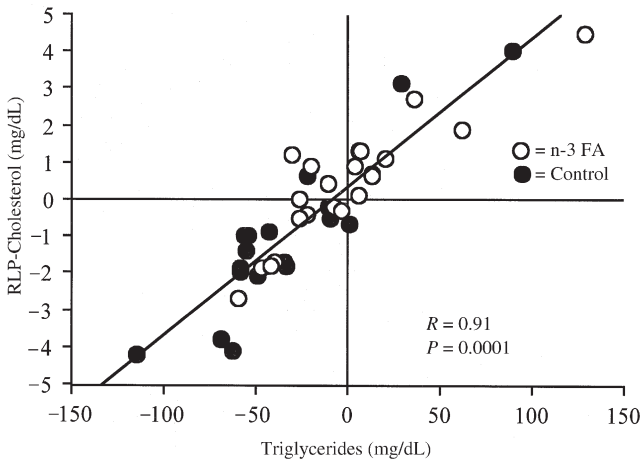

FIG. 1. Correlation between changes from baseline in serum triglycerides and remnant-like particle (RLP)-cholesterol at week 8. The study protocol is described briefly in the footnotes to Table 3.

TABLE 4
Serum Lipid Concentrations[a,b]

	Total cholesterol		HDL-cholesterol		LDL-cholesterol		TG		RLP-cholesterol	
	Control	n-3 FA	Control	n-3 FA	Control	n-3 FA	Control	n-3 FA	Control	n-3 FA
	mg/dL									
Week 0	216 ± 28	206 ± 29	56 ± 11	51 ± 9	131 ± 23	123 ± 29	146 ± 44	162 ± 54	5.1 ± 1.6	5.6 ± 2.3
Week 4	223 ± 38	210 ± 36	60 ± 14	57 ± 1	135 ± 25	123 ± 34	147 ± 42	138 ± 58*†	5.1 ± 1.8	5.3 ± 2.8
Week 8	219 ± 34	203 ± 33	60 ± 13	55 ± 1	135 ± 25	122 ± 31	144 ± 53	129 ± 59**††	5.4 ± 2.0	4.6 ± 2.0**†
Week 12	221 ± 39	210 ± 34	61 ± 15	57 ± 1	130 ± 29	122 ± 32	150 ± 58	134 ± 67*†	6.1 ± 2.1†	5.4 ± 2.6*

[a]Values are means ± SD. Baseline values between the groups did not differ. Significant intergroup differences with baseline as covariate are shown by *$P < 0.05$ and **$P < 0.01$ (Mann–Whitney's U test). Significant intra-group differences compared with baseline are shown by $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.005$ (Wilcoxon signed-ranks test).
[b]See Table 3 for a brief description of the study protocol.

DISCUSSION

As noted in the Results section, the distribution of guesses of randomization status was relatively well-distributed among control, "hard to tell," and EPA milk in both groups, which means that the fish odor of the active soybean milk was well masked.

Administration of fish oil-containing soybean milk significantly increased the EPA levels of RBC, despite the low daily dose of EPA (Table 3). On the contrary, DHA did not change to any appreciable extent in the n-3 FA group. This was probably due to a low content of DHA in the active soybean milk compared with baseline DHA intakes in the study subjects, i.e., 670–830 mg/d. These intakes of DHA were comparable to the reported Japanese n-3 FA intakes of 680 mg DHA/d and 390 mg EPA/d (20). The high baseline DHA levels in RBC (Table 3) might be another reason for the stable DHA values.

The n-3 LC-PUFA have rather constantly been shown to be effective in reducing blood TG levels (21). In the present study, serum total, LDL-, and HDL-cholesterol levels were unchanged in the n-3 FA group. These findings are consistent with most fish oil studies on blood lipids as reviewed by Harris (21); administration of 1.1–7 g n-3 LC-PUFA in placebo-controlled, parallel-design studies slightly increased LDL levels by 5%, but did not change HDL-cholesterol levels and decreased TG levels by a mean of 25%.

Higgins *et al*. (22) recently showed that a dose of n-3 LC-PUFA similar to that used in the present study was able to reduce plasma TG levels when administered for 16 wk. However, they did not measure RLP-cholesterol levels. On the other hand, RLP-cholesterol levels were reduced through administration of 0.9–1.8 g EPA ethyl ester in open trials (8,9) in which the reductions in RLP-cholesterol were surprisingly high (77–80%). This was in marked contrast to the effects noted in the present study (a difference of 1.2 mg/dL between the two groups with baseline as covariate). This difference may be explained by the differences in study styles (open vs. double-blind), doses of n-3 FA, and study subjects. In addition, the mean baseline RLP-cholesterol values in their studies [14.5 mg/dL (8) and 12.7 mg/dL (9)] were very high compared with ours (5.1–5.6 mg/dL).

The last blood samples were collected in the middle of December, when it had already started snowing in the Toyama Prefecture where all of the study subjects lived. Mild exercise reduces blood RLP-cholesterol levels without changing body fat (23). Consequently, it might be possible that the increase in RLP-cholesterol levels in the control group toward the end of the study (Table 4) was related to a decrease in their physical activity due to the cold weather and shorter daylight hours in the Toyama Prefecture at the time of the last blood sampling. In this context, the stable levels in RLP-cholesterol in the n-3 FA group at the end of the study suggest RLP-cholesterol-lowering effects of the active food throughout the study.

The assay system employed in the present study for measuring RLP has been reported to be valid in many aspects (24). The thickness of the common carotid artery intima-media correlated better with RLP-cholesterol than with LDL-cholesterol or plasma TG levels (25). In addition, Kugiyama *et al*. (26) measured fasting serum RLP-cholesterol of 135 patients with coronary artery disease and followed them for up to 36 mon. They found that patients with the highest tertile had a significantly higher relative risk, namely, 6.12, of developing coronary events than those with the lowest RLP-cholesterol tertile as calculated with a multivariate Cox hazard model. Furthermore, it was recently indicated from cycle 4 of the Framingham Heart Study that RLP-cholesterol was an independent risk factor for cardiovascular disease in women, and provided significantly more information than did TG (27). Consequently, evidence for RLP-cholesterol as an independent risk factor for CHD is accumulating (24).

In the present study, we used essentially the same amount of n-3 LC-PUFA as in the GISSI-Prevenzione Study, in which fish oil ethyl esters reduced sudden death by 45%, to determine whether this small amount of n-3 LC-PUFA could significantly affect RLP-cholesterol. Although the detailed proof of the relationship between the reduction of RLP-cholesterol and prevention of sudden death is lacking, it is worth noting the following *in vitro* observations with regard to RLP. First, Saniabadi *et al*. (28) reported the platelet-aggregating effects of RLP. They found that the addition of RLP to whole-blood samples containing minimum lipoprotein remnants induced significant platelet aggregation; these effects were observed around and well below the physiologic concentrations, namely, concentrations of 0.08–3.2 mg/dL of RLP-cholesterol with the peak aggregation at 0.32 mg/dL. Interestingly, platelet aggregation with RLP was observed only if there were RBC, and aggregates were found on the surface of RBC (28). In contrast, other lipoproteins did not markedly affect platelets (28). In addition, the additive effects on platelet aggregation were observed with 0.06 μM ADP (28). Knöfler *et al*. (29) also found a strong potentiation by RLP of ADP-induced platelet aggregation, although they were not able to detect any direct platelet stimulation by RLP alone. Second, RLP-cholesterol was taken up by macrophages, and it is likely that they accumulated cholesterol esters and became foam cells in the vessel wall (30). Third, postmortem plasma lipid values were measured in 119 subjects 20–69 yr old, who suddenly and unexpectedly died and were autopsied (10). RLP-cholesterol values of 93 subjects (12.4 mg/dL) who died from acute myocardial infarction and chronic ischemic heart failure were significantly elevated compared with nonvascular sudden deaths (5.4 mg/dL) primarily from trauma, accident, and suicide (10). Taken together, it is conceivable that the reduction in sudden death induced by fish oil in the GISSI-Prevenzione Study might be due at least in part to a reduction in RLP-cholesterol.

The present study was not planned to elucidate the mechanisms of TG and/or RLP-cholesterol-lowering effects of fish oil. However, Figure 1 and the very high correlation at weeks 4 and 12 merit some discussion. RLP are a mixture of VLDL

metabolites and chylomicron remnants; both are metabolites of TG-rich lipoproteins. It is therefore likely that the availability of substrates for RLP production, as reflected by the total TG concentration, was lowered by n-3 LC-PUFA, and that RLP-cholesterol production was lowered accordingly. Zheng *et al*. (31), in fact, reported a decline in the secretion of TG and apo B from rat hepatocytes after exposure of the cells to fish oil.

In conclusion, it is possible that a low dose of n-3 LC-PUFA might be able to significantly reduce the serum levels of TG and RLP-cholesterol; this is of interest in considering why sudden death is infrequent in people consuming fish or n-3 LC-PUFA.

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Fish Consumption and Blood Lipids in Three Ethnic Groups of Québec (Canada)

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ABSTRACT: The purpose of this study was to compare fish intake and plasma phospholipid concentrations of n-3 fatty acids, in particular of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), among representative population samples of Québecers, James Bay Cree, and Inuit of Nunavik (Canada). The relationships between these concentrations and cardiovascular disease (CVD) risk factors were also investigated and compared in the three populations. In 1990–1992, the study subjects had participated in the extensive Santé Québec health surveys conducted in southern Québec, James Bay, and Nunavik. Significant differences in levels of CVD risk factors were found among these three populations. Globally, Inuit showed the lowest risk status for CVD compared with Cree and Québecers, despite the high prevalence of cigarette smoking and obesity. Daily fish intakes varied significantly among the three groups, averaging 13, 60, and 131 g for Québecers, Cree, and Inuit, respectively. Concentrations of EPA + DHA in plasma phospholipids were highest among Inuit (8.0%), second-highest among Cree (3.9%), and lowest among Québecers (1.8%). When the three populations were grouped together, there was a positive association between concentrations of EPA + DHA stratified into quartiles and HDL cholesterol, with a significant relation in quartile 4 (EPA + DHA ≥4.04%). An inverse relation was also found between EPA + DHA and triacylglycerols in quartile 4. Our results indicate that increased consumption of fish as a source of n-3 fatty acids is beneficially associated with levels of HDL cholesterol and triacylglycerols.

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Cardiovascular diseases (CVD) are the leading cause of mortality and morbidity in Québec (Canada), and large interregional and ethnic differences exist in cardiovascular mortality rates and risk factors among the residents of Québec. The territory of Québec extends as far north as the 60th parallel and includes Cree communities of the James Bay region located between the 49th and 55th parallels, and Inuit communities of Nunavik, located north of the 55th parallel. Both of these Aboriginal populations historically have experienced much lower rates of ischemic heart disease (IHD) than the southern Québec population (mainly Caucasian). During the period 1992–1996, the age-standardized mortality rate (per 100,000 person-years) for IHD (CIM-9, 410–414) was 66.3 among Inuit, 92.8 for the Cree, and 140.2 for the Québec population as a whole (1). Epidemiologic data have demonstrated that n-3 polyunsaturated fatty acids (PUFA) may protect against heart disease (2–5). This protective effect is associated with a high intake of fish and marine products that are rich in n-3 fatty acids, eicosapentaenoic (EPA), and docosahexaenoic (DHA) acids. Studies reporting the beneficial effects of fish consumption have been conducted mainly among populations consuming large quantities of fish (6–8). Fish consumption is relatively low among Québecers, whereas among Aboriginal populations, consumption of traditional foods generally comprises a large amount of fish and marine products. However, in recent decades, changes in lifestyle and dietary patterns, including a decrease in the consumption of traditional foods, have been documented among James Bay Cree and, more recently, among Inuit of Nunavik (9–12). As observed in many Aboriginal populations, the abandonment of aspects of a traditional lifestyle and diet has been associated with increased prevalence of CVD and of their risk factors such as obesity, high blood pressure, and diabetes. In historical sequence, the Cree population has shown these Western shifts in lifestyle and diet for a longer period of time than Inuit in Nunavik. It can be hypothesized that the gradient in CVD mortality rates observed among Québecers, Cree, and Inuit is related to differences in lifestyle, with a special emphasis on diet. Our research team recently published detailed results of fatty acid profiles and the relations between n-3 fatty acids and CVD risk factors for each of these three populations (Québecers, Cree, and Inuit) (13–15). The goal of the present study was to group together and compare data collected among these three populations. The first specific objective was to compare fish intake and plasma phospholipid concentrations of EPA + DHA in the three populations. The second specific objective was to compare the relations between EPA + DHA and plasma HDL cholesterol and triacylglycerols.

SUBJECTS AND METHODS

Study design. Between 1990 and 1992, Santé Québec, an organization of the Québec Health and Social Services Ministry, conducted a series of health surveys among southern Québecers,

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Abbreviations: BMI, body mass index; CVD, cardiovascular disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IHD, ischemic heart disease; PUFA, polyunsaturated fatty acids.

James Bay Cree, and Inuit of Nunavik. The primary objective of these surveys was to collect information on physical, social, and psychosocial health with particular focus on "heart health" (16–19). This information was gathered in several stages, and all surveys followed a common standardized protocol. Face-to-face interviews were conducted at home by nurses using a questionnaire discussing lifestyle habits and sociodemographic characteristics. The same participants were invited to attend a clinical session that involved the collection of anthropometric and physiologic measurements such as blood pressure, and a blood specimen. Another face-to-face interview was conducted by a nutritionist or a nurse to collect information on dietary habits. For the present study, information on the demographic portrait and levels of CVD risk factors was obtained from the Santé Québec data files. Furthermore, from 1992 to 1996, our team used stored frozen plasma samples to determine plasma phospholipid fatty acid concentrations among these participants. Detailed results of these fatty acid analyses and their relations with CVD risk factors were published recently (13–15).

Study population. The target population of the Santé Québec health surveys comprised noninstitutionalized men and women between 18 and 74 yr of age. Three population samples were covered by the surveys, which aimed to collect information at the regional level with participants constituting a stratified probabilistic sample of each population. The statistical sampling design, developed by the Québec Bureau of Statistics, was described elsewhere (16). Our study population included 1460 Québecers, 917 James Bay Cree, and 426 Inuit of Nunavik, for whom concentrations of plasma phospholipid fatty acids were determined.

Plasma lipids, glucose, and insulin. Concentrations of plasma total cholesterol, triacylglycerols, LDL cholesterol, and HDL cholesterol were analyzed according to methods of the Lipid Research Clinics. Cholesterol and triacylglycerol concentrations were determined in plasma and in lipoprotein fractions using an Auto-Analyzer II (Technicon Instruments, Tarrytown, NY). The HDL fraction was obtained after precipitation of LDL in the infranatant with heparin and manganese chloride. Plasma glucose and insulin were measured according to methods described elsewhere (14,20).

Plasma phospholipid fatty acids. Plasma samples used for fatty acid analyses were stored at –80°C until time of analysis. All fatty acid analyses were conducted in the same laboratory at Guelph University, Canada. For the determination of the fatty acid composition in plasma phospholipids, 200-μL aliquots of plasma were extracted after the addition of chloroform/methanol (2:1, vol/vol), in the presence of a known amount of internal standard (diheptadecanoyl phospholipid) (21). The total phospholipid was isolated from the lipid extract by thin-layer chromatography using heptane/isopropyl ether/acetic acid (60:40:3, by vol) as the developing solvent. After transmethylation, using BF_3/methanol, the fatty acid profile was determined by capillary gas–liquid chromatography. Concentrations of fatty acids were expressed as percentages of phospholipid fatty acids.

Blood pressure. Using a standardized technique (22), trained survey nurses recorded two blood pressure measurements during the home visit and two additional readings at the clinical visit. The blood pressure values reported in this paper are based on the mean of the four measurements.

Lifestyle assessment and anthropometry. The trained survey nurses administered a standard questionnaire covering lifestyle habits (including, e.g., sociodemographic characteristics, alcohol intake, smoking status). Anthropometric measurements were recorded during the clinical visit. Height, waist, and hip girth measurements were recorded to the nearest centimeter, and weight to the nearest 100 g. In this study, body mass index (BMI) and waist girth were considered to measure obesity and abdominal fat accumulation (23).

Dietary assessment. Fish intake data were obtained using a 24-h dietary recall administered by nurses and nutritionists during the face-to-face interviews conducted in participants' homes (24–26). Fish intake comprised intake of fish, shellfish, and marine mammals. Models of standardized portions were used to define and describe amounts of food eaten by the participants.

Data analysis. Statistics presented in this paper were obtained from weighted data. Each respondent was given a value (weight) corresponding to the number of subjects he or she represented in his/her respective population (16). Thus, all results presented were weighted and representative of each of the three populations. Raw *n* values are presented for information only.

Means adjusted for sex and age and standard errors (SEM) were used to present characteristics of the three populations, and ANOVA was performed to compare means. Logistic regression was used to compare the prevalence of CVD risk factors adjusted for sex and age. The association between plasma phospholipid concentrations of EPA + DHA and HDL cholesterol and triacylglycerols was assessed by multiple linear regression analysis. The regression analyses were conducted on subjects free of prescribed drugs for CVD problems such as hypercholesterolemia, high blood pressure, diabetes, and heart disease. Adjustments were made for potential confounding effects of age, sex, BMI, waist girth, smoking, and alcohol intake. The potentially modifying effect of participants' population of origin on the relation between n-3 fatty acids and CVD risk factors was verified by using an interaction term. The three populations were grouped together and the association between concentrations of EPA + DHA was stratified into quartiles; HDL cholesterol and triacylglycerols were also assessed by multiple linear regression analysis. All statistical analyses were performed with the SAS software package (SAS Institute, Cary, NC) (27), and statistical significance was set at $P \leq 0.05$.

RESULTS

The study population was comprised of 2803 subjects between 18 and 74 yr of age (1323 men and 1480 women) (Table 1). The composition of the study population with respect to ethnicity was as follows: 52% Québecers (n = 1460, mean age = 40.1 yr), 33% Cree (n = 917, mean age = 35.2 yr), and 15% Inuit (n = 426, mean age = 38.2 yr). Characteristics of

TABLE 1
Sample Size of Study Population According to Ethnicity, Sex, and Age

Ethnic group	Men				Women				Total	(%)
Age (yr)	18–34	35–49	50–74	Total	18–34	35–49	50–74	Total		
n	711	278	334	1323	811	334	335	1480	2803	
Québecers	387	120	215	722	397	151	190	738	1460	(52)
James Bay Cree	243	103	76	422	293	117	85	495	917	(33)
Inuit of Nunavik	81	55	43	179	121	66	60	247	426	(15)

the three populations are presented in Table 2. Mean age- and sex-adjusted concentrations of total and LDL cholesterol were highest in Québecers, second-highest in Inuit, and lowest in Cree. Inuit had the highest concentrations of HDL cholesterol and the lowest ratio of total to HDL cholesterol compared with Cree and Québecers. Triacylglycerol concentrations were highest among Québecers, intermediate among Cree, and lowest among Inuit. Significant differences in mean systolic and diastolic blood pressures were found between Inuit and other populations, with Inuit having the lowest mean blood pressures. There were no differences among the three populations with respect to mean concentrations of plasma glucose. In contrast, higher concentrations of insulin were observed among Cree, followed by Québecers and Inuit, respectively. In particular, Cree women had elevated insulin concentrations (mean = 124 pmol/L, data not shown). Waist girth and BMI were highest among Cree, intermediate among Inuit, and lowest among Québecers. There was a large and statistically significant difference between Inuit and Québecers for the prevalence of cigarette smoking, i.e., Inuit smoked twice as much as Québecers (65 vs. 29%), with the prevalence of smoking among Cree intermediate between the two other groups. Québecers had the greatest proportion of occasional and habitual drinkers, followed by Inuit, whereas Cree had the greatest proportion of nondrinkers (including ex-drinkers). Many more Québecers used medication for CVD problems than Inuit and Cree participants.

Data from the 24-h dietary recalls revealed that on the day before each survey, the mean fish intake was 13 g [95% confidence interval (CI): 12.7–14.4] for Québecers, 60 g (95% CI: 47.5–71.9) for Cree, and 131 g (95% CI: 110.8–152.1) for Inuit (Fig. 1). Fish intake varied significantly among the three populations and increased with age in all populations. Plasma phospholipid concentration of EPA + DHA was highest among Inuit (8.0%, 95% CI: 7.5–8.4), second-highest among Cree (3.9%, 95% CI: 3.8–4.0), and lowest among Québecers (1.8%, 95% CI: 1.7–1.8). Concentrations of EPA + DHA increased significantly with age, and this increase was more pronounced among Cree and Inuit (Fig. 2).

For the next analyses, 399 subjects were excluded because they used medication for CVD problems. For the remaining

TABLE 2
Characteristics of Québecers, James Bay Cree, and Inuit of Nunavik Who Participated in the Santé Québec Health Surveys[a,b,c]

	Québecers (*n* = 1460)		Cree (*n* = 917)		Inuit (*n* = 426)		
Characteristic	Mean	(SEM)	Mean	(SEM)	Mean	(SEM)	*P*-value
Total cholesterol (mmol/L)	5.3	(0.02)	5.0	(0.03)	5.3	(0.05)	<0.0001
LDL (mmol/L)	3.3	(0.02)	3.1	(0.03)	3.2	(0.04)	<0.0001
HDL (mmol/L)	1.3	(0.01)	1.3	(0.01)	1.5	(0.02)	<0.0001
Total/HDL cholesterol	4.3	(0.03)	4.2	(0.04)	3.8	(0.06)	<0.0001
Triglycerides (mmol/L)	1.6	(0.02)	1.4	(0.03)	1.2	(0.04)	<0.0001
Systolic blood pressure (mm Hg)	122.6	(0.36)	124.9	(0.47)	116.4	(0.67)	<0.0001
Diastolic blood pressure (mm Hg)	76.1	(0.23)	77.2	(0.30)	75.5	(0.43)	0.001
Glucose (mmol/L)	5.3	(0.04)	5.4	(0.05)	5.4	(0.07)	0.21
Insulin (pmol/L)	79.3	(1.71)	109.5	(2.35)	61.4	(3.32)	<0.0001
Waist girth (cm)	83.7	(0.32)	101.4	(0.42)	88.0	(0.62)	<0.0001
Body mass index (kg/m^2)	25.0	(0.13)	30.9	(0.16)	27.3	(0.24)	<0.0001
			Prevalence (%)				
Smoking status							
Smoker	29.1		36.0		64.7		<0.0001
Nonsmoker	70.9		64.0		35.3		
Alcohol intake							
None	11.7		69.7		52.3		<0.0001
Occasional or regular drinkers	88.3		30.0		47.7		
CVD medication							
Yes	22.9		12.0		5.1		<0.0001
No	77.1		88.0		94.9		

[a]Mean adjusted for sex and age (SEM); *P*-value obtained from ANOVA.
[b]Prevalence adjusted for sex and age (%); *P*-value obtained from logistic regression.
[c]Abbreviation: CVD, cardiovascular disease.

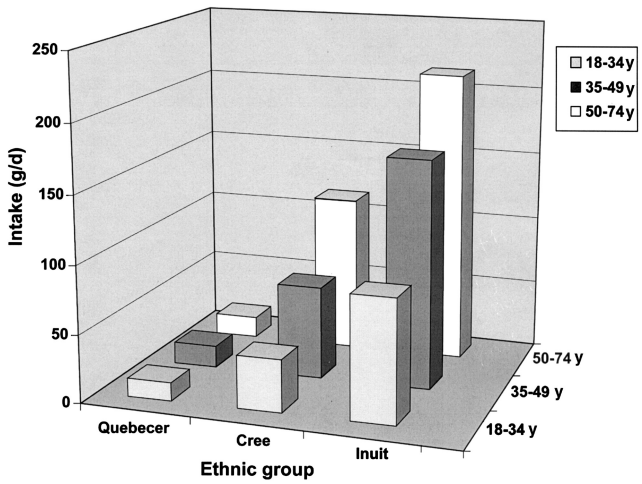

FIG. 1. Mean intake of fish and marine products by age group among Québecers, James Bay Cree, and Inuit of Nunavik.

subjects (n = 2404), the association between concentrations of EPA + DHA and HDL cholesterol and triacylglycerols was examined. Globally, the relationship between EPA + DHA and HDL cholesterol and triacylglycerols varied significantly according to population group ($P < 0.0001$ for HDL cholesterol and triacylglycerols). However, when stratifying EPA + DHA into quartiles, there was no modifying effect of population group on the relation between EPA + DHA and HDL cholesterol and triacylglycerols, ($P > 0.09$ for HDL and $P > 0.43$ for triacylglycerols). We found that HDL cholesterol increased with the increase in EPA + DHA, and the relation was significant in quartile 4 (Table 3). In contrast, triacylglycerol concentrations were inversely associated with EPA + DHA in quartile 4.

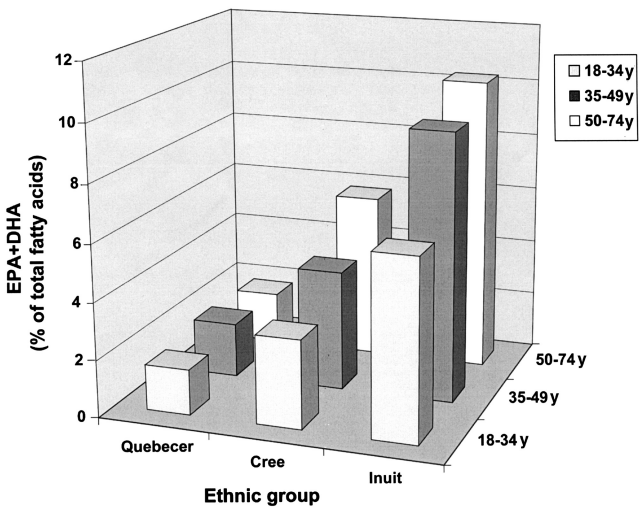

FIG. 2. Mean plasma phospholipid concentrations of eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) by age group among Québecers, James Bay Cree, and Inuit of Nunavik.

TABLE 3
Regression Coefficients[a] (β values) of EPA + DHA in Relation to HDL Cholesterol and Triacylglycerols as Dependent Variables

	HDL		Triacylglycerols	
Quartiles of EPA + DHA	β coefficient	(*P*)	β coefficient	(*P*)
Quartile 1 (≤1.62)	0.088	(0.59)	1.060	(0.002)
Quartile 2 (1.62–2.35)	0.017	(0.95)	–0.178	(0.81)
Quartile 3 (2.36–4.03)	0.284	(0.22)	–0.962	(0.09)
Quartile 4 (≥4.04)	0.601	(<0.001)	–0.817	(<0.001)

[a]Regression coefficients and *P*-values obtained from multiple linear regression analysis; one model for each cardiovascular disease risk factor; each model included age, sex, body mass index, waist girth, smoking, and alcohol intake. Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

DISCUSSION

Our results show large cross-ethnic differences in plasma phospholipid concentrations of n-3 fatty acids among Québecers, James Bay Cree, and Inuit of Nunavik. We expected to observe such great differences among the three populations because their respective fish and marine product consumptions differed markedly. Fish and marine products are captured and consumed in large quantities all year around by Cree and Inuit, whereas Québecers generally consume small quantities of fish. This may reflect a south–north gradient in nutritional habits; Québecers have greater access to processed and market foods and are not necessarily as traditionally and culturally tied to fish consumption as these two Aboriginal groups in the province. Moreover, Québecers generally consume commercial fish such as cod, plaice, and haddock (28), which are lean-flesh fishes with low concentrations of EPA and DHA (Blanchet, C., and Dewailly, É., unpublished data). Québecers also consume fish from aquaculture operations such as salmon and trout for which the n-3 fatty acid concentrations may greatly vary depending on their feeding patterns (29–31). In fact, the plasma phospholipid level of n-3 fatty acids observed among Québecers closely approached that of U.S. citizens (29,30). In contrast, concentrations of n-3 fatty acids of Nunavik Inuit are probably among the highest reported to date, as are those of their counterparts throughout the circumpolar north (32–35). Traditional food consumption, obtained by hunting, fishing, trapping, and gathering activities, is still very common among Inuit populations, and because they reside in predominantly coastal locations, they have great access to marine resources. Inuit consume high quantities of fish and marine mammals, which are made up primarily of fatty-flesh and n-3 fatty acid-rich species (12).

James Bay Cree had fish intake and plasma phospholipid concentrations of n-3 fatty acids intermediate between the two other populations. Cree are not traditionally marine resource harvesters, consuming more freshwater fish and terrestrial mammals; for a number of reasons, they have demonstrated these shifts in consumption to a more Westernized diet for a longer period of time than the Inuit population of Nunavik (15,26,36,37). However, the level of n-3 fatty acids observed among James Bay Cree is certainly much higher

than that of the majority of Westernized populations. Furthermore, in a recent study, we examined the profile of n-3 fatty acids according to geographical place of residence of James Bay Cree (15). Results showed that fish intake and concentrations of EPA + DHA were significantly higher among coastal residents (3.8%; 95% CI: 3.7–3.9) than those in inland communities (3.2%; 95% CI: 3.0–3.3), which likely reflected the greater accessibility and availability of fish in coastal areas.

The relation between fish intake and plasma phospholipid concentrations of n-3 fatty acids could not be examined directly in this study because fish intake data were obtained from a single 24-h dietary recall, which is not representative of regular long-term consumption habits (38). Nevertheless, our results showed that fish intake varied significantly among these three populations, with figures for fish intake among Inuit being approximately two and ten times greater than that of Cree and Québecers, respectively. Consequently, we observed that the population level of fish and marine product consumption was reflected in plasma concentrations of EPA and DHA. In the three populations, fish intake and plasma concentrations of EPA and DHA increased significantly with age; this association was stronger among Cree and Inuit. These observations are consistent with other dietary surveys conducted among northern Aboriginal populations, which have indicated positive correlations between age and traditional food consumption (12,33,34,39–41). Older Aboriginal people consume fish, marine products, and wild animals more frequently than younger adults and youth. The greater availability of market foods in many Aboriginal regions appears to be attracting young people more quickly than older individuals.

A number of factors such as high blood pressure, elevated plasma lipids and lipoproteins, diabetes, obesity, and diets high in saturated and *trans*-fatty acids increase the risk of CVD (42–46). In this study, we observed significant differences in CVD risk factor levels among the three populations. For HDL cholesterol, the ratio of total to HDL cholesterol, triacylglycerols, systolic and diastolic blood pressures, and insulin, Inuit, despite the high prevalence of cigarette smoking and obesity, had the lowest risk status for CVD compared with Cree and Québecers. Moreover, it is well known that the Inuit population is younger than that of Cree or south Québecers and that CVD risk increases with age. The adjustment for age of CVD risk factor levels did not change the lower CVD risk level observed among the Inuit population compared with Cree and Quebec populations. High triacylglycerol and low HDL cholesterol concentrations are now well recognized as key risk factors for CVD (43,46). A number of intervention studies have demonstrated the triacylglycerol-lowering effect of n-3 fatty acids (47–52). Some studies have also observed an HDL-increasing effect of n-3 fatty acids (49,53,54). Surveys conducted in animals and humans suggest that the suppression of the hepatic VLDL and triacylglycerol production is the primary mechanism by which n-3 fatty acids reduce triacylglycerol levels (43). This effect is mediated by increased fatty acid oxidation and a decrease in fatty acid synthesis, which in turn decreases fatty acid availability for triacylglycerol synthesis (43,53). The mechanism by which n-3 fatty acids increase HDL may be related to the decrease in lipid transfer protein activity, favoring an increase in larger HDL (HDL_2 particles), which are considered to be the most antiatherogenic HDL subtype (53–55).

Detailed statistical analyses published previously by our team revealed that positive association between n-3 fatty acids and HDL concentrations was consistent among Inuit and Cree populations (13–15). Among Québecers, only EPA and the ratio of EPA to arachidonic acid were positively associated with HDL cholesterol (13). Moreover, no consistent and inverse association between n-3 fatty acids and triacylglycerols was found among Québecers, whereas inverse relations were observed among Cree and Inuit. It seems likely that the levels of fish intake or n-3 fatty acid concentrations among Québecers were not high enough to show a significant and potential beneficial effect of n-3 fatty acids on plasma triacylglycerols, whereas the strength of the inverse association observed among Cree and Inuit likely reflected their higher fish and marine product consumption. Moreover, statistical analyses performed in the present study in using quartiles of EPA + DHA suggested a threshold value in plasma phospholipids (quartile 4) at which a potential beneficial effect of EPA + DHA on HDL and triacylglycerols could be observed. In fact, only 0.4% of Québecers had plasma phospholipid concentrations of EPA + DHA included in quartile 4, whereas 89% had concentrations lower than the median (quartiles 1 and 2). In contrast, the higher EPA + DHA concentrations of James Bay Cree and Inuit allowed the inclusion of 31% of the Cree and 84% of the Inuit in quartile 4.

In the present study, we cannot estimate the daily intake of EPA + DHA associated with a plasma EPA + DHA concentration in quartile 4. However, it was reported that a consumption of a minimum of 1 g/d of n-3 fatty acids can be expected to significantly lower triacylglycerols (43). Our results agree with these findings, i.e., a minimum intake or concentration of n-3 fatty acids is required to obtain an effect on triacylglycerols or HDL cholesterol. The daily fish intake of the Québecer, Cree, and Inuit populations provided ~170, 700–900, and 2115 mg of EPA + DHA, respectively. Recently, an American group of nutrition scientists recommended a combined average EPA + DHA intake of 650 mg for optimal health and CVD prevention (56). Numerous studies have indicated that long-term consumption of fish (up to 2–3 servings/wk) is associated with lower primary and secondary heart attack rates and death from CVD (3,4,47,57–61). Although our study design differed from that of those prospective studies, data on CHD mortality rates among the three study populations suggest that the traditional diets of Cree and Inuit, which are rich in n-3 fatty acids, contribute to their relatively favorable IHD mortality rate. However, as the Cree and Inuit lifestyle and diet become increasingly Westernized, their relatively low level of risk for IHD may be lost. Hence, the promotion of a healthy nutritional diet among these populations should include nutritious market foods and traditional foods of marine

origin, in particular among young Arboriginal people, who are less likely to eat traditional foods than their parents. Québecers must also be encouraged to increase their consumption of fish and marine products, especially species rich in n-3 fatty acids.

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Phytosterols and Human Lipid Metabolism: Efficacy, Safety, and Novel Foods

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ABSTRACT: Plant sterols have been known for several decades to cause reductions in plasma cholesterol concentrations. These plant materials have been granted a conditional health claim in the United States regarding their effects in the prevention of cardiovascular disease and are being sold in functional foods in several countries in Europe as well as in the United States and Australia. It is generally suggested that daily consumption of ~2 g of plant sterols can lower cholesterol concentrations as part of a dietary prevention strategy. However, phytosterols have been added and tested for their cholesterol-lowering effects mainly in spreads. Consumption of these high-fat foods seemingly flies in the face of current recommendations for the promotion of heart health, which suggest lowering total fat and energy intake to maintain weight. Hence, new food formulations are being evaluated using phytosterols incorporated into low-fat and reduced-fat food items. The purpose of this review is to examine the cholesterol-lowering efficacy of plant sterols, focusing on novel food applications, their mechanism of action, and safety. These novel food formulations include new solubilization processes that lead to improved uses for plant sterols, as well as new foods into which phytosterols have been incorporated, such as breads, cereals, and beef. Such new foods and formulations should pave the way for greater use of phytosterols in heart health promotion, increasing the longer-term potential for the creation of innovative functional foods containing plant sterols and their derivatives.

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Plant sterols have long been known to decrease plasma cholesterol concentrations. The new Therapeutic and Lifestyle Changes guidelines of the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) now encourage the use of plant sterols as therapeutic dietary options before resorting to drug treatment to lower plasma cholesterol concentrations (1). However, plant sterols, due to their lipophilic nature, have traditionally been incorporated into high-fat foods such as spreads, seemingly a contradiction in light of current approaches to maintain heart-healthy diets and lifestyles. The NCEP ATP III guidelines suggest reducing total fat intake to 25–35% of total energy intake and maintaining energy balance (1). Therefore, novel food applications are emerging in an attempt to incorporate phytosterols into lower-fat foods. The objective of this review is to examine the efficacy of phytosterols for cholesterol reduction, with emphasis on new food formulations as well as mechanisms of action and safety.

EFFECTS OF PHYTOSTEROLS AND PHYTOSTANOLS ON PLASMA LIPID LEVELS: STUDIES USING SPREADS

A recent meta-analysis of 18 clinical trials showed that plant sterol consumption, either as phytosterols or as hydrogenated phytostanols incorporated into spreads, led to reductions in LDL cholesterol (LDL-C) concentrations of 0.33–0.50 mmol/L, depending on the dose given and the age of the subjects (2). This absolute reduction is equivalent to a decrease of 8–13% in LDL-C, which would translate into a 25% lower risk of cardiovascular disease (3). Although the meta-analysis grouped studies of plant sterols and stanols, earlier research examining the effects of plant sterols proposed that the hydrogenated phytostanols provided better cholesterol-lowering potential than the nonhydrogenated phytosterols (4–7). In a 12-mon trial, consumption of 2.6 g/d of sitostanol in margarine reduced total cholesterol (TC) and LDL-C concentrations by 10.2 and 14.1%, respectively (7). Smaller TC and LDL-C reductions of 5.4 and 7.5%, respectively, were noted in children consuming margarine providing 1.5 g phytostanol/d for 3 mon (8).

Phytosterols have also been shown to decrease TC and LDL-C by 5 and 9%, respectively, in normocholesterolemic subjects (9) and by 17% in children with familial hypercholesterolemia (10). In adult men with elevated cholesterol levels, a controlled diet enriched with a blend of esterified phytosterols containing mainly sitosterol and campesterol resulted in 9.1 and 15.5% declines in TC and LDL-C concentrations, respectively, relative to an identical control diet not containing plant sterols (11). These trials show that phytosterols, when incorporated into high-fat spreads, produce significant cholesterol reductions.

More recently, trials have been conducted to compare directly the efficacy of phytosterol and phytostanol consumption, as part of margarines or butter, in reducing plasma cholesterol concentrations (12–15). Jones *et al*. (13) showed that when administered in comparable dosages and in esterified forms, phytosterols possessed slightly greater efficacy in reducing LDL-C

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Abbreviations: ABCG, ATP-binding cassette transporter G; apo, apolipoprotein; LDL-C, LDL cholesterol; NCEP ATP, National Cholesterol Education Program Adult Treatment Panel; TC, total cholesterol.

concentrations than phytostanols. However, the same effect was not observed by others (12,14,15), who found similar cholesterol-lowering efficacy between plant sterol ester and plant stanol ester spread consumption. In a controlled feeding clinical trial (14), consumption of free phytosterols, phytostanols, or a 1:1 mix of phytosterols and phytostanols all caused similar decreases of 7.8–13.1% in TC and 11.3–16.0% in LDL-C concentrations. Although the trial of Jones *et al.* (13) favored phytosterols over phytostanols, all trial results agree that plant sterols, with and without hydrogenation, result in a TC and LDL-C lowering of ~6 and 13%, respectively, relative to control in short-term trials (12–15). It is now generally accepted that plant sterol esters, stanol esters, and free sterol/stanol mixtures, when consumed in high-fat spreads, possess similar efficacy for lowering TC and LDL-C (3,16,17).

DOSAGE

Most recent trials producing cholesterol reductions with plant sterols and stanols have used dosages ranging from 0.8 to 3 g/d. In several dose-response trials, doses as low as 0.8 g/d were efficacious in lowering TC and LDL-C concentrations (6,18,19). Similarly, there was no difference in the extent of cholesterol-lowering between a phytosterol ester dose of 1.1 and 2.2 g/d (20) or a dose of 1.5 and 3 g phytosterols/d (21). In the study by Christiansen *et al*. (21), margarines were consumed for a period of 6 mon and doses of 1.5 and 3 g phytosterols/d produced reductions in TC concentrations of 8.9 and 8.3%, respectively, and in LDL-C concentrations of 11.3 and 10.6%, for the lower and higher doses, respectively, compared with control. As part of an NCEP Step I diet, doses of 1.1 and 2.2 g phytosterol esters/d reduced TC and LDL-C concentrations by 5.2–6.6% and 7.6–8.1%, respectively, vs. control (20). It was demonstrated recently that a margarine enriched with 2.9 g of phytosterol esters, providing ~1.8 g phytosterols/d, was more effective in reducing LDL-C concentrations in subjects consuming a high-cholesterol, high-fat, or high saturated fat diet (22). Subjects who consumed more energy and had average and high campesterol to cholesterol ratios also had greater reductions in LDL-C than subjects who consumed less energy and were low cholesterol absorbers. Therefore, when consumed as part of high-fat spreads, and in diets containing 30–35% of energy as fat, plant sterol doses of 1.1–3 g/d seem to have similar cholesterol-lowering efficacy.

Although it has been recommended that 2 g plant sterols/d be consumed to achieve cholesterol-lowering (1), equivalent reductions may also be possible with smaller doses. Very small intakes of 150 and 300 mg of phytosterols added to sterol-free corn oil in a single test meal reduced cholesterol absorption by 12.1 and 27.9%, respectively, and may contribute to the cholesterol-lowering benefits of plant oils (23). In a randomized, crossover trial, 740 mg phytosterols/d, incorporated into butter, produced reductions in TC and LDL-C concentrations of 10 and 15%, respectively, compared with a control diet (24). In that trial, the processing method used to disperse finely ground particles of plant sterols evenly in butter may have enhanced their efficacy.

Small doses of phytosterols, naturally occurring in a vegetarian diet, may also lead to significant reductions in circulating cholesterol concentrations (25). In a study examining the effects of high fruit and vegetable consumption on plasma lipid concentrations, it was found that consuming a diet resembling our early ancestral diet, i.e., one containing exclusively fruits, vegetables, and nuts, provided ~1–1.4 g phytosterols/d (26). Consumption of this vegetarian diet led to reductions in TC and LDL-C of 15 and 27% compared with a control, low saturated fat diet. Although several components of the high fruit and vegetable diet may have contributed to the reductions in cholesterol, it is believed that this level of phytosterol intake may be sufficient to effectively lower circulating cholesterol concentrations. It is possible that natural sources of plant sterols may have greater efficacy at dosages lower than those resulting from artificial (man-made) enrichment of foods with phytosterols, perhaps due to the nature of the molecular distribution in foods. Optimal dispersion of phytosterols in certain natural food matrices may further reduce intestinal cholesterol absorption, possibly through enhanced stimulation of the ATP-binding cassette transporter G (ABCG) or greater displacement of cholesterol from micelles within the intestinal lumen (27). Ingestion of phytosterols with other components found in certain natural foods (perhaps soluble fibers such as β-glucans or plant membrane components such as those found in dietary lecithin) may enhance the bioavailability and serum cholesterol-lowering efficacy of phytosterols.

Trials examining the effects of plant sterols in spreads have found that optimal reductions in cholesterol concentrations are obtained with 1.6 g plant sterols/d (18,19). In the meta-analysis by Law (2), trials using <2 g plant sterols/d gave reductions of 0.4–0.5 mmol/L in LDL-C concentrations, whereas trials using doses >2 g/d had LDL-C reductions of 0.43–0.54 mmol/L in subjects in the same age range. Therefore, it is generally accepted that phytosterol intakes of ~2 g/d are sufficient to elicit a hypocholesterolemic response. Nevertheless, lower quantities of plant sterols, 750–1000 mg/d, have been shown to produce cholesterol-lowering (24,26). This level of phytosterol consumption is similar to dietary intakes of the great ape and hominoid periods (26). It can therefore be speculated that high circulating cholesterol concentrations are consequences of a lack of dietary plant sterols and that phytosterols, in small quantities, may be essential dietary components. The addition of phytosterols into foods may thus fill a dietary deficiency in our modern diets (Fig. 1).

NOVEL FOOD APPLICATIONS FOR PLANT STEROLS AND STANOLS

Plant sterols and stanols, and their esterified derivatives, have been studied mainly when incorporated into dietary fats such as spreads (http://www.benecol.com; http://www.takecontrol.com). Results from a previous trial (22) seem to indicate

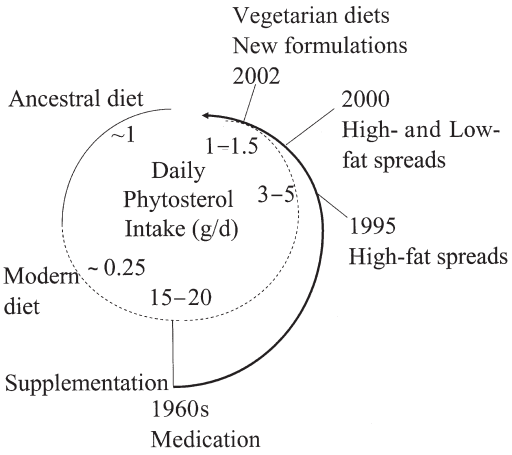

FIG. 1. Historical perspective of phytosterol intakes and recommended supplementation levels (g/d) for cholesterol reduction relative to dietary form.

greater cholesterol-lowering benefit of plant sterols in subjects who consume a less desirable diet, providing a strong rationale for incorporating plant sterols into high-fat foods. However, because the aim of phytosterol supplementation is to lower cholesterol, and knowing that cholesterol concentrations are also responsive to dietary fat intakes, it seems counterintuitive to use a high-fat food item as a vehicle for a lipid-reducing agent. Trials have thus been conducted to examine the effects of plant sterols in low-fat spreads (20,28,29), food items more advisable for incorporation into a heart-healthy diet.

Hallikainen and Uusitupa (28) investigated the effect of a low-fat margarine containing 40% of energy from fat as a vehicle for wood- and vegetable oil-based plant stanols on plasma lipid concentrations. In this supplementation trial, ~2.3 g plant stanols/d decreased TC by 8.0–10.6% and LDL by 8.5–13.7% vs. control. Apolipoprotein (apo) B concentrations decreased by 8.5–14.0% in the plant stanol-supplemented groups compared with the control. Another recent trial also observed cholesterol-lowering in a comparison of groups supplemented with 1.1 or 2.2 g phytosterol esters/d with a control group not consuming phytosterol esters (20). Consumption of both phytosterol ester-supplemented fat-reduced margarines resulted in similar TC and LDL-C lowering of 5.2–6.6% and 7.6–8.1%, respectively. As was noted by Hallikainen and Uusitupa (28), apoB concentrations were reduced with intakes of phytosterol esters, showing that phytosterol ester consumption not only lowers cholesterol concentrations but also diminishes the number of atherogenic particles in circulation. However, another trial testing the efficacy of phytosterol esters incorporated into low-fat spreads and salad dressings failed to produce any difference in degree of cholesterol-lowering with doses of 0, 3, 6, and 9 g phytosterols/d (29). It was hypothesized that phytosterols from a reduced-fat salad dressing may not have been well incorporated into micelles and that compliance may not have been optimal. It is also possible that the amount of fat in the salad dressing, 28% of energy content, was too low to effectively solubilize the phytosterols and hence diminished their cholesterol-lowering efficacy.

Lately, methods have been developed and tested in attempts to make the use of plant sterols in low-fat foods more applicable (Table 1). For example, microcrystalline suspensions of plant sterols in rapeseed oil allow for incorporation of up to 30% of plant sterols into a food without any chemical reactions or additives such as emulsifiers, and have been shown to reduce TC and LDL-C concentrations by 8.3–8.9% and 10.6–11.3%, respectively (21). This extent of cholesterol-lowering is similar to that observed using phytosterol esters dispersed in high-fat spreads. Solubilization of phytosterols in DG as opposed to TG also has been proposed as a method of formulating phytosterols for incorporation into foods. It was found that phytosterols are more soluble in DG than in TG (6 vs. 1.3%, respectively), and result in significant decreases in TC and LDL-C concentrations when consumed in a small dose of 500 mg phytosterols/d in 10 g of mayonnaise (30). Lecithin micelles can also be used to increase the intestinal bioavailability of phytostanols (27). Incorporating phytostanols into lecithin micelles can decrease cholesterol absorption by ~34 and 37% at doses of 300 and 700 mg, respectively. Such formulations can result in very small intakes of phytostanols, producing measurable reductions in cholesterol concentrations, although the trial by Ostlund *et al*. (27) could not establish this effect due to its short duration. Also, in animal trials, an analog of a hydrophilic phytostanol ester reduced TC and LDL-C concentrations and prevented weight gain in gerbils without affecting their food and water intakes (31).

These newer formulation methods for phytosterols increase their potential for incorporation into low-fat foods, thereby augmenting the number of functional foods that can be produced using plant sterols. Low-fat functional foods using phytosterols would also be more valuable in the promotion of healthy eating, especially in a population at higher risk of cardiovascular disease. In addition, formulations that improve phytosterol bioavailability and action may allow smaller doses to be as effective for cholesterol-lowering as the currently recommended doses. Trials are thus needed to assess dose-responsiveness with these new formulations and to determine whether smaller doses than those currently recommended can produce similar cholesterol reductions in long-term treatment. Only one trial to date has compared the cholesterol-lowering efficacy of a new formulation method, DG solubilization, with a conventional TG solubilization method (30). TC and LDL-C concentrations were reduced by 4.7 and 7.6%, respectively, with consumption of 500 mg/d of DG-solubilized plant sterols, compared with no change in TC concentrations and a nonsignificant reduction of 3.6% for LDL-C with consumption of the TG-solubilized plant sterols. Consumption of plant sterol solubilized in DG resulted in significantly greater change in TC concentrations than plant sterols solubilized in TG.

Recently, the effects of plant sterols on plasma lipids were examined when plant sterols were esterified to n-3 FA from an EPA and DHA concentrate (32). After 4 wk of consumption,

TABLE 1
New Formulations and Food Applications for Phytosterols and Their Cholesterol-Reducing Potential[a]

Formulation/food	Reference	Plant sterol (g/d)	Lipid reduction vs. control
Microcrystalline suspension	21	1.5	TC 8.9%; LDL-C 11.3%
		3.0	TC 8.3%; LDL-C 10.6%
DG solubilization	30	0.5	TC 4.7%
			LDL-C 4.5% (NS)
Lecithin micelles	27	0.3	Cholesterol absorption 34%
		0.7	Cholesterol absorption 37%
Hydrophilic analog (animal trial)	31	1 and 2% w/w	TC 43% LDL-C 100%
Esterification to n-3 FA (animal trial)	32	0.25% w/w	TC 36% Non-HDL-C 38% TG 29%
Low-fat spread	28	2.3	TC 8–10.6% LDL-C 8.5–13.7%
Low-fat spread and dressing	29	3	TC 4.9% (NS); LDL-C 3.7% (NS)
		6	TC 0.9% (NS); LDL-C 1.5% (NS)
		9	TC 4.6% (NS); LDL-C 6.7% (NS)
Corn fiber oil (animal trial)	33	1% w/w[b]	TC 30%; LDL-C 32%
		2% w/w	TC 49%; LDL-C 55%
		3% w/w	TC 53%; LDL-C 57%
Corn fiber oil (animal trial)	34	1% w/w	TC 13.2% Non-HDL-C 14.4%
Beef	39	2.7	TC 9.3% LDL-C 14.6%
Low-fat yogurt	43	3	TC 8.7% LDL-C 13.7%
Low-fat yogurt	42	1	TC 4.4%[c] LDL-C 6.2%
Breakfast cereal, bread, margarine (1:1:1)	40	2.4	TC 8.5%[d] LDL-C 13.6%
Bread, sausage, yogurt	41	0.9	TC 4.4%; LDL-C 5.4%
		1.9	TC 6.2%; LDL-C 7.9%
		4.2	TC 5.4%; LDL-C 8.1%

[a]Abbreviations: TC, total cholesterol; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; NS, nonsignificant.
[b]Plant sterol content calculated as 20% of total corn fiber oil consumption.
[c]Versus control, no statistics reported by authors.
[d]Versus baseline, baseline values not provided by authors.

guinea pigs fed the test diet containing plant sterols esterified to the long-chain n-3 FA had a 28% reduction in TG concentrations compared with controls, as well as lower concentrations of TC and thromboxane A_2. Esterification of plant sterols to FA known for their beneficial effects on blood lipids could increase the scope of action of phytosterols. For instance, in the trial by Ewart *et al*. (32), when esterified to n-3 FA, plant sterols were able to fulfill the dual action of lowering cholesterol concentrations as well as TG levels, a component of plasma lipids that is usually not altered by plant sterol supplementation.

Corn fiber oil, which contains ~13.9 g phytosterols/100 g, has also been shown to reduce cholesterol concentrations in animal models (33,34). Consumption of corn fiber oil by guinea pigs led to concentrations of TC and LDL-C that were 30–53% and 32–57%, respectively, lower than control (33). However, similar intakes of corn fiber oil reduced TC and non-HDL-C concentrations by 13 and 14%, respectively, vs. control in hamsters (34). In that trial, non-HDL-C reductions were similar to reductions obtained when fortifying corn oil with sterols and stanols. It can thus be hypothesized that the cholesterol-lowering ability of corn fiber oil stems from its phytosterol content.

The phytosterols in corn fiber oil are largely esterified to ferulic acid, an antioxidant (3). As hypothesized earlier, esterifying phytosterols to other active ingredients, or mixing it with other oils with functional properties, can increase the health benefits obtained from phytosterol consumption. For example, mixing phytosterols with medium-chain TG oil, which is known for its effects on energy metabolism and satiety (35), has been shown to produce cholesterol reductions (36,37). Therefore, a combination of medium-chain TG oil and phytosterols could enhance energy metabolism, leading to a negative energy balance, while also improving the plasma lipid profile. Consumption of such a concoction could improve cardiovascular disease twofold, by preventing weight gain and by lowering circulating cholesterol concentrations. Another possible approach to reducing weight gain and improving lipid concentrations may be through consumption of the new hydrophilic phytostanol analog (38). Although this

new plant sterol formulation has not been studied in humans, preliminary results suggest that weight gain may be blunted without altering food and water intake in gerbils.

Phytosterols have also been incorporated into novel foods, including low-fat and non-fat food items (Table 1). One such new food application of phytosterols is their incorporation into beef (39). Subjects were fed lean ground beef containing phytosterol esters or no plant sterols at lunch for a period of 28 d. The phytosterol-containing beef provided 2.7 g plant sterols/serving and produced reductions of 9.3 and 14.6% in TC and LDL-C concentrations, respectively. There was no change in blood lipid concentrations in the control group eating beef that did not contain plant sterols. This group also assessed LDL particle size in subjects consuming placebo or plant sterol-containing beef, but found no effect of treatment on particle size and proportion of small and large LDL. Results from this trial show that plant sterols can be added to low-fat animal products to produce beneficial plasma lipid profiles.

Few studies have examined the effects of low-fat and non-fat phytosterol-containing foods (40–43). It is believed that solubilization of phytosterols is necessary to observe a hypocholesterolemic effect (16). Because plant sterols are lipophilic, their efficacy in low-fat foods was thought to be unlikely. Nevertheless, all studies examining the effects of low-fat foods containing phytosterols have reported significant cholesterol-lowering of 7–13% with phytosterol consumption (40–43). However, some methodological limitations prevent firm conclusions from being drawn from these trials. For instance, in the trial by Nestel *et al*. (40), one-third of the phytosterols, 0.8 g/d, was provided in margarine. This level of phytosterol supplementation has previously been shown to favorably alter plasma lipid profile and may have been solely responsible for the decrease in cholesterol concentrations observed. In another trial (41), the fat content of foods was not provided. It is possible that the quantity of fat intrinsic to the foods in which phytosterols were incorporated was sufficient to solubilize the plant sterols and ensure their efficacy. In the study by Volpe *et al*. (42), results from the treatment and placebo groups were not compared; thus, it is not known whether there was a statistically significant difference in the blood lipid levels of subjects consuming a yogurt enriched with 1 g phytosterols/d compared with a yogurt not containing phytosterols. Substantial decreases in TC and LDL-C concentrations were observed by Mensick *et al*. (43) when ~3 g/d of esterified stanols was provided in low-fat yogurts. TC and LDL-C concentrations decreased by 8.7 and 13.7%, respectively, relative to placebo. In this trial, because stanols were esterified, it is uncertain whether similar results would be obtained using free stanols.

MECHANISMS OF CHOLESTEROL-LOWERING ACTION OF PLANT STEROLS/STANOLS

The exact mechanism by which plant sterols exert their hypocholesterolemic action is not clearly understood. It has been shown that plant sterols decrease intestinal cholesterol absorption (13,44,45) while de-suppressing its synthesis (13). In an early study comparing the effects of phytosterols and phytostanols on cholesterol absorption, a phytosterol infusion decreased intestinal cholesterol absorption from 32 to 16%, whereas a phytostanol infusion reduced cholesterol absorption from 29 to 5% (44). It was thus concluded that phytostanols are more effective in inhibiting cholesterol absorption than phytosterols, perhaps supporting the early notion that phytostanols were more effective than phytosterols for cholesterol-lowering. However, it was observed recently that both plant sterol and stanol esters reduce cholesterol absorption to the same extent, from 56 to 38 and 39%, respectively, when consumed blended with butter as part of a high-fat, high-cholesterol diet (45). Similar results were obtained with consumption of phytosterol and phytostanol ester margarines as part of controlled diets (13) and with phytostanol ester margarine supplementation (46). Decreased cholesterol absorption seems to stimulate cholesterol synthesis, and it has been shown that both plant sterol and stanol esters increase cholesterol synthesis by ~53 and 38%, respectively, relative to a control diet (13). However, the extent to which cholesterol synthesis is enhanced with plant sterol and stanol consumption does not seem to compensate entirely for the decrease in cholesterol absorption (13), hence the reduced circulating levels of cholesterol.

Margarines containing phytostanol esters have also been shown to increase the fecal output of cholesterol as neutral sterols (36%), but not as bile acids (46). A recent report demonstrated increased cholesterol esterification with infusion of a phytostanol ester formula compared with a control formula (47). Cholesterol may be esterified to a greater extent due to preferential hydrolysis of plant sterols over cholesterol (47). Because esterified cholesterol is less well solubilized into micelles than free cholesterol, its absorption is less efficient, leading to enhanced cholesterol excretion (Fig. 2).

Another possible mechanism for phytosterol action may be through the ATP-binding cassette transporters. Two separate genes, ABCG-5 and ABCG-8, are involved in the intestinal absorption of sterols and promote their excretion in bile (48,49).

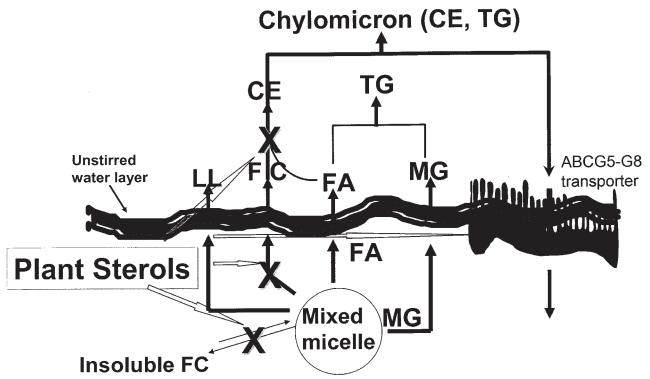

FIG. 2. Plant sterols may exert their action through prevention of free cholesterol incorporation into micelles, prevention of micellar absorption across the intestinal mucosa, or prevention of free cholesterol esterification to cholesterol esters. Abbreviations: CE, cholesterol esters; FC, free cholesterol; LL, lysolecithin.

In addition, mutations in any one of those genes are associated with sitosterolemia (48,49), a disorder characterized by enhanced phytosterol absorption. Bile from individuals affected with sitosterolemia is poor in cholesterol, further showing that the genes involved in selective sterol absorption may also be involved in cholesterol output into bile (48,50). It is believed that the products of ABCG-5 and ABCG-8, sterolin-1 and sterolin-2, respectively, are both involved, either as heterodimers or as tightly coupled proteins, in the selective transport of cholesterol in and out of enterocytes as well as in sterol excretion into bile (51). It may therefore be hypothesized that plant sterols exert their action on cholesterol metabolism by stimulating the ABCG-5/ABCG-8 complex, resulting in increased cholesterol excretion (50). A polymorphism in ABCG-5 and ABCG-8 may affect the ability of plant sterols to displace cholesterol from intestinal micelles and may thus explain the high degree of variability observed in response to phytosterol consumption among individuals.

SAFETY

Animal and human experiments have not reported any serious side effects with consumption of phytosterols in spreads at low doses (52). A previous concern with the use of plant sterols in high doses was their effect on sex organ weight, sperm concentration, and sex hormone secretion in animals (53). However, in a series of safety evaluations of phytosterols, it was demonstrated that phytosterol esters did not have any estrogenic effect *in vitro* and *in vivo* in rabbits (54), nor did it affect reproductive performance in rats over two generations (55).

In humans, daily consumption of 8.6 g phytosterols provided in margarine for 3–4 wk did not affect gut microflora and female sex hormone levels (56), nor did it produce increased concentrations of sterol oxides (57). In addition, it was reported that phytosterols and phytosterol esters showed no evidence of mutagenicity when assessed using a bacterial mutation assay, a chromosome aberration assay in human peripheral blood lymphocytes, and in a mammalian cell gene mutation assay (58). It can thus be concluded that plant sterols are safe for consumption at low doses and do not result in deleterious effects in reproduction or cancer risk.

Another concern regarding the use of plant sterols is their possible negative effect on fat-soluble vitamin concentrations (15,19). However, Raieni-Sarjaz *et al*. (59) demonstrated that intakes of ~2 g of phytosterol or phytostanol esters in margarine did not alter concentrations of vitamins D and K, α- and β-tocopherol, α- and γ-carotene, lycopene, and lutein, after adjusting for TC reductions. Hendriks *et al*. (19) also observed no effect of plant sterol esters on levels of vitamins D and K, as well as the ratio of α-tocopherol to TC, but this group found a significant decrease in α-tocopherol concentrations. In this dose-response trial, it was concluded that 1.6 g plant sterols was the daily dose that would produce optimal cholesterol-lowering with minimal effects on fat-soluble vitamin concentrations. Over a 1-yr period, it was found that phytostanol ester supplementation did not affect vitamin D and retinol concentrations but led to decreases in α-tocopherol and α- and β-carotene (60). However, after adjusting for TC concentrations, only β-carotene concentration was lowered compared with baseline and control. It was concluded that the reductions in β-carotene were not a concern because retinol concentrations remained stable.

A recent trial examining the effects of phytosterol esters in low-fat spreads on plasma lipid and fat-soluble vitamin concentrations found significant reductions in *trans*-β-carotene with intakes of 1.1 and 2.2 g phytosterol esters/d (20). In this trial, the higher phytosterol dose also reduced lycopene and α-carotene concentrations and the lower dose decreased lutein concentrations. However, all carotenoid concentrations, except for lycopene, remained in the reference concentration range. Also, after correcting for TC concentrations, only reductions in *trans*-β-carotene remained significant. These results are similar to those obtained by Hallikainen and Uusitupa (28), who found significant reductions in β-carotene and α-tocopherol concentrations before correcting for TC concentrations but not after the corrections for the change in TC were made. Phytostanol esters were also shown to reduce absolute concentrations of α-tocopherol, β-carotene, lycopene, β-cryptoxanthin, phytofluene, and β- and γ-tocopherol (61); however, the reductions were affected by the frequency of administration of phytostanol esters, either once or three times per day. Again, after correction for LDL-C concentrations, none of the antioxidant concentrations was significantly different from the control.

Recently, a study examining the effects of simultaneous fruit and vegetable supplementation with plant sterol and stanol consumption found that when subjects consumed ≥5 servings of fruits and vegetables/d, including one serving from a list of high-carotenoid foods, changes in lutein, retinol, α-tocopherol, lycopene, and α- and β-carotene concentrations were not altered after adjusting for TC concentrations (62). It can thus be concluded that plant sterol intakes, in the context of a balanced diet rich in fruits and vegetables, would not lead to reductions in plasma fat-soluble antioxidant concentrations. Furthermore, most studies have shown that reductions in fat-soluble vitamin concentrations were due mainly to reductions in LDL particles and that concentrations remained within the reference range (28,59,61).

Few adverse clinical events have been reported with consumption of plant sterols. Most adverse events have been related to respiratory and gastrointestinal problems (20,29). In the study by Davidson *et al*. (29), of all adverse events reported, less than one-quarter were thought to be related to plant sterol intake, whereas in the trial by Maki *et al*. (20), the number of adverse events was not different between phytosterol-consuming and placebo groups. In addition, none of the adverse events, mostly respiratory complaints, was related to phytosterol intake.

In a trial conducted on stroke-prone spontaneously hypertensive rats, it was found that consumption of olive oil and canola and soybean oils enriched with phytosterols decreased the life span of rats compared with consumption of soybean

oil and fat, mimicking the Canadian FA intake profile (63). In addition, rats consuming canola oil, canola oil plus phytosterols, soybean oil plus phytosterols, and corn oil had lower platelet counts and red cell deformability indices than rats consuming soybean oil, olive oil, and the fat mimicking Canadian FA intake. The authors suggested that in susceptible populations, such as phytosterolemic patients, phytosterol consumption may be detrimental to health. However, because the increased consumption of canola oil, an oil rich in phytosterols, in Canada has not been accompanied by an increase in intracerebral hemorrhages, consumption of this plant sterol-rich oil seemed to be advisable, according to the authors, to decrease the risk of coronary heart disease, a leading cause of death in this country (63). Also, recent data from our group showed that consumption of a plant sterol-rich diet for 1 mon does not affect red cell membrane fragility in mildly hypercholesterolemic but otherwise healthy adults (Jones, P.J., Raeini-Sarjaz, M., Kendall, C.W.C., Vidgen, E., Trautwein, E.A., Lapsley, K.G., Marchie, A., Cunnane, S.C., and Connelly, P.W., unpublished data).

In conclusion, plant sterols can be safely incorporated into diets for cholesterol-lowering. Both stanol and sterol esters are listed as "generally recognized as safe" for consumption by the general population (3). Their consumption, at a level of ~1.6 g/d, incorporated into high-fat spreads, has been shown to produce significant reductions in plasma TC and LDL-C concentrations and to do so without adverse health effects. Smaller intakes of plant sterols, formulated so as to improve their solubility in foods may be efficacious in providing protection against increases in circulating cholesterol concentrations. New emerging food incorporation strategies for phytosterols may improve the available variety of functional foods containing phytosterols. The addition of phytosterols into various foods, such as low-fat meats and dairy and low-cholesterol egg products, could also help reduce serum cholesterol concentrations and risk of cardiovascular disease. Furthermore, combining phytosterols with other functional ingredients such as n-3 or medium-chain FA, could increase their scope of action to include TG-lowering, further cholesterol reductions, thrombogenesis risk reduction, and stimulation of thermogenesis. However, caution must be exerted when allowing various foods to be enriched with plant sterols because some individuals would be consuming high doses. Although high intakes of phytosterols have not produced adverse effects, it is not known whether chronic consumption of high levels of phytosterols would have some toxic effects.

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Modulation of Body Composition and Immune Cell Functions by Conjugated Linoleic Acid in Humans and Animal Models: Benefits vs. Risks

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ABSTRACT: We have reviewed the published literature regarding the effects of CLA on body composition and immune cell functions in humans and in animal models. Results from studies in mice, hamsters, rats, and pigs generally support the notion that CLA reduced depot fat in the normal or lean strains. However, in obese rats, it increased body fat or decreased it less than in the corresponding lean controls. These studies also indicate that *t*10,*c*12-CLA was the isomer that reduced adipose fat; however, it also increased the fat content of several other tissues and increased circulating insulin and the saturated FA content of adipose tissue and muscle. Four of the eight published human studies found small but significant reductions in body fat with CLA supplementation; however, the reductions were smaller than the prediction errors for the methods used. The other four human studies found no change in body fat with CLA supplementation. These studies also report that CLA supplementation increased the risk factors for diabetes and cardiovascular disease including increased blood glucose, insulin, insulin resistance, VLDL, C-reactive protein, lipid peroxidation, and decreased HDL. Most studies regarding the effects of CLA on immune cell functions have been conducted with a mixture of isomers, and the results have been variable. One study conducted in mice with the purified *c*9,*t*11-CLA and *t*10,*c*12-CLA isomers indicated that the two isomers have similar effects on immune cell functions. Some of the reasons for the discrepancies between the effects of CLA in published reports are discussed. Although significant benefit to humans from CLA supplementation is questionable, it may create several health risks in both humans and animals. On the basis of the published data, CLA supplementation of adult human diets to improve body composition or enhance immune functions cannot be recommended at this time.

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CLA is a collective term for isomers of linoleic acid that have conjugated double bonds. All of the positional and geometric isomers of CLA can now be purified, and their mass spectra have been reported (1). Most of the published studies have used a mixture of CLA isomers that contained the two major forms, *cis*9,*trans*11-CLA (*c*9,*t*11-CLA) and *trans*10,*cis*12-CLA (*t*10,*c*12-CLA), and a number of minor isomers. However, the exact isomeric composition of the CLA used in many studies conducted before 1998 may not be accurate because the methylation procedures used for analysis involved heating with methanolic hydrochloric acid. That can cause interconversion of different isomers. Furthermore, the chromatographic columns used had poor isomer resolution (2).

Feeding a mixture of CLA isomers to animal models has been reported to alter chemically induced carcinogenesis (3–14), atherogenesis (15–17), diabetes (18), body composition (19–43), and immune cell functions (44–54). These studies suggest that there may be possible health benefits from CLA supplementation of animal diets. However, the data regarding most of the health benefits are controversial and also raise concerns regarding adverse effects of CLA. Only a limited number of CLA supplementation studies have been conducted in humans (55–68), and the results reported are variable. Effects of CLA on body composition in animal models, lipid metabolism, anticarcinogenic effects, and the mechanisms involved have been the topic for several recent reviews (69–73). Here, we will briefly review the effect of CLA on body composition in animal models but will focus on its effects on body composition in humans, immune functions in humans and animal models, and possible adverse effects.

EFFECT OF CLA ON BODY COMPOSITION IN ANIMAL MODELS

CLA supplementation has been found to reduce depot fat in several animal species, including mice, hamsters, rats, and pigs (19–43). Most of these experiments were conducted with growing animals by supplementing their diets with a mixture of CLA isomers ranging from 0.25 to 2.0 weight%. Effects on body composition could be noticed within 2 wk of CLA supplementation. In general, animals consumed the feed *ad libitum*, and one study found a reduction in food intake in animals fed CLA-containing diets (24). In CLA studies conducted with mice, lean body mass either increased (20), remained unchanged (22), or was not examined (25). Supplementing a mixture of CLA isomers (1% for 8 mon) to diets of mice caused a complete loss of brown adipose tissue, but also

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Abbreviations: BMI, body mass index; Con A, concanavalin A; DEXA, dual energy X-ray absorptiometry; IL, interleukin; INF, interferon; LPS, lipopolysaccharide; MLN, mesenteric lymph node; PGE_2, prostaglandin E_2; PHA, phytohemagglutinin; TNF-α, tumor necrosis factor-α.

caused 3.6- and 1.6-fold increases in liver and spleen masses, respectively (24,25). Those changes were associated with an increase in the amount of circulating insulin and a decrease in the amount of circulating leptin (23–25). In another study with mice, *t*10,*c*12-CLA (0.5% for 8 wk), but not *c*9,*t*11-CLA reduced retroperitoneal fat by 50%; however, the liver mass and liver lipids were increased by 200 and 500%, respectively, compared with the corresponding values in mice fed the control diet (43). This and other studies in mice and hamsters show that the *t*10,*c*12-CLA, but not *c*9,*t*11-CLA was the isomer responsible for reduction in adipose fat (19,26). Whether the fat is stored in liver, spleen, and muscle instead of being stored in adipose tissue or whether there is indeed a reduction in total body fat is still an open question. Risks associated with increased insulin resistance and hepatic lipid storage raise concern regarding the use of CLA to reduce adipose fat.

Supplementing the diets of Sprague-Dawley rats with a mixture of CLA isomers (3% for 3 wk) caused a 27% reduction in body fat and an 11% increase in lean body mass compared with the corresponding values in rats fed a control diet (31). In other strains of rats, results varied between the lean and obese rats. For example, in the Otsuka Long Evans Tokushima fatty rats, CLA supplementation (1% for 4 wk) reduced the perirenal, epididymal, and omental fat pads by 36, 54, and 26%, respectively, compared with the corresponding fat pads in rats fed the control diet (29); however, in Zucker obese rats, CLA supplementation (0.5% for 8 wk) caused a 15% increase in both inguinal and retroperitoneal fat pads (32). In the lean Zucker rats, CLA supplementation caused an 11 and 24% decrease in inguinal and retroperitoneal fat pads, respectively. Those results indicate that effects of CLA vary with the genotype of the animal.

The effects of CLA on body composition in pigs have been variable. CLA supplementation reduced body fat in some (34,37–40) but not in other (35,36,42) studies. One of the most likely reasons for this discrepancy was that growing pigs fed high-fat diets did not respond to CLA, whereas finisher pigs fed conventional low-fat diets did. Other reasons, including differences in the concentration of 10*t*,12*c*-CLA and the methods used to evaluate body fat, may also have contributed to these controversial results. Studies in pigs have also reported that CLA supplementation increased the saturated FA content and decreased the unsaturated FA content of adipose tissue and longissimus muscle (35,37). This improved the firmness of the meat, which may be desirable; however, it may make the meat more atherogenic to the consumer.

In conclusion, the effectiveness of CLA in altering body composition has been variable in different animal species, and risks are associated with its use. Reduction in fat mass has been attributed to increased energy expenditure and loss in excreta (21), increased adipocyte apoptosis (25), and a reduction in cell size (30). In mice fed diets containing *t*10,*c*12-CLA, the mRNA for leptin and adiponectin in the adipose tissue were decreased by 82 and 11%, respectively, compared with the corresponding values in mice fed a control or *c*9,*t*11-CLA-containing diet (43). These results, together with the reduction in circulating leptin concentrations observed in several other studies, suggest a possible role for these hormones in mediating the effects of CLA on body composition. Other details regarding the mechanisms by which CLA alters body composition can be found in a recent review (72).

EFFECT OF CLA ON BODY COMPOSITION IN HUMANS

Eight human studies have been published that report the effects of CLA on human body composition (Table 1; Refs. 55–64,68). Four of these studies were conducted in subjects with normal body weights [body mass indices (BMI) <25 kg/m^2], whereas the other four were conducted in overweight or obese subjects. The amount of CLA consumed ranged from 1.7 to 6.8 g/d; it was a variable mixture of different isomers, except for one study that used purified *t*10,*c*12-CLA (63). Only one of these studies was conducted in a metabolic unit; all of the other studies were among free-living subjects.

We conducted a 94-d study in a metabolic unit with healthy women (n = 17; mean age, 28.7 yr; BMI, 22.7 kg/m^2) (55,56). A 5-d dietary menu was used throughout the study, and all subjects participated in two daily walks of two miles each. For the first 30 d, all subjects supplemented their diets with six 1-g capsules of sunflower oil (placebo). For the next 64 d, the placebo supplement was replaced with 6 g of Tonalin for 10 women; the other 7 women continued to take the placebo capsules. Tonalin provided 3.9 g total CLA/d, with the *c*9,*t*11-CLA and *t*10,*c*12-CLA isomers each providing ~1 g; the remainder was comprised of other isomers. Body weight, fat mass (determined by total body electrical conductivity), and lean body mass did not change between days 30 and 94, the intervention period, in both groups. Energy expenditure, fat oxidation, rate of lipolysis, and FA reesterification were also not altered by CLA supplementation. No obvious health risks appeared to be associated with CLA supplementation, except a 20% increase in circulating insulin and a similar decrease in leptin concentrations (56). These changes in hormones are consistent with those discussed in animal models above and must be monitored closely in future human studies with CLA.

Another study with normal-weight subjects was conducted in Norway by Thom *et al*. (57). Men (n = 10) and women (n = 10) equally divided between the CLA and placebo groups participated in a 12-wk study. Subjects in the CLA group were administered a supplemental mixture of CLA isomers (1.8 g/d), whereas those in the control group were given a placebo (hydrogel). All subjects did standardized exercises in a gym for 90 min, three times a week. Body fat, as measured by IR interactance, decreased by 4% in the CLA group but did not change in the control group. Body weight did not change in either group. A third study with normal-weight subjects was conducted in Sweden by Smedman and Vessby (58). Men (n = 27) and women (n = 26), 23–63 yr old, were divided into two groups; one group supplemented their diets with 4.2 g/d CLA, whereas the other group supplemented with an equivalent amount of olive oil for 12 wk. The CLA used in

TABLE 1
Effect of CLA on Human Body Composition (BC)[a]

CLA type/ amount (g/d)	Time	*n*	Age (yr)	BMI (kg/m^2)	BC method	BW change (kg)	FM change (kg, cm, or %)	Comments	Reference
Sunflower oil placebo	9 wk	7	29 ± 7	22 ± 3	TOBEC	+0.5	+0.01	Diet, exercise, and all other	55
CLA mix, 3.9		10	27 ± 6	23 ± 2		– 0.2	–0.2	activity controlled in MRU.	
Hydrogel placebo	12 wk	10	28 ± 3	23 ± 3	Infrared interactance	+0.2	+0.4	Exercise 90 min, 3 times/wk. Prediction error of method >	57
CLA-mix, 1.8		10	28 ± 3	23 ± 2		–1.9	–3.4	change in BF.	
Olive oil placebo	12 wk	24	48 ± 10	25 ± 4	Skinfold	+0.2	–0.4%	% BF ↓3.8% in CLA group,	58
CLA-mix, 4.2		26	43 ± 13	26 ± 4	thickness	+0.4	–1.1%	no change in BW, BMI, or SAD.	
Olive oil placebo	28 d	Total 23	23 ± 1	25.2	DEXA	– 0.1	+0.1	No change in BW, BMI,	68
CLA-mix, 6						+0.1	+0.5	fat mass or % BF	
Placebo, unknown	6 mon	40	NA	Obese	Underwater	–2.5	–1.0	Subjects restricted food intake,	59
CLA-mix, 2.7		40		Obese	weighing	–2.5	–1.0	increased exercise.	
Olive oil placebo	12 wk	10	44 ± 13	28 ± 2	DEXA	+1.4	+1.5	Amount of exercise varied	60
CLA-mix, 1.7		12	47 ± 14	30 ± 3		–0.4	–1.2	among different groups;	
CLA-mix, 3.6		8	43 ± 10	28 ± 2		–0.4	–1.7	60% of subjects showed AE.	
CLA-mix, 5.1		11	48 ± 11	29 ± 3		–0.1	–0.4		
CLA-mix, 6.8		11	46 ± 13	30 ± 3		–0.8	–1.3		
Olive oil placebo	4 wk	10	52 ± 8	32 ± 2	SAD	–0.4	ΔSAD, 0	No change in BW, BMI, or WHR.	61
CLA-mix, 4.2		14	54 ± 6	32 ± 3		–0.3	–0.6 cm		
Olive oil placebo	12 wk	19	53 ± 10	30 ± 2	SAD + BIA	+0.1	–0.6 cm	Changes in SAD, % BF.	63
CLA-mix, 3.4		19	51 ± 7	30 ± 2		–0.5	–0.9 cm	BMI not different among the	
*t*10,*c*12-CLA, 3.4		19	55 ± 7	31 ± 3		–0.9	–0.9 cm	three groups.	

[a]Abbreviations: AE, adverse effects; BF, body fat; BIA, bioelectrical impedance analysis; BMI, body mass index; BW, body weight; DEXA, dual energy X-ray absorptiometry; FM, fat mass; MRU, metabolic research unit; SAD, sagittal diameter; TOBEC, total body electrical conductivity; WHR, waste-to-hip ratio.

that study contained ~38% of each of the *c*9,*t*11-CLA and *t*10,*c*12-CLA, with the remainder made up of other FA. At the end of the study, body weight, BMI, waist-to-hip ratio, and sagittal abdominal diameter did not change in either group. The percentage of body fat as determined by skinfold thickness decreased by 1.2% in the control group and 3.8% in the CLA group; the difference between the two groups was significant ($P = 0.05$). In the fourth study, experienced, resistance-trained men ($n = 23$) were divided into two groups; one group supplemented their diets with olive oil (9 g/d for 28 d) and the other with CLA (6 g/d + olive oil 3 g/d). Body composition as determined by dual energy X-ray absorptiometry (DEXA) showed no change in fat-free mass, fat mass, and percentage of body fat in either group (68). Thus, two of four studies with normal-weight subjects reported a reduction in body fat with CLA supplementation, but the changes observed were smaller than the prediction errors for the methods used to determine body composition.

One study with obese subjects (BMI, 30 kg/m^2) was conducted with men and women ($n = 80$) at the University of Wisconsin–Madison (59). Half of the subjects supplemented their diets with 2.7 g/d of a mixture of CLA isomers for 6 mon, whereas the other half supplemented with a placebo. Subjects in both groups were encouraged to increase their exercise level and reduce food intake. At the end of the study, body weight decreased by 2.5 kg and fat mass (underwater weighing) decreased by 1 kg in both groups. These changes could not be attributed to CLA and were most likely the result of reduced food intake and increased exercise. The second study with overweight subjects was conducted in Norway by Blankson *et al.* (60). Men and women ($n = 47$ BMI, 28–30) were divided into five groups and received different amounts of CLA (0, 1.7, 3.4, 5.1, and 6.8 g/d) supplements for 12 wk. The placebo used was olive oil (9 g/d). CLA supplements provided equal amounts of the *c*9,*t*11-CLA and *t*10,*c*12-CLA, and the total fat supplement in all groups was comparable to the placebo group. Subjects in all groups were asked to participate in either light or intensive exercise. The number of subjects participating in two levels of exercise varied among different CLA groups. Fat mass, as determined by DEXA, decreased significantly in the groups taking CLA supplements of 3.4 or 6.8 g/d, but not in those taking 1.7 or 5.1 g/d. The mean amount of fat lost was 1.7, 0.4, and 1.3 kg in those taking 3.4, 5.1, and 6.8 g CLA, respectively. The authors claim that CLA intake of 3.4 g/d reduced body fat. This conclusion was weakened because the decrease in body fat was not significant in the group administered 5.1 g CLA. Furthermore, the changes in fat mass seen were within the prediction error for DEXA (1.65 kg). Lean body mass increased (0.88) significantly only in the group administered 6.8 g CLA. This is the group that reported maximum increase in the number of hours spent with intensive exercise. Those confounding variables make it difficult to distinguish whether the increase in lean body mass was due to increased exercise or the CLA supplement. Of the 60 study

participants, 36 exhibited side effects, including fatigue, gastrointestinal problems, and relapse of asthma. The authors claimed that there was no difference in adverse effects between the control and CLA groups. However, with the small number of subjects, it is difficult to prove or disprove this claim; it is obvious, though, that the smallest number of adverse effects occurred in the control group and the greatest number in the group with the highest supplement of CLA. Future studies should address this safety issue.

Two studies with obese subjects were conducted in Sweden (61,63). In one of these studies (61), 24 abdominally obese men (39–64 yr; BMI, 32 kg/m^2) participated in a double-blind randomized control trial for 4 wk. Fourteen men were given 4.2 g CLA/d, and 10 men received a placebo (olive oil). The CLA used contained ~38% of each of the *c*9,*t*11-CLA and *t*10,*c*12-CLA, with the rest made up of minor CLA isomers and other FA. At the end of 4 wk, the mean sagittal abdominal diameter in the CLA group decreased from 29.5 to 28.9 cm, whereas it remained unchanged at 29.2 cm in the control group. In the second study (63), abdominally obese men with metabolic syndrome (n = 60; 35–65 yr) were divided into three groups and received 3.4 g/d of FA supplements for 12 wk. The supplements used were olive oil (placebo), a mixture of CLA isomers as used in their earlier study, or CLA enriched with the *t*10,*c*12-CLA isomer alone. Bioelectrical impedance was used to calculate total body fat, and sagittal abdominal diameter was used to approximate central fat. Total adiposity and BMI were not altered with either of the CLA supplements, but central fat tended to decrease with both CLA supplements. The changes seen were smaller than the prediction error for the methods used.

Collectively, results from human studies regarding the effects of CLA on body composition were quite variable. Four of the eight studies indicated a possible reduction in body fat with CLA supplementation, but the other four reported no change. If the reported changes in body fat resulted from CLA intake, then discrepancies among studies may be the result of differences in the isomer composition or amount and duration of CLA supplement. It is also possible that these changes were the result of confounding variables, including food, exercise, and the prediction errors for the methods used. Long-term controlled studies are required to determine whether indeed CLA intake reduces body fat in humans. Before such studies are undertaken, it is important to address the potential risks associated with CLA supplementation.

EFFECT OF CLA ON IMMUNE FUNCTIONS IN ANIMAL MODELS

There are less than a dozen published studies examining the effects of CLA supplementation on immune cell functions in animal models (Table 2). The amount of CLA fed ranged from 0.1 to 1.5 weight% of the diet, and the feeding duration ranged from 2 to 8 wk. The CLA mixture used was reported to contain ~40% each of the *c*9,*t*11 and *t*10,*c*12 isomers with the remainder made up of the minor components. Accuracy of the isomer composition reported may be debated for some of the studies. Growing animals were used in most of the studies and

TABLE 2
Effect of CLA on Immune Cell Functions in Animal Models[a]

Index	CLA amount/type (wt% of diet)	Time (wk)	Species	*n*/group	Effect	Comments	Reference
DTH	0.5, CLA-mix	4	Rat	7	Increased	Foot-pad swelling 24 h post-PHA	45
DTH	1.0, CLA-mix	8	Mice	10	NC	Urd incorporation into ear 16 h post-DFNB	47
Listeria resistance	0.5, CLA-mix	4	Mice	4–5	NC	Infer no decrease in cellular immunity	48
SPC proliferation	1.0, CLA-mix	8	Mice	10	Increased or NC	Responses varied to PHA and Con A	47
SPC proliferation	0.1, 0.3, or 0.9, CLA-mix	6	Mice	8	NC	PHA, Con A, and LPS used for stimulation	46
SPC proliferation	0.5, purified 9/11 and 10/12	12	Mice	12	NC	Con A and LPS used	54
IL-2 secretion	1.0, CLA-mix	8	Mice	10	Increased in young not old	Con A: 24 h	47
IL-2 secretion	0.1, 0.3, or 0.9, CLA-mix	6	Mice	8	NC	Con A: 24 h	46
IL-2 secretion	0.5, purified	8	Mice	12	NC	Con A: 4, 24, 48 h	54
IL-4 secretion	0.5, purified	8	Mice	12	Decreased by both isomers	Con A: 4, 24, 48 h	54
IL-6 secretion	0.5, purified	8	Mice	12	Increased by both isomers	LPS: 4, 24, 48 h	
TNF and secretion	0.5, purified	8	Mice	12	Increased by both isomers	LPS: 4, 24 , 48 h	54
Serum Ab	0.5 or 1.0, CLA-mix	3	Rat	5	Increased IgA, IgG, IgM Decreased IgE	Similar effect seen in splenic and MLN cells	49
Serum Ab	0.05–0.5, CLA-mix	3	Rat	5	NC	*In vitro* CLA decreased Ig secretion by SPC	50

[a]Abbreviations: Ab, antibody; Con A, concanavalin A; DFNB, difluoronitrobenzene; DTH, delayed-type hypersensitivity skin response; IL, interleukin; LPS, lipopolysaccharide; MLN, mesenteric lymph node; NC, no change; PHA, phytohemagglutinin; SPC, splenocyte; TNF, tumor necrosis factor; Urd, deoxyuridine; 9/11, *cis*9,*trans* 11-CLA; 10/12, *trans*10,*cis*12-CLA.

food intake was not controlled. Effects of CLA varied from stimulation to inhibition, depending on the function examined or even for the same function examined in different studies. Here, we will discuss the *in vivo* and *ex vivo* effects of CLA on immune status and response in animal models.

Three studies examined the effects of CLA supplementation on splenocyte proliferation in response to T- and B-cell mitogens (46,47,54). In the first study, 0.3 or 0.9 weight% CLA was fed for 3 or 6 wk to 8-wk-old, female Balb/c mice. After 3 wk, splenocyte proliferation in response to the T-cell mitogen, phytohemagglutinin (PHA), was 146 and 192% in the 0.3 and 0.9 CLA groups, respectively, compared with the control group (safflower oil), but at 6 wk, the three groups did not differ (46). CLA supplementation did not alter splenocyte proliferation in response to another T-cell mitogen, concanavalin A (Con A), or the B-cell mitogen, lipopolysaccharide (LPS), after 3 or 6 wk of supplementation. The second study (47) used 4- or 24-mon-old C57BL/6N mice; their diets were supplemented with 0 or 1% CLA for 8 wk. The proliferation of splenocytes isolated from the young mice fed the CLA-containing diet was approximately twice that in the control mice when Con A concentrations of 0.5 and 5.0 were used; at a Con A concentration of 1.5 mg/L, however, the difference in proliferation between the control and CLA groups was not significant. In the old mice, the splenocyte proliferation in the CLA group was twice that of the control group at a Con A concentration of 1.5 mg/L, but at the other two concentrations, the CLA and control groups did not differ. Proliferation in response to PHA was tested at concentrations of 5, 20, and 40 mg/L. It was not different between the control and CLA groups at all PHA concentrations in both the young and old mice, except at 40 mg/L, where it was significantly greater (50% more) for the young mice fed the CLA diet. Similarly, results regarding splenocyte proliferation in response to the B-cell mitogen, LPS, varied with its concentration. In the third study, 8-wk-old female mice were fed diets containing purified isomers of CLA (*c*9,*t*11- or *t*10,*c*12-CLA, 0.5% for 8 wk); splenocyte proliferation in response to several different concentrations of Con A and LPS did not differ between the control and CLA groups (54). In view of the variability in the data from these studies, it is difficult to determine whether CLA indeed enhanced T- or B-cell proliferation in response to mitogens.

The above-mentioned three studies also examined *ex vivo* interleukin (IL)-2 production by splenocytes stimulated with Con A. In one study (46), IL-2 production after 3 wk of CLA supplementation was 126, 230, and 192% in the 0.1, 0.3, and 0.9% CLA groups, respectively, compared with the corresponding value in the control group; at 6 wk, the four groups did not differ. In the other study, IL-2 secretion was significantly increased (188% of control) after CLA supplementation in young but not old mice (47). Supplementation of purified isomers of CLA did not alter IL-2 secretion when tested at 4, 24, and 48 h in culture with two different concentrations of Con A (54). Secretion of the Th-2 cytokine, IL-4, was not different among the three groups at 4 h after treatment with Con A, but it was significantly reduced (30–70%) by both isomers at 24 and 48 h. The period of time for which the splenocytes were treated with the mitogens seems critical in detecting the effects of CLA on the secretion of Th2 and inflammatory cytokines. These studies also reported that CLA supplementation had no effect on lymphocyte cytotoxicity (46) and natural killer cell (47) activities. Results from another study showed that 0.5% CLA supplementation, for 4 wk, did not alter *in vivo Listeria* infection (48). CLA feeding increased foot swelling in response to PHA in rats (45), but did not alter the ear responses after 2,4-dinitrofluorobenzene in mice (47). Whether these discrepancies are due to the lack of a CLA effect on lymphocyte functions or to the differences in experimental protocols cannot be established from the information available.

Results from CLA studies on lymphocyte functions in rats are also variable. Supplementing the diets of male 4-wk-old Sprague-Dawley rats with 0.5 or 1.0% CLA for 3 wk led to a significant (6 and 22%) reduction, respectively, in the *in vitro* secretion of IgE, by mesenteric lymph node (MLN) cells and significant increases (200–700%) in the secretion of IgG, IgA, and IgM compared with the corresponding values in the rats fed control diet (49). The effect of CLA supplementation on the secretion of Ig by splenocytes and on their serum concentrations was much smaller than that seen with MLN. The ratio between the CD4+ and CD8+ cells in the MLN was not altered by CLA supplementation, suggesting that the effect of CLA may be directly on the B cells. In a subsequent study (50), the same authors examined the effects of a range of CLA concentrations (0.0, 0.05, 0.10, 0.25, and 0.50%) on both the serum levels of Ig and their *in vitro* secretion by splenocytes. *Ex vivo* secretion of IgA, IgG, and IgM increased as the concentration of CLA in the diet was increased with the maximum (two- to threefold increase) attained at 0.1 or 0.25%. However, there was no effect on the serum concentration of the three Ig at all CLA concentrations tested. Results of the first study from this group showed a twofold increase in serum concentration of IgG with a CLA dose of 0.5%, whereas in the second study, this dose of CLA had no effect on serum IgG. Rats of the same strain and age and the same feeding duration were used in the two studies; thus, these factors could not account for the discrepancy between the results. The authors did not comment on this discrepancy but suggested that CLA supplementation may reduce the risk of food allergies. Antibody response in rats and chicks to BSA and sheep red blood cells was not affected by CLA feeding (45). Overall, these results do not support the claim that CLA feeding improves antibody response in animals.

Only a few studies have examined the effects of CLA on monocyte/macrophage cell functions. Peritoneal macrophages isolated from the rats fed CLA exhibited more than twice phagocytic activity compared with those isolated from rats fed the control diet (45). In another study, addition of CLA (1%) to a soybean oil-based rat diet caused a >50% reduction in *ex vivo* secretion of IL-6 by resident peritoneal macrophages, but it had no effect when added to a menhaden oil-based diet (51). In this study, CLA feeding also decreased the basal but not the

LPS-simulated secretion of tumor necrosis factor-α (TNF-α) in both the soybean oil- and menhaden oil-based diets. CLA feeding had no effect on the *ex vivo* production of IL-1 and prostaglandin (PG)E_2 by rat peritoneal macrophages (51) and mouse splenocytes (47). Supplementing the diets of mice with purified isomers of CLA had similar effects on the *ex vivo* secretion of inflammatory cytokines (54). Secretion of TNF-α by the cultured splenocytes decreased between 4 and 48 h, whereas that of IL-6 increased. TNF-α secretions were significantly increased (>30%) by both the *c*9,*t*11- and *t*10,*c*12-CLA isomers at 4 h, but not at 24 or 48 h, after treatment with LPS. IL-6 secretion was increased in both CLA groups by 30–50% at 24 and 48 h, but not at 4 h. Differences in the effects of CLA on the secretion of inflammatory cytokines between mice and rats may be due to species differences, different cell types, or the diets; however, they may also be due to the duration of cell culture. We found that the period of time for which cells were cultured was critical in detecting CLA effects on the inflammatory and Th2 cytokines.

In summary, the effects of CLA on immune functions in animal models have been variable. Differences may be due to a number of factors, including species, strain, age, and health of the animals used, FA and antioxidant nutrient composition of the diets, feeding regimen, amount and duration of CLA supplementation, culture conditions, including the type and amount of serum and the duration of the cell culture. None of the animal studies reported here cultured cells in autologous sera, and most examined the response variables at a single time point. Results from one study that used multiple time points and mitogen concentrations indicated that the two isomers had similar effects on a number of immune cell functions; neither of the isomers altered splenocyte proliferation or production of the Th1 cytokine IL-2; both isomers decreased production of the Th2 cytokine IL-4 and increased production of the inflammatory cytokines TNF- and IL-6. Further studies with chemically defined CLA isomer mixtures and standardized experimental protocols are required to resolve the controversies regarding the effects of CLA on immune cell functions.

EFFECT OF CLA ON IMMUNE CELL FUNCTIONS IN HUMANS

There are only four published reports in which the effects of CLA supplementation on human immune cell functions were examined (Table 3). The first was a metabolic unit study that we conducted (65,66). As discussed above, this study was conducted with 17 healthy women. For the first 30 d of the study, all subjects consumed a basal diet supplemented with sunflower oil capsules (6 g/d). The supplement for 10 women was replaced with Tonalin (6 g/d) for study days 31–94, whereas for the remainder 7 women, a placebo supplement was used throughout the study. Tonalin supplement provided 3.9 g CLA/d; it was comprised of *t*10,*c*12-CLA (22.6%), *c*11,*t*13-CLA (23.6), *c*9,*t*11-CLA (17.6), *t*8,*c*10-CLA (16.6), and other isomers (19.6). Immune cell functions were evaluated by using fasting blood samples drawn three times before the start of CLA supplementation (study days 15, 22, and 29) and three times at the end of CLA supplementation (study days 78, 85, and 92). Delayed hypersensitivity skin response to a battery of seven recall antigens was determined on study days 30 and 90. All subjects were immunized with influenza vaccine on study day 65, and the serum antibody titers were determined using the blood samples drawn on study day 65 (preimmunization) and 92 (postimmunization).

The total CLA concentration in circulating white blood cells (WBC) on study day 90 increased eightfold compared with the concentration on study day 30 (0.12–0.96% of total FA); however, it did not significantly alter the concentration of other FA. CLA supplementation did not alter the number of circulating total WBC, lymphocytes, granulocytes, monocytes, or the various subsets of lymphocytes. It had no effect on proliferation of lymphocytes cultured with PHA, Con A, or influenza vaccine, *in vitro* production of cytokines [IL-2 , interferon (INF)-γ, IL-1, and TNF-α], or the production of inflammatory eicosanoids, PGE_2 and leukotriene B_4, when cells were cultured in medium containing 10% autologous sera. Serum influenza antibody titers and delayed hypersensitivity skin response were also not affected by CLA supplementation. Overall, results of this study suggest that short-term supplementation with a modest level of CLA to healthy adult women had no beneficial effect on human immune response. This is consistent with the effects on body composition and energy metabolism discussed earlier. Results from another study indicated no change in serum concentrations of TNF-α and IL-6 when overweight/obese volunteers supplemented their diets with CLA (3.4 g/d, isomer mixture or purified *t*10,*c*12-CLA for 12 wk) or an equivalent amount of olive oil (64). In a study with experienced resistance-trained men, CLA supplementation (CLA mixture 6 g/d for 28 d) caused a 25% decrease in the ratio of circulating neutrophils to lymphocytes, whereas the ratio in the olive oil-supplemented subjects did not change (68). From the data available, it is not possible to determine whether the reduction in this ratio was due to a decrease in neutrophil numbers or an increase in lymphocyte numbers. In this study, CLA supplementation also did not significantly affect gains in bench or leg press. Whether higher amounts or longer duration of CLA supplementation would affect human immune cell functions remains to be determined; however, increasing the duration may also increase the health risks.

Another human study examined the effects of two different mixtures of CLA isomers (80:20 or 50:50) on antibody response to hepatitis B vaccination, delayed hypersensitivity skin response, and other indices of immune cell functions in healthy men (67). The total amount of CLA supplemented was 1.7 g/d for 84 d. CLA supplementation did not alter delayed hypersensitivity skin response or any of the other indices of immune cell functions tested. Mean antibody titers against hepatitis B did not differ among the three groups; however, the number of subjects attaining antibody titers >10 IU/L compared with the number of subjects with antibody titers <10 IU/L was significantly ($P < 0.05$) greater in the 50:50 group than in the 80:20 or placebo group. The validity of the claim that such arbitrary titers are seroprotective may be questionable, particularly when they are only two- to three-

TABLE 3
Effect of CLA on Human Immune Cell Functions[a]

Index	CLA (g/d)	Time (wk)	Subject type (n)	Effect	Comments	Reference
DTH to 6 Ag	3.9, CLA-mix	9	Healthy women CLA (10) Placebo (7)	↑	No difference between placebo and CLA	65
Serum Ab to influenza	3.9, CLA-mix	9	Healthy women CLA (10) Placebo (7)	↑	No difference between placebo and CLA	65
No. of circulating WBC	3.9, CLA-mix	9	Healthy women CLA (10) Placebo (7)	NC	NC in total or subsets of WBC or lymphocytes	65
PBMNC proliferation	3.9, CLA-mix	9	Healthy women CLA (10) Placebo (7)	NC	Cultured in autologous sera and PHA or influenza Ag	65
Secreted IL-1, IL-2, TNF2	3.9, CLA-mix	9	Healthy women CLA (10) Placebo (7)	NC	Cultured in autologous sera and PHA or LPS	65
Intracellular IL-2, IFN, and TNF	3.9, CLA-mix	9	Healthy women CLA (10) Placebo (7)	NC	Flow cytometry	65
Secretion of PGE_2 and LTB_4	3.9, CLA-mix	9	Healthy women CLA (10) Placebo (7)	NC	Cultured in autologous sera and LPS	65
Serum Ab to HepB	1.7, 50:50 or 80:20 mix of 9/11:10/12	12	Healthy men (25/group)	?	Mean antibody titer and number of responders not different; twice as many subjects with Ab > 10 IU/L in 50:50 than other two groups; infer 10/12 increased HepB response. Many other indices did not change.	67
Serum TNF and IL-6	3.4, CLA-mix or purified 10/12	12	Metabolic syndrome men, 19/group	NC	Many aspects of glucose and lipid metabolism deteriorated by 10/12.	64
Neutrophil/lymphocyte ratio	6.0, CLA-mix	4	Resistance-trained men, 11 or 12/group	↓25%	No change in placebo group.	68

[a]Ag, antigen; HepB, hepatitis B; LTB_4, leukotriene B_4; PBMNC, peripheral blood mononuclear cells; PGE_2, prostaglandin E_2; WBC, white blood cells; for other abbreviations see footnotes to Tables 1 and 2.

fold higher than the preimmunization titers. Because the 50:50 mixture contained more of the *t*10,*c*12-CLA isomer than the 80:20 mixture, the authors concluded that *t*10,*c*12-CLA enhanced the antibody response to hepatitis B, whereas the *c*9,*t*11-CLA did not alter that response. Those results are not consistent with the observed effects of CLA on influenza antibody titers (65). Whether this is antigen specific, sex specific, or the result of differences in CLA isomers used must be resolved in future studies.

ADVERSE HEALTH EFFECTS OF CLA

The adverse effects of CLA supplementation of animal and human diets are summarized in Table 4. Although there is a reduction in adipose fat in several animal species, there is a concomitant increase in liver, spleen, and muscle fat in the animal models. The consequences of increased fat on the functions of these organs must be monitored. Studies in pigs indicate that CLA supplementation increased the saturated fat content of the muscle; this improved the firmness of the meat, but also made it more atherogenic. Results from studies in mice and humans indicate an increase in the concentration of blood sugar and insulin, insulin resistance, VLDL, and a reduction in blood leptin and HDL. In overweight human subjects, *t*10,*c*12-CLA caused a several-fold increase in lipid peroxidation and a twofold increase in serum C-reactive protein. These changes indicate that CLA supplementation could promote an increased tendency toward diabetes and cardiovascular disease. Against this background of risks and no known benefits of CLA for humans, the wisdom of its supplementation to humans is questionable. Furthermore, its supplementation to animal diets may also increase health risks to humans who consume foods from such animals. It seems that most of the health risks are attributable to the *t*10,*c*12-CLA, and that the *c*9,*t*11-CLA may be safe for human consumption. If *c*9,*t*11-CLA is found to have anitcarcinogenic or other health benefits in humans as claimed in animals, future studies should concentrate on this isomer. The risks and benefits of many other CLA isomers are unknown and may be of interest.

CONCLUSIONS AND FUTURE DIRECTIONS

Results from animal studies indicate that the *t*10,*c*12-CLA isomer reduced body fat in several different species, but raise safety concerns dealing with the deposition of fat in other tissue, altering the FA profiles of the tissues, as well as serum insulin and leptin concentrations. Although the effects of CLA on immune cell functions have been quite variable, the two isomers seem to have similar effects. This is in contrast to the differences in their effects on body composition. Results from human studies regarding the effects on both body composition and immune cell functions have been variable. These studies also raise safety concerns, in particular con-

TABLE 4
Adverse Effects of CLA in Animals and Humans[a]

Index	CLA amount/type	Duration	Species	Effect	Comments	Reference
Liver weight	1%, CLA-mix	4 d to 8 mon	Mice	3.6 × control	May impair liver function	25
Spleen weight	1%, CLA-mix	4 d to 8 mon	Mice	1.6 × control	May impair spleen function	25
Liver weight	0.5%, CLA 9/11	8 wk	Mice	Not different than control	May impair liver function	43
	0.5%, CLA 10/12	8 wk	Mice	2 × control		
Liver lipids	0.5%, CLA 10/12	8 wk	Mice	5 × control	Fat stored in liver instead of AT	43
Serum insulin	1.0%, CLA-mix	4 d to 8 mon	Mice	4 × increase in fasting insulin 8 × increase in fed state	Indicated increased insulin resistance	43
Serum leptin	1.0%, CLA-mix	4 d to 8 mon	Mice	↓49% (fasting) ↓79% (fed)	Leptin infusion Corrected insulin resistance	25
Muscle FA composition	0.25, 0.5, 1.0 or 2%, CLA-mix	48 d	Pigs	Linear increase with increased CLA in 16:0 and 18:0 content	This increased meat firmness but will have adverse health effects	36
Muscle FA composition	1%, CLA-mix	7 wk	Pigs	Increased saturated FA	This increased meat firmness but will have adverse health effects	35
Fat pad weights	0.5%, CLA-mix	5 wk	Obese Zucker rats	↑15% (obese) ↓5% (lean)	Did not reduce fat in obese rats	32
Serum insulin	3.9 g, CLA-mix	9 wk	Healthy women	↑20% (day 63)	Increased insulin resistance	56
Serum insulin	3.4 g, CLA-mix or purified 10/12	12 wk	Obese men	3 × increase with 10/12 than placebo	Increased insulin resistance or mix	63
Fasting glucose	3.4 g, CLA-mix or purified 10/12	12 wk	Obese men	↑4% with 10/12	Decreased insulin sensitivity	63
Serum HDL	3.4 g, CLA-mix or purified 10/12	12 wk	Obese men	↓4% with 10/12	Lipid profile changed	63
Serum VLDL	3.4 g, CLA-mix or purified 10/12	12 wk	Obese men	↑11% with 10/12	Favored CVD	63
Lipid peroxidation	3.4 g, CLA-mix or purified 10/12	12 wk	Obese men	↑578% in urinary 8-Iso, PGF_2 with 10/12	Increased lipid peroxidation increases CVD	64
Serum CRP	3.4 g, CLA-mix or purified 10/12	12 wk	Obese men	↑110% with 10/12, ↑41% with mix	Increased inflammation	64

[a]Abbreviations: AT, adipose tissue; CRP, C-reactive protein; CVD, cardiovascular disease; PGF_2, prostaglandin F_2.

cerning glucose and lipid metabolism as well as lipid peroxidation. Controlled studies with purified isomers of CLA should be conducted to determine which isomer(s) may be responsible for health benefits as well as risks to human health. Results will have to be confirmed independently in different laboratories. Dose–response studies should be conducted to determine the minimum concentration of CLA required to produce the desired effects. The amounts of CLA that have been effective in changing body composition in growing animals would be 30–60 g/d for a 60-kg person, and thus could not be considered nutritional. The most urgent issue is to determine the benefits from CLA supplementation to humans as well as the risks associated with it. To avoid risks associated with high concentrations and long duration of CLA intake, it would be preferable to conduct initial studies with nonhuman primates. Once this information is available, future studies will have to determine the mechanisms by which CLA alters human physiologic functions.

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Brain Levels of *N*-Acylethanolamine Phospholipids in Mice During Pentylenetetrazol-Induced Seizure

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ABSTRACT: The *N*-acylethanolamine phospholipids (NAPE) are precursors for *N*-acylethanolamines (NAE), including anandamide (20:4-NAE), which is a ligand for the cannabinoid receptors. Previously, NAPE were believed to be found only in injured tissue, e.g., after neurodegenerative insults. Neuronal injury may occur in response to seizure activity. Therefore, we investigated the effect of pentylenetetrazol (PTZ)-induced seizures in PTZ-kindled mice on the level of NAPE in the brain. Male NMRI mice were kindled with PTZ injections 3 times/wk, thereby developing clonic seizures in response to PTZ. Mice were killed within 30 min after the clonic seizure on the test day (12th injection) and the brains were collected. Eight species of NAPE were analyzed as the glycerophospho-*N*-acylethanolamines by high-performance liquid chromatography-coupled electrospray ionization mass spectrometry. No effect of the PTZ kindling on the NAPE levels in murine brains was observed. Total NAPE in control mice cortex ($n = 4$) was 16.4 ± 3.0 μmol/g wet weight of which 20:4-NAPE accounted for 3.6 mol%, and the major species was 16:0-NAPE, accounting for 52.1 mol%. Determination of the activity of NAPE-hydrolyzing phospholipase D and of *N*-acyltransferase in brain membrane preparations from adult and 3-d-old mice revealed an enzyme pattern in the adult mice that was favorable for NAE accumulation as opposed to NAPE accumulation. Thus, there was no difference in NAPE levels; at present, however, this does not exclude that NAE may accumulate during seizure.

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N-Acylethanolamine phospholipid (NAPE) is now recognized as a naturally occurring phospholipid in the rat brain (1,2); it accumulates in the brain of young rats in response to various forms of injury (3,4). The lack of accumulation in the adult rat brain may be caused by an age-dependent enzyme pattern, i.e., lower activity of the NAPE-forming enzyme, *N*-acyltransferase, and higher activity of the NAPE-degrading enzyme, NAPE-hydrolyzing phospholipase D (4). It has been suggested that the formation of *N*-acylethanolamines (NAE), including the formation of anandamide and the saturated NAE, may constitute a neuroprotective response (5). Activation of cannabinoid receptors has been found to be neuroprotective *in vivo* in several animal models of brain injury (6–11), although blockade of the cannabinoid receptor also can be neuroprotective (12). *N*-Palmitoylethanolamine has been found to be anticonvulsant against pentylenetetrazol (PTZ)-induced seizure and in electroshock seizure in mice (13).

Prolonged seizure activity induces nerve cell death in selectively vulnerable neurons, most likely mediated by excessive Ca^{2+} influx and failure of intracellular Ca^{2+} buffering and ATP-dependent Ca^{2+} extrusion (14). Increased intracellular levels of Ca^{2+} can initiate NAPE formation in neurons (1,2,15,16).

Thus, we measured the levels and *N*-acyl composition of NAPE in the brains of PTZ-kindled adult mice. Furthermore, we estimated the activity of the two NAPE-metabolizing enzymes, *N*-acyltransferase and NAPE-hydrolyzing phospholipase D, in the brains of adult and young mice.

MATERIALS AND METHODS

Material and reagents. All commercial chemicals were used as received. CH_3OH, $CHCl_3$, dimethyl sulfoxide, acetonitrile [mass spectrometry (MS)-grade] and thin-layer chromatography (TLC) plates were from Merck (Darmstadt, Germany). Methanol and methyl *tert*-butyl ether (both MS-grade) were from Romil (Cambridge, United Kingdom). Triton-X-100 was from Boehringer Mannheim (Mannhein, Germany). Ammonia solution was from Aldrich (Steinheim, Germany). 1,2-Di[1′-^{14}C]-decanoyl-*sn*-glycero-3-phosphocholine (67 Ci/mol) was obtained from Amersham Pharmacia Biotech (Amersham, United Kingdom). $ZrOCl_2$ was purchased from Riedel-de Haën (Seelze, Germany). All other chemicals were from Sigma Chemical (St. Louis, MO). PTZ was from Nomeco (Copenhagen, Denmark). NMRI mice were from our own stable or from M & B Ltd. (Ry, Denmark).

Animals. Male mice ($n = 16$; 24–26 g) were used for the kindling experiment. Eight mice (PTZ-mice) were kindled with PTZ, and the other eight mice were used as controls. The mice were housed separately in two cages with free access to food and water under a 12-h light/dark cycle, lights on at

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Abbreviations: BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; GPNAE, glycerophospho-*N*-acylethanolamine; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NAE, *N*-acylethanolamine; NAPE, *N*-acylethanolamine phospholipid; PTZ, pentylenetetrazol; TLC thin-layer chromatography.

0600 h. They were acclimated for 1 wk before experimentation to compensate for transportation stress-induced alterations in behavior. PTZ-mice were kindled with PTZ injections [35 mg/kg intraperitoneally in saline (10 mL/kg)] 3 times/wk for 4 wk, thereby developing clonic seizures in response to PTZ. Mice were killed within 30 min after the clonic seizure on the test day (12th injection) and the brains were excised, cooled immediately on ice, and stored at –80°C. Control mice were injected with saline and were killed along with the PTZ-treated mice. Ethical permission for the studies was granted by the animal welfare committee, appointed by the Danish Ministry of Justice, and all animal procedures were carried out in compliance with the EC Directive 86/609/EEC and with Danish law regulating experiments on animals.

For the enzyme measurements, brains from 4 adult males, 3 adult females, and 4 × 8 three-day-old mouse pups were collected and brain membrane preparations were isolated as described previously (17). Protein determination was performed by the method of Bradford modified for membrane samples (boiling with NaOH) (18).

MS analysis. The thawed brains (0–4°C) were split into cortices and noncortex brains. To obtain enough tissue for the sample preparation procedure (>150 mg), the tissues were pooled two by two, resulting in four sample groups (each $n = 4$). Procedures for sample preparation and high-performance liquid chromatography (HPLC)-coupled MS analysis are described elsewhere in detail (19). In short, lipids were extracted along with 500 pmol internal 17:0-NAPE standard [*N*-heptadecanoyl-1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine, synthesized as described previously (20)]. The chloroform phase was applied to a strong cation-exchange solid phase column (1000 mg silica; IST, Mid Glamorgan, United Kingdom) and the NAPE-containing effluent fraction was subjected to a mild acid and alkaline-catalyzed hydrolysis, thereby generating glycerophospho-*N*-acylethanolamine (GPNAE). GPNAE was desalted/purified on a C18 reversed-phase solid-phase extraction column (200 mg silica; IST) and stored in methanol at –20°C for later analysis. Immediately before analysis, the solvent was evaporated and redissolved in 100 µL methanol. All organic solvents used in the assay contained 0.02% (wt/vol) butylated hydroxytoluene as antioxidant. Separation of individual GPNAE species was accomplished by injecting the sample *via* a 20-mL loop onto a reversed-phase C18 HPLC column (150 × 2.0 mm; Phenomenex, Cheshire, United Kingdom), using a gradient of methanol/acetonitrile/H_2O ramped from 5:5:90 (by vol) to 55:30:15 (by vol) in 10 min with a continuous flow of 500 µL/min. The eluent was directed to an electrospray ionization mass spectrometer equipped with an ion-trap mass analyzer (ThermoQuest, San Jose, CA) and operated in the negative ionization mode. The mass spectrometer was operated in the single-ion monitoring mode, and determination of a range of GPNAE molecular ions was achieved by multiple single-stage mass spectrometer, monitoring *m/z* values (mass to charge) equivalent to the molecular weight of each anionic species. The response was calculated as integrated peak areas of the molecular ion base peaks. This MS assay was shown earlier to reveal a limit of quantification at 1 pmol and standard curve linearity up to 10 nmol (3).

Enzyme assays. For the *N*-acyltransferase assay, 5 µg membrane protein was incubated (in duplicate) with 1.36 nmol of 1,2-di[1′-^{14}C]-decanoyl-*sn*-glycero-3-phosphocholine (73,500 dpm/nmol) for 0, 10, 20, and 30 min at 37°C in a total volume of 250 µL of 60 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP)-buffer (pH 8.25) containing 3 mM dithiothreitol, 5 mM $CaCl_2$, and 0.01% Triton X-100. Lipids were extracted and separated on TLC with $CHCl_3$/CH_3OH/aqueous-NH_3 (80:20:2, by vol) as the mobile phase. Radioactive spots were located and quantified using a PhosphorImager scanner (STORM; Molecular Dynamics, Sunnyvale, CA). The activity of *N*-acyltransferase was expressed as pmol of ^{14}C-labeled NAPE formed/min/mg protein because the activity was linear with protein concentration. There was no formation of lysoNAPE during the incubations.

For the NAPE-hydrolyzing phospholipase D assay, 125 µg membrane protein was incubated (in duplicate) with 2.1 nmol 1,2-dilauroyl-*sn*-glycero-3-phospho(*N*-[1′-^{14}C]palmitoyl)ethanolamine [prepared as described previously (4)] for 0, 30, 60, and 90 min at 37°C in a total volume of 200 µL BTP-buffer (pH 8.0) containing 2 mM dithiothreitol, 0.04% Triton X-100, 10 mM phenylmethylsulfonyl fluoride, and 5 µL dimethyl sulfoxide. The reaction was stopped by the addition of 1 mL of ice-cold methanol. Then 100 µL of 0.2 M $ZrOCl_2$ in 0.1 M NaOH, 150 µL of 0.1 M NaH_2PO_4, and 200 µg of boiled membrane fraction were added as described in detail elsewhere (21). After centrifugation, 700 µL of each supernatant containing the product NAE was counted by liquid scintillation counting. The activity of NAPE-hydrolyzing phospholipase D was expressed as pmol of *N*-[1-^{14}C]palmitoyl-ethanolamine formed/min/mg protein because the activity was linear with protein concentration.

RESULTS

The total NAPE content (mean ± SEM) of mouse cortex ($n = 4$) and noncortex brain ($n = 4$) was 16.4 ± 3.0 and 19.8 ± 5.0 nmol/g tissue in PTZ-treated mice and control mice, respectively. As can be seen in Table 1, there was no difference between cortices of PTZ-treated mice and control mice, in either the total content or the mol% of individual *N*-acyl species of NAPE. The anandamide precursor constituted 3.6–3.8 mol% of total NAPE species, and the major species contained palmitic acid in the *N*-acyl position. There was no difference between noncortex brains of control mice and PTZ-mice (data not shown) or compared with the cortices from the same mice.

The enzyme activities revealed 2.6-fold higher *N*-acyltransferase activity and 12-fold lower NAPE-hydrolyzing phospholipase D activity in the brains of young mice compared with brains from adult mice (Fig. 1.) There were no differences between enzyme activities in adult females and adult males (data not shown); thus, they were pooled in Figure 1.

TABLE 1
NAPE Levels in Brain Cortex from Mice with PTZ-Induced Convulsions and from Control Mice[a,b]

N-Acyl species	Control mice (n = 4)		PTZ mice (n = 4)	
	(pmol/g)	(mol%)	(pmol/g)	(mol%)
14:0	102 ± 22	0.63	121 ± 41	0.67
16:0	8728 ± 2655	52.1	9176 ± 351	51.6
16:1	374 ± 86	2.3	507 ± 92	2.8
18:0	2634 ± 183	16.4	2797 ± 243	15.8
18:1	3266 ± 691	20.1	3467 ± 356	19.4
18:2	349 ± 54	2.2	437 ± 89	1.4
20:4	576 ± 147	3.6	681 ± 210	3.8
22:6	403 ± 45	2.5	609 ± 160	3.4
Total	16430 ± 3043	100	17828 ± 1011	100

[a]Values are means ± SEM.
[b]Abbreviations: NAPE, *N*-acylethanolamine phospholipid; PTZ, pentylenetetrazol.

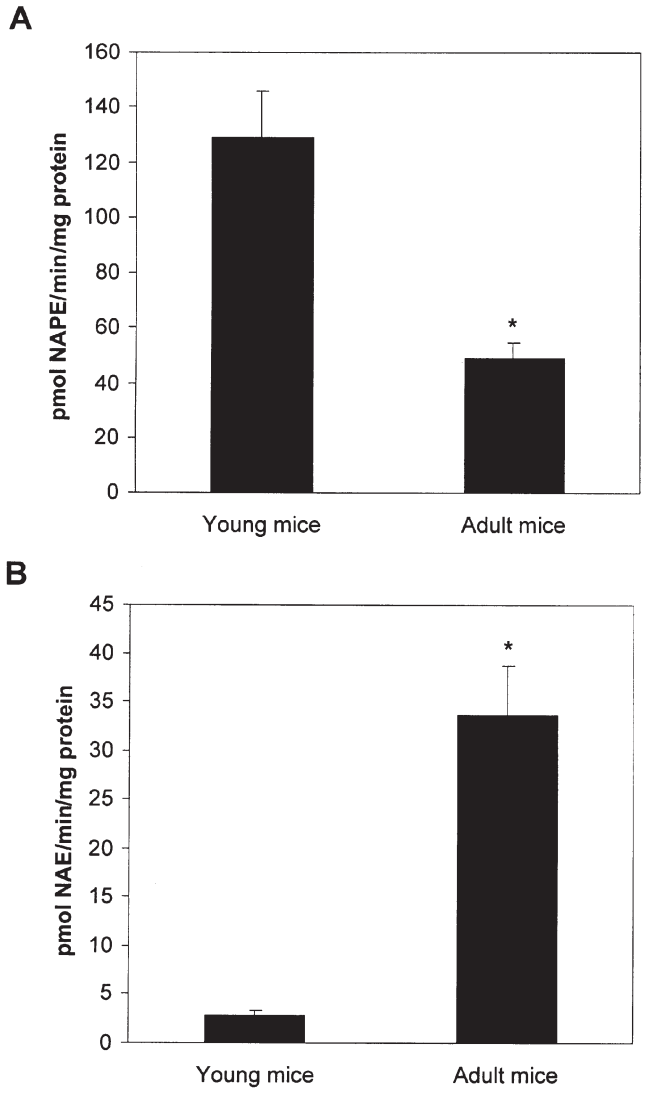

FIG. 1. Comparison of enzyme activity of (A) *N*-acyltransferase and (B) *N*-acylethanolamine phospholipid (NAPE)-hydrolyzing phospholipase D in brain membrane fraction of young (3-d-old; n = 4) and adult (n = 6–7) mice, respectively. *N*-Acyltransferase activity was measured as pmol of *N*-[1-^{14}C]decanoyl species of NAPE formed/min/mg protein from 1,2-di[1′-^{14}C]decanoylphosphatidylcholine and endogenous ethanolamine phospholipids. NAPE-hydrolyzing phospholipase D activity was measured as *N*-[1-^{14}C]palmitoylethanolamine formed/min/mg protein from 1,2-dilauroyl-*sn*-glycero-3-phospho(*N*-[1′-^{14}C]palmitoyl)ethanolamine (NAPE). Enzyme activities were linear with respect to protein concentration and time. Statistical differences were assessed by one-tailed *t*-test, $P < 0.0001$.

DISCUSSION

To investigate the influence of seizure on the concentration of NAPE in mouse brain, seizures were induced in mice using PTZ. We found no difference in the level or *N*-acyl composition of NAPE in the cortex or in the rest of the brain between PTZ-kindled mice and control mice. This could be due to a true lack of difference in the turnover of this phospholipid between the two groups of mice during seizure, or the PTZ-induced seizures were not severe enough to induce an accumulation of NAPE. On the other hand, in adult rats, brain ischemia cannot induce measurable NAPE formation as opposed to brain ischemia in young rats. This is probably due to an age-related change of enzyme pattern that may favor formation of NAE instead of NAPE in the brain of adult rats (4). Ischemia-induced NAE accumulation has been found in the adult brain of cows, sheep, and pigs (22,23), but no data are available for the adult rodent brain. Such a developmental change in NAPE-metabolizing enzymes may be specific for the brain because it was not seen for the enzymes in rat heart (17). Our observation of a comparable pattern of NAPE-metabolizing enzymes in rats and mice (Fig. 1) may suggest that the lack of NAPE accumulation in adult PTZ-kindled mice could also be due to an enzyme activity pattern in the brain of adult mice favoring NAE accumulation instead of NAPE accumulation.

Brain levels of NAPE have been reported previously only for rats (1,3,24,25). The present data are the first to be reported for mouse brain. It is noteworthy that 20:4-NAPE in the mouse brain account for 3.6 mol% of NAPE, whereas it accounts for only 0.4–1.1 mol% in the rat brain, whether young or adult (3,24–26). Rats tend to have higher levels of arachidonic acid in the brain lipids than do mice (27). Although *N*-palmitoylethanolamine is an anticonvulsant in PTZ-induced seizures in mice (13), the present data do not suggest an increased formation of its precursor, 16:0-NAPE, during seizure. In contrast, picrotoxinin-induced seizures are reported to cause accumulation of a range of 2-monoacylglycerols in the adult rat brain, including the endogenous cannabinoid receptor ligand, 2-arachidonoyl glycerol (28). In future studies, it would be desirable to measure NAE levels, including 20:4-NAE, during seizure activity because the enzyme pattern of the adult mouse brain may favor NAE formation instead of NAPE formation.

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Dietary Intakes and Food Sources of Omega-6 and Omega-3 Polyunsaturated Fatty Acids

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ABSTRACT: Both n-6 and n-3 polyunsaturated fatty acids (PUFA) are recognized as essential nutrients in the human diet, yet reliable data on population intakes are limited. The aim of the present study was to ascertain the dietary intakes and food sources of individual n-6 and n-3 PUFA in the Australian population. An existing database with fatty acid composition data on 1690 foods was updated with newly validated data on 150 foods to estimate the fatty acid content of foods recorded as eaten by 10,851 adults in the 1995 Australian National Nutrition Survey. Average daily intakes of linoleic (LA), arachidonic (AA), α-linolenic (LNA), eicosapentaenoic (EPA), docosapentaenoic (DPA), and docosahexaenoic (DHA) acids were 10.8, 0.052, 1.17, 0.056, 0.026, and 0.106 g, respectively, with long-chain (LC) n-3 PUFA (addition of EPA, DPA, and DHA) totaling 0.189 g; median intakes were considerably lower (9.0 g LA, 0.024 g AA, 0.95 g LNA, 0.008 g EPA, 0.006 g DPA, 0.015 g DHA, and 0.029 g LC n-3 PUFA). Fats and oils, meat and poultry, cereal-based products and cereals, vegetables, and nuts and seeds were important sources of n-6 PUFA, while cereal-based products, fats and oils, meat and poultry, cereals, milk products, and vegetable products were sources of LNA. As expected, seafood was the main source of LC n-3 PUFA, contributing 71%, while meat and eggs contributed 20 and 6%, respectively. The results indicate that the majority of Australians are failing to meet intake recommendations for LC n-3 PUFA (>0.2 g per day) and emphasize the need for strategies to increase the availability and consumption of n-3-containing foods.

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Considering the importance of different fatty acids in relation to health, especially the n-3 polyunsaturated fatty acids (n-3 PUFA), there is a paucity of information on current intakes and the contribution of different food groups to their intakes. Moreover, there is considerable variation in recommended intakes for PUFA in adults (Table 1). Recommended intakes for LA range from 4.4–20 g/d; LNA ranges from 1.35–2.2 g/d, and LC n-3 PUFA range from 0.16–1.6 g/d (Table 1; 1–8). Hence, there are large differences in existing recommendations, and the feasibility of their adoption depends on current intakes, for which reliable information is lacking.

Sinclair *et al.* (9) estimated the intake of LC n-3 PUFA by analying the plasma phospholipid fatty acids in 108 healthy adult male and female Australians and calculated this to be about 0.1 g/d. In a study by Mann *et al.* (10) based on Australian dietary intake data from 1983, adults were consuming approximately 100 mg/d of arachidonic acid (AA, 20:4n-6) and a similar amount of EPA and DHA combined (10). Ollis *et al.* analyzed weighed food records from 83 men and women residing in the Illawarra region of New South Wales in Australia and estimated the intake of LC n-3 PUFA to be 0.18 g/d (11). However, this study was only a small-scale study using limited food composition fatty acid data. This raises the question of validity of the results being representative of the Australian adult population. Since then we have established an updated fatty acid database (12) and used it to extend the 1995 National Nutrition Survey nutrient database (AUSNUT) (13) to contain a total of 32 individual and subtotals of fatty acids (14).

The aim of this study was to apply this extended AUSNUT database (14) to food consumption data measured in the 1995 National Nutrition Survey (NNS) to determine more reliably the total intakes and food sources of fatty acids in the Australian diet.

METHODS

The fatty acid database was developed using the existing Nutrient Table (NUTTAB) released in 1995 (NUTTAB95) fatty acid supplement database supplied by Food Standards Australia and New Zealand (FSANZ), as g fatty acid/100 g edible portion (two decimal places). A total of 449 foods from a total of 1116 foods in the existing database were eliminated. This number included many meat and egg entries that failed to report the presence of LC PUFA and other food items where fatty acids had been misidentified. In most cases, this

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Abbreviations: α-linolenic acid (α-LNA, 18:3n-3); arachidonic acid (AA, 20:4n-6); docosahexaenoic acid (DHA, 22:6n-3); docosapentaenoic acid (DPA, 22:5n-3); eicosapentaenoic acid (EPA, 20:5n-3); European Academy of Nutritional Sciences (EANS); Food Standards Australia New Zealand (FSANZ); International Society for the Study of Fatty Acids and Lipids (ISSFAL); linoleic acid (LA, 18:2n-6); long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA, 20:5n-3, 22:5n-3, and 22:6n-3); National Health and Medical Research Council (NHMRC); National Heart Foundation (NHF); National Nutrition Survey (NNS); megajoules (MJ); monounsaturated fatty acids (MUFA); n-3 polyunsaturated fatty acids (n-3 PUFA); n-6 polyunsaturated fatty acids (n-6 PUFA); saturated fatty acids (SFA).

TABLE 1
Various International Recommendations for Entire Populations for Polyunsaturated Fatty Acid (PUFA) Intakes (g/d and % energy)

	Ref.	LA	Total n-6 g/d (% energy)	LNA n-3	LC n-3
NHMRC Australia, 1992[b]	1	—	12 (6)	2.0 (1)	—
BNF, 1992[c]	2	12 (6)	—	2.0 (1)	1.0 (0.5)
Japanese, 1996[b,c]	3	10–12 (5–6)	—	2.0 (1)	1.6 (0.8)
EANS, 1998	4	—	—	2.0 (1)	0.21 (0.11)
Simopolous *et al.*, 1999	5	4.4–6.7 (2.2–3.4)	—	2.2 (1.1)	0.65 (0.33)
NHF of Australia, 1999	6	—	16–20 (8–10)	≥2.0 (1)	0.16–0.43[d] (0.08–0.22)
Omega-3 working group Germany, 2002	7	—	—	—	≥0.3 (0.15)
United States/Canada, 2002[b]	8	14–15[e] (7)	—	1.35 (0.68)	<0.135[f] (0.07)

[a]Abbreviations: LA, linoleic acid; LNA, linolenic acid; LC, long-chain; NHMRC, National Health and Medical Research Council; BNF, British Nutrition Foundation; EANS, European Academy of Nutritional Sciences; NHF, National Heart Foundation; —, not reported.
[b]Government agencies.
[c]Based on average intakes.
[d]Estimated from two fish meals per week (lean – fatty fish).
[e]Average estimate of male and female intakes.
[f]10% of LNA intake.

was due to the low sensitivity and poor resolving power of packed column gas chromatography analyses that were in use at the time of measurement. More recent fatty acid composition data on approximately 400 other foods from 27 reputable Australian research studies or industry projects were added to the database. The details of the database and contributors have been published elsewhere (12). The database has been incorporated into dietary analysis software in Australia and can be viewed at http://www.xyris.com.au.

Australians' fatty acid intakes, and the food sources of these fatty acids, were estimated using FSANZ's DIAMOND dietary exposure program by applying the fatty acid extension of the Australia Nutrient (AUSNUT) database (containing nutrient data for 4,554 foods) to the individual 24-h dietary intakes of 13,858 respondents in the 1995 National Nutrition Survey (NNS) (15). Dietary data were collected from February 1995 until March 1996 and thus cover all seasons (15).

The DIAMOND program is a custom application developed and used by FSANZ (FSANZ, Canberra, ACT, Australia) to estimate actual or theoretical dietary exposures to chemicals in food. In this study, the DIAMOND computation procedure first calculated the intakes of individual and subtotal fatty acids corresponding to the single 24-h food consumption record of each Survey respondent in the population group of interest. Summary statistics for each fatty acid such as mean (mg/d) and median intakes (mg/) were then generated for each Survey population group. To account for total energy intake, the estimated means were divided by the reported total energy intake for that age and sex category and expressed as mg/d/MJ. Food sources of individual fatty acids were calculated on the basis of the contribution of each major food group to the total of each fatty acid intake for adults aged over 19 yr.

It should be noted, however, that the results were not adjusted for intra-individual variation over time, nor were population weights applied. This means that there are likely to be small differences between our results for fatty acid subtotals such as total PUFA and the official published NNS data. First, extremes of intake are likely to be exaggerated when derived from a single 24-h period, rather than being averaged over a longer period of time. Second, the distribution of age/sex categories in the Survey sample was not adjusted to represent the whole Australian population. However, this is unlikely to make a significant difference to the mean and median results. More information on the NNS data and adjustments of the data can be found in the Technical Paper, NNS, Confidential Unit Record File 1995, Australian Bureau of Statistics, Canberra.

RESULTS

Table 2 shows the estimated mean intakes (in mg/d and mg/d/MJ) of the individual and total n-6 PUFA, the individual and total n-3 PUFA, and the total LC n-3 PUFA for all age groups (males and females combined). Adults consume more LC n-3 PUFA than children 18 yr and younger, whether expressed as mg/d or mg/d/energy (Table 2).

Table 3 shows the estimated mean fatty acid intakes [in mg/d and mg/d/energy (MJ)] for adult females and males. Adult males consume more fatty acids (mg/d) compared with females in the same age category. When total energy intake is taken into account, these differences disappear. It is not possible to determine the fatty acid intake as mg/d/energy (MJ) for the 19–64-yr-old category, as the energy intake for the 19–64 yr age category is not available. However, it is interesting to note that adults aged 65 and over have higher total intakes of EPA (33%) and DHA (26%) and hence total LC n-3 PUFA (27%) compared with the adults (19 yr and over). This would suggest that adults aged 65 and over also have higher intakes per MJ energy than the 19–64 yr as the 65 yr and over age category is a subset of the 19 yr and over age category. In essence, the older adults (65 yr and over) consume more LC n-3 PUFA than younger adults. It is also interesting to note

TABLE 2
PUFA Intakes for All Ages[a,b]

	All persons Age (yr)													
	Fatty acid intake (mg/d)							Fatty acid intake [mg/d/energy (MJ)]						
	2–3	4–7	8–11	12–15	16–18	19+	19–64	65+	2–3	4-7	8–11	12–15	16–18	19+	65+
n	383	799	739	653	433	10851	8891	1960	383	799	739	653	433	10851	1960
Energy intake (MJ)	6.3	7.4	9.0	10.1	11.2	9.2	ND	7.3	6.3	7.4	9.0	10.1	11.2	9.2	7.3
n-6 PUFA															
18:2	6100	7500	9900	10700	11700	10800	11200	9100	960	1008	1100	1059	1047	1169	1247
20:2	2	3	5	6	5	6	6	5	0.3	0.4	0.6	0.6	0.4	0.6	0.7
20:3	16	23	27	31	35	38	39	32	2.5	3.1	3.0	3.1	3.1	4.1	4.4
20:4	16	22	35	44	44	52	54	43	2.5	3.0	3.9	4.4	3.9	5.6	5.9
22:4	0.1	0.3	0.3	0.5	0.4	0.7	0.8	0.6	0.02	0.04	0.03	0.05	0.04	0.08	0.08
Σ n-6 PUFA	6200	7600	10000	10800	11800	10900	11300	9200	976	1021	1111	1069	1056	1180	1261
n-3 PUFA															
18:3	680	810	1080	1220	1290	1170	1210	980	107	109	120	121	115	127	134
20:5	10	19	30	32	41	56	55	59	1.6	2.6	3.3	3.2	3.7	6.1	8.1
22:5	5	10	17	22	20	26	27	25	0.8	1.3	1.9	2.2	1.8	2.8	3.4
22:6	24	47	60	63	77	106	106	106	3.8	6.3	6.7	6.2	6.9	11.5	14.5
Σ LC n-3 PUFA	40	76	106	117	138	189	188	191	6.3	10	12	12	12	20	26
Σ n-3 PUFA	720	880	1180	1330	1430	1360	1400	1170	113	118	131	132	128	147	160
Σ PUFA (this study)	6920	8480	11180	12130	13230	12260	12700	10370	1098	1145	1242	1200	1181	1333	1420

[a]All values are expressed as the mean values.
[b]Abbreviations: LC, long chain; LC n-3 PUFA is the sum of 20:5, 22:5, and 22:6; ND, not determined; for other abbreviation see Table 1.

that the comparison of total PUFA intake is within 5% of the PUFA intakes determined in the original NNS analysis (Ref. 15, Table 9).

The results show that adult Australians (age 19+) are consuming on average 10.8 and 0.052 g of LA and AA, respectively, per day. The n-3 PUFA daily intakes were 1.17, 0.056, 0.026, and 0.106 g of LNA, EPA, DPA, and DHA, respectively. Collectively, the LC n-3 PUFA intake was 0.189 g per day and total n-3 PUFA intake was 1.36 g per day (Table 2). However, the median intakes are much lower than the mean intakes (Table 4), as LC n-3 PUFA intake is skewed and not normally distributed. A high proportion of Australians had no intake of the LC n-3 PUFA on the day of dietary data collection, while some people had very high intakes on the day of dietary collection.

Food sources of fatty acid classes are shown in Tables 5–7 inclusive. The major food source contributing to total fat intake (Table 5) is the meat group followed by cereal-based products and milk products. Fish and seafood contribute only 3% of total fat intake, which is slightly lower in adult males (2%) (separate data for male and females are not shown). The main contributor to saturated fat intake (Table 5) is milk and milk products (27%) followed by meat (21%) and cereal-based products (19%). Fish and seafood contribute only 2% of total saturated fatty acid (SFA) intake. The main contributor to monounsaturated fatty acid (MUFA) intake (Table 5) is the meat group (28%) with males (30%) being higher than females (25%) (data not shown). Cereal-based products (19%) and fats and oils (11%), milk and milk products (10%), and vegetable products and dishes (10%) also contribute to MUFA intake. The main contributor to PUFA intake (Table 5) is the fats and oils group (21%) followed by meat (15%), cereal-based products (15%), cereals and cereal products (14%), and vegetable products and dishes (11%). Females obtain less PUFA from meat (14%) compared with males (16%), but they obtain more PUFA from fish and seafood (5%) compared with males (4%) (data not shown).

Table 6 shows the percentage contribution of the different food groups to n-6 PUFA intakes. As expected, fats and oils are the main source of LA, while meat and fish are the major sources of AA.

Table 7 shows the percentage contribution of the different food groups to n-3 PUFA intakes. Cereal-based products and dishes (24%), fats and oils (16%), meat, poultry, and game products and dishes (15%) are the main sources of LNA and total n-3 PUFA intakes. As expected, fish and seafood are the main food source of LC n-3 PUFA (71%), but surprisingly, meat contributed 20% to the intakes of these LC n-3 PUFA.

The relative contribution of total n-6 PUFA, LNA, and the LC n-3 PUFA intakes per food group are shown in Figure 1. While nearly all foods groups listed contribute to the intakes of n-6 PUFA and LNA, only three main food sources contribute to LC n-3 PUFA intakes, namely, fish and seafood, meat, and eggs, with fish and seafood being the major contributor (Fig. 1).

TABLE 3
PUFA Intakes for All Adult Males and Females

	Fatty acid intake (mg/d)						Fatty acid intake [mg/d/energy (MJ)]			
	19+		19–64		65+		19+		65+	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
n	3742	4236	2840	3178	902	1058	3742	4236	902	1058
Energy intake (MJ)	11.0	7.5	ND	ND	8.5	6.4	11.0	7.5	8.5	6.4
n-6 PUFA										
18:2	12800	9100	13200	9400	10500	7900	1158	1216	1234	1241
20:2	8	5	8	5	7	4	0.7	0.7	0.8	0.6
20:3	46	31	47	31	36	29	4	4	4	5
20:4	65	40	68	41	51	36	6	5	6	6
22:4	0.9	0.6	1.0	0.6	0.7	0.4	0.08	0.08	0.08	0.06
Σ n-6 PUFA	12900	9200	13400	9400	10600	7900	1167	1230	1246	1241
n-3 PUFA										
18:3	1380	990	1440	1020	1120	860	125	132	132	135
20:5	66	47	66	46	68	53	6	6	8	8
22:5	32	21	33	21	28	21	3	3	3	3
22:6	124	90	125	89	119	95	11	12	14	15
Σ LC n-3 PUFA	222	159	224	156	214	170	20	21	25	27
Σ n-3 PUFA	1600	1150	1660	1170	1330	1030	145	154	156	162
Σ PUFA (this study)	14500	10350	15060	10570	11930	8930	1318	1380	1403	1395
Σ PUFA[c]	14700	10400	ND	ND	11600	8800	1336	1387	1365	1375
n-6:n-3 ratio	8	8	8	8	8	8	8	8	8	8

[a]All values are expressed as the mean values.
[b]For abbreviations see Tables 1 and 2.
[c]*Source:* Reference 14.

DISCUSSION

This study has estimated the mean adult Australian intake of total fat as 76 g per day of which 32 g is saturated fat, 27 g monounsaturated fat and 12.2 g polyunsaturated fat. This compares favorably with intakes reported in the original analysis of the NNS (15) using NUTTAB95. Until recently, the supporting Australian nutrient software program did not allow for the determination of individual fatty acids, such as the n-6 and n-3 PUFA. However, with the development of a database of fatty acid composition of Australian foods (12), it is now possible to accurately assess the intake levels of individual fatty acids in the Australian population. This study shows that the mean adult Australian n-6 PUFA intake is 10.9 g per day of which 10.8 g is LA and 0.052 g is AA. The mean Australian n-3 PUFA intake is 1.36 g per day of which 1.17 g is LNA and 0.189 g is LC n-3 PUFA. These results compare favorably with an earlier estimation that determined the mean or median intakes of total n-6 PUFA, LNA, LC n-3 PUFA, and total n-3 PUFA to be 9.9, 1.01, 0.180, and 1.2 g, respectively (11). However, the median intakes show that 50% of the population is consuming no more than 0.029 g of LC n-3 PUFA, which is comparable to the mean estimated intakes (0.039 g of LC n-3 PUFA) of adolescents in Rhode Island (16) but falls well short of the range of recommendations mentioned in the Introduction and the current Japanese intake of 1.6 g per day (3). It appears that older Australians are consuming nearly twice as much LC n-3 PUFA [mg/d/energy (MJ)] as that of persons aged 18 years and younger (Table 2), probably due to the adults consuming more fish and seafood products and dishes (15).

As highlighted in the introduction, there appear to be conflicting recommendations regarding the recommended daily intake (RDI) of PUFA. The rationale for decreasing our intakes of n-6 PUFA is to maximize the utilization of n-3 PUFA, as LA and LNA compete for the same desaturase and elongase enzymes (17,18). Moreover, it has been shown more recently that the incorporation of LC n-3 PUFA into membranes depends on the background fatty acid intake (19,20). For example, the LC n-3 PUFA are incorporated into membrane phospholipids to a

TABLE 4
The Mean and Median Intakes of PUFA by Australian Adults (≥19 yr old)[a]

Fatty acid	Mean intakes	Median intakes	Median/mean (%)
n	10,851	10,851	
n-6 PUFA			
18:2	10.8	9.0	83
20:2	0.006	0.001	10
20:3	0.039	0.008	20
20:4	0.052	0.024	46
22:4	0.001	0.000	0
Σ n-6 PUFA	10.9	9.01	83
n-3 PUFA			
18:3	1.17	0.95	81
20:5	0.056	0.008	14
22:5	0.026	0.006	23
22:6	0.106	0.015	14
Σ LC n-3PUFA	0.189	0.029	15
Σ n-3 PUFA	1.36	1.08	74
Total PUFA	12.24	10.28	84

[a]For abbreviations see Tables 1 and 2.

TABLE 5
Food Sources for Total Fat (TF), Saturated Fatty Acids (SFA), Monounsaturated Fatty Acids (MUFA), and PUFA for All Persons (≥19 yr old)

Major food group name	TF	SFA	MUFA	PUFA
	(%)			
Meat, poultry, and game products and dishes	23.10	21.26	27.88	15.20
Cereal-based products and dishes[a]	18.27	19.11	19.09	15.23
Milk products and dishes	18.04	27.48	10.01	4.03
Vegetable products and dishes	8.94	6.96	9.80	11.23
Fats and oils	8.84	9.13	11.22	20.87
Cereals and cereal products[b]	6.73	3.33	4.96	13.70
Savory sauces and condiments	2.73	1.71	3.14	4.66
Seed and nut products and dishes	2.57	1.04	3.42	4.73
Fish and seafood products and dishes	2.55	1.80	2.60	4.44
Confectionery and health bars	2.31	3.20	1.92	0.75
Egg products and dishes	2.20	1.64	2.57	1.73
Soup	1.22	1.10	1.21	1.14
Snack foods	1.13	1.25	1.37	1.15
Nonalcoholic beverages	0.48	0.43	0.21	0.05
Fruit products and dishes	0.37	0.11	0.17	0.19
Legume and pulse products and dishes	0.27	0.12	0.20	0.73
Sugar products and dishes	0.09	0.16	0.11	0.07
Miscellaneous	0.08	0.11	0.07	0.05
Alcoholic beverages	0.05	0.07	0.04	0.01
Special dietary foods	0.01	0.01	0.02	0.02

[a]These foods include biscuits, cakes, pies, fried rice, pizza, vol-au-vents (puff pastry), quiche, gnocchi, lasagna, commercial hamburgers, croissants, and pancakes (15).
[b]These foods include bread, muffins, rice, pasta, and breakfast cereals (15).
For other abbreviation see Table 1.

greater extent if the background intakes of fatty acids are primarily monounsaturated with limited intake of n-6 PUFA (19). This may have implications for subsequent eicosanoid production, as eicosanoids produced from the n-6 PUFA AA have mainly vasoconstrictor, pro-aggregatory, and pro-inflammatory actions, whereas eicosanoids produced from n-3 PUFA EPA result in primarily vasodilation and anti-inflammatory actions (21,22). Hence, there is a necessity to balance these fatty acids in our cell membranes, which can be achieved by modifying dietary intakes.

Our Paleolithic ancestors consumed much higher intakes of n-3 PUFA compared with our current intakes. It is estimated that the diet of Paleolithic hunter-gatherers contained 8.8 g/d of LA and 12.6 g/d of LNA, giving an n-6/n-3 PUFA intake ratio of approximately 0.7 for the 18-carbon length PUFA (23). Their LC PUFA intakes were estimated to be 1.8 g/d for AA and 1.1 g/d for LC n-3 PUFA, giving an n-6/n-3 ratio for LC PUFA of 1.6. The total n-6 and n-3 PUFA intakes were 10.6 g/d and 13.7 g/d, respectively, giving an overall n-6/n-3 PUFA intake ratio of 0.8 (23). This ratio is much lower than our estimate of 8:1 for the n-6/n-3 PUFA intake ratio reported in this study. If we were to readdress the balance, we need either to reduce the n-6 PUFA intake or to increase the n-3 PUFA intake or both.

Given that our Paleolithic ancestors consumed approximately 1.1 g of LC n-3 PUFA per day (23), Greenland Eskimos consume 14% of total fatty acids as LC n-3 PUFA per day (24), and Japanese consume 1.6 g per day (3), it should be possible to substantially increase our LC n-3 intake. Epidemiological evidence links high intakes of LC n-3 PUFA to longevity (25), and experimental evidence shows that taking <1 g of LC n-3 PUFA per day can reduce total death, nonfatal myocardial infarction, and stroke in people with recent (<3 mon) myocardial infarction (26).

On the other hand, the American Heart Association and the National Heart Foundation (NHF) of Australia (6) recommend doubling our current intakes of n-6 PUFA from 5% to

TABLE 6
Food Sources for the n-6 PUFA

Major food group name	18:2	20:2	20:3	20:4	22:4	Σ n-6
	(%)					
Meat, poultry, and game products and dishes	14.94	86.46	23.97	70.15	11.14	15.27
Cereal-based products and dishes[a]	14.73	0.41	9.87	1.00	0	14.64
Milk products and dishes	3.18	0.01	0	0.12	0	3.16
Vegetable products and dishes	11.64	1.50	0	0	0	11.54
Fats and oils	21.85	6.52	0	0	0	21.66
Cereals and cereal products[b]	14.15	1.67	1.13	0.24	0	14.03
Savory sauces and condiments	4.87	0.01	0	1.13	0	4.84
Seed and nut products and dishes	5.38	0	0	0	0	5.33
Fish and seafood products and dishes	3.24	2.90	2.04	27.21	88.62	3.36
Confectionery and health bars	0.84	0	0	0	0	0.83
Egg products and dishes	1.58	0.04	62.93	0.16	0.24	1.78
Soup	1.23	0	0.06	0	0	1.22
Snack foods	1.29	0.40	0	0	0	1.28
Nonalcoholic beverages	0.04	0	0	0	0	0.04
Fruit products and dishes	0.20	0.04	0	0	0	0.20
Legume and pulse products and dishes	0.69	0.04	0	0	0	0.69
Sugar products and dishes	0.07	0	0	0	0	0.07
Miscellaneous	0.04	0	0	0	0	0.04
Alcoholic beverages	0.01	0	0	0	0	0.01
Special dietary foods	0.02	0	0	0	0	0.02

[a]These foods include biscuits, cakes, pies, fried rice, pizza, vol-au-vents (puff pastry), quiche, gnocchi, lasagna, commercial hamburgers, croissants, and pancakes (15).
[b]These foods include bread, muffins, rice, pasta, and breakfast cereals. For abbreviation see Table 1.

TABLE 7
Food Sources for the n-3 PUFA

Major food group name	18:3	20:5	22:5	22:6	Σ LC[c]	Σ n-3[c]
	(%)					
Meat, poultry, and game products and dishes	15.26	20.58	49.21	11.77	19.6	15.86
Cereal-based products and dishes[a]	23.8	0.15	0.40	4.35	2.55	20.85
Milk products and dishes	11.40	0	0	0	0	9.82
Vegetable products and dishes	9.81	2.22	0	0	0.66	8.54
Fats and oils	16.31	0	0	0	0	14.05
Cereals and cereal products[b]	12.86	0.05	0	0.33	0.20	11.10
Savory sauces and condiments	3.54	0	0	0.37	0.21	3.08
Seed and nut products and dishes	0.43	0	0	0	0	0.37
Fish and seafood products and dishes	3.54	75.87	50.36	73.12	70.78	12.86
Confectionery and health bars	0.53	0	0	0	0	0.46
Egg products and dishes	0.55	1.12	0.04	10.02	5.98	1.30
Soup	0.30	0	0	0.01	0.01	0.26
Snack foods	0.10	0	0	0	0	0.09
Nonalcoholic beverages	0.18	0	0	0	0	0.15
Fruit products and dishes	0.16	0	0	0	0	0.14
Legume and pulse products and dishes	1.01	0	0	0.03	0.02	0.87
Sugar products and dishes	0.08	0	0	0	0	0.07
Miscellaneous	0.08	0	0	0	0	0.07
Alcoholic beverages	0.04	0	0	0	0	0.03
Special dietary foods	0.03	0	0	0	0	0.02

[a]These foods include biscuits, cakes, pies, fried rice, pizza, vol-au-vents (puff pastry), quiche, gnocchi, lasagna, commercial hamburgers, croissants, and pancakes (15).
[b]These foods include bread, muffins, rice, pasta, and breakfast cereals.
[c]Σ LC is the sum of 20:5, 22:5, and 22:6; Σ n-3 is LNA and Σ LC; for other abbreviations see Table 1.

8–10% of total energy. The rationale is that, when saturated fat is replaced by n-6 PUFA in the diet, the risk of coronary heart disease decreases (6). However, the decreased risk is attributable to the reduction in saturated fat; increasing the consumption of n-6 PUFA without a concomitant decrease in saturated fat has not been shown to be of benefit. Importantly, the resultant displacement of n-3 PUFA from membranes by n-6 PUFA may produce an undesirable imbalance in precursors of eicosanoid production (18,20,27).

In terms of food sources, our study has shown that males eat more meat than females; hence, they have greater intakes of SFA, MUFA, and PUFA from meat, including the LC n-3 PUFA (22 vs. 16% for males and females, respectively). As females eat more fish (as a percentage of total energy) than

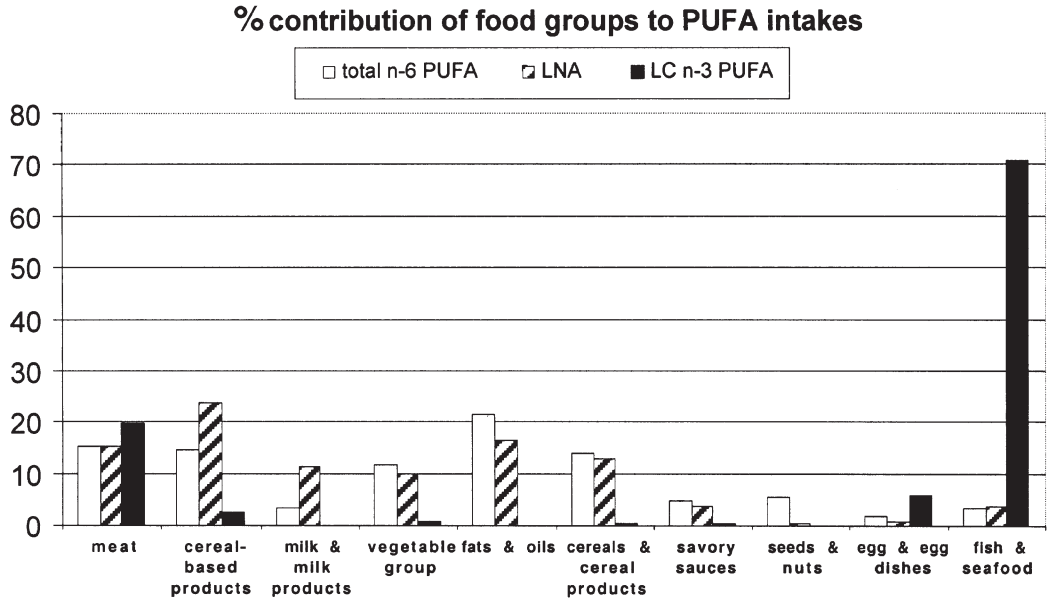

FIG. 1. The percentage contribution of the different food groups to PUFA intakes by adult Australians (>19 yr). Abbreviations: PUFA, polyunsaturated fatty acid; LNA, linolenic acid; LC n-3 PUFA, 20:5n-3, 22:5n-3, and 22:6n-3.

males, they obtain 74% of LC n-3 PUFA from fish, whereas males obtain 68% LC n-3 PUFA from fish. However, a great proportion of people do not consume fish or seafood products at all. Another way to increase the consumption of n-3 PUFA is to consume foods that have been fortified with the LC n-3 PUFA. Such foods may meet the criteria for the n-3 nutrition claim.

The former Australia New Zealand Food Authority, now known as Food Standards Australia New Zealand (FSANZ), recently introduced criteria in the Food Standards Code for voluntary nutrition claims in relation to the omega fatty acid content of foods (28). Nutrition claims in relation to n-3 PUFA content of a food, "other than fish or fish products that have no added saturated fatty acids," must not be made "unless the total of saturated fatty acids and *trans* fatty acids is less than 28 percent of the total fatty acid content of food; or the food contains no more than 5 g of saturated fatty acids and *trans* fatty acids per 100 g of the food" (28). Further, a claim that a food is a "source" of n-3 PUFA must not be made unless the food also "contains no less than 200 mg α-linolenic acid per serving or 30 mg total EPA and DHA per serving," whereas for a claim of a "good source" of n-3 PUFA, the food must contain no less than 60 mg total EPA and DHA per serving (28). Members of the food industry wishing to produce food products containing n-3 PUFA can utilize the n-3 nutrition claim and consumers will have more of a choice regarding food sources of LC n-3 PUFA. One may hope that this increased food choice will translate into increased consumption of LC n-3 PUFA.

In summary, adult Australians are consuming 10.8 g LA, 0.052 g AA, 1.17 g LNA, 0.056 g EPA, 0.026 g DPA, 0.106 g DHA, and collectively the LC n-3 PUFA of 0.189 g per day. The total n-6 PUFA is 10.9 g per day, and the total n-3 PUFA intake is 1.36 g per day. The mean n-6 PUFA intakes compare favorably to the recommendations by the NHMRC of Australia but fall short of NHF of Australia (6) and U.S./Canadian (8) recommendations and far exceed those recommendations by Simopolous *et al.* (5) (Table 1). The mean LNA intake is comparable to the U.S./Canadian recommendations but is approximately half the recommended intakes by the other organizations (Table 1). The mean LC n-3 PUFA intake compares favorably to some recommendations (4,6,8) but falls short of other international recommendations (2,5–7), especially the Japanese intakes of 1.6 g per day (3) (Table 1). The main food sources for n-6 PUFA include the fats and oils group, meat, cereals and cereal-based products, and vegetable products and dishes, while the main food sources for n-3 PUFA include cereal-based products and dishes, fats and oils, meat, poultry, and game products and dishes. The main food sources for the LC n-3 PUFA are fish and seafood, followed by meat and eggs.

In conclusion, we have identified intakes and food sources of both n-6 and n-3 PUFA for the Australian adult population. Our intakes of LC n-3 PUFA could be increased and this may be achieved by the introduction of foods fortified with LC n-3 PUFA.

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Fat, Fishing Patterns, and Health Among the Bardi People of North Western Australia

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ABSTRACT: Research into the resource use strategies of the Bardi Aboriginal People of One Arm Point, Western Australia, found that they maximize the consumption of specific beneficial marine FA. The Bardi assess the relative fatness of fish and animal species in their environment, procuring fish and marine species only when they are considered to be at their fattest stage: during specific seasons; at specific physiological life stages, or through on-site evaluation. In June 1999 and September 2000, samples of fish, dugong, oyster, and turtle were collected by Bardi fishermen, focusing specifically on species considered to be high in fat content and very popular among the Bardi. Nine species were analyzed for total lipids and FA profile, which were determined by capillary GLC. Comparative lipid analysis established that the Bardi hunters' selection process between species and within species and the selection of specific fish fat deposits increase the levels of beneficial FA made available to the community. Bardi fishing and hunting patterns meet a demand for fat within the community and may protect many species of fish whose spawning season is inversely related to the accumulation of the specific gut fat deposits sought by the Bardi. These fat deposits make up for the relatively low levels of fat in the flesh of tropical fish. The Bardi model provides important insights into the nature of human–environment interaction and expands our understanding of the role that warmer-water fisheries can play in human health.

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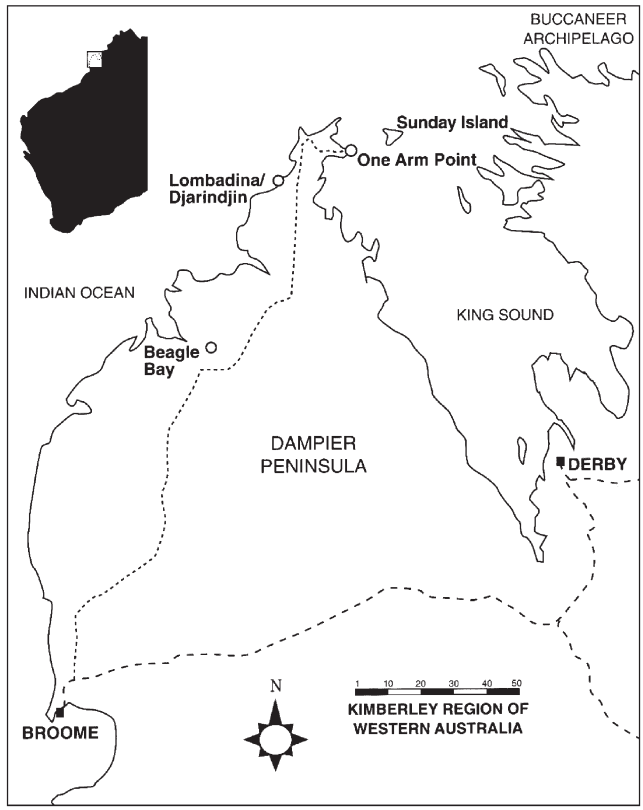

FIG. 1. Map of Australia and detail map of One Arm Point.

The study of coastal communities with high fish consumption has contributed greatly to the understanding of the role played by marine fats in human health (1–6). These studies have largely been carried out among fish consumers in higher latitudes consuming cold-water fish. There have been few studies investigating the lipid profiles of tropical fish and their health potential among tropical fish consumers. We previously reported that a group of coastal Australian Aboriginal people, the Bardi of north Western Australia (Fig. 1), pattern almost all of their fishing activity to maximize the lipid content of harvested resources by following well-established cues to guide their hunting and fishing behavior (7).[1] Fatness was indeed manifest at the times the Bardi people chose to hunt and fish. In some species, the seasonal change in physiology is quite dramatic, with fish that are specifically isolated and consumed by the Bardi displaying significant fat deposits in the gut (Fig. 2). The obsession with species fatness and focused fat consumption by the Bardi posited some interesting questions as to the nature of fats in tropical fisheries. In 1999, in conjunction with the Center for Ocean and Human Health and the Centre pour l'Océan et la Santé at CHUQ (Québec, Canada), a small study was initiated to ascertain whether any potential health benefits are associated with Bardi use of fatty tropical marine resources.

BACKGROUND

Fatness as a determinant of fishing behavior. It is well known that Aboriginal people follow seasonal patterns of resource

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[1]The Bardi are a group of Australian Aborigines located on the northwestern coast of Australia, some 300 km north of the town of Broome. Located on the most northern tip of the Dampierland Peninsula at latitude 16°30′ south, the Bardi and their predecessors have exploited this remarkable environment with its huge 30-foot (9.1-m) tidal range for over 27,000 yr (8).

Abbreviations: MFA, monounsaturated FA; SFA, saturated FA.

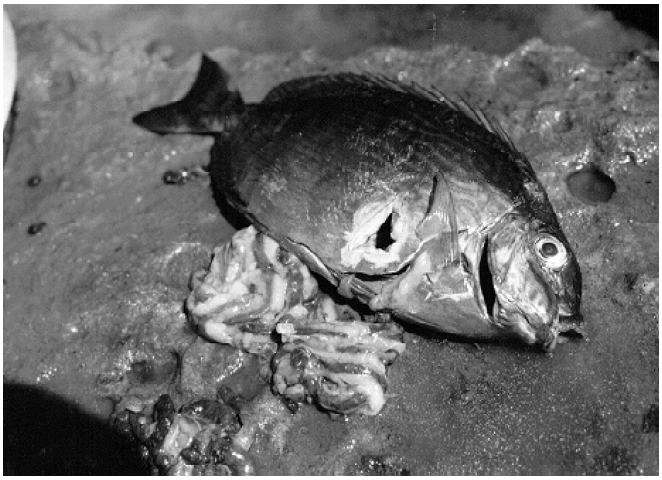

FIG. 2. *Siganus lineatus* displaying fat deposit on intestine.

use (9–12). These seasonal shifts between resources have been catalogued and mapped for most of the existent aboriginal groups. Much in the way we would anticipate, resource use among Australian Aborigines shows seasonal shifts. Many floral resources, though perhaps present year-round, frequently become edible or produce significant edible quantities only at specific times, so their exploitation follows patterns predicated on seasonal changes. Animals can be consumed year-round, so exploitation patterns should reflect shifts in availability. However, among the Bardi, many marine resources are available year-round but are exploited only at specific times. Hunting and fishing patterns among the Bardi do not necessarily reflect shifts in the availability of the resources *per se* but follow shifts and changes in the physiology of the resources related to the relative levels of fat they contain.

A preference for fatness in terrestrial resources among Aboriginal people is well known, and anthropologists have indicated that seasonal shifts in hunting and fishing patterns do take into account the fatness of the resource concerned (13). Among the Bardi, fatness is the determining criterion in food choices over a wide range of maritime resources (7,12,14). For many species of fish, turtles, and shellfish, Bardi exploitation patterns follow specific guidelines relative to perceived levels of fat. These guidelines take into account differences in observable fatness (fish or turtles with specific fat deposits, rays or sharks with big livers) and more subtle shifts in the taste or texture of resources that are related to broad annual seasonal environmental shifts, specific life-cycle stages, monthly tidal changes, or localized ecological considerations.

The shift to certain resources on a seasonal basis is one of the most dramatic features of Bardi fishing. There is no single season for fatness that all species follow, but many species present significant seasonal physiological changes broadly related to a seasonal change in water temperature. When discussing Bardi seasonal shifts in marine resource use, the Bardi often make reference to the fatness of specific species of fish. Some fish are said to be fat only at specific times of the year. Previous research by Smith and Kalotas (11) indicated that there is a general period during which fish are said to be fat and fishing intensifies:

" 'Bargana' is the 'cold season' when people start to light night fires. Strong, often unpleasant southeast winds blow, and the season is often called southeast time. Bargana is said to have begun when pandanus nuts are red. This is dugong hunting season, many fish are fat, night fishing is common though tides are rubbish for reefing" (Ref. 11, p. 324).

For those resources for which an identifiable period for fatness cannot be established, the Bardi use specific techniques to select resources at the point of capture in order to harvest for the most fat.

Rock oysters are preferably eaten during certain tidal phases when they are said to have more taste. Green turtles *(Chelonia mydas),* which are exploited year-round for their green fat, undergo an on-site, generally pre-capture analysis to assess fatness. In daylight, turtles are evaluated for fatness by an assessment of the quality of rolls of skin between the top and bottom of the carapace. During night-hunting expeditions for green turtle, their fatness is assessed by their breath odor pre-capture as they surface to breathe behind boulders in the tidal flow.

Resources that do not possess any particular taste or fatness are labeled as "rubbish": too dry or tasteless to be enjoyed.

Research by Rouja (7) established that fish that are present year-round are harvested exclusively between March and September, during the dry season, when the water is cooler and significant deposits of fat have accumulated in their gut.[2] The Bardi expectation of seasonal fat accumulations in fish does in fact match actual changes in the physiology of targeted fish, with fish demonstrating seasonal accumulation and loss of significant fat deposits in the gut. The fluctuation in the fat content in many fish is related primarily to spawning and reproductive cycles, with many accumulating fat stores in cooler-water months when they are not spawning. The Bardi are deliberately avoiding the spawning season of these fish "to let them do their business" (spawn), (Bardi Elder Douglas Wiggan) by concentrating on harvesting their fat accumulations. This avoidance of spawning fish also has been noted among the Bardi's southerly neighbors the Yawuru (12).[3] Few species of fish are considered to be consistently fat all year, those that are being highly valued and facing cultural restrictions on their capture and consumption. The value placed on these fish, such as ring-tailed surgeonfish, is not for fat deposits in the gut but for the taste of fat or oil in their flesh. However, even these are said to be better-tasting in the cool dry season when some, like ring-tailed surgeonfish, also display fat deposits in the gut.

The deposition of fat in the gut varies from species to species: The fat of golden lined spinefoot is attached to the

[2]Rays (rajiformes), also available year-round, are only harvested from November through February in the wet season. In this case, only those that are judged to be no more than 2 yr old and possessing yellow lips are considered to have the fattest livers.

[3]The work of Johannes (15) in Palau, a semi-anthropological study of fishing cultures that deals with the issue of fish physiology as a determinant of fishing behavior, offers an interesting contrast to this fishing pattern. In Palau, Johannes found the opposite association between spawning and fishing behavior, with spawning leading to an intensification in fishing.

peritoneum in association with the intestine (Fig. 2). In others, such as mangrove jack, it lines the back wall of the mesenteric cavity, and in others, it lines the length of the gut along the inside of the belly of the fish (sweetlips). In some fish, it is manifest in all three ways. The first type of fat, which is between and attached to the intestine of the golden lined spinefoot, is one of the most valued and the most difficult to consume as it is in association with the pungent contents of the intestine. These contents must first be removed before the fat can be cooked and eaten. The Bardi run the sharp edge of a fingernail or the tip of another small, sharp object along the tripe and push out the festering contents, rinsing repeatedly the mass of the intestine and fat in the ocean until clean. This is a painstaking process, taking up to 10 min per fish, with usually a good number of gut fat deposits from individual fish being processed at once. Most fat is cooked in the same way: Either it is reduced to liquid in a small baler shell or tin can set on the coals, which is then drunk and into which fish flesh is dipped, or, if attached to the intestine, it is wrapped around a stick and roasted over a cooling fire.

In the present study, FA were measured in marine species most often eaten by the Bardi, with particular attention given to the n-3 FA and monounsaturated FA. Our objective was to investigate whether Bardi strategies for resource use specifically increase the intake of beneficial n-3 FA in the Bardi diet.

EXPERIMENTAL PROCEDURES

In June 1999 and September 2000, samples of fish, shellfish, turtle, and dugong were collected by Bardi fishermen, focusing specifically on species considered to be high in fat content. These species were selected for laboratory analyses. Samples were collected from the four predominantly seasonal fatty fish species. Flesh or meat was taken at the time of initial processing or butchering. Samples of flesh with two distinct gut samples, liver, and fat (sometimes with associated intestine) were taken separately. Fat samples were taken postpreparation, after they had been cleaned and once they had been prepared for cooking, to ensure that they would represent the fats consumed by the Bardi.

Samples were stored at −20°C until they were analyzed. In all, nine marine species (Table 1) and a total of 42 samples were analyzed for total lipids and FA at the University of Guelph (Canada) by Dr. Bruce J. Holub. Ten grams of fish tissue was extracted following the addition of chloroform/methanol (2:1, vol/vol), in the presence of a known amount of internal standard tritridecanoin, according to Bligh and Dyer (16). Following transmethylation, using BCl_3/methanol, the FA profile was determined by capillary GLC by using a 60-m DB-23 column, 0.32 mm i.d. × 0.15 μm film thickness, using a Varian 3400 gas chromatograph.

TABLE 1
List of Species, Harvested by the Bardi, Analyzed for FA Composition

Common name	Bardi name	Species
Brown sweetlips	Mardal	*Plectorhinchus celebicus*
Golden lined spinefoot	Barbal	*Siganus lineatus*
Golden trevally	Giral	*Gnathandon speciosus*
Mangrove jack	Maran	*Lutjanus argentimaculatus*
Ring-tailed surgeonfish	Gambal	*Acanthurus grammoptilus*
Small silver bream	Gulurr	*Acanthropagus latus*
Rock oyster	Niwarda	*Saccostrea*
Dugong	Odorr	*Dugong dugon*
Green turtle	Gulil	*Chelonia mydas*

Data analysis. Arithmetic means of FA concentrations in animal tissues were calculated if two or more samples were analyzed. Data analyses were performed with the Statistical Analysis System software package (SAS Institute, Cary, NC).

RESULTS

FA composition of the flesh in species harvested by the Bardi. The total amount of FA in the flesh of species differed significantly (Table 2). FA in fish species are known to differ markedly from one species to another. The fish flesh appears to be relatively lean, the total amount of FA ranging from 0.3 to 2.5 g/100 g of flesh. In contrast, the flesh of dugong, which is a marine mammal, showed the highest amount of FA (31 g/100 g), followed by the green turtle (19.6 g/100 g). The flesh of ring-tailed surgeonfish had the highest amount of FA of all fish samples, followed by golden lined spinefoot, whereas mangrove jack and small silver bream had the lowest amount of FA in their flesh. Ring-tailed surgeonfish flesh also showed the highest amount of saturated FA (SFA) in fish, again followed by golden lined spinefoot. However, dugong and green turtle meat had the highest concentration of SFA of all species analyzed. The fat content of rock oysters varied greatly according to specific tidal phases, rock oysters taken during spring tides being four times higher in total FA (4565 mg/100 g) than rock oysters taken during neap tides (1044.1 mg/100 g).

The flesh of dugong also showed the highest concentration of monounsaturated FA (MUFA) (17.7 g/100 g), followed by the green turtle (7.9 g/100 g). Oleic acid (data not shown) was the predominant FA of the MUFA series. For fish species, the concentration of MUFA in the flesh varied from 57 to 502 mg/100 g. PUFA were in higher amounts than MUFA in the flesh of fish and in rock oysters, whereas the inverse was observed in the flesh of dugong and green turtle. Dugong and rock oyster showed the highest ratios of n-3 to n-6 FA. This ratio varied from 0.5 to 1.6 in the flesh of fish species. Except for ring-tailed surgeonfish, the amount of DHA was higher than that of EPA in fish flesh. The flesh of small silver bream and mangrove jack was particularly low in n-3 FA. Arachidonic acid (20:4n-6) was the predominant PUFA in the flesh of brown sweetlips, golden trevally, and small silver bream. EPA and DHA also were present in small amounts in the meat of dugong and green turtle, and the amount of arachidonic acid was relatively high in the flesh of green turtle. The n-3 FA composition of dugong flesh included high amounts of n-3 FA despite low concentrations of EPA, DHA, and 22:5n-3. However, the amount of a precursor of the n-3 FA series

TABLE 2
FA Composition[a] of the Species Harvested by the Bardi (mg/100 g, raw)

		FA (series)							n-3 series				n-6 series	
Species	(*n*)	Total FA	SFA[b]	MUFA[c]	PUFA	PUFA, n-3 series[d]	PUFA, n-6 series[e]	n-3/n-6	18:3	20:5 (EPA)	22:5	22:6 (DHA)	18:2	20:4
Brown sweetlips														
Flesh	1	701.1	309.7	145.8	245.6	120.0	125.6	1.0	4.9	29.3	27.9	53.7	13.3	75.3
Fat (gut)	1	338.1	148.4	57.5	132.2	40.2	92.0	0.4	0	6.7	9.6	22.0	4.8	69.9
Liver	1	5788.1	2534.8	1305.1	1958.2	1017.8	940.4	1.1	31.9	208.3	270.6	459.9	85.0	522.7
Golden lined spinefoot														
Flesh	2	1870.5	836.6	389.9	644.0	403.7	240.4	1.6	42.3	64.4	125.7	141.8	52.4	90.9
Fat (gut)	4	27195.3	13613.4	5602.9	7979.1	5186.4	2792.7	1.9	880.8	842.1	1594.2	1221.4	884.4	871.5
Liver	3	5244.8	2650.2	868.8	1725.9	1070.9	655.0	1.6	32.7	76.8	238.5	679.5	53.0	345.5
Golden trevally														
Fat (gut)	1	1042.2	482.2	156.3	402.7	244.6	158.1	1.5	7.3	37.0	38.8	154.2	18.6	85.7
Liver	1	1791.3	831.5	271.7	688.0	391.2	296.8	1.3	10.4	68.8	52.9	248.3	23.5	176.2
Mangrove jack														
Flesh	1	323.4	127.8	57.2	138.4	63.4	75.0	0.8	0.6	8.9	6.1	47.8	3.9	49.5
Fat (gut)	1	31425.7	15558.3	10028.5	5838.9	2921.8	2917.1	1.0	318.5	644.2	711.9	935.5	506.9	1196.2
Liver	1	4976.6	1917.3	1365.8	1693.4	1043.2	650.2	1.6	37.7	98.8	142.1	732.0	64.0	376.6
Ring-tailed surgeonfish														
Flesh	2	2539.3	1271.2	502.1	766.1	432.9	333.1	1.3	81.5	153.2	66.0	78.3	95.2	131.6
Fat (gut)	1	16528.1	8606.1	3401.8	4520.2	2365.4	2154.8	1.1	462.7	972.6	355.5	307.7	824.9	656.9
Liver	2	5046.4	2548.7	772.8	1724.8	997.4	727.4	1.4	94.0	218.8	152.8	465.5	139.5	363.2
Small silver bream														
Flesh	1	385.2	150.9	69.9	164.4	58.1	106.3	0.5	2.9	14.1	22.9	17.0	5.3	78.1
Fat (gut)	1	58432.7	28792.3	19808.0	9827.4	5320.3	4507.1	1.2	1167.7	1171.1	1985.0	477.4	1140.2	2371.7
Rock oyster neap tide	1	1044.1	505.9	167.8	370.4	259.8	110.6	2.3	11.8	132.4	11.8	98.2	26.1	49.7
Rock oyster spring tide	1	4565.0	2064.0	911.5	1589.6	1105.5	484.1	2.3	73.7	503.8	56.9	461.6	127.9	225.7
Dugong														
Flesh	3	31048.5	11262.3	17700.8	2085.4	1483.8	601.6	2.5	1157.0	32.0	118.5	33.2	473.7	60.9
Green turtle														
Meat and fat	2	19606.0	10530.3	7867.5	1208.3	581.9	626.4	0.9	123.8	113.1	281.4	26.2	164.5	237.8
Fat	2	69047.0	36072.8	29338.1	3636.1	1787.1	1849.1	1.0	348.8	310.4	923.6	89.3	505.4	650.9
Liver	3	18432.9	10116.1	7442.2	874.6	314.7	559.9	0.6	26.6	112.5	121.1	41.5	89.0	347.9
Kidney	1	13591.2	4751.4	6269.4	2570.4	903.7	1666.7	0.5	23.0	53.9	728.5	89.1	29.5	416.0

[a]SFA, saturated FA; MUFA, monounsaturated FA.
[b]SFA: (8:0 + 10:0 + 12:0 + 14:0 + 15:0 + 16:0 + 18:0 + 20:0 + 22:0 + 24:0).
[c]MUFA: (14:1 + 16:1 + 18:1 + 20:1 + 22:1 + 24:1).
[d]PUFA, n-3 series: (18:3 + 18:4 + 20:3 + 20:4 + 20:5 + 22:5 + 22:6).
[e]PUFA, n-6 series: (18:2 + 18:3 + 20:2 + 20:3 + 20:4 + 22:2 + 22:4 + 22:5).

(α-linolenic acid: 18:3n-3) was very high (1157 mg/100 g). This is probably a consequence of the herbivorous diet of the dugong. For rock oysters, the amount of EPA was higher than that of DHA. We observed again that the n-3 and n-6 FA content varied significantly according to the season.

FA composition of fat fish harvested by the Bardi. The total amount of FA present in the gut fat of fish species differed markedly. The reason is unclear but may be related to enclosed gut tissue when sampling the fat of fishes. In the fat fish, the most abundant FA were saturated, followed by PUFA. However, MUFA were higher than PUFA in the fat of small silver bream and mangrove jack. With the exception of golden lined spinefoot and golden trevally, for which n-3 FA were in higher amounts than n-6 FA, the distribution of n-3 and n-6 FA were similar in the gut fat of most of the species studied. The fat of golden lined spinefoot, mangrove jack, ring-tailed surgeonfish, and small silver bream contained large amounts of EPA and DHA when compared with others species. Moreover, the content in 18:3n-3 and 22:5n-3 of the fat of golden lined spinefoot and small silver bream was particularly high, whereas these FA were in small amounts in the fat of brown sweetlips and golden trevally. Finally, the fat of small silver bream, mangrove jack, golden lined spinefoot, and ring-tailed surgeon fish showed high amounts of linoleic acid (18:2n-6) and arachidonic acid. SFA, followed by MUFA, were the predominant FA in the fat of the green turtle, and the n-3/n-6 ratio was equal to 1. The most abundant n-3 FA in the fat of green turtle was 22:5n-3, whereas 18:2n-6 was the predominant FA of the n-6 series.

FA composition of the liver in species harvested by the Bardi. Except for golden trevally, for which the liver appeared to be leaner than that of other fish species, the amount of total FA in the liver of fish was relatively constant and amounted to approximately 5 g/100 g. In the liver of all fish species studied, SFA followed by PUFA were the most abundant FA. In the liver of green turtle, SFA were the major FA followed by MUFA, the concentration of PUFA representing only 5% of total FA. Generally, in the liver of fish, n-3 FA were in slightly higher amount than n-6 FA. In contrast, the liver of

green turtle showed twice as much n-6 FA as n-3 FA, arachidonic acid being the predominant FA of the n-6 series. Finally, DHA was the most abundant FA of the n-3 series in the liver of most species.

Contribution of Bardi selective fishing practices to the intake of MUFA and n-3 FA. Data allowed us to estimate the intake of MUFA and n-3 FA by Bardi consumers when consuming golden lined spinefoot, small silver bream, and dugong. It was assumed that a meal of fish was a mix of flesh (200 g) and gut fat (15 g). A meal of dugong was composed of flesh (or meat with associated fat) only. Hence, one meal of golden lined spinefoot provides 1.5 g of MUFA, 1.6 g of n-3 FA, and 721 mg of EPA + DHA. One meal of small silver bream provides 3.1 g of MUFA, 914 mg of n-3 FA, and 309 mg of EPA + DHA. Finally, one meal of dugong provides 35.4 g of MUFA, 3.0 g of n-3 FA, and 130 mg of EPA + DHA. These estimates reveal that the marine species consumed by the Bardi are good sources of MUFA and n-3 FA, especially when the fat of the gut is consumed with the flesh of the fish.

DISCUSSION

n-3 FA represent 15 to 23% of total FA in the flesh of all fish studied. Eating only the flesh would provide a limited amount of n-3 FA per meal. However, the deposits of fat in the gut of fish contain important amounts of these FA. Hence, our analyses showed that the consumption of mesenteric (gut) fat of the fish species studied may contribute to a significant intake of n-3 and n-6 FA for the Bardi. Furthermore, dugong and green turtle showed very high levels of MUFA. This is probably related to their herbivorous diet. It appears that Bardi have access to a major source of EFA as well as PUFA (including n-3 and n-6 FA) and MUFA, over a range of aquatic resources. Overall fat concentrations in the flesh of fish were low, but these matched the expectations of Bardi fishermen who said they were generally "not fat," "dry," or "skinny." Research among coastal communities in higher latitudes supports that diets rich in fish and marine mammals have beneficial effects on cardiovascular risk, and these benefits are attributed to the large n-3 FA content of colder-water seafood (1,4–6,17–22).

The Inuit diet also contains a higher content of MUFA (1). It is likely that the n-3 FA and MUFA create a particularly favorable condition that reduces the risk of ischemic heart disease in populations with high intake of fat from fish and marine mammals (23). Studies among Australian Aboriginal people support that a health benefit is also gained from moving Aboriginal people away from elements of the adopted modern Western diet back to traditional diets, specifically as it beneficially affects type 2 diabetes (24–28). O'Dea and Sinclair showed that some health benefit is gained from moving people away from Western foods and returning them to a diet of traditional marine foods, and found elevated levels of arachidonic acid in fish from northern Australia (27,29,30).

Our research among the Bardi suggests that the focus by the Bardi on tropical marine fats and specifically on seasonal fat deposits in the gut of many fish, high in DHA and EPA, may be intrinsically beneficial to health and mirror those benefits attributed to fish and enjoyed by consumers of fish in colder latitudes.

In every case where we have been able to get adequate data on species harvested by the Bardi, their assessment of the relative fatness of fish and marine species has been correct. There are measurable differences in the relative fatness of different parts of the fish (flesh vs. gut fat deposits) and between fish (golden spinefoot vs. golden trevally). In addition, the flesh of species considered fat year-round, such as ring-tailed surgeonfish, is significantly fatter than the flesh of seasonally fat fish.

For those seasonally fat fish species analyzed in this study, the intake of n-3 FA is directly related to the amount of fatty tissue taken from the gut of the fish and consumed by the Bardi. If the Bardi only ate the flesh of fish, they would miss out on a very rich source of beneficial n-3 FA and MUFA. Fishing peoples in tropical temperate fisheries are aware of seasonal shifts in fish gut morphology. However, the entire gut contents are generally discarded when the fish are gutted. The mesenteric or gut fat deposits have received no special attention (with the exception of the liver). The general feeling, that beneficial fats are low in tropical fish, is probably due to the evaluation of lipid profiles of tropical fish flesh without paying attention to seasonal shifts in fat accumulation and without accounting for fat deposits in the gut of the fish.

Analysis of rock oysters taken "at the right time" by Bardi fishermen, during spring tidal phases, and those taken when they were considered to be "rubbish" by Bardi fishermen, during neap tides, shows significant differences in fat levels. Contents in rock oysters harvested during spring tides were over four times higher in total FA and n-3 and n-6 FA than those sampled during neap tides. Many shellfish do in fact have a spring peak in protein and fat content that appears to be related to the timing of upwelling events or specifically to particularly large tidal fluxes (31).

In the case of hunting for turtle at night (bingarr hunting), where harvesting decisions based on maximizing fatness rely on the judgment of the halitosis of the turtle taking resting breaths, the Bardi are probably rejecting those turtles who have used up their fat reserves (perhaps by traveling long distances). In effect, these turtles are demonstrating ketosis, identifiable as a strong sweet-smelling acetone odor on the breath of the animal. This has recently been noted by Chukokta whalers smelling the breath and meat of emaciated whales (32). The Bardi have associated this halitosis with poor fat quantity and quality in greenback turtles and reject them when it is smelled.

In conclusion, we can put forward that Bardi strategies for resource use employ selective fishing and hunting practices that maximize the consumption of specific beneficial marine FA in a warm-water fishery. This maximization is achieved through a particular attention to mesenteric fat deposits in fish and evaluations of fatness in turtles, dugong, and shellfish.

When we consider that it is estimated that the Bardi received 70% of their food intake from the sea prior to contact (33), the diet of the Bardi must have been very high in beneficial marine FA. The degree of specialization and the myriad of complex stratagems (only a few of which have been outlined here) used by the Bardi to seek out and maximize this specific fat intake suggest that there is some risk associated with not securing these fats for consumption. It was reported that the incidence of low birthweight increased during the wet season in these communities (34). Interestingly, it is expected that the intake of n-3 FA is lower during the wet season. High n-3 FA intake during pregnancy has been associated with longer gestation and higher birthweight and optimal neurological development (35,36). Dietary practices that have evolved through homeostatic mechanisms should indicate beneficial relationships between ingested materials, their chemical properties, and human physiological processes, revealing those that go beyond energetic maintenance, some of which act as integral parts of physiological processes and others that have a more typical medicinal function (37).

The fishing behavior of the Bardi fishermen represents a culturally sanctioned resource use strategy that takes into account the concrete observable consequences of their behavior (fishing success, good taste, avoidance of seasonal spawners) and perhaps the adaptive value of the behavior (maximization of beneficial FA intake). Like many indigenous people throughout the world, the Bardi face a general health crisis caused by a range of debilitating personal and community challenges. Elaborating and supporting the benefits of traditional food among the Bardi are critical at this juncture.

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High-DHA Eggs: Feasibility as a Means to Enhance Circulating DHA in Mother and Infant

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ABSTRACT: Dietary DHA enhances infant attention and visual development. Because the DHA content of red blood cells and plasma lipids varies approximately threefold in pregnancy, maternal DHA status may influence subsequent infant function. It would be feasible to study the effects of higher maternal DHA intake on infant development if dietary intake of DHA could be increased by a reliable means. This study was designed to determine whether women provided with one dozen high-DHA hen eggs (135 mg DHA/egg) would consume the eggs and have higher blood DHA levels than women consuming ordinary eggs (18 mg DHA/egg). The study was a randomized, double-masked comparison of the effect of eggs with different concentrations of DHA on intake and blood lipid DHA content of women and their infants. A third nonrandomized group ate few eggs. In this study, DHA intake reported from eggs was eightfold higher in the high-DHA egg group compared to the ordinary egg group. Including all groups, DHA intake ranged from 0 to 284 mg/d. In this intake range, maternal blood lipid DHA content at enrollment best predicted DHA content at delivery, accounting for 36.5 and 51.7% of the variance in ordinary and high-DHA egg intake groups, respectively. The high-DHA vs. ordinary egg groups had similar maternal and cord blood lipid DHA, but there was a positive relationship between maternal plasma phospholipid DHA and daily DHA intake from eggs controlled for study duration ($r = 0.278$, $P = 0.048$). DHA intake and birth weight were also correlated ($r = 0.299$, $P = 0.041$). High-DHA eggs were well accepted and increased DHA intake. Other benefits of DHA intake during pregnancy were also suggested.

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Numerous studies have reported higher early visual acuity development (1–5) and other aspects of development (6–9) in preterm infants fed formulas with, compared to without, DHA. In conjunction with a body of work in nonhuman primates made deficient during fetal life (10,11), these studies have been used to imply the importance of the third trimester for normal brain DHA accumulation.

During the last intrauterine trimester and early postnatal life, brain DHA accumulation is quite variable within cultural groups (12,13). Likewise, the degree of variability in blood lipid DHA content as a biochemical indicator of apparent DHA status is approximately threefold among pregnant women (14), newborn infants (15), and formula-fed infants fed identical milk-based formulas (16). There is evidence that variability is much greater among cultural groups (14). The high degree of variability and evidence that circulating DHA declines in women during pregnancy (17) suggest that DHA accumulation by some term infants could be less than optimal for the developing newborn.

Maternal milk DHA (18) and maternal DHA levels during pregnancy (19) can be increased by increasing dietary DHA; maternal and infant DHA levels at birth are related (14,17), and DHA in maternal circulation at birth has been related to measures of infant attention (20). It is plausible that increasing DHA intake during pregnancy could enhance neural function of infants. Before these studies can be done, however, it is necessary to identify food sources of DHA that women will consume and to determine their impact on measures of apparent maternal and infant DHA status.

The objective of this study was to test the feasibility of consuming high-DHA hen eggs compared to ordinary eggs to increase blood lipid levels of DHA during the last trimester of pregnancy in a group of mainly African-American women. Although ocean fish contain high amounts of DHA, our experience with this population of women suggested that ocean fish were rarely, if ever, consumed. On the other hand, they usually consumed eggs, suggesting that eggs were a good food to use as an intervention to increase DHA. The secondary objectives of the study were (i) to evaluate the relationship of maternal DHA levels at enrollment and at delivery and (ii) to obtain data on pregnancy outcome to ensure that use of the high-DHA eggs did not pose any obvious risk to pregnant women.

MATERIALS AND METHODS

Subjects. Women were eligible for the study if they were between 24 and 28 wk pregnant by obstetric assessments (either date of last menstrual period or ultrasound), were between the ages of 16 and 35 yr at the time of enrollment, were accessible by telephone, and planned to deliver at the Regional Medical Center (Memphis, TN). Exclusion criteria for the study included any chronic illness, pregnancy-induced hypertension, pre-eclampsia, or pregnancy-induced diabetes at the time of enrollment. Women were excluded if they had more than four prior pregnancies, because we were concerned they would have too little time to comply with the study compared to women caring for fewer children.

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Abbreviations: AA, arachidonic acid; PL, phospholipid; RBC, red blood cell; TFA, total fatty acid.

Women eligible for the study were told that we would like to tell them about a study that involved eating eggs. If they said they ate eggs, they were asked for informed consent to be randomized to ordinary or high-DHA eggs under a protocol approved by the Institutional Review Board of the University of Tennessee–Memphis. Women who said they did not eat eggs or ate them only occasionally were asked for informed consent to collect the same blood samples and information about their medical and diet history as the randomized groups, but they were not provided eggs.

OmegaTech, Inc. (Boulder, CO) supplied both ordinary and high-DHA eggs (Gold Circle Farms). The method of producing the high-DHA eggs has been described (21). Refrigerated eggs were delivered to the clinical site every 2 wk. The ordinary and high-DHA eggs had white shells but came in cartons of different colors. Carton color remained the same throughout the study. At the clinical site, the eggs were labeled with the name and address of each woman, placed in ice packs, and delivered by courier. Each subject was given careful instructions to refrigerate the eggs immediately and to cook them before use. Subjects were told their assigned carton color and asked to inform the investigators immediately if the eggs delivered to them were not that color. The eggs were shown to have the same sensory properties in prior studies conducted by OmegaTech, Inc. In addition, we conducted our own sensory tests with our clinic nurses, who were blinded to the egg source. All felt that the eggs tasted and appeared like those they usually consumed.

A total of 73 women signed an informed consent approved by the University of Tennessee–Memphis Institutional Review Board. Of these, 52 women were randomized to the two egg groups: 25 to the regular egg group and 27 to the high-DHA egg group. Another 21 women consented to the study but were not randomized and were not given eggs (low-egg-intake group). Fifty-three women had maternal and cord blood collected at the time of delivery: 19 ordinary egg group, 18 high-DHA egg group, and 16 nonrandomized, low-egg-intake group.

Subjects in the ordinary egg, high-DHA egg, and low-egg-intake groups, 79, 83, and 75%, respectively, were mostly of African-American descent. Other characteristics of the study population are shown in Table 1. The groups did not differ significantly with respect to any recorded characteristic except age. Women assigned to consume ordinary eggs were significantly older than those in the high-DHA egg group ($P < 0.05$). In all groups, the mean age was skewed toward the lower end of the range eligible for the study.

Experimental design. Blood was drawn from each woman after she was enrolled. Information was obtained on her current weight and height, weight before pregnancy, age, and gestational age. At the time they were enrolled in the study, women completed a food frequency questionnaire that included adjustment questions that permitted more refined analyses of fat intake (22). Although the nutrient database did not include DHA content of fats consumed, available data for DHA content of individual foods were added to the database so that individual pre-study DHA intake could be estimated. In turn, DHA intake of each woman during the study was estimated by subtracting her pre-study DHA intake from eggs from her pre-study DHA intake and adding her estimated study DHA intake from eggs. Women who ate eggs regularly were assigned to either ordinary or high-DHA eggs using a randomization in blocks of six to ensure that the groups remained relatively balanced in number of subjects enrolled.

Our plan was to enroll 75 women (~25/group) with the expectation that we could retain ~20 women in each group. We estimated that we would lose 20% of each group through failure to eat eggs or to deliver at our hospital. We estimated that 20 women per group would allow us to estimate the range of DHA intake from eggs that could be expected if women were provided eggs as a source of DHA and how intake would affect blood lipid DHA. Enrollment was stopped after 73 women

TABLE 1
Characteristics of the Study Population[a]

	Low eggs ($n = 16$)	Regular eggs ($n = 19$)	OmegaTech eggs ($n = 18$)
Race (B/W)	12/4	15/4	15/3
Age (yr)	21.3 ± 4.8	24.8 ± 7.8[a]	19.9 ± 4.1[b]
Height (cm)	163.2 ± 7.7	160.6 ± 5.5	161.6 ± 11.4
Pregnancy no.	1.8 ± 0.9	2.3 ± 1.9	1.9 ± 1.1
Pre-study DHA intake (mg/d)	81 ± 29	104 ± 32	51 ± 19
Pre-pregnancy wt (kg)	64.8 ± 19.8	61.5 ± 11.5	68.6 ± 19.1
Enrollment wt (kg)	69.3 ± 16.5	72.6 ± 16.9	78.2 ± 20.2
Delivery wt (kg)	81.0 ± 19.0	78.3 ± 15.4	84.3 ± 20.6
Pregnancy wt gain (kg)	12.9 ± 3.8	15.1 ± 6.6	15.3 ± 7.9
Study duration (wk)	12.9 ± 2.4	12.7 ± 2.7	12.8 ± 1.6
Start egg intake (no/wk)	1.4 ± 1.0 (0–3)	4.0 ± 3.3 (0–14)	4.9 ± 3.7 (0–12)
Study egg intake (no/wk)	2.7 ± 3.5 (0–12)	8.9 ± 3.4 (2–14)	10.7 ± 5.8 (1–25)
Gestation at start (wk)	26.1 ± 2.0	26.7 ± 1.4	27.0 ± 1.1
Gestation at delivery (wk)	38.7 ± 1.6	38.5 ± 2.2	39.3 ± 1.6

[a]ANOVA was carried out with *post hoc* Tukey's test for differences between means ($P < 0.05$). Groups with different letter designations differed significantly. OmegaTech (Boulder, CO).

had consented to enter the study, because one of the investigators (E.B.) was scheduled to continue training elsewhere.

During the course of the study, women were sent two dozen eggs every 2 wk by courier. After the first delivery, they were interviewed before each subsequent delivery and asked how many eggs they had consumed. In addition, the unused eggs were returned by the courier, counted, the number recorded, and the eggs destroyed. Subjects were asked to keep a written record of their egg intake on forms supplied to them and to return these with the uneaten eggs; however, few were compliant with this request. The phone interviews were the only regular contact between the investigators and subjects until the subjects were admitted to the hospital to deliver.

When a woman came to deliver, she was evaluated by nursing personnel. If the decision was made to admit her to the hospital, blood was drawn by the phlebotomist and an investigator was paged. Each delivery was attended by one of the investigators, who collected cord blood on ice and weighed the placenta. After the delivery, infant weight, gestational age, size for gestational age, and any problems developed by the mother or infant were recorded.

Blood samples. Maternal blood (2 mL) was drawn from an arm vein and added to EDTA to prevent coagulation. Cord blood was removed from the cord vein and treated in the same way. All blood samples were placed on ice immediately after they were drawn and either transferred to the laboratory immediately or, in the case of samples obtained during the night, refrigerated in the hospital until they could be transferred to the laboratory on ice in the morning. In the laboratory, the red blood cells (RBC) were separated from the plasma by centrifugation at 5°C. The plasma was removed and stored under nitrogen at −70°C until analysis. The RBC were washed three times (0.15 M NaCl, 1 mM EDTA), and the supernatant was discarded after centrifugation. RBC were stored like plasma until analysis.

Analytical methods. Plasma and RBC lipids were extracted by the procedure of Dodge and Phillips (23) using chloroform and methanol. Methanol contained 50 mg/L of BHT as an antioxidant. The lipid extracts of plasma and RBC were washed with 0.15 M KCl according to Folch *et al.* (24), and the organic solvent phase was vaporized under nitrogen. Phospholipids (PL) and TAG (the latter only for plasma) were fractionated by TLC (Silica gel G plates, 10 × 20 cm; Analtech, Inc., Newark, DE) in hexane/diethyl ether/acetic acid (80:20:1). TAG migrated with an $R_f > 0.5$, and PL remained at the origin. Both TAG and PL were removed completely from the plate and transmethylated with boron trifluoride methanol (Sigma Chemical Co., St. Louis, MO) to yield FAME (25).

Individual FA were separated on a Varian 3300 gas chromatograph with a 100-m SP-2560 100 capillary column (Supelco, Bellefonte, PA). The instrument was programmed for column, injector, and detector temperatures of 175, 225, and 275°C, respectively. Helium was used as a carrier gas at a flow rate of 21 cm/s. Individual peaks were integrated automatically with a programmable Varian 4290 recorder/integrator. The peaks were identified by comparison to authentic standards. Standards of defined concentration were analyzed periodically to verify column integrity (NHI-C, NHI-F, NHI-C, and NHI-D; Supelco, Inc.), and biologic mixtures of FA containing the major FA of interest were analyzed daily (PUFA 1 and 2; Supelco, Inc.). DHA eluted at approximately 60 min. To quantify the amount of FA/mL of plasma in PL and TAG, 17:0 was added to PL and TAG isolated from a known amount of plasma (26).

Data analyses. Data were analyzed using SPSS for Windows (version 9). Characteristics of the study population, DHA and AA contents of plasma TAG and PL, and RBC PL were determined as means ± SD. The change in maternal DHA and AA levels from enrollment to delivery was also calculated in the plasma TAG and PL, and RBC PL fractions. The three groups were compared by ANOVA. Means were compared by Tukey's test with a level of significance set at $P < 0.05$. Partial correlations, to correct for birth order, were calculated to compare maternal DHA and birth weights, and to relate maternal and cord plasma PL DHA and daily egg DHA intake, while controlling for study duration. The relationship between maternal plasma PL DHA at enrollment and at delivery was calculated by using simple linear regression. Because the regression lines of the three groups did not differ, only the regression line for the total group is shown. The same applied to maternal and cord plasma PL DHA vs. study DHA intake from eggs.

RESULTS

Effect of study eggs on DHA intake. The number of eggs that women reported consuming doubled in all groups during the study (Table 1). The randomized groups did not differ from each other in study egg intake, but, as expected, egg intake was higher in the randomized groups than in the group that was not randomized to treatment. The mean daily DHA intake from eggs in each group at enrollment and delivery is shown in Figure 1. As expected, the mean daily DHA intake from eggs in the high-DHA egg group (183.9 ± 71.4 mg DHA) was significantly higher than in the ordinary egg group (35.1 ± 13.2 mg DHA) and in the low-egg-intake group (10.8 ± 4.0 mg DHA) ($P < 0.0001$). The DHA intake from eggs varied from 27.6 to 264.9 mg in the high-DHA egg group and from 0 to 36.0 mg DHA in the ordinary egg and low-egg-intake groups, reflecting the number of eggs consumed during the study as well as the DHA content of the type of egg consumed.

Effect of egg intake on FA composition of maternal and newborn blood lipids. The relative FA composition [g/100 g total FA (TFA)] of plasma TAG and PL, and RBC PL is presented in Table 2. Plasma TAG DHA levels were higher in the infants from mothers who consumed ordinary or high-DHA eggs, compared to infants from mothers in the low-egg-intake group ($P < 0.05$). The plasma PL arachidonic acid (AA) levels were also higher in the newborns from mothers consuming ordinary or high-DHA eggs compared to infants of mothers in the low-egg-intake group ($P < 0.05$). At delivery, the maternal RBC AA levels were lower in women consuming high-DHA eggs than

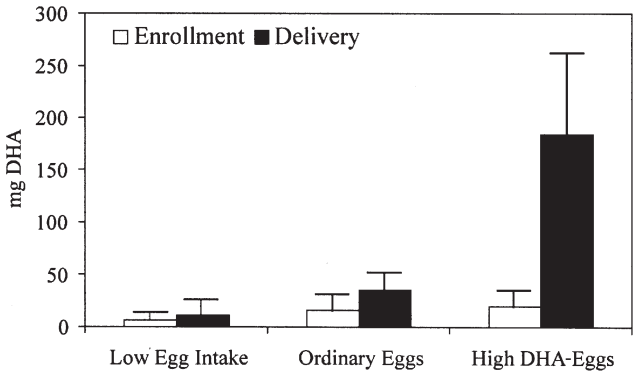

FIG. 1. Daily DHA intake from eggs during the last trimester of pregnancy at enrollment and at delivery.

in those consuming ordinary eggs ($P < 0.05$), and maternal RBC PL AA decreased significantly during the study in the high-DHA egg group but not in the other groups ($P < 0.05$). No effect of type of eggs on AA in infant blood lipid classes was observed. Table 3 describes the FA concentration (μg/mL plasma) of TAG and PL in women and infants. The differences between the groups were not statistically significant.

Simple linear regression analysis indicated that maternal plasma PL DHA at enrollment explained 51.7 and 36.5% of the variability in maternal plasma PL DHA at delivery in the high-DHA and ordinary egg groups, respectively. DHA from eggs consumed during the study accounted for <8% of the variability in maternal DHA levels at delivery, and infants' DHA levels at birth. On the other hand, maternal DHA at birth explained 34.8 and 25.7% of the infants' variability in DHA at birth in the ordinary and high-DHA egg groups, respectively. The number of pregnancies accounted for 27.2% of the variability in cord DHA levels in the ordinary-egg group, but <2% in the high-DHA-egg group.

Gestation and fetal growth. We recorded and present gestation and newborn size at birth even though the study was not powered to detect a clinically meaningful difference in these outcomes. The mean weight, length, and head circumference of infants in the high-DHA egg group were greater than in the ordinary egg group, and gestation was 5.6 d longer. When controlled for birth order, there was a positive association at enrollment between birth weight and maternal plasma PL DHA ($r = 0.289$; $P = 0.048$) and RBC PL DHA ($r = 0.279$; $P = 0.057$), but there was no association with birth weight and maternal PL DHA at delivery. A statistically significant, positive correlation was found between birth weight and total estimated DHA intake ($r = 0.299$, $P = 0.041$). A statistically nonsignificant trend was found between birth weight and study DHA intake from eggs when controlled for study duration ($r = 0.275$, $P = 0.058$).

Relationship between maternal and cord plasma DHA and daily egg DHA intake. The relationship between maternal and cord plasma PL DHA, and the daily egg DHA intake are depicted in Figures 2A and 2B, respectively. There was a positive relationship between the daily study DHA intake from eggs and both maternal plasma PL DHA (Fig. 2A) and cord plasma PL DHA (Fig. 2B) after controlling for study duration ($r = 0.278$, $P = 0.048$ and $r = 0.241$, $P = 0.095$, respectively).

Relationship between maternal plasma PL DHA at enrollment and at delivery. The relationship between maternal plasma PL DHA at enrollment and at delivery is depicted in Figure 3. There was a strong relationship between maternal plasma PL DHA at enrollment and at delivery ($r = 0.581$; $P < 0.0001$).

Relationship between maternal and cord plasma and RBC DHA at delivery. The relationships between DHA in maternal blood lipids at delivery and those in the same lipid class in cord blood are shown in Figure 4. Plasma and RBC PL DHA were strongly correlated between maternal and infants at delivery ($r > 0.370$). The highest correlation was found between

TABLE 2
FA Composition (g/100 g total FA) of Plasma TAG and Total Phospholipids (PL), and of Red Blood Cell (RBC) PL in Maternal and Newborn Blood[a]

	Plasma TAG		Plasma PL		RBC PL	
	AA	DHA	AA	DHA	AA	DHA
Low egg intake ($n = 16$)						
3rd trimester	1.91 ± 0.79	0.37 ± 0.14	12.12 ± 1.48	3.55 ± 0.30	16.57 ± 1.22	4.36 ± 0.50[a]
Delivery	1.73 ± 0.66	0.37 ± 0.10	11.94 ± 1.81	3.32 ± 0.64	16.58 ± 1.70[a,b]	4.46 ± 0.67
Change	–0.18 ± 0.99	0.00 ± 0.16	–0.19 ± 1.38	–0.23 ± 0.62	0.01 ± 1.65[a,b]	0.09 ± 0.63
Newborn	5.14 ± 2.52	1.01 ± 0.41[a]	19.11 ± 1.75[a]	4.80 ± 0.83	20.00 ± 1.23	5.52 ± 0.61
Low-DHA eggs ($n = 19$)						
3rd trimester	1.54 ± 0.65	0.41 ± 0.22	12.88 ± 2.29	4.07 ± 0.91	16.41 ± 0.96	5.00 ± 0.76[b]
Delivery	1.64 ± 0.67	0.36 ± 0.10	13.01 ± 2.18	3.69 ± 0.59	17.26 ± 1.26[a]	4.78 ± 0.92
Change	0.10 ± 0.62	–0.05 ± 0.22	0.13 ± 1.63	–0.38 ± 0.62	0.86 ± 1.64[a]	–0.22 ± 0.62
Newborn	6.65 ± 3.01	1.62 ± 0.58[b]	21.61 ± 1.75[b]	5.42 ± 1.01	21.17 ± 2.05	6.18 ± 0.97
High-DHA eggs ($n = 18$)						
3rd trimester	1.76 ± 0.70	0.35 ± 0.13	12.66 ± 1.68	3.70 ± 0.77	16.49 ± 1.27	4.82 ± 0.76[a,b]
Delivery	1.63 ± 0.80	0.42 ± 0.23	11.98 ± 1.65	3.73 ± 1.08	15.51 ± 1.32[b]	4.84 ± 1.22
Change	–0.19 ± 1.09	0.07 ± 0.22	–0.60 ± 1.27	0.05 ± 0.70	–1.01 ± 1.05[b]	0.04 ± 1.12
Newborn	5.38 ± 2.41	1.70 ± 0.89[b]	21.37 ± 2.44[b]	5.53 ± 1.32	20.57 ± 1.38	6.36 ± 0.76

[a]Values are reported as mean ± SD. Statistics: ANOVA with *post hoc* Tukey's test for differences between the means of groups. Groups with different superscript roman letters differ significantly from each other ($P < 0.05$).

TABLE 3
FA Composition (μg/mL) of Plasma TAG and Total PL in Maternal and Newborn Blood[a]

	Plasma TAG		Plasma PL	
	AA	DHA	AA	DHA
Low egg intake (n = 16)				
3rd trimester	13.26 ± 5.46	2.57 ± 1.04	119.00 ± 15.99	35.09 ± 5.63
Delivery	13.66 ± 8.99	2.84 ± 1.32	113.03 ± 27.44	31.52 ± 8.46
Change	0.39 ± 10.13	0.26 ± 1.42	–5.97 ± 20.10	–3.58 ± 8.53
Newborn	17.56 ± 11.97	3.63 ± 3.04	148.13 ± 27.35	37.16 ± 8.43
Low-DHA eggs (n = 19)				
3rd trimester	11.45 ± 5.27	2.97 ± 1.43	125.40 ± 29.03	39.01 ± 8.31
Delivery	12.71 ± 5.99	2.99 ± 1.63	123.46 ± 30.37	34.93 ± 7.23
Change	1.21 ± 6.39	0.02 ± 2.07	–1.95 ± 21.35	–4.09 ± 8.18
Newborn	13.52 ± 5.85	3.40 ± 1.57	161.18 ± 36.65	40.59 ± 10.83
High-DHA eggs (n = 18)				
3rd trimester	12.35 ± 5.40	2.39 ± 0.85	128.57 ± 24.36	37.43 ± 8.49
Delivery	11.22 ± 5.50	2.85 ± 1.80	117.23 ± 24.48	35.90 ± 9.53
Change	–1.58 ± 6.37	0.42 ± 1.66	–11.34 ± 30.91	–1.53 ± 12.01
Newborn	14.64 ± 7.40	5.26 ± 4.10	164.93 ± 32.39	45.71 ± 14.96

[a]Values are reported as mean ± SD. Statistics: ANOVA with *post-hoc* Tukey test for differences between the means of groups. See Table 1 for abbreviation.

maternal and cord RBC PL DHA level at delivery (r = 0.534; P < 0.0001). We did not find a significant correlation between maternal and cord plasma TAG DHA levels.

Safety monitoring. Table 4 presents the outcome data that were collected for safety monitoring of the groups. The pregnancy outcomes did not suggest any safety concerns for eating high-DHA eggs during the third trimester of pregnancy. If anything, the data suggested that the high-DHA-egg group might have a better outcome than the other two groups. The high-DHA egg group had fewer preterm deliveries (5.6%) than the ordinary egg (25%) and low-egg-intake (26%) groups. No low birth weight infants (<2500 g) were born in the high-DHA-egg group, but the prevalence was 12.5 and 26% in the low-egg-intake and ordinary egg groups, respectively. The reasons for admission to special or intensive care nurseries instead of the routine well-baby care nursery included: low-egg-intake group (n = 3)—one breech, one preterm, and one difficult delivery; ordinary egg group (n = 6)—one for effects of maternal gestational diabetes, two for premature birth, one for maternal chorioamnionitis, one for absence of spontaneous respiration (41 wk); and high-DHA-egg group (n = 2)—one for maternal chorioamnionitis and meconium, one with oligohydraminos and an Apgar of 3 at 1 min. The placenta weights also tended to be higher in the high-DHA egg group (760 ± 86 g) compared to the low-egg-intake (658 ± 122 g) and ordinary egg (663 ± 159 g) groups.

DISCUSSION

Although the mean DHA intake of the high-DHA-egg group was relatively small, it was close to the 200 mg/d of preformed long-chain n-3 FA suggested for healthy populations

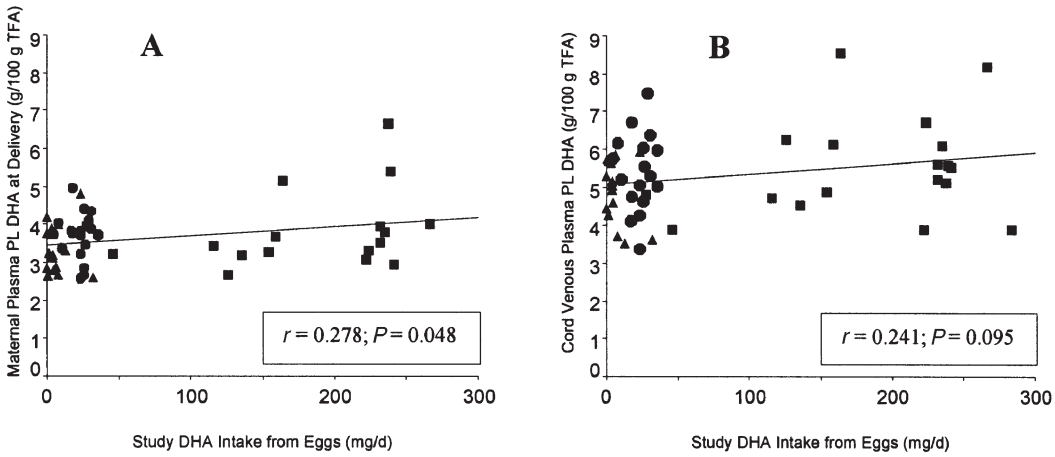

FIG. 2. Correlation between maternal (A) and cord (B) plasma DHA levels, and daily egg DHA intake in pregnant mothers with low egg intake (▲), and who consumed low-DHA eggs (●) and high-DHA eggs (■). PL, phospholipid; TFA, total FA.

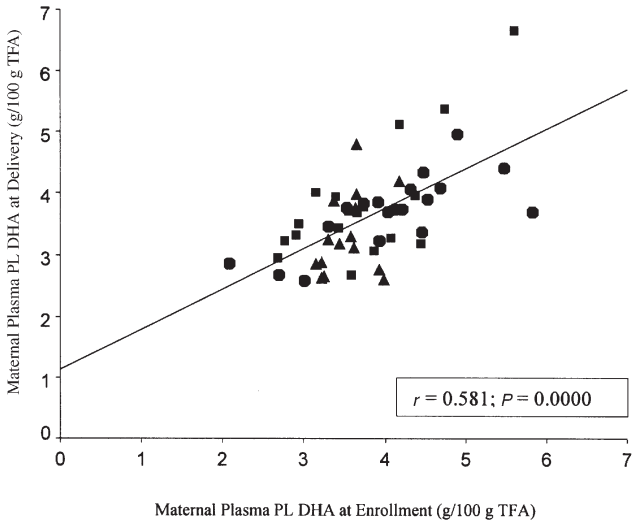

FIG. 3. Correlation between maternal plasma DHA levels at enrollment and at delivery in pregnant mothers with low egg intake (●), and who consumed low-DHA eggs (▲) and high-DHA eggs (■). See Figure 2 for abbreviations.

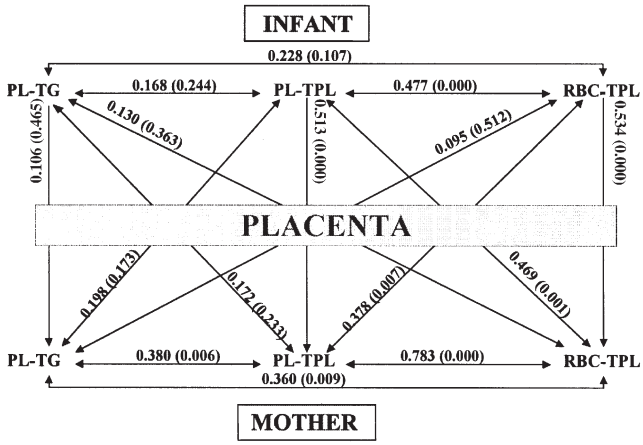

FIG. 4. Correlation coefficients (*P* values) between various measures of maternal and infant measurements of DHA in blood lipids: plasma (PL), TG, PL, total phospholipids (TPL), and red blood cell (RBC) TPL at delivery.

(27). This represented a 4- to 10-fold increase in DHA intake per day compared to the pre-study DHA intake based on our estimate of DHA intake from dietary reports collected at the beginning of the study. Despite this, we found no significant effect of consuming high-DHA eggs on blood lipid DHA of pregnant women or their newborns. Other investigators who have provided DHA and EPA from fish oil during lactation and pregnancy have found significant increases in DHA in milk (18) or neonatal blood lipids (19), but the amounts of DHA consumed were many-fold higher than in the present study. Moreover, Connor *et al.* (19) did not find a large increase in plasma DHA even with a fivefold increase in DHA intake (1.1 g/d).

Because we analyzed DHA in blood lipids of the same women at the end of the second trimester and at delivery, we were able to observe that DHA in circulating lipids tracked well for individual women, i.e., women with high DHA at enrollment tended to remain high and women with low DHA tended to remain low. The variability in maternal plasma DHA status at birth was mostly explained by the maternal DHA status at enrollment (>36%), and to a much lesser extent by DHA intake from study eggs (<8%).

Although we did not find a significant difference in blood lipid DHA between women consuming ordinary and high-DHA eggs, we do not consider this too surprising for several reasons. First, DHA intake was relatively low, even with high-DHA eggs. Second, the group consuming ordinary eggs did consume DHA from eggs. Third, the eggs were provided only during the last one-third of pregnancy. Finally, most women consumed DHA from foods other than eggs in variable amounts, and pre-study estimates of DHA intake suggested that women randomized to the high-DHA-egg group

TABLE 4
Preliminary Safety Monitoring[a]

	Low egg intake (n = 16)	Low-DHA eggs (n = 19)	High-DHA eggs (n = 18)
33–37 wk gestation	4/16 (25%)	5/19 (26%)	1/18 (5.6%)
LBW (<2500 g)	2/16 (12.5%)	5/19 (26%)	0/18 (0%)
Not routine hospital	3/16 (18.8%)	4/19 (21%)	2/18 (16.7)
Meconium	3/16 (18.8%)	4/19 (21%)	3/18 (17%)
Meconium to ICU/SCN	0/16 (0%)	0/19 (0 %)	2/18 (11%)
C-section	4/16 (25%)	6/19 (32%)	2/18 (11%)
Maternal antibiotics	7/16 (44%)	6/19 (32%)	3/18 (17%)
Baby length (cm)	47.8 ± 4.8	48.8 ± 2.1	49.2 ± 2.5
Baby head circ (cm)	34.1 ± 2.0	33.3 ± 1.7	34.3 ± 0.9
Baby head circ (>37 wk)	34.3 ± 2.2	34.2 ± 0.9	34.3 ± 0.9
Baby weight (g)	3100 ± 494	3008 ± 578	3232 ± 463
Baby weight (>37 wk)	3226 ± 429	3232 ± 478	3220 ± 455
Placenta weight (g)	658 ± 122	663 ± 159	760 ± 86
Gestational diabetes	0/16 (0%)	3/19 (16%)	0/18 (0%)
Pre-eclamsia	0	0	1/18 (5.6%)

[a]LBW, low birth weight; ICU, intensive care unit; SCN, special care nursery.

consumed less DHA than women in the other two groups. The high correlation between maternal plasma PL DHA at enrollment and delivery suggests that the FA analysis itself was not imprecise. In addition, we feel confident that we obtained a reasonable estimate of DHA intake from eggs, because we interviewed women about their estimated egg intake every 2 wk and also counted eggs returned. Women chose their egg intake and were not made to feel that they needed to eat all of the eggs they were provided.

Researchers who evaluated blood lipid DHA during pregnancy found dramatic differences among women from a number of European countries (14). The population with the lowest apparent DHA status was Hungary and that with the highest was Finland. Blood lipid DHA of women in our study was a bit lower than, but neonatal plasma DHA levels in our study were similar to, those reported for pregnant women and neonates in Hungary. The observation could suggest that incoming maternal DHA accumulates preferentially in the fetus when maternal DHA levels are low.

Hornstra and his co-workers (14,17,28) found that DHA concentration in blood lipids increased 30 to 40% over prepregnancy levels and remained high until the infant was delivered. Their international comparative study found the proportional increase to be greatest in the group that already had the highest circulating PL DHA, i.e., the group from Finland (14). The finding could be used to suggest that circulating DHA during pregnancy reflects prior DHA stores. It is tempting to speculate that the increase in DHA in plasma lipids during pregnancy is a means of increasing the amount of maternal DHA available for transfer to the fetus.

We found, as have others (17), that maternal and infant PL DHA are highly correlated regardless of the blood lipid class evaluated (Fig. 4). Thus, pregnancy itself (14,17,28), as well as relatively higher maternal blood DHA levels due to cultural patterns of food intake (14), is likely to equate to higher infant DHA accumulation. From developmental studies of preterm (1–9) and term (29–33) infants fed formulas with DHA, there is considerable evidence to suggest that the fetus could benefit developmentally from being exposed to higher preformed DHA from its mother.

One of the most surprising findings of the present study was the suggestion that DHA intakes in the range of 200 mg/d might be able to increase gestation and neonatal size. Birth weight was ~200 g higher in the high-DHA egg group compared to the ordinary egg group, and we found a positive association between total dietary DHA intake and DHA intake from eggs, and birth weight. DHA intake from eggs accounted for nearly 8% of the variance in birth weight, a large amount to be accounted for by one easily modifiable factor given that birth weight is influenced by many factors, many not easily modifiable. Olsen and co-workers (34,35) have reported previously that gram amounts of DHA and EPA from fish oil prolonged gestation (34) and increased birth weight and length of gestation among Danish women who had a prior preterm delivery (35).

A limitation of the study was that we had to rely on indirect measures of egg intake, so it was not possible to determine the difference between actual and assessed egg intake. We also had limited information concerning background DHA intake during the study, and it appeared that pre-study DHA intakes might be higher among women in the ordinary-egg group compared to the high-DHA group. However, baseline DHA content in blood lipids appeared to be equivalent in the two randomized groups. Another limitation of the study was the absence of data on smoking and alcohol use, which could actually increase DHA in infant blood lipids relative to the infant's mother (36). Although the groups differed in maternal age and weight, we are unaware of any reason why this could influence blood DHA content. Maternal age and weight could influence study outcomes such as gestation and birth weight, and they would need to be controlled or adjusted for statistically in any study designed to measure the effect of an intervention on gestation or birth weight. Those outcomes are reported here for possible interest of some readers.

The study raised no safety concerns on pregnancy outcomes (Table 4) after mothers were exposed to high-DHA eggs during the last trimester of pregnancy. Based on the results of this study, we hypothesized that women who consumed high-DHA eggs during the last trimester of pregnancy would have a longer gestation and higher birth weight infants. We have recently completed a randomized clinical trial of 350 women in another American city, again in a majority African-American population (37).

In conclusion, the study provided evidence that high-DHA eggs could increase DHA intake among pregnant women. Even though the amount consumed had no significant effect on blood levels of DHA, the study provided the first evidence that quite low intake of n-3 FA from DHA alone could increase length of gestation and birth weight.

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Dietary Intake of Fish vs. Formulations Leads to Higher Plasma Concentrations of n-3 Fatty Acids

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ABSTRACT: The n-3 fatty acids from fish appear to be more efficacious, in terms of cardioprotection, than equivalent amounts provided as capsules. Volunteers were given, for 6 wk, either 100 g/d of salmon, providing 383 mg of EPA and 544 mg of DHA, esterified in glycerol lipids, or 1 or 3 capsules of fish oil/d, providing 150 mg of EPA and 106 mg of DHA or 450 mg of EPA and 318 mg of DHA, as ethyl esters. Further, we reevaluated data from a previous study carried out with the same design, i.e., with 3 and 6 capsules/d of fish oil, providing 1290 and 2580 mg/d EPA and 960 and 1920 mg/d DHA. Marked increments in plasma EPA and DHA concentrations (μg/mg total lipid) and percentages of total fatty acids were recorded at the end of treatment with either n-3 capsules or salmon. Net increments of EPA and DHA in plasma lipids were linearly and significantly correlated with the dose after capsule administration. Further, increments in plasma EPA and DHA concentration after salmon intake were significantly higher than after administration of capsules. The same increments would be obtained with at least two- and ninefold higher doses of EPA and DHA, respectively, if administered with capsules rather than salmon. We provide experimental evidence that n-3 fatty acids from fish are more effectively incorporated into plasma lipids than when administered as capsules and that increments in plasma concentrations of EPA and DHA given as capsules are linearly correlated with their intakes.

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Several "minor components" of the diet are indispensable for a number of vital processes. The essentiality of various nutrients and recognition of their healthful effects have, occasionally, promoted their utilization as formulated preparations for clinical studies. However, clinical trials have often proved such preparations to be less effective than what was anticipated by epidemiologic data based on the dietary intakes of the same bioactive compounds (1,2).

The n-3 fatty acids (FA) are essential components of functionally important cell membranes, e.g., in the cardiovascular and nervous systems, and they must be derived from the diet. The average Western diet provides <200–300 mg/d n-3 FA, mostly from fish, out of a total fat intake of >100 g/d. Several epidemiologic and clinical studies demonstrated the cardioprotective activities of these minor components (3,4). Controlled studies have also shown that fish rich in n-3 FA is cardioprotective (5) even when consumed in small amounts, i.e., a few grams/d (6). These data suggest that fish consumption is more efficacious in terms of biological effects than administration of formulated preparations, e.g., capsules, that provide comparable amounts of n-3 FA (6–8). Despite this apparent contradiction, the comparative effects on plasma n-3 status of the administration of n-3 when taken with food or as capsules have not been addressed specifically to date. Yet given the popularity of n-3 prescription in secondary prevention, this comparison may bear important therapeutic consequences.

We investigated the relationships between consumption of given amounts of salmon or administration of capsules containing EPA and DHA acids ethyl esters, i.e., the most widely used pharmaceutical form of n-3 administration, and n-3 plasma concentrations in healthy subjects. These compounds are derived exclusively from the diet, and to assess their status in the plasma compartment, conventional measurements of their plasma levels as percentages of total FA may not be completely adequate. In fact, this value provides information on relative changes, i.e., increments in certain FA are associated with reductions in others. Also, their measurements in selected lipid classes, e.g., in phospholipids (PL) as conventionally performed, may provide incomplete information because although DHA is preferentially associated with PL, EPA is appreciably associated also with other lipid classes such as cholesterol esters. Therefore, we included measurements of their concentrations in plasma lipids [μg/mg total lipids (TL)], in turn establishing the magnitude of the circulating n-3 FA pool and its increase after administration.

For comparative purposes, we also reanalyzed and included the results of a previous study, carried out by following exactly the same protocol, in which different preparations of EPA and DHA were administered to healthy individuals (9).

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Abbreviations: cps, capsule; FA, fatty acids; PL, phospholipids; TL, total lipids.

STUDY DESIGN

This study conforms with the ethical standards on human experimentation of the University of Milan and is in accordance with the Helsinki Declaration of 1975 as revised in 1983.

Normolipidemic, healthy volunteers (ages, 26–38 yr; mean body mass index, 21.6 kg/m^2; mean total cholesterol concentration, 190.5 mg/dL; mean triglyceride concentration, 95.5 mg/dL) were recruited from within our Department, gave informed consent to the study, and were instructed to abstain from fish consumption during the 2 wk (T_{-2}) preceding the treatment, which was carried out for 6 wk. We did not control for alcohol intake, although subjects were instructed not to drink more than one alcoholic beverage per day.

Eight subjects (4 men and 4 women) were given 100 g/d of smoked salmon (Chinook, Tourin, Italy), which provided 383 mg of EPA and 544 mg of DHA (EPA/DHA 0.70). Two groups of four subjects each (6 men and 2 women) took 1 or 3 capsules of fish oil per day (Now Foods, Bloomingdale, IL), providing 150 mg of EPA and 106 mg of DHA or 450 mg of EPA and 318 mg of DHA as ethyl esters, respectively (EPA/DHA 1.41). These subjects were instructed to take the capsules with the main meal. No placebo oil was used because the aim of the study was to compare the effects of n-3 FA intake through two different sources. Also, n-3 FA are exclusively derived from the diet and therefore, unless ingested, no change will ever occur in their plasma lipids levels.

Blood was drawn by venipuncture from fasting subjects at –2, 0, and 6 wk (T_{-2}, T_0, and T_6) using heparin as anticoagulant; plasma was separated by centrifugation. In the previous study (9), performed by following the same protocol, three (1290 mg EPA and 960 mg DHA) or six (2580 mg EPA and 1920 mg DHA) 1-g capsules/d (Pharmacia, Upjohn, Milan, Italy) were given to two groups of eight healthy subjects each. The EPA/DHA ratio was 1.34. In the previous paper, however, relationships between intakes and plasma concentrations were not reported (9).

Quantification of administered n-3 FA. To evaluate the amount of n-3 FA in the different sources, several 50-g samples of salmon were ground, and five aliquots of the final mixture were extracted according to the method of Folch *et al.* (10). The profile of fatty acid methyl esters was subsequently analyzed and quantified by gas–liquid chromatography, as fully described by Marangoni *et al.* (11). Conditions were as follows: column Omegawax 320 (Supelco, Bellefonte, PA), 30 m, i.d. 0.32 mm, film thickness 0.25 μm. Gas chromatographic run programming: 1.2 min at 125°C, to 205°C at 2.5°C/min, 205 min for 20 min, to 220°C at 5°C/min, at 220°C for 30 min with programmable temperature vaporizing injector and flame ionization detector. Calibration standards were purchased from Nu-Chek-Prep (Elysian, MN). The n-3 FA contents in the capsules were also controlled by quantitative gas–liquid chromatography after derivatization to methyl esters (11).

Analysis of plasma FA. Lipids were extracted from plasma according to the method of Folch *et al.* (10) and quantified by microgravimetry. FA methyl esters were prepared from TL extracts, and plasma concentrations were determined by gas chromatography made quantitative by the use of 19:0 and 21:0 as internal standards (11). We report the values both as percentage of total plasma FA and as mg/mg total plasma lipids (TL).

Statistical analysis. Statistical analysis was carried out using a nonparametric method (Wilcoxon's matched pairs signed rank test) to allow for the limited number of participants (12). A value of $P < 0.05$ was considered significant. Statistics were performed by use of SPSS 11.0 for Windows (Chicago, IL).

RESULTS

The FA composition and contents of salmon and capsule lipids and their individual lipid classes are reported in Table 1. The major FA were 16:0 and 18:1, whereas n-3 FA (EPA, docosapentaenoic acid, and DHA) represented about one-third of total FA. The n-6 FA were found in small amounts, whereas among the monounsaturates, 20:1 and 22:1 were present in appreciable concentrations.

The total amounts of the n-3 FA, in the form of ethyl esters, in a 1-g capsule, were: Now Foods, USA EPA, 150 mg/capsule and DHA 106 mg/capsule; Pharmacia-Upjohn EPA 430 mg/capsule and DHA 320 mg/capsule.

In plasma, there was no appreciable change in triglyceride and cholesterol levels (not reported) after treatment, an expected finding because the subjects were normolipidemic.

In plasma lipids, there was no appreciable change in EPA and DHA percentage values and concentrations between T_{-2} and T_0; therefore, we report only differences between T_0 and

TABLE 1
Lipid Content and Fatty Acid Composition of Salmon and Capsules Administered to Volunteers[a]

Fatty acid	Salmon (%)	Capsules (%)
14:0	4.7	5.1
16:0	15.9	16.8
18:0	3.5	3.7
16:1	6.9	9.2
18:1	16.3	12.6
20:1	6.2	1.4
22:1	5.8	1.3
18:2	3.2	1.8
20:4	0.8	1.3
18:3	0.9	1.2
20:5	11.4	22.7
22:5	4.9	2.5
22:6	16.1	16.1
n-6	4.4	3.4
n-3	35.5	46.1
n-3/n-6	8.0	13.5
Unsaturation index	236	274
Total n-3 in salmon (mg/100 g)		Total n-3 in capsules (mg/capsules)
EPA	383	150
DHA	544	106

[a]The unsaturation index is Σ (% of each fatty acid × number of double bonds).

T_6 for all five treatments. Basal EPA ranged between 0.44 and 0.67% of total plasma FA in the different groups of the five studies, whereas DHA ranged between 1.38 and 1.97%. After 6 wk, the percentage values of EPA increased from 0.97 to 4.03 percentage points in the different experimental groups, whereas DHA increased from 0.91 to 3.07 percentage points.

Measurements of plasma FA concentrations allow the evaluation of the net changes associated with treatments. The initial plasma concentrations in the different groups ranged between 0.97 and 3.2 μg/mg TL for EPA and between 6.03 and 6.63 μg/mg TL for DHA (Table 2, which also reports FA concentrations at the end of treatments). DHA concentrations were substantially higher and more uniform throughout the various groups than those of EPA. The mg/mL plasma concentrations (not reported) were somewhat more variable than the values expressed as mg/mg TL. However the basal (T_0) values for DHA were rather uniform, ranging from 26.9 ± 7.2 (SD) in the salmon group to 35.9 ± 4.5 in the group taking 6 capsules from the previous study (9). Despite the different initial EPA and DHA plasma concentrations in the different groups, increments (μg/mg plasma TL) of both FA were linearly correlated with the doses after 6 wk of capsule administration ($R^2 = 0.98$ and 0.92, respectively (Figs. 1 and 2, respectively). Such increments were much smaller for DHA (Fig. 2) than for EPA (Fig. 1) (slopes: 1.34×10^{-3} and 9.74×10^{-3}, respectively). It can be extrapolated from the equations that increasing the intake by 100 mg/d for a 6-wk period results in a 0.97 mg/mg TL increment for EPA and in a 0.151 mg/mg increment for DHA. The increments of plasma EPA and DHA observed 6 wk after the consumption of salmon were substantially greater than those that can be extrapolated from the curves for equivalent intakes of these FA as capsules. In fact, to obtain the same increment in plasma EPA induced by 383 mg/d for 6 wk in salmon by using capsules, a dose of 800 mg, i.e., more than twofold, would be required; as for DHA, a dose of 4858 mg, i.e., almost ninefold compared with 544 mg given with salmon, would be required by using capsules.

DISCUSSION

The initial values of EPA and DHA expressed as mg/mg TL and especially as mg/mL plasma allow the assessment of the basal n-3 FA status. The values were rather uniform in the case of DHA, which can be synthesized from the precursor EPA through a rate-limiting and tightly controlled process involving peroxisomal reactions; this is more relevant in terms of both the quantitative aspects and the physiologic roles. Based on evaluations of plasma volume/body weight (i.e., 60 mL/kg body mass) and mg TL/mL plasma values, it can be calculated that the basal amounts of circulating DHA are ~1.6–2.1 mg/kg body weight.

This study, comparing different n-3 FA intakes, provides two new sets of information. First, by increasing and maintaining the intakes of EPA and DHA for a 6-wk period, in groups of normolipidemic subjects and using the same type of formulation, i.e., ethyl esters, there was a progressive increment of plasma levels that has a linear relationship with dose. It is of interest that the increments were related exclusively to the doses, and were independent of the initial concentration value. The situation in essence resembles what happens when a certain volume of fluid is added to a container in which some fluid is already present: The added volume simply adds up to the previous one and the increment is independent of the initial value. The data on the effects of salmon consumption were obtained with a single level of intake. The possibility that a similar relationship occurs with increasing intakes of salmon warrants further investigation.

Second, net increments of n-3 FA, notably those of DHA, after 6 wk of salmon consumption were much higher than after capsule administration. Plasma EPA increments after a fish meal providing a dose of 383 mg/d correspond to those obtained with a more than double dose of EPA administered as capsules, and the difference is even greater in the case of DHA (an almost ninefold greater dose would be required using capsules rather than fish to obtain the same increment in plasma).

The greater "bioavailability" of n-3 FA from fish than from capsules could be attributed to the association of n-3 with a larger amount of fat, as part of 100 g of ingested food, ensuring administration in a diluted form. In addition, EPA and DHA in fish are esterified mainly in the *sn*-2 position of triacylglycerols and glycerophospholipids, which represent a significant proportion of fish lipids. The glycerol *sn*-2 position is to a large extent preserved from hydrolysis during digestion and intestinal absorption of exogenous fat (13).

Conversely, capsules provide a small lipidic bolus and, in the eventual absence of a concomitant intake of other fats, although the subjects were instructed to do so, the processes of lipid absorption may not be adequately activated. The results

TABLE 2
Plasma Concentrations of EPA and DHA Before (T_0) and After (T_6) 6 Wk of Administration of Either Salmon or Fish Oil Capsules[a,b]

		EPA T_0	EPA T_6	DHA T_0	DHA T_6
		(mg fatty acid/total lipids)			
Fish	($n = 8$)	1.68 ± 0.66	10.58 ± 3.14[c]	6.41 ± 1.15	15.11 ± 2.25[c,d]
1 capsule current study	($n = 4$)	1.58 ± 0.39	4.21 ± 1.62[c]	6.69 ± 1.47	8.77 ± 1.71
3 capsules current study	($n = 4$)	0.97 ± 0.42	6.28 ± 2.01[c]	6.03 ± 1.48	9.08 ± 2.24
3 capsules previous study (9)	($n = 8$)	2.87 ± 0.37	16.59 ± 0.28[c]	6.16 ± 0.32	9.54 ± 0.69[c]
6 capsules previous study (9)	($n = 8$)	3.28 ± 0.34	29.57 ± 3.01[c]	6.51 ± 0.37	11.41 ± 0.4[c]

[a]See study design section for experimental details.
[b]Data are means ± SD.
[c]Different from T_0, $P < 0.05$.
[d]Different from 3 capsules in current and previous studies $P < 0.05$.

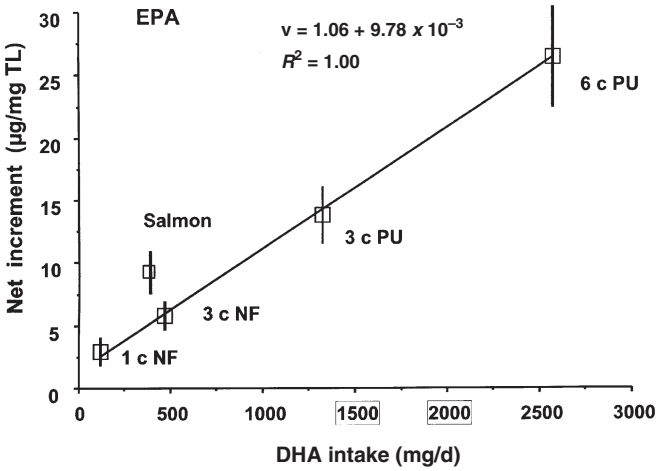

FIG. 1. Plasma net increments of EPA after supplementation of healthy volunteers with either fish oil capsules or salmon. Data are means ± SD. NF refers to the capsule data from the current study (1 and 3 capsules of fish oil per day from Now Foods, USA, Bloomingdale, IL), whereas PU refers to capsule data reported in Tremoli *et al.* (9) (3 and 6 capsules of fish oil per day from Pharmacia Upjohn, Milan, Italy). TL, total lipids; c, capsules.

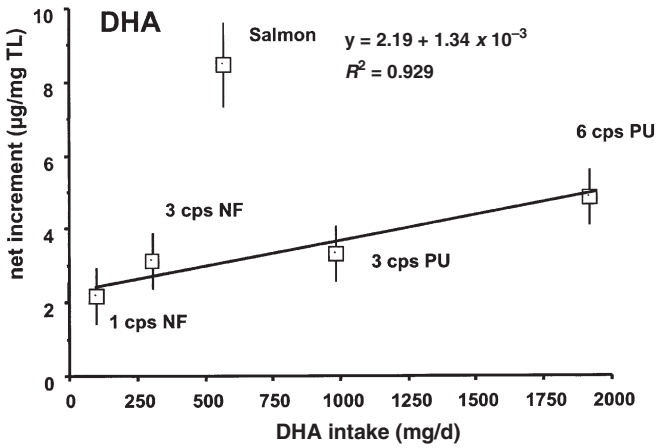

FIG. 2. Plasma net increments of DHA after supplementation of healthy volunteers with either fish oil capsules or salmon. Data are means ± SD. NF refer to the capsule data from the current study (1 and 3 capsules of fish oil/d from Now Foods, USA), whereas PU refers to capsules data reported in Tremoli *et al.* (9) (3 and 6 capsules of fish oil per day from Pharmacia Upjohn). TL, total lipids; cps, capsules. For manufacturers

clearly indicate that fish is more efficient than capsules in providing n-3 FA. In turn, these data might explain why fish consumption, even with low doses and infrequent consumption, is highly protective toward cardiovascular disease. From our data, it could be postulated that 10–20 g salmon/d would effectively raise plasma DHA levels.

It should be considered, however, that treatments were carried out only for 6 wk and that n-3 FA tend to be maintained in plasma and cell lipids for long time periods after their intake is interrupted (11); therefore, long-term and regular intakes of n-3 FA with capsules might attain plasma levels comparable to those obtained with fish intake. A "pharmacological" treatment may be recommended when fish consumption is not accepted or is not feasible.

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A Biomarker of n-3 Compliance in Patients Taking Fish Oil for Rheumatoid Arthritis

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ABSTRACT: Dietary fish oil supplements have been shown to have benefits in rheumatoid arthritis (RA), other inflammatory diseases, and in cardiovascular disease. As with any medical advice, variability will exist with regard to adherence and consequent biochemical or pharmacophysiologic effects. The aim was to explore the utility of plasma phospholipid EPA as a measure of n-3 PUFA intake and response to standardized therapeutic advice given in an outpatient or office practice setting, to increase dietary n-3 PUFA, including a fish oil supplement. Patients with early RA were given verbal and written advice to alter their dietary n-3 PUFA intake, including ingestion of 20 mL of bottled fish oil on juice daily. The advice included instructions to increase n-3 PUFA and to avoid foods rich in n-6 PUFA. Every 3 mon, blood samples were obtained for analysis of plasma phospholipid FA. Plasma phospholipid EPA was used as the primary index of n-3 PUFA intake. A diverse response was seen, with about one-third of patients achieving a substantial elevation of plasma phospholipid EPA over the 12-mon study period. A third had little change, with the remainder achieving intermediate levels. Data obtained longitudinally from individual patients indicated that substantial elevations of EPA (>5% total plasma phospholipid FA) could be maintained for more than 3 yr. Plasma phospholipid EPA is a convenient measure of adherence to advice to take a dietary n-3 PUFA-rich fish oil supplement. This measure may prove a useful adjunct to intention to treat analyses in determining the effect of dietary fish oil supplements on long-term outcomes in arthritis and other chronic inflammatory diseases. It may also provide a guide to the effectiveness of therapeutic and preventive messages designed to increase n-3 PUFA intake.

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Fish oil is a rich source of long-chain n-3 PUFA, which has been shown to reduce symptoms in rheumatoid arthritis (RA) (1), to increase the interval between relapses in Crohn's disease (2), and to reduce progression to renal failure in IgA nephropathy (3). Dietary n-3 PUFA have also been shown to reduce cardiovascular risk factors and events, especially sudden cardiac death (reviewed in Ref. 4). Reduction in the latter has been shown to correlate with erythrocyte EPA (20:5n-3) levels (5).

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Abbreviations: AA, arachidonic acid; HUFA, highly unsaturated FA; IL, interleukin; RA, rheumatoid arthritis; TNF, tumor necrosis factor.

The benefits of n-3 PUFA in inflammatory diseases are generally delayed, with symptomatic improvement in RA typically occurring 2–3 mon after commencing treatment (1). For this reason, a biochemical measure of effective adherence with advice to take fish oil and otherwise modify dietary PUFA intake may be especially useful in monitoring this aspect of therapy. This study explores the use of a measure of n-3 PUFA intake in patients with recent onset RA, in whom a fish oil supplement had been recommended in both hospital outpatients and private clinic settings.

SUBJECTS AND METHODS

Subjects. The studies undertaken were approved by the Royal Adelaide Hospital Ethics of Research Committee. Patients attending the Royal Adelaide Hospital Early Arthritis Clinic were recruited to the study, after their written informed consent was obtained. The Clinic approach is based in the Outpatients Department of the hospital but is also applied by participating specialists in their private rooms. The subjects fulfilled American College of Rheumatology criteria for RA (6) and had disease duration of less than 6 mon before the time of entry. Subjects were entered into a program of combination therapy with methotrexate, hydroxychloroquine, and sulfasalazine (unless known to be sulfonamide intolerant). Doses of these agents were increased in the event of persistent active disease observed at reviews performed every 6 wk, according to criteria determined by the protocol. If necessary, further medication with gold sodium thiomalate, then leflunomide therapy, were added in a predetermined treatment hierarchy. All patients were given advice to take a fish oil supplement, to increase n-3 PUFA-rich foods, and to avoid n-6 PUFA-rich foods. Patients with active disease were reviewed every 6 wk and those with minimal disease activity or fulfilling remission criteria every 3 mon. At each visit, the subjects completed a Vital Activities and Lifestyle Index form, which was an adaptation of that of Pincus *et al*. (7). Additional questions included inquiry into the dose and form of fish oil supplementation.

Fish oil preparation and dietary advice. The fish oil preparation was a bottled cod liver oil purchased from Melrose Laboratories (Mitcham, Victoria, Australia). The oil contains 10% EPA and 10% DHA (22:6n-3) of total FA, 625 IU/mL retinol, and 80 IU/mL cholecalciferol. Patients were instructed to float

20 mL of this oil on 30 mL of juice in a small glass and to swallow in a single gulp in a manner designed to avoid contact of the oil with the lips. The swallow was followed immediately by drinking a small amount of juice (~50 mL) from a separate glass. It was advised that the dose be taken with a solid meal without further fluid. Using this method, the dose can be taken without tasting the oil and without a repeating fishy taste. This method has the advantage of providing a fish oil supplement at a fraction of the cost of fish oil capsules and with less reflux. Some patients found the prospect of taking the bottled (i.e., unencapsulated) oil unacceptable. These patients were advised to take fish oil capsules as an alternative. They were advised that the dose of long-chain n-3 PUFA in the 20-mL dose was roughly equivalent to 13 standard fish oil capsules containing 30% long-chain n-3 PUFA (typically EPA 18%, DHA 12%). Some patients who started out with bottled fish oil switched to fish oil capsules (typically 1–2 daily), having been made aware of this option by others without a full appreciation of volume/dose equivalence. These patients were appraised of the number of capsules required, after which some opted to take the bottled oil. Each patient was given a diet information sheet that outlined the "two-glass" method for taking the fish oil supplement. Suggestions for n-3 PUFA-rich foods included 15 mL freshly ground linseed as a breakfast food with milk as a drink or with fruit and cereal. Canola-based spreads and oils were recommended and safflower-, sunflower-, and corn oil-based products proscribed. It was recommended that at least two meals of fish be consumed per week. A checklist of desirable and undesirable prepared foods was provided.

Collection of blood samples. Random peripheral venous blood samples were collected in heparinized tubes. Plasma lipids were extracted and the phospholipid fraction was separated by TLC. FAME were prepared before GLC analyses of FA as described previously (8).

Correlation between mononuclear cell phospholipid EPA and plasma phospholipid EPA. This correlation was examined using data from a previously published dietary intervention study in healthy volunteers (9). Briefly, parallel groups of subjects consumed a diet in which visible fats (spreads, cooking oils, dressings) were provided. These were prepared using either flaxseed oil (n-3 rich) or safflower oil (n-6 rich). The test diets were consumed over a 6-wk period. During the second 3-wk period, all subjects consumed a fish oil supplement (EPA 18%, DHA 12%, 6 g) that delivered 2 g long-chain n-3 PUFA daily. Venous blood samples were taken at baseline, 3 and 6 wk and analyzed for mononuclear cells and plasma phospholipids.

Kinetics of plasma phospholipid EPA levels after dosing. Three Early Arthritis Clinic patients known to have stable elevations of plasma phospholipid EPA (EPA > 5% of total FA) and 3 healthy subjects not consuming an n-3 PUFA-rich diet or supplements were studied. After an overnight fast, a blood sample was taken before a dose of fish oil (EPA 10%, DHA 10% w/w) 20 mL. Further blood samples were taken 15, 30, and 60 min later, and then at 2, 4, 6, and 24 h. The samples were taken into lithium heparin, and the plasma phospholipid fraction and erythrocytes were analyzed for phospholipid FA.

Computation of time-averaged levels of plasma phospholipid FA over a 12-mon period. Blood samples were taken from patients at intervals of 6–12 wk over the 12-mon period. Commencing with the first sample after providing dietary advice, usually taken at 6 wk, the area under the curve for FA of interest over the remainder of the 12-mon period was calculated using all results available. This value was then divided by the number of weeks defining the area to obtain the time-averaged value.

RESULTS

Correlation between mononuclear cell and plasma phospholipid EPA. There was a close correlation between phospholipid EPA in peripheral blood mononuclear cells and plasma (r = 0.97) (Fig. 1).

Kinetics of plasma and erythrocyte phospholipid EPA after dosing with fishing oil. After a single 20-mL dose of fish oil by fish oil-naïve volunteers, there was an increase in plasma phospholipid EPA from a low predose level (0.6–1.6%) to EPA levels of 1.6–3.0%, i.e., ~1.5 times the predose value. A similar pattern of increase was seen in all of the normal subjects. By contrast, there was little or no consistent change in plasma phospholipid EPA levels in patients after the test dose of fish oil (Fig. 2). There was essentially no change in erythrocyte phospholipid EPA throughout the 24-h observation period after the test fish oil dose in either the fish oil-treated patients or fish oil-naïve volunteers (Fig. 2).

Plasma phospholipid EPA and DHA levels in rheumatoid subjects given advice to take a daily fish oil supplement. At 12 mon, 30 of 64 subjects (47%) stated they were taking bottled fish oil regularly and a further 9 (14%) stated they were taking capsules. At entry, the mean value of plasma phospholipid EPA was 1.2 ± 0.14 (mean ± SEM) as a percentage of total FA. The value in these patients contrasts with baseline values (0.7 ± 0.05%) observed in a previously reported dietary study in healthy volunteers (9). At the 12-mon analyses, there were significant increases in mean plasma phospholipid EPA to 3.4 ± 0.3% as a percentage of total FA. A similar change was seen at 6 mon (Fig. 3). Levels in individual patients could be variable, consistently elevated, or essentially unchanged (Fig. 4.) However, the

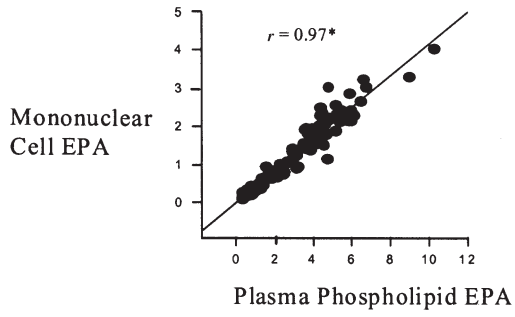

FIG. 1. Correlation between plasma phospholipid EPA and mononuclear cell EPA (expressed as a percentage of total phospholipid FA) in healthy subjects.

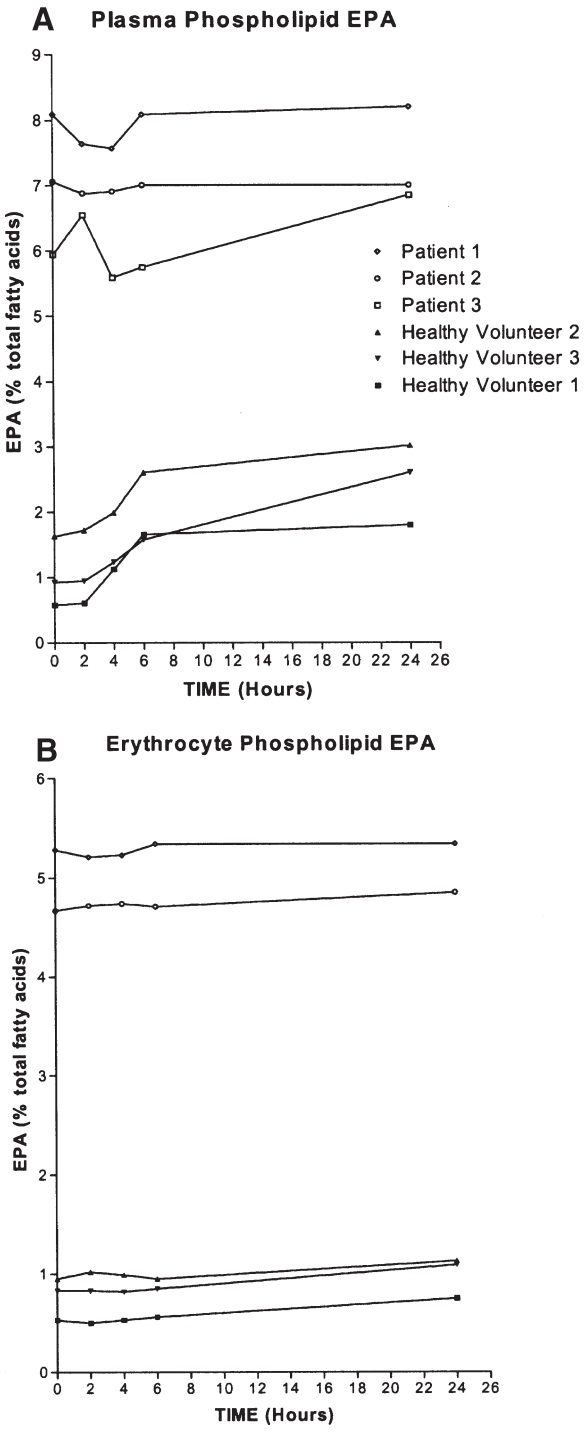

FIG. 2. Kinetics of (A) plasma phospholipid EPA and (B) erythrocyte phospholipid EPA after a dose of fish oil, containing 2 g EPA and 2 g DHA, in fish oil-naïve healthy volunteers and rheumatoid subjects engaged in long-term treatment that included ingestion of the same dose of fish oil on a daily basis. One patient specimen for erythrocyte FA analysis was not analyzed due to a laboratory mishap.

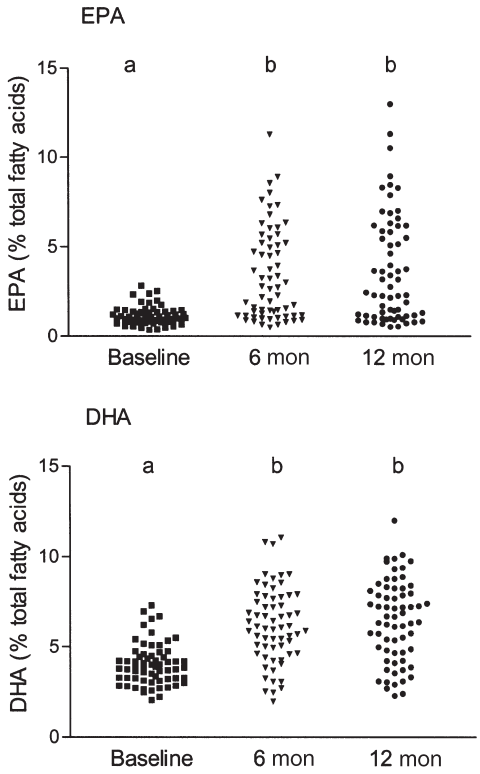

FIG. 3. Plasma phospholipid EPA and DHA in patients with recent onset rheumatoid arthritis, who were advised to take a daily fish oil supplement containing 2 g EPA and 2 g DHA. Results are shown for analyses of samples taken at baseline and 6 and 12 mon after commencing treatment. Columns with different letters are significantly different from each other (ANOVA followed by Tukey's multiple comparison test, $P < 0.05$).

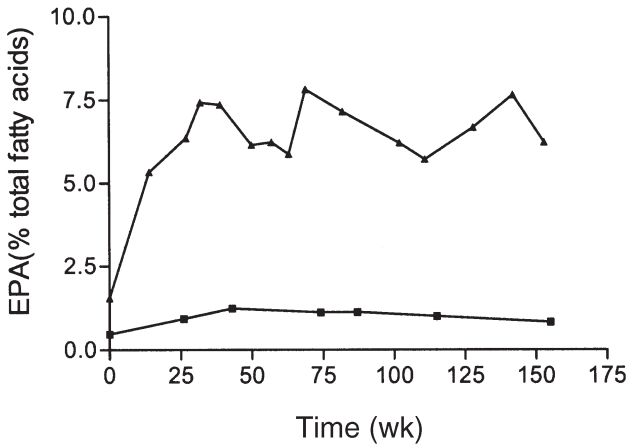

FIG. 4. Longitudinal observations of plasma phospholipid EPA over a 3-yr period in two patients with recent onset rheumatoid arthritis, who were each advised to ingest a daily fish oil supplement containing 2 g EPA and 2 g DHA.

distribution of time-averaged values for individuals indicated that the phospholipid levels achieved varied substantially with about a third showing a marked change, a third little change, and the remainder an intermediate difference (Fig. 5). DHA levels also increased from baseline values, although the proportionate increases were not as large as for EPA (Fig. 3).

Effect of dietary fish oil intake on arachidonic acid (AA) as a proportion of total highly unsaturated FA (HUFA). Dietary

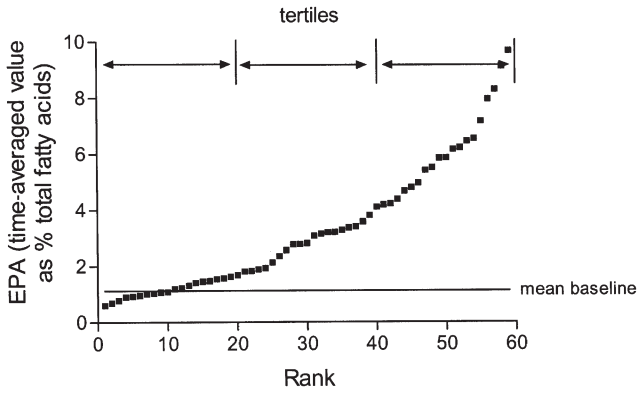

FIG. 5. Time-averaged levels of EPA as a percentage of total plasma phospholipid FA for individual patients computed from an area under the curve calculation based on at least four estimates per patient over a 12-mon period. Mean level at baseline of 1.13% is shown.

fish oil supplementation is thought to exert anti-inflammatory effects at least in part by displacing AA from the pool of HUFA in the *sn*-2 position of membrane phospholipids, from which it is released by phospholipase A_2 to provide substrate for eicosanoid-forming enzymes (10). EPA acts as an alternate substrate and inhibitor of AA metabolism. In the highest tertile for plasma phospholipid EPA, AA as a percentage of HUFA was depressed by 37%, whereas essentially no effect was seen in the tertile with the lowest EPA level during treatment (Table 1).

DISCUSSION

Numerous short-term randomized controlled trials have shown reduced symptoms in RA in response to treatment with a fish oil supplement (1). Although these studies have been analyzed appropriately on an intention to treat basis, in longer-term studies of outcomes in more usual clinic settings, a biochemical measure of tissue enrichment with n-3 PUFA may be more informative. This is especially important because the symptomatic response to fish oil is delayed in a dose-dependent manner for up to 6 wk to 4 mon after the introduction of treatment (1). Furthermore, production of interleukin (IL)-1β and tumor necrosis factor (TNF)α by peripheral blood mononuclear cells is inversely related to EPA levels in these cells (11), which in turn correlates with plasma phospholipid EPA as shown here. The above cytokines have been implicated in upregulating processes that cause inflammatory tissue damage (12). Thus, there is reason to believe that increasing tissue EPA levels may reduce long-term tissue damage in RA. Accordingly, monitoring plasma phospholipid EPA could be useful in treatment with anti-inflammatory doses of fish oil. A plasma phospholipid EPA level of 3.2% correlates with 1.5% mononuclear cell EPA. At this level, a significant reduction of IL-Iβ and TNFα synthesis is seen (11). Long-term elevations to above this level were found in more than half of the patients in the present study.

Lands *et al.* (10) suggested a ratio to express the proportions of n-6 and n-3 HUFA, i.e., n-6 and n-3 C_{20} plus C_{22} FA, in tissue and plasma phospholipids. The intent is to highlight the availability of AA as a substrate for proinflammatory and prothrombotic eicosanoids. In response to the dietary advice used in this study, a significant reduction of AA as a proportion of total HUFA was seen in rank order in all tertiles for increase in plasma EPA. This ratio could be used as an alternative or complement to EPA as a proportion of total plasma phospholipid PUFA because both are obtained from the same GLC analysis.

The baseline plasma phospholipid EPA in the patients was slightly higher than values observed in healthy volunteers (9). This may reflect a tendency for patients to self-medicate with fish oil, typically at modest doses (one or two 1-g capsules daily). The data show substantial differences in plasma phospholipid EPA in the patients after being given standardized advice to increase their n-3 PUFA intake during long-term management of RA. Both variations in compliance with advice to take fish oil or to follow the suggested diet and variations in metabolism may contribute to these differences. The diversity in the observed biochemical responses highlights the potential utility of monitoring plasma phospholipid FA in guiding long-term n-3 supplementation during management and assessment of outcomes in chronic inflammatory diseases such as RA. Comparison of the kinetics of plasma phospholipid EPA and erythrocyte EPA after a dose of fish oil in a small group of patients with stable, high plasma phospholipid EPA values and in

TABLE 1
Comparison of Time-Averaged Values for Arachidonic Acid (AA) with Baseline Values of AA Grouped by EPA Tertiles[a]

Tertiles according to rank EPA as % total plasma phospholipid FA[b]	Baseline EPA for each tertile as % total plasma phospholipid FA[c]	Mean (±SD) AA as % total plasma phospholipid HUFA[b]	Baseline AA for each EPA tertile as % total plasma phospholipid HUFA[c]	% Change from baseline of AA as % total HUFA
Lowest (0.61–1.63)	0.95 ± 0.54	46.0 ± 5.39	49.31 ± 7.06	–5.6 ± 8.9[d]
Middle (1.69–3.81)	1.28 ± 0.47	39.0 ± 4.95	48.21 ± 4.61	–18.3 ± 9.2[d]
Highest (4.11–9.69)	1.15 ± 0.50	32.43 ± 6.29	49.65 ± 6.12	–33.7 ± 12.0[d]

[a]Values are ranges and means ± SD.
[b]Time-averaged values of EPA and AA calculated over the course of the trial. HUFA, highly unsaturated FA.
[c]No significant difference between group values for each tertile (ANOVA).
[d]$P \leq 0.05$, paired *t*-tests for comparison between baseline and time-averaged values of AA.

fish oil-naïve volunteers confirmed the discriminatory value of random plasma phospholipid EPA analyses. However, the data suggest that erythrocyte EPA may have an advantage in being less directly affected by an individual dose. Thus, erythrocyte EPA may be a better measure of long-term compliance than plasma phospholipid EPA.

The use of biochemical and physiologic measures to guide treatments designed to achieve improved long-term outcomes rather than short-term symptomatic effect has many precedents. Obvious examples include blood pressure, plasma glucose, and cholesterol. The plasma EPA measurement, in common with these analyses, is a continuous, not a dichotomous, variable; as such, the extent of the desired effects may be proportionate to the actual level achieved rather than mere achievement of some arbitrarily chosen threshold level. Although the correlation between sustained elevations in plasma phospholipid EPA and improved long-term outcomes in RA remains to be established, the results of these studies confirm the feasibility of using a blood measurement of n-3 PUFA intake during therapy with fish supplements, or other dietary interventions that deliver long chain n-3 PUFA. In addition to RA, the index could also be applied in the treatment of other inflammatory diseases such as Crohn's disease, IgA nephropathy, and psoriasis. Other potential applications include guidance of n-3 fortification designed to improve long-term function of renal allografts and prophylaxis against cardiovascular events.

This study, showing the long-term feasibility of using bottled fish oil taken on juice, used cod liver oil because it was the only readily available bottled fish oil at the time of study commencement. The approach is far less expensive and more convenient than the use of fish oil capsules for anti-inflammatory therapy. The 20-mL dose of cod liver oil used in this study is equivalent to a daily intake of 13 standard (unconcentrated) fish body oil capsules at about one-tenth the cost. However, cod liver oil contains vitamins A and D, which in the dose given exceed recommended daily intakes. This may be especially problematic in countries in which foods and milk products are fortified with these vitamins. Although treatment over 12 mon did not increase plasma vitamin A and increased plasma vitamin D within the desirable range, the intakes of vitamins A and D that occur with cod liver oil can be avoided by using fish body oils. Although it is now our practice to use a bottled body fish oil preparation, the data obtained with cod liver oil are presented here to demonstrate the feasibility of the approach and to give impetus to making inexpensive bottled body fish oils more generally available. When taking a standard fish body oil (EPA 18%, DHA 12% w/w) on juice, a dose of 15 mL delivers an adequate anti-inflammatory dose, which can be taken easily in a single swallow. There is no advantage in using more concentrated fish oils, which are far more expensive, because there is no practical advantage in reducing the volume of the bolus. This contrasts with the use of fish oil concentrates in capsules, which reduces the number of capsules that need to be taken, but at a substantial price premium.

Although the influence of fish oil supplements on long-term outcomes in RA remains to be established, studies that show reduced use of nonsteroidal anti-inflammatory drugs indicate a safety benefit (reviewed in Ref. 1). The use of an anti-inflammatory dose of fish oil as outlined here should also yield a cost advantage, although this may not be the case with the more expensive option of encapsulated fish oil. The advent of biological agent therapies that block TNFα and IL-1 action provides a rationale for concurrent therapy with fish oil, which reduces production of these cytokines (11,12). The addition of an inexpensive bottled fish oil supplement on juice may thereby provide an adjunct, making the use of these very expensive agents more cost efficient. The respective experiences with fish oil and TNFα blockers in Crohn's disease highlights the potential pharmacoeconomic benefits of using fish oil. Belluzzi and coworkers (2) showed that fish oil supplements given over a 12-mon period reduced relapse on Crohn's disease from 69 to 28% of patients. The number needed to treat to prevent a relapse (assuming conservatively one relapse per subject) is thus 2.4. Using our approach of taking bottled fish oil, the dose used of 2.7 g can be delivered at a cost of Aus$65/yr. An outlay of Aus$156 would be needed to prevent one relapse. A single infusion of the therapeutic TNFα-blocking antibody infliximab 5 mg/kg treatment in the course of treatment for a relapse of Crohn's disease costs ~Aus$3,000 and a course of several infusions may be required to settle a relapse (13). Similar cost relativities are likely to pertain elsewhere.

In summary, the advice to take fish oil in RA yielded a substantial enrichment in n-3 PUFA intake in ~50% of patients. Plasma phospholipid and erythrocyte EPA could be used to identify those who are adherent and biochemically responsive to advice to increase dietary n-3 PUFA intake. Values observed can thereby be used to provide positive feedback in those who are responsive and to identify those who are not responsive or consistently adherent to advice, to whom encouragement and further education regarding benefit can be directed. Also, the measurement could prove more sensitive in establishing putative benefits than intention to treat analyses, particularly in longer-term studies and in the routine clinical settings, where effective delivery of advice and adherence to it may be especially variable.

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Influence of Dietary Long-Chain PUFA on Premature Baboon Lung FA and Dipalmitoyl PC Composition

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ABSTRACT: One of the major survival challenges of premature birth is production of lung surfactant. The lipid component of surfactant, dipalmitoyl PC (DPPC), increases in concentration in the period before normal term birth *via* a net shift in FA composition away from unsaturates. We investigated the influence of dietary DHA and arachidonic acid (AA) on lung FA composition and DPPC concentration in term and preterm baboons. Pregnant animals/neonates were randomized to one of four groups: breast-fed (B), term formula-fed (T^-), preterm formula-fed (P^-), and preterm fed formula supplemented with DHA-AA (P^+). Breast milk contained 0.68%wt DHA and the P^+ group formula contained 0.61%wt DHA. In the preterm groups (P^- and P^+), pregnant females received a course of antenatal corticosteroids. At the adjusted age of 4 wk, neonate lung tissue was harvested, and FA composition and DPPC were analyzed. Palmitate was ~28%wt of lung total FA and no significant differences were found among the four treatment groups. In contrast, DPPC in the B group lung tissue was significantly greater than DPPC in the unsupplemented groups, but not compared with the P^+ group. The B and P^+ groups were not significantly different in DHA and AA, but were different compared with the unsupplemented (T, P^-) groups. These results indicate that LCP supplementation increases lung DHA and AA, without compromising overall lung 16:0 or DPPC. The shift in FA composition toward greater unsaturation in the groups consuming LCP supported improved surfactant lipid concentration in preterm neonate lungs.

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Human lung development is a complex process under developmental control that starts early in fetal life and continues until after birth (1–3). Pulmonary immaturity is one of the most common survival problems in premature infants (4), and the mortality rate is as high as 60% (5). It is associated with decreased synthesis and secretion of pulmonary surfactant, resulting in the development of respiratory distress syndrome (RDS) and related complications (5–7).

Lung surfactant is necessary to decrease surface tension at the air–liquid interface of the alveoli to allow expansion of the lungs (7–9). Lower alveolar surface tension reduces the energy required to expand the lungs, prevents the collapse of the alveolus during respiration, and facilitates pulmonary gas exchange, especially at the end-expiration (2,4).

Lung surfactant is composed of ~90% lipid and 10% protein. Eighty percent of the surfactant lipids are present as PC; of this, dipalmitoyl PC (DPPC) constitutes 60% of the total lung surfactant PC. The balance of pulmonary surfactant lipids is composed of phosphatidylglycerol, PE, sphingomyelin, and PI (4). The two saturated acyl chains (palmitate, 16:0) in DPPC enable the lipid to form a tightly packed monolayer with a low surface tension (10,11) and, unlike unsaturated FA, are highly resistant to oxidation in the high oxygen tension at the air–liquid interface in the lung. In the weeks before normal term birth, total lung FA composition shifts away from unsaturates toward saturates, specifically 16:0, in apparent preparation for secretion of highly saturated surfactant lipids.

We recently completed a study of the influence of prematurity and long-chain polyunsaturate (LCP) supplementation on the metabolism of LCP in baboon neonates. LCP supplementation is now widely recommended for formula-fed human infants to enhance brain and retinal development, and premature baboons are a well-studied model for human RDS (12–14). LCP supplementation, specifically with DHA and arachidonic acid (AA), is intended to support the demands of the central nervous system for LCP postnatally, and may be expected to alter FA concentrations of other tissues. Because the FA composition of many tissues is related to dietary FA levels, increases in lung LCP due to supplementation might be at the expense of palmitate available for DPPC synthesis, thereby compromising DPPC concentrations. Such an effect could unintentionally exacerbate RDS in preterm infants consuming LCP-supplemented formula intended to support optimal neural development.

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Abbreviations: AA, arachidonic acid; CS, cesarean section; DLPC, dilauryl-PC; DPPC, dipalmitoyl-PC; LCP, long-chain polyunsaturated FA; RDS, respiratory distress syndrome.

In this paper, we report on the analysis of lung tissues from 4-wk-old adjusted-age baboons born ~24 d preterm and fed formula without or with supplemental LCP. We compare them with a breast-fed group and a term formula-fed group as controls. The total FA composition in lung, as well as the DPPC concentrations, is reported to establish whether inclusion of AA (20:4n-6) and DHA (22:6n-3) in formula has an influence on the overall FA concentration and surfactant concentration of neonatal baboon lung.

MATERIALS AND METHODS

The protocol for this study is described in detail elsewhere (15), and a brief outline will be presented here. The Cornell Institutional Animal Care and Use committee (IACUC) approved the animal care protocol and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved the animal care facility.

Animals and diets. Pregnant baboons ($n = 22$; *Papio cynocephalus*) were transported in the first half of gestation to Cornell University (Ithaca, NY), housed in separate cages, and fed controlled diets. Pregnant female/neonate pairs were randomly assigned to one of these four groups: term, breast-fed (B) $n = 5$; term, formula fed (T^-) $n = 7$; preterm, formula fed (P^-) $n = 5$; preterm, formula + LCP fed (P^+) $n = 5$. Twelve baboons delivered spontaneously (182 ± 2 d gestational age). From these, five neonates were assigned to the B group and nursed for 4 wk. The remaining seven, the T^- group, were transferred to the primate nursery within 12 h of birth and fed commercially available infant formula (Enfamil; Mead Johnson, Evansville, IN) containing no LCP for 4 wk. For the P^+ and P^- pregnant females, a course of antenatal betamethasone, 175 mg/(kg body wt·d), was administered at 152 ± 4 d of gestation (normal term = 182 d) 48 and 24 h before cesarean section (CS). The P^- group included five premature neonates fed Enfamil, the identical formula used for the T^-group. The P^+ group ($n = 5$) was fed formula reconstituted from powdered Enfamil, blended with a microencapsulated DHA/AA powder, kindly provided by Mead Johnson Nutritionals.

Sampling. Baboon lungs were collected at 28 d after delivery for the term neonates, and at 49 d after CS for the preterm neonates so that all baboons were of the same postconceptual age of ~210 d, or 28 d adjusted age. At necropsy, lungs were cut into 1-g samples and immediately flash frozen in liquid nitrogen, then transferred to a –80°C freezer where they were kept until analysis. No attempt was made to remove surfactant by lavage was made; thus, FA concentrations reflect that of the tissue and surfactant.

Lipid analysis. Total lipids were extracted from baboon lung homogenates using a modification of the method of Bligh and Dyer (16), with freshly prepared diheptadecanoyl-PC (Matreya, Pleasant Gap, PA) added as an internal standard to each sample before homogenization. FA were derivatized to FAME with 14% BF_3 in methanol, and the FAME were dissolved in heptane with BHT as an antioxidant (17).

The total FA concentrations were determined by GC (HP 5890 GC with FID) using a BPX-70 (SGE, Austin, TX) capillary column (60 m × 0.32 mm × 0.25 μm film thickness) and H_2 carrier gas. Response factors for each FA were obtained using an equal weight FAME mixture and calibrated with methyl heptadecanoate as an internal standard. Double bond positions of FA were determined by acetonitrile chemical ionization tandem MS analysis with a Varian Saturn 2000 ion trap gas chromatograph (Walnut Creek, CA) (18).

DPPC was analyzed by an electrospray tandem MS method using a Sciex API-III+ (Thornhill, Ontario, Canada), as described in detail elsewhere (Ziadeh *et al.*, unpublished data). Briefly, crude lung lipid extracts obtained from the Bligh and Dyer procedure were diluted in solvent [methanol/acetonitrile, 90:10 (vol/vol)] with 100 μL of 1% (vol/vol) trifluoroacetic acid. Dilauryl-PC (DLPC) was added quantitatively as an internal standard to samples, and the signal was calibrated using a standard curve over the range 0.1 to 100 pmol/μL in DPPC. Selected reaction monitoring was used to quantify on the transition m/z 734.5 → 184 for DPPC and m/z 622.5 → 184 for the internal standard DLPC. Intraday accuracy and precision were <5% for standards, and all data presented here were run consecutively on a single day.

Statistics. Data are expressed as means ± SD. A one-way ANOVA with Tukey's pairwise test was used to declare significant differences at $P < 0.05$ using SPSS release 10.0 for Windows (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

Animal characteristics. The average conceptual age, estimated from ultrasound measurements *in utero*, was 183.2 ± 11.6 d for the term groups and 155.0 ± 5.6 d for the preterm groups. Birthweights averaged 852 ± 136 and 621 ± 92 g for term and preterm baboons, respectively; by *t*-test, these were significantly different as expected. At the time of killing, the T^- group mean weight was ~1148 ± 113 g compared with ~1035 ± 221 g for the P^+ group, and 1065 ± 180 g for P^-; however, these differences were not significant.

Diets. Summary data for the FA composition of breast milk (B), unsupplemented (P^-, T^- groups), and supplemented (P^+) formulas are shown in Table 1; more complete FA analyses are reported elsewhere (15). The unsupplemented formula was devoid of LCP, whereas the supplemented formula contained twice as much AA and a similar amount of DHA as found in the breast milk of baboons in the B group. Breast milk contained significant amounts of n-3 LCP precursors to 22:6n-3, totaling 0.97%, particularly 20:5n-3 and 22:5n-3. In adult humans, the rate-limiting step of 22:6n-3 synthesis appears to be Δ6 desaturation of 18:3n-3, and these n-3 LCP are likely to serve as more efficient precursors for 22:6n-3 than 18:3n-3 (19). The LCP concentrations in the supplemented formula were 0.6%energy for AA (1.21 ± 0.09%wt) and 0.3%energy DHA (0.61 ± 0.03%wt). These figures are about twice that of the higher end in U.S. infant formulas (15) with a similar ratio of 2:1. The DHA concentration of this formula was very similar to that in baboon breast milk (0.68 ± 0.22%wt).

TABLE 1
Summary of the FA Composition in the Three Neonate Baboon Diets[a,b]

FA	Neonatal baboon diets		
	Baboon breast milk (B)	Unsupplemented formula (T^-/P^-)	LCP-supplemented formula (P^+)
Σ SFA	45.1 ± 9.5	30.2 ± 1.7	32.4 ± 3.3
Σ MUFA	31.2 ± 5.1	44.7 ± 1.4	43.0 ± 0.15
18:2n-6	27.23 ± 3.99	22.35 ± 0.42	20.11 ± 0.14
18:3n-3	2.12 ± 0.53	2.33 ± 0.02	2.06 ± 0.05
20:4n-6	0.62 ± 0.12	ND	1.21± 0.09
22:6n-3	0.68 ± 0.22	ND	0.61± 0.03
Σ n-6	20.7 ± 8.9	22.7 ± 0.42	21.8 ± 0.21
Σ n-3	3.08 ± 0.97	2.33 ± 0.02	2.85 ± 0.11
Σ LCP (n-6)	1.60 ± 0.13	ND	1.48 ± 0.16
Σ LCP (n-3)	1.23 ± 0.27	ND	0.79 ± 0.09

[a]Values are means ± SD.
[b]Abbreviations: B, breast-fed; T^-, term without long-chain polyunsaturates (LCP); P^+, preterm with LCP; P^-, preterm without LCP; SFA, saturated FA; MUFA, monounsaturated FA; ND, not detected.

Lung FA composition. The FA composition of baboon lungs for the breast-fed (B), unsupplemented (P^-, T^-), and supplemented (P^+) groups are shown in Table 2, with 16:0, AA, and DHA shown graphically in Figure 1. There were no significant differences in 16:0 among group treatments, although the groups consuming AA/DHA, B and P^+, had more 16:0 than the groups with diets devoid of AA/DHA. About 28% of total FA in baboon neonate lung was 16:0 across all groups. Stearic acid (18:0) was significantly higher in formula (T, P^+, P^-) groups, at ~23%, than in the B group, at ~17% of total FA. There were no differences in DHA and AA between the B and P^+ treatment groups, and no significant differences between the T^- and P^- treatment groups. Significant differences were found between B/P^+ (LCP groups) and unsupplemented T^-/P^- groups.

Our finding that lung 16:0 concentration is not affected by AA/DHA consumption indicates that supplementation does not alter overall 16:0 availability for synthesis of DPPC. In the weeks before normal term birth, the FA composition of PC shifts toward saturates in a process that has been called "remodeling." This process requires 16:0, which is synthesized *de novo* (13). The nonsignificant trend in mean 16:0 concentrations suggests that AA/DHA supplementation in the P^+ group was more similar to the B group than either the T^- or P^- group, both of which had lower 16:0 concentrations.

DPPC was significantly greater in the B group than the unsupplemented groups (Fig. 2). Supplementation restored DPPC concentrations to an intermediate value such that they were no longer significantly different from any of the groups. These data indicate that the influence of prematurity on DPPC is small compared with supplementation. Moreover, diets containing DHA and AA enhance lung surfactant lipid concentration,

TABLE 2
Lung FA Composition for Each Baboon Neonate Group[a,b]

FA	Baboon feeding group			
	B	T^-	P^-	P^+
14:0	1.87 ± 0.11^c	2.74 ± 0.22^d	3.27 ± 0.29^e	3.43 ± 0.21^e
16:0	29.0 ± 2.5	27.9 ± 1.2	26.2 ± 1.3	28.8 ± 1.8
18:0	17.1 ± 0.44^c	23.8 ± 1.4^d	23.56 ± 0.56^d	22.9 ± 0.74^d
20:0	0.51 ± 0.12	0.50 ± 0.04	0.47 ± 0.05	0.46 ± 0.02
22:0	0.48 ± 0.07^c	0.73 ± 0.06^d	0.70 ± 0.07^{d,e}	0.62 ± 0.03^c
16:1	1.24 ± 0.10	1.23 ± 0.48	1.52 ± 0.78	1.19 ± 0.71
18:1	10.3 ± 1.1^c	9.39 ± 0.44^c	9.64 ± 0.54^c	7.73 ± 0.31^d
20:1n-9	0.62 ± 0.04^c	0.88 ± 0.05^{d,e}	0.98 ± 0.09^e	0.80 ± 0.10^d
18:2n-6	10.3 ± 1.12^c	9.56 ± 0.36^c	9.44 ± 0.53^c	7.81 ± 0.29^d
18:3n-6	0.35 ± 0.04	0.34 ± 0.06	0.34 ± 0.03	0.29 ± 0.03
20:2n-6	0.85 ± 0.06^{d,e}	0.92 ± 0.06^{c,e}	0.99 ± 0.07^c	0.73 ± 0.12^d
20:3n-6	2.15 ± 0.36^c	2.32 ± 0.17^c	2.06 ± 0.11^c	1.27 ± 0.14^d
20:4n-6	15.4 ± 0.93^c	13.8 ± 1.1^d	14.6 ± 0.75^{c,d}	16.0 ± 0.55^c
22:4n-6	2.14 ± 0.12^c	2.52 ± 0.28^d	2.93 ± 0.20^e	3.02 ± 0.22^e
18:3n-3	0.25 ± 0.07	0.34 ± 0.06	0.28 ± 0.07	0.27 ± 0.05
20:5n-3	1.45 ± 0.21^c	0.54 ± 0.10^d	0.47 ± 0.08^d	0.47 ± 0.09^d
22:5n-3	2.17 ± 0.39^c	1.14 ± 0.16^d	1.31 ± 0.22^d	0.59 ± 0.05^e
22:6n-3	3.85 ± 0.15^c	1.36 ± 0.22^d	1.22 ± 0.19^d	3.64 ± 0.19^c
Σ SFA	49.0 ± 2.5^c	55.7 ± 1.9^d	54.2 ± 1.4^b	56.25 ± 2.0^d
Σ MUFA	12.1 ± 1.1^c	11.5 ± 0.65^c	12.1 ± 0.95^c	9.72 ± 0.79^d
Σ n-6	31.2 ± 1.5^c	29.8 ± 1.2^{c,d}	30.4 ± 0.94^{c,d}	29.1 ± 0.67^d
Σ n-3	7.72 ± 0.48^c	3.38 ± 0.29^d	3.28 ± 0.31^d	4.96 ± 0.22^e
Σ n-6/Σ n-3	4.04 ± 0.31^c	8.73 ± 0.83^d	9.24 ± 0.91^d	5.86 ± 0.30^e
Σ LCP (n-6)	20.6 ± 1.0	19.6 ± 1.2	20.6 ± 0.79	21.0 ± 0.62
Σ LCP (n-3)	7.47 ± 0.47^c	3.0 ± 0.29^d	3.00 ± 0.30^d	4.69 ± 0.22^e

[a]Values are means ± SD.
[b]For abbreviations see Table 1.
[c–e]Means with different superscripts are significantly different, $P < 0.05$.

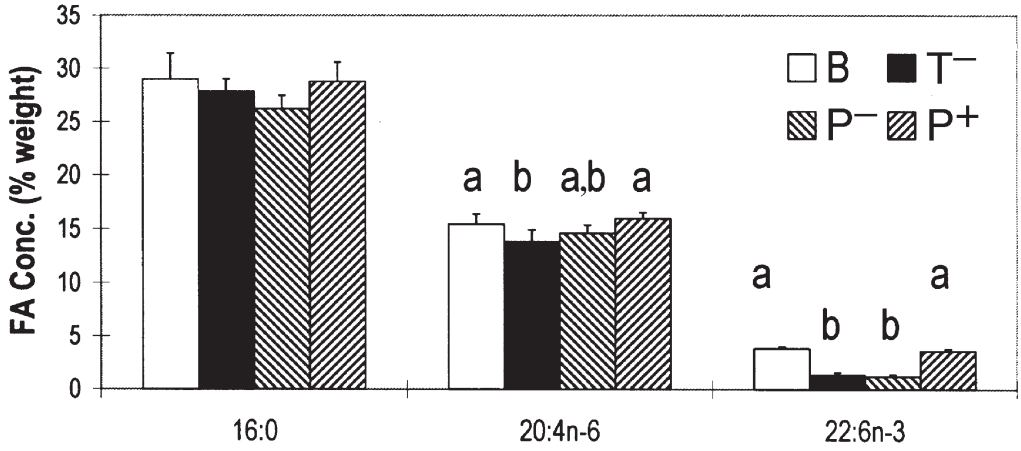

FIG. 1. Lung FA composition in baboon neonates for 16:0, 20:4n-6, and 22:6n-3. Values are means ± SD. Within a FA, group values without a common letter are significantly different, $P < 0.05$. B, breast-fed; T^-, term without long-chain polyunsaturated (LCP); P^+, preterm with LCP; P^-, preterm without LCP.

indicating that the lung is more mature when diets with LCP are consumed compared with those devoid of LCP.

In 1959, Avery and Mead (6) discovered that pulmonary surfactant deficiency is the major cause of RDS in preterm infants. Liggins proposed a positive effect of glucocorticoids on fetal maturation in lambs (8,20). In 1972, Liggins and Howie (21) reported the benefits of administering corticosteroids to mothers who were at risk of preterm delivery. Antenatal corticosteroids significantly decrease the incidence of RDS, intraventricular hemorrhage, and infant death (5), and are recommended for women presenting in danger of premature labor (22). An important cause of RDS is the deficiency of surfactant due to immaturity of the pulmonary tissue, specifically the type II alveolar cells, which are the primary producers of lung surfactant.

In this study of baboon neonatal lung, 16:0 constituted ~28% of the total FA, the major FA contained in lung surfactant lipid. This value was not significantly altered by dietary LCP supplementation, similar to results found in a piglet model of LCP supplementation (23). In contrast, DHA and AA concentrations in baboon neonatal lung were significantly elevated in the groups consuming DHA and AA. DHA was elevated almost threefold in these groups, rising from ~1.3 to ~3.7%. AA is a major component of lung tissue, with a concentration of 15% in breast-fed and supplemented neonates. The significant decline in AA was only ~1–2% in the unsupplemented groups, comparable to the magnitude of the decline in DHA concentration. These data are evidence of the tight control of AA concentrations found in tissues.

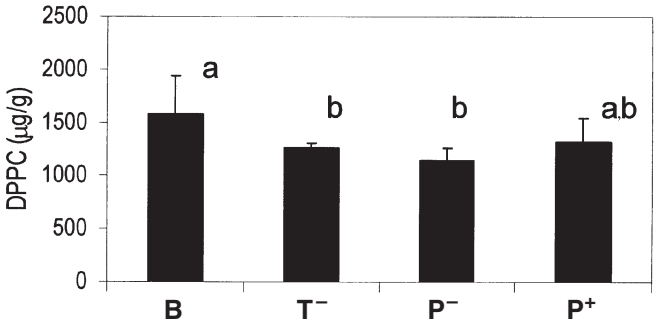

FIG. 2. Dipalmitoyl PC (DPPC) concentrations in whole lung homogenates. For diet codes see Figure 1. Values are mean ± SD. Bars that do not share a common letter are significantly different, $P < 0.05$.

Supplementation induced significant declines in 18:1, 20:1, 18:2n-6, 20:2n-6, 20:3n-6, and 22:5n-3 in the P^+ group compared with the P^- group. The magnitude of these changes ranged from ~20 to 60% and was more pronounced for 20:3n-6 and 22:5n-3. In all of these cases, the P^- group values were paralleled by the T^- group values, and none of the concentrations of these six FA were significantly different between the two unsupplemented groups. The only difference in the formulas consumed by the P^-/T^- groups and the P^+ group was the presence of LCP in the latter; thus, comparisons of all FA concentrations among the three formula groups are meaningful. Comparisons with the B group are inappropriate because many breast milk FA are significantly different than those in formulas. Owing in part to this factor, we cannot speculate whether the changes in these six FA with supplementation have a positive or negative effect, or indeed whether they have any physiologic relevance.

In contrast, DPPC concentration was enhanced in breast-fed baboon lungs compared with unsupplemented neonates, and supplemented formula partially restored lung DPPC. DPPC concentrations in membranes are normally very low; thus, lung DPPC concentrations represent primarily surfactant lipid. These data show that shifts in lung FA concentration due to higher dietary LCP enhance surfactant concentrations, rather than compromising them.

We conclude that AA/DHA LCP supplementation does not significantly influence total lung 16:0 in 4-wk-old adjusted-age baboon neonates. DHA values tripled in supplemented preterm neonates, and AA underwent a modest but significant increase. DPPC, the major lung surfactant, increased with LCP supplementation, showing that shifts in lung FA composition toward more unsaturation are not at the expense of surfactant production. Overall, our data support the hypothesis

that LCP supplementation improves lung surfactant concentration in preterm primates.

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Docosapentaenoic Acid Does Not Completely Replace DHA in n-3 FA-Deficient Rats During Early Development

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ABSTRACT: The reciprocal replacement of DHA by docosapentaenoic acid (DPAn-6) was studied in rats that consumed an n-3 FA-deficient or n-3 FA-adequate diet. Dams were fed the two experimental diets from weaning and throughout pregnancy and lactation. Their pups were then fed the respective diets after weaning. Cortex FA analysis was performed at various times (0, 5, 10, 20, 50, and 91 d) after birth to determine whether DPAn-6 completely replaced DHA in the n-3-deficient group. Cortical DHA levels were significantly lower (average 86%) in the n-3-deficient rats. DPAn-6 increased significantly in the n-3-deficient rats starting with a 6.5-fold increase at day 0 up to a 54-fold increase at day 91 compared with the n-3-adequate group. However, this significant increase did not completely replace the loss of DHA at postnatal days 5, 10, and 20 in which there was still an 11.5, 10.3, and 8.0% deficit in the sum of DHA and DPAn-6, respectively, in the n-3-deficient group. Once docosatetraenoic (DTA) and arachidonic acids (AA) were included in the sum (DHA + DPAn-6 + DTA + AA), the levels between the two groups were similar. These results suggest that not only DPAn-6 but also other n-6 FA, including DTA and AA, replace DHA in n-3-deficient rats. The lack of total 22-carbon (22C) FA in the brain during the rapid membrane biogenesis that occurs during early development could be a factor in the nervous system functional deficits associated with n-3 FA deficiency.

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DHA (22:6n-3), a long-chain PUFA (LC-PUFA) of the n-3 family, is highly concentrated and retained in the central nervous system when α-linolenic acid (LNA; 18:3n-3) and/or DHA is present in the diet (1,2). A severe depletion of DHA in the brain, induced by an n-3 FA-deficient (n-3 Def) diet, is associated with abnormal visual development and learning-related deficits in rodents (3–10).

When rats consume an n-3 Def diet for several generations, tissue DHA levels decrease and docosapentaenoic acid (DPAn-6; 22:5n-6) levels increase (11,12) due to the competition between the n-3 and n-6 families for elongation and desaturation enzymes and the change in the available substrates (13–16). High levels of brain DPAn-6, a member of the n-6 FA family, are present only in the DHA-deficient tissue, presumably to maintain an optimal amount of these highly unsaturated FA in the brain. Other polyunsaturated n-6 FA such as docosatetraenoic acid (DTA; 22:4n-6) and arachidonic acid (AA; 20:4n-6) also increase in response to an n-3 FA deficiency. Many studies describe a complete reciprocal replacement of DHA with DPA-n-6 (11,12,17) such that there are no significant differences between an n-3 Def brain and an n-3 FA-adequate (n-3 Adq) brain in terms of total DHA plus DPAn-6. It is thus intriguing that deficits in central nervous system functions are detectable in n-3 Def animals because DPAn-6 has an identical structure to DHA except for the absence of a double bond in the n-3 position. This suggests that even if DHA is completely replaced by DPAn-6, DHA is the critical FA necessary for optimal function. However, in large part, only adult central nervous system DHA and DPAn-6 levels have been discussed (11,12) in the reports in which reciprocal replacement was claimed. This raises the question whether DPAn-6 completely replaces DHA at critical early developmental periods. If not, it is possible that the decrease in total 22-carbon (22C) FA or total PUFA during early brain development may account, at least in part, for the abnormal functional outcomes in n-3 Def rodents. Green *et al.* (18) determined the accumulation of DHA in the rat brain from embryonic day 12 to postnatal day 16, where the dams were consuming only an n-3-Adq diet. Otherwise, few papers describe the fatty acyl composition during brain development, much less the reciprocal replacement of DHA with DPAn-6 during development.

The purpose of this study was to determine whether the DHA in cortex, an area with a high neuronal content, is completely reciprocally replaced by DPAn-6 at various developmental stages including days 0, 5, 10, 20, 50, and 91 in n-3 Def rats compared with n-3 Adq rats. A further question was: If DHA is not completely replaced by DPA, what other FA are increased in the n-3 Def rat cortex?

MATERIALS AND METHODS

Animals and study design. This study was performed under a protocol approved by the NIAAA, NIH Animal Care and Use

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Abbreviations: AA, arachidonic acid; 22C, 22-carbon; DPAn-6, docosapentaenoic acid; DTA, docosatetraenoic acid; LC-PUFA, long-chain PUFA; LNA, α-linolenic acid; n-3 Def, n-3 FA-deficient; n-3 Adq, n-3 FA-adequate.

Committee. Two cohorts of Long Evans (Charles River, Portage, MI) female rats arrived at our animal facility when they were 21 d old. They were immediately matched for weight and randomized into one of two dietary groups. All rats were maintained in our animal facility under conventional conditions with controlled temperature (23 ± 1°C) and illumination (12 h; 0700–1900 h). Food and water were consumed on an *ad libitum* basis. At ~11 wk of age, the female rats (F1 generation) were mated with chow-fed (NIH 31) males. The male offspring (F2 generation) of these females were used for this study. Only males were used in this study to minimize possible effects of hormonal factors.

Different stages of brain development were of interest in this study; thus, days 0 and 5 were selected to represent the newborn stage. For brain growth spurt stages (19), days 10 and 20 were selected, and days 50 and 91 were selected to represent the mature, adult brain. Our goal was to obtain six male rats at each time point. Because of our stringent requirement that each pup in each group be from a separate litter, $n = 6$ was obtained in all groups with the exception of the day 5-adequate and day 20-deficient groups, which each contained five pups, the day 0-deficient and day 50-adequate groups, which each contained four pups and the day 0-adequate group, which contained three pups. Rat pups were killed by decapitation. The whole brains were removed and weighed; the cortex was dissected out and frozen at –80°C before lipid extraction and FA analysis.

Experimental diets. The AIN-93 diet was the base for the two custom-made experimental diets (Dyets, Bethlehem, PA). To achieve the lowest level of n-3 FA possible in this diet, several ingredient substitutions were made. Vitamin-free casein was used instead of unprocessed casein because the latter contains n-3 FA. Much of the cornstarch, which also contains n-3 FA, was replaced with maltose-dextrin and other carbohydrates (Table 1). Both diets contained 10 wt% fat, and the only difference between the two diets was the addition of a small amount of flaxseed (source of 18:3n-3) and algal oils (source of DHA; Martek Biosciences, Columbia, MD) to the n-3 Adq diet to supply n-3 FA. The n-3 Def diet contained hydrogenated coconut oil in place of the flaxseed and algal oils. Table 1 shows the complete FA profile of each experimental diet.

FA analysis. The cortex samples were thawed and lipids were extracted using the method of Folch *et al.* (20). Subsequent to lipid extraction, a small aliquot of each total lipid extract was used for FA analysis. Transmethylation was performed with BF_3-methanol by a modification of the method of Morrison and Smith (21) as described by Salem *et al.* (22). Methyl esters were then quantified on a gas chromatograph (Hewlett-Packard, model 5890/series II, Palo Alto, CA) equipped with an FID and fused-silica capillary column (DB-FFAP; 30 m × 0.25 mm × 0.25 μm; J&W, Folsom, CA). The carrier gas (hydrogen) linear velocity was 50 cm/s, and injector and detector temperatures were set to 250°C with the oven temperature program as follows: 130–175°C at 4°C/min, 175–210°C at 1°/min, and then to 245°C at 30°C/min with a final hold for 15 min at 245°C. The tissue FA were identified by comparison with the retention times of a standard mixture of FA (462; Nu-Chek-Prep, Elysian, MN), and the concentrations of each FA were determined by reference to an internal standard (ethyl ester 22:3n-3; 100–500 μg depending on tissue size), which was added to the sample before lipid extraction.

TABLE 1
Composition of the Diets

Ingredient	(g/100 g diet)	
Casein, vitamin free	20	
Carbohydrate[a]	60	
Cellulose	5	
Salt and mineral mix	3.5	
Vitamin mix	1	
L-Cystine	0.3	
Choline bitartrate	0.25	
Fat	10	
Fat sources	n-3 adequate	n-3 deficient
Hydrogenated coconut oil	7.43	8.1
Safflower oil	1.77	1.9
Flaxseed oil	0.5	—
DHASCO[b]	0.3	—
FA[c]		
Total saturated	73.0	80.0
Total monounsaturated	4.6	3.5
18:2n-6	15.2	15.5
Σn-6	15.2	15.5
18:3n-3	3.0	0.05
22:6n-3	1.5	ND[d]
Σn-3	4.5	0.05
18:2n-6/18:3n-3	5.0	310.0
n-6/n-3	3.4	310.0

[a]The carbohydrate was composed of 20, 15, 15, and 10 g/100 g diet of dextrose, cornstarch, maltose-dextrin, and sucrose, respectively.
[b]DHASCO: 46% DHA (from Martek Bioscience, Columbia, MD).
[c]Presented as the percentage of total FA weight.
[d]ND, not detected.

Statistical analysis. Data were analyzed using Statistica (StatSoft, Tulsa, OK). The differences in FA concentrations between the two experimental groups at each time point were analyzed using the Student's *t*-test. Differences were considered significant when $P \leq 0.05$.

RESULTS

There were no differences in body, brain, or liver weights between the n-3 Adq and n-3 Def rats at day 0, 5, 10, 20, or 50. There were also no differences in brain or liver weights at day 91. However, at day 91, there was a significant difference in body weight between the two groups. Body weights at day 91 were 589 ± 42 and 511 ± 34 g (mean ± SD, $P < 0.01$, $n = 6$) for the n-3 Adq and n-3 Def groups, respectively.

Cortex total FA concentrations were not different between the groups at any time point. Cortex DHA levels were significantly lower ($P < 0.000001$) in the n-3 Def group compared with the n-3 Adq group at all time points (Fig. 1). The most significant decrease in cortex DHA in the n-3 Def group was seen at

day 10 in which DHA was 88% lower ($P < 0.000001$) compared with the n-3 Adq group. The average loss of cortex DHA over all time points was 86% in the n-3 Def group. As expected, cortex DPAn-6 levels were significantly higher ($P < 0.000002$) in the n-3 Def rats compared with the n-3 Adq rats at all time points. At day 0, DPAn-6 was 6.5-fold higher ($P < 0.000001$) in the n-3 Def rats. The increase in DPAn-6 in the n-3 Def rats was 7.6, 12.2, 22.8, 36.7, and 53.8-fold compared with the n-3 Adq rats on days 5, 10, 20, 50, and 91, respectively. This increase over time was due mainly to the decrease in DPAn-6 in the n-3 Adq group as the rats aged, rather than an increase in DPAn-6 in the n-3 Def group. In other words, the DPAn-6 as a percentage of FA decreased slightly in the n-3 Adq rats as they matured, and the DPAn-6 decreased slightly or stayed constant in the n-3 Def rats as they matured.

Previous work indicated a "reciprocal replacement" of DHA with DPAn-6 in the brains of adult rats given a diet low in n-3 fats (11,12). Therefore, the sum of the DHA and DPA-n-6 in the n-3 Def rat brain would be expected to be similar to that in the n-3 Adq rat brain. Cortex DHA plus DPAn-6 levels in the n-3 Adq and n-3 Def rats are shown in Figure 2. On days 5, 10, and 20, the DHA plus DPAn-6 levels were significantly lower in the n-3 Def rats compared with the n-3 Adq rats, indicating that DPAn-6 did not completely replace DHA in the n-3 Def rats. There was 11.5 ($P < 0.005$), 10.3 ($P < 0.0001$), and 8.0% ($P < 0.01$) less DHA plus DPAn-6 on days 5, 10, and 20 in the n-3 Def rats, respectively.

Cortex DTA levels were significantly higher ($P < 0.05$) at all time points except day 0 in the n-3 Def rats. When the DTA levels were summed together with DHA and DPAn-6, the total 22C was still significantly lower at days 5 and 10 ($P < 0.05$) in the n-3 Def rats (Fig. 3). There were no significant differences between groups on any other days analyzed.

AA levels were significantly higher ($P < 0.02$) at most time points (all except day 5) in the n-3 Def group. Summed cortex DHA, DPAn-6, DTA and AA levels in the two groups are shown in Figure 4. When the levels of these four FA are considered, the PUFA content of the n-3 Def cortex was equal to or even greater (at later time points) than that of the n-3 Adq cortex, indicating that AA completes the DHA replacement by the C22 n-6 polyunsaturates during early development.

There were no differences in the cortex levels of the saturated FA, 16:0 and 18:0, between the two groups; however, there was a significantly higher percentage ($P < 0.03$) of 18:1n-9 in the n-3 Adq group at days 5, 10, 50, and 91 (data not shown).

The complete fatty acyl composition at the 10-d time point is presented in Table 2. These data illustrate all of the features presented above. There were highly significant losses in DHA and

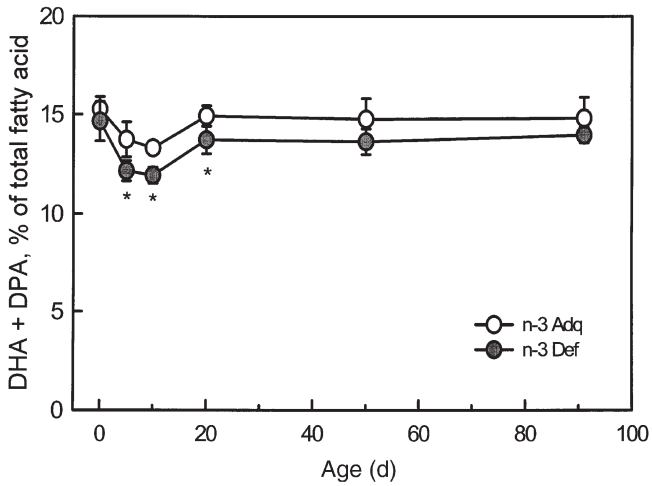

FIG. 2. Effects of an n-3 FA deficiency on DHA + docosapentaenoic acid (DPAn-6) levels in rat cortex over 91 d. For each time point, the sum of DHA + DPAn-6 was calculated for each rat and then averaged with the sums from the other rats at that time point. *Significantly different from the n-3 Adq group ($P < 0.05$). For abbreviations see Figure 1.

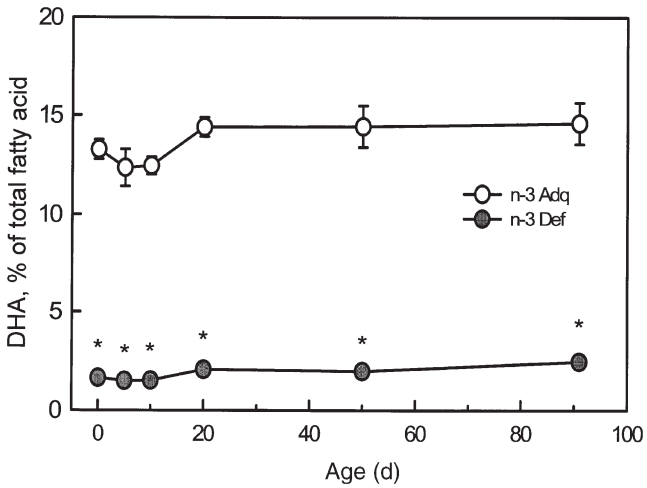

FIG. 1. Effects of an n-3 FA deficiency on the DHA levels in rat cortex over 91 d. DHA levels are presented as the mean percentage of total FA ± SEM. DHA levels at each time point were significantly different between the two groups ($P < 0.05$). n-3 Adq, n-3 FA-adequate; n-3 Def, n-3 FA-deficient.

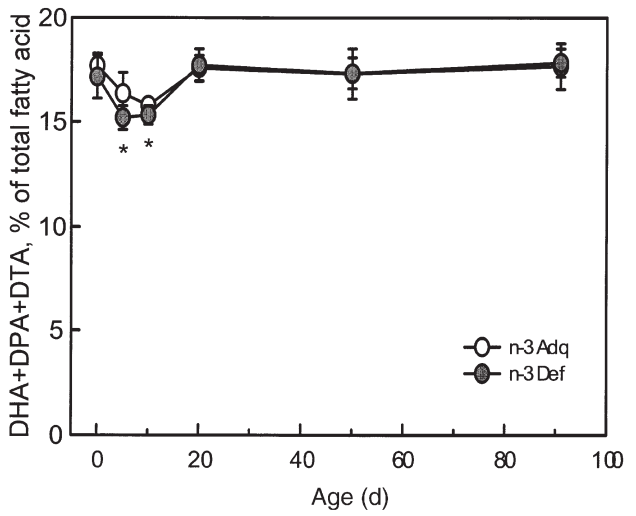

FIG. 3. Effects of an n-3 FA deficiency on DHA + docosapentaenoic acid (DPAn-6) + docosatetraenoic acid (DTA) levels in rat cortex over 91 d. For each time point, the sum of DHA + DPAn-6 + DTA was calculated for each rat and then averaged with the sums from the other rats at that time point. *Significantly different from the n-3 Adq group ($P < 0.05$). For abbreviations see Figure 1.

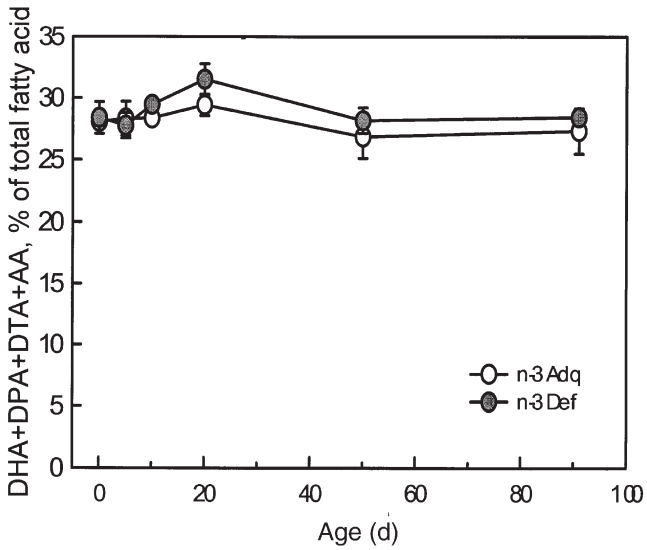

FIG. 4. Effects of an n-3 FA deficiency on DHA + docosapentaenoic acid (DPAn-6) + docosatetraenoic acid (DTA) + arachidonic acid (AA) levels in rat cortex over 91 d. For each time point, the sum of DHA + DPAn-6 + DTA + AA was calculated for each rat and then averaged with the sums from the other rats at that time point. No significant differences were found for this variable. For abbreviations see Figure 1.

22:5n-3 and increases in AA, DTA, and DPAn-6 in the n-3 Def group compared with the n-3 Adq group. It was interesting that the shorter-chain and less unsaturated 18- and 20-C n-6 polyunsaturates actually decreased in the n-3 Def group. There was also a slight decrease in 18:1n-9 with an accompanying increase in 18:1n-7 in the n-3 Def group.

TABLE 2
Rat Cortex Fatty Acyl Composition in the Second Generation of Rats Consuming an n-3-Adequate (n-3 Adq) or n-3-Deficient (n-3 Def) Diet at 10 days of Age[a]

FA	n-3 Def ($n = 6$)	n-3 Adq ($n = 6$)
Nonessential		
14:0	1.8 ± 0.05	1.8 ± 0.11
16:0	29.3 ± 0.41	29.4 ± 0.18
18:0	13.9 ± 0.14	14.2 ± 0.18
20:0	0.05 ± 0.03	0.05 ± 0.001
16:1n-7	1.7 ± 0.01*	1.6 ± 0.02
18:1n-9	8.9 ± 0.10**	9.3 ± 0.04
18:1n-7	2.4 ± 0.06	2.3 ± 0.02
20:1n-9	0.13 ± 0.04	0.17 ± 0.03
24:1n-9	1.4 ± 0.29	0.79 ± 0.19
n-6 Polyunsaturates		
18:2n-6	0.84 ± 0.02***	1.13 ± 0.03
18:3n-6	0.09 ± 0.026	0.12 ± 0.040
20:2n-6	0.13 ± 0.003***	0.18 ± 0.007
20:3n-6	0.31 ± 0.017***	0.61 ± 0.025
20:4n-6	14.1 ± 0.10***	12.6 ± 0.13
22:4n-6	3.38 ± 0.03***	2.47 ± 0.07
22:5n-6	10.4 ± 0.16***	0.9 ± 0.06
n-3 Polyunsaturates		
22:5n-3	0.04 ± 0.002***	0.28 ± 0.18
22:6n-3	1.5 ± 0.03***	12.4 ± 0.13
Total FA (mg/g wet weight)	18.2 ± 0.58	17.8 ± 0.45

[a]Rats were 10 d of age at the time of killing. Data are expressed as wt% of total FA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$ vs. n-3 Adq group (Student's *t*-test).

DISCUSSION

The n-3 Def diet was successful in producing rats with low brain DHA. Despite the extreme differences in n-3 FA between the groups, the body and organ weights did not differ except for body weight on day 91. Because brain weights were not different between groups at any time point and body weights were not different at other time points, this single body weight difference at day 91 is not expected to affect the results or the interpretation significantly.

In the adult rats (days 50 and 91), depleted DHA was reciprocally replaced by DPAn-6, as reported in previous investigations (11,12). However, DPAn-6 did not completely reciprocally replace DHA in rat cortex total lipids during early development (days 5, 10, and 20). Levels of LC-PUFA were "restored" in the n-3 Def rat cortex at the early time points only when increases in DTA and AA, the other dominant n-6 FA in cortex, were also considered.

Although it was reported previously that DPAn-6 can replace DHA in adult nervous system tissues, the present results indicate that DPAn-6 is not completely replacing DHA during early developmental periods. Availability of adequate LC-PUFA supplies may be most critical during these early days because DPAn-6 cannot replace DHA in terms of the function of these tissues (23–25). For example, when rhodopsin is reconstituted in liposomes composed of DPAn-6 phospholipids, phosphodiesterase activity after light activation is significantly less than that in membranes composed of DHA-phospholipids (26).

Clearly, in this study, n-6 metabolism did not proceed at a rate such that it was capable of synthesizing enough 22C long-chain n-6 FA to completely replace the depleted DHA at days 5, 10, and 20. That this occurred only at these initial time points is no doubt related to the explosive brain growth during this time period. Eventually, n-6 FA elongation/desaturation can provide adequate DPAn-6 to replace all of the lost DHA. Only after DTA and AA, a 20C FA, were considered was the LC-PUFA level fully replete in the n-3 Def brain. This raises the question of what would happen in terms of functional outcomes if 22C n-6 FA were adequate during early development. Other questions raised include whether any 22C unsaturated FA is adequate to support the functional requirements of neural cells during this time or whether DHA is specifically required.

The critical experiments required to answer the aforementioned questions include a study in which DPAn-6 is fed to pregnant and lactating n-3 FA Def dams. The aim would be to maintain brain levels of 22C FA throughout all critical periods of development, thus providing replacement of depleted DHA with preformed DPAn-6 rather than relying on *in vivo* n-6 metabolism. Subsequently, functional outcomes would have to be assessed to determine whether supplying adequate 22C FA to the brain during early development prevents the previously described effects of an n-3 FA deficiency. The persistence of losses in nervous system function in that case would strongly support the conjecture that DHA is specifically required for optimal function.

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Enhancement of G Protein–Coupled Signaling by DHA Phospholipids

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ABSTRACT: The effect of phospholipid acyl chain and cholesterol composition on G protein–coupled signaling was studied in native rod outer segment (ROS) disk and reconstituted membranes by measuring several steps in the visual transduction pathway. The cholesterol content of disk membranes was varied from 4 to 38 mol% cholesterol with methyl-β-cyclodextrin. The visual signal transduction system [rhodopsin, G protein (G_t), and phosphodiesterase (PDE)] was reconstituted with membranes containing various levels of phospholipid acyl chain unsaturation, with and without cholesterol. ROS membranes from rats raised on n-3 fatty acid–deficient and –adequate diets were also studied. The ability of rhodopsin to form the active metarhodopsin II conformation and bind G_t was diminished by a reduction in the level of DHA (22:6n-3) acyl chains or an increase in membrane cholesterol. DHA acyl chain containing phospholipids minimized the inhibitory effects of cholesterol on the rate of rhodopsin-G_t coupling. The activity of PDE, which is a measure of the integrated signal response, was reduced in membranes lacking or deficient in DHA acyl chains. PDE activity in membranes containing docosapentaenoic acid (DPA, 22:5n-6) acyl chains, which replace DHA in n-3 fatty acid deficiency, was 50% lower than in DHA-containing membranes. Our results indicate that efficient and rapid propagation of G protein–coupled signaling is optimized by DHA phospholipid acyl chains.

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Many of the functions associated with biological membranes, e.g., signal transduction, ion movement, or energy conversion, are carried out by membrane proteins. A wide range of diseases, psychiatric disorders, and dietary conditions are likely due to changes in membrane phospholipid composition and cholesterol content (see Refs. 1 and 2 for recent reviews), which in turn lead to altered protein function and subsequent illness. Most biological membranes undergo constant remodeling and renewal, and their lipid composition is readily altered by changes in the lipid precursor components made available by diet and metabolism. Thus, diet and disease can readily alter the lipid composition of membranes, although, in general, only genetic mutations alter protein function directly. To understand how the function of biological membranes is impaired or altered by changes in membrane lipid composition, it is necessary to understand how membrane lipids and proteins interact with each other and among themselves, so as to carry out a wide range of biological functions.

One of the principal compositional variables of biological membranes subject to dietary influence is the content of highly polyunsaturated phospholipid acyl chains. A large body of research demonstrates the need for high levels of polyunsaturated fats, particularly n-3 polyunsaturates, for optimal infant development and performance in later life (3–5). The conclusions drawn from decades of research on nutrition strongly suggest that specific, but unidentified, biochemical processes are optimized by the presence of n-3 polyunsaturates and/or compromised by their absence. The phospholipids of neuronal and retinal cells are rich in highly unsaturated acyl chains, especially those of DHA. Much previous work demonstrated that DHA-containing phospholipids enhance the formation of the active metarhodopsin II (MII) conformation of photoactivated rhodopsin (6–8). In this report, we demonstrate that multiple aspects of G protein–coupled signal transduction require DHA phospholipid acyl chains for optimal functional efficacy and that this requirement for DHA phospholipid acyl chains is related to the unique acyl chain packing properties imparted by DHA.

The G protein–coupled motif is a fundamental and widespread mode of intracellular signaling; it includes the sensory pathways for vision, olfaction, taste, touch, and a variety of neurotransmitters, including dopamine, serotonin, γ-amino butyric acid (GABA), and histamine (9). Each of these chemical and physical agents acts upon a unique integral membrane receptor protein, which is embedded in a lipid membrane. Many of the membranes that contain significant amounts of G protein–coupled signaling activity, such as neuronal and retinal tissues and the olfactory bulb, contain high levels of the n-3 polyunsaturated acyl chain derived from DHA (10,11). Dietary n-3 fatty acid deficiency leads to the replacement of DHA phospholipid acyl

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Abbreviations: DPA, docosapentaenoic acid, 22:5n-6; DPH, 1,6-diphenyl-1,3,5-hexatriene; DTPA, diethylenetriamine pentaacetic acid; ERG, electroretinogram; f_v, membrane free volume parameter; GABA, γ-amino butyric acid; G_t, transducin; K_a, MII·G_t association constant; K_{eq}, MI-MII equilibrium constant, [MII]/[MI]; MBCD, methyl-β-cyclodextrin; MI, metarhodopsin I; MII, metarhodopsin II; PDE, phosphodiesterase; Rh*, fraction of rhodopsin molecules that absorb a photon; ROS, rod outer segment; TBS, Tris basal salt.

chains with docosapentaenoic acid (DPA) acyl chains in these membranes (12). The functional significance of DHA is demonstrated by impaired visual response (13,14), learning deficits (11,15), loss of odor discrimination (16), and reduced spatial learning (17) associated with n-3 fatty acid deficiency. These findings suggest that a high level of DHA in membrane phospholipids is required for optimal function of a number of diverse signaling pathways. A common feature of these pathways is the central role of G protein–coupled signaling.

Receptors in the G protein–coupled superfamily are integral membrane proteins, consisting of seven transmembrane helices and their respective connecting loops. The ligand-binding site on the receptor is formed by transmembrane helices and generally lies near the midpoint of the membrane; hence, the conformational changes accompanying receptor activation would be expected to have a dependence on the physical properties of the membrane lipid bilayer. The G protein and effector proteins are generally peripheral proteins, bound to the membrane by a combination of an isoprenoid chain-lipid bilayer interaction (18,19) and electrostatic forces (20). The interaction of the G protein with the receptor occurs in the hydrophilic region of the protein, external to the membrane bilayer (21,22). In this study, we asked whether the composition and physical properties of the hydrophobic core of the membrane might affect the interaction between the receptor and the G protein that occurs external to the membrane bilayer.

After absorption of light by 11-*cis* retinal, rhodopsin exists as an equilibrium mixture of an active conformation, MII, and an inactive conformation, metarhodopsin I (MI) (see Ref. 23 for a review). Each MII activates several hundred transducin (G_t) molecules, which then activate the effector enzyme, a cGMP-specific phosphodiesterase (PDE). The activated PDE catalyzes the hydrolysis of cGMP, triggering closure of cGMP-gated Na^+/Ca^{2+} channels in the plasma membrane, leading to hyperpolarization of the rod outer segment (ROS) and the visual response. In this study, we reconstituted rhodopsin, G_t, and PDE into large, unilamellar vesicles containing varied levels of unsaturated acyl chains and cholesterol. In addition, we purified ROS disk membranes from rats raised on either an n-3 fatty acid-deficient or -adequate diet. Our results demonstrate that the degree of unsaturation in the acyl chain and the level of cholesterol in the membrane significantly affect MII formation, MII·G_t coupling efficiency and speed, and PDE activity. Because the visual signaling system is the prototype member in the superfamily of G protein–coupled signaling systems, our findings regarding the effect of lipid composition and cholesterol on receptor-G protein coupling may well serve as a general demonstration of the modulation of cell signaling by changes in membrane composition.

EXPERIMENTAL PROCEDURES

Sample preparation. Cholesterol and methyl-β-cyclodextrin (MBCD) were purchased from Sigma Chemical (St. Louis, MO). The cholesterol-MBCD complex was prepared by premixing cholesterol and MBCD as solids (weight ratio of 1:20) followed by solubilization in degassed Tris basic salt (TBS) buffer consisting of 10 mM Tris, 60 mM NaCl, 30 mM KCl, and 50 μM diethylenetriamine pentaacetic acid (DTPA), pH 7.5. The solution was sealed in argon and shaken at room temperature overnight. The final solution was filtered through a 0.45-μm filter and assayed for final cholesterol concentration. The cholesterol CII assay kit was from Wako (Wako Chemicals, Richmond, VA). The bicinchoninic acid and Coomassie Plus protein assay kits were from Pierce (Pierce Chemical, Rockford, IL). Bovine ROS were isolated from frozen retinas (James and Wanda Lawson, Lincoln, NE) (24) and intact rod disk membranes were isolated from ROS by centrifugation on a ficol gradient (25). For reconstitution in vesicles with defined lipid composition, rhodopsin was solubilized in octylglucoside and purified on a concanavalin A affinity column (26). Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL) and their purity was ascertained by HPLC. Large unilamellar vesicles containing rhodopsin at a ratio of 1 rhodopsin to 100 phospholipids were prepared using the rapid dilution technique (27). After dialysis to remove detergent, all vesicle preparations were suspended in pH 7.5 Tris basal salt (TBS) buffer consisting of 10 mM Tris, 60 mM NaCl, 30 mM KCl, and 50 mM DTPA. G_t was prepared from ROS as a hypotonic extract (28) and stored in pH 7.5 TBS buffer with 30% glycerol at –20°C for no longer than 2 wk. All preparation of phospholipids was carried out in an argon-filled glove box and in thoroughly degassed buffers due to the susceptibility to oxidation of polyunsaturated phospholipids. The phospholipid, cholesterol, and rhodopsin content of each reconstituted vesicle preparation was determined by independent phosphate (29), cholesterol, and rhodopsin (27) assays, respectively.

Samples for equilibrium absorbance measurements and PDE activity measurements were suspended in TBS buffer at 7.5 μM rhodopsin. Samples for fluorescence measurements were made immediately before use by diluting a concentrated vesicle or ROS disk membrane stock solution to 150 μM phospholipid, and adding 0.5 μL of 1,6-diphenyl-1,3,5-hexatriene (DPH) in tetrahydrofuran to yield a final phospholipid to DPH ratio of 300:1.

Cholesterol-depleted disk membranes were prepared by incubating disk membranes with 10 mM MBCD in TBS buffer, pH 7.5. Samples were incubated at room temperature in the dark for 2 h on a shaker. Measurements of disk cholesterol content at several time points indicated that 2 h was a sufficient incubation time to reach equilibrium for cholesterol exchange between disk membranes and MBCD (30). The MBCD-treated disk membranes were then separated from MBCD by centrifugation followed by two additional washes in TBS buffer. The membrane pellet and MBCD-containing supernatant were assayed for cholesterol, phospholipids (29), and rhodopsin (27), and the mole fraction of cholesterol to total phospholipids in disk membranes was determined. Cholesterol-enriched disk membranes were prepared similarly, except that disk membranes were incubated with cholesterol-

loaded MBCD (0–1.2 mM cholesterol in 10 mM MBCD). The following mol% samples were used in this study: 4, 12, 15, and 38 mol%. The 15 mol% cholesterol sample comprised native disk membranes without MBCD treatment.

Measurements. The MI-MII equilibrium in the absence and presence of G_t was measured in isotonic buffer at pH 7.5 using a series of equilibrium absorption spectra, as previously described (31). The amount of additional MII formed in the presence of G_t is proportional to the amount of $MII \cdot G_t$ complex formed; thus, $MII \cdot G_t$ binding constants were determined from a series of measurements with varying ratios of rhodopsin to G_t. The kinetics of both MII and $MII \cdot G_t$ formation were assessed by measuring the transient absorption at 380 nm using a flash photolysis system constructed in the laboratory. The kinetic data were analyzed in terms of a microscopic photoreaction model, as described previously (32).

PDE activity was assayed using a continuous pH method (33) with minor modifications. Samples were preincubated with the pH electrode in a thermoregulated 1-mL quartz cuvette and bleached by a light pulse that was attenuated with neutral density filters to obtain the desired bleaching level of rhodopsin. The bleaching flash was triggered after acquisition of a preflash baseline.

Fluorescence lifetime and differential polarization measurements were performed with a K2 multifrequency cross-correlation phase fluorometer (ISS, Urbana, IL) as previously described (34). For lifetime measurements, 12 modulation frequencies were used, logarithmically spaced from 5 to 200 MHz, and differential polarization measurements were made at 15 modulation frequencies logarithmically spaced from 5 to 200 MHz. Both total intensity decay and differential polarization measurements were repeated with each membrane composition a minimum of three times. Measured polarization-dependent differential phases and modulation ratios for each sample were combined with the measured total intensity decay to yield the anisotropy decay, $r(t)$. Anisotropy decays of DPH were analyzed using the Brownian rotational diffusion model (35). This model characterizes the anisotropy decay of DPH in terms of the orientational distribution function, $f(\theta)$, and the diffusion coefficient for rotation about the long axis of DPH. The orientational distribution function was used to derive the membrane free volume parameter, f_v, which is proportional to the overlap of $f(\theta)$ with a random orientational distribution (35,36).

Analysis. All data analysis was performed with NONLIN (37) with subroutines specifying the particular functions required for the different types of measurements written by the authors. NONLIN uses the Gauss–Newton method to perform nonlinear least-squares analysis while accounting for the SD of the measurement associated with each independent data point. Measurement SD was obtained from the noise in the preflash signal, in the case of the kinetic absorption and PDE activity measurements, or from the statistical analysis performed by the acquisition software in the case of the equilibrium absorption measurements and all fluorescence measurements. NONLIN returns the optimized parameter values as well as asymmetric confidence intervals corresponding to 1 SD. The asymmetry of all parameter values was <15%; thus, the high and low SD were averaged to obtain the reported values of 1 SD. All measurements were repeated 3–6 times and each trial was analyzed independently. The results of separate trials were averaged by weighting the results of each trial with its associated SD. Statistical significance was assessed from *P*-values resulting from Student's *t*-test with two-tailed distributions and two-sample unequal variance.

RESULTS

The effect of membrane cholesterol on both rhodopsin activation and acyl chain packing was investigated in ROS disk membranes by using MBCD to vary the membrane cholesterol content (30). An inverse correlation between the membrane cholesterol content and the MI-MII equilibrium constant (K_{eq}) was observed at 37°C. In control disk membranes, the cholesterol concentration was 15 mol% and K_{eq} was 0.81 ± 0.08 at 37°C. In disk membranes depleted of cholesterol to a level of 4 mol%, K_{eq} was 1.00 ± 0.10, whereas in membranes enriched to 38 mol% cholesterol, K_{eq} was reduced to 0.73 ± 0.11. Variation in the cholesterol content of disk membranes also resulted in substantial changes in the motional and orientational properties of the hydrophobic fluorescence probe DPH. A series of studies demonstrated that the overall orientational order of DPH in a phospholipid bilayer is well summarized by the parameter f_v, a measure of phospholipid acyl chain packing free volume (35,38). At 37°C, reduction of membrane cholesterol to 4 mol% increased f_v by 45% relative to control disks, whereas enrichment of cholesterol to 38 mol% reduced f_v by 30% relative to control disks. The values of K_{eq} and f_v at 37°C produced by variation in membrane cholesterol are linearly related, as shown in Figure 1. Previous measurements show that K_{eq} and f_v are linearly related with respect to changes in temperature and membrane cholesterol content (39,40) for rhodopsin reconstituted with a number of specific PC. The data summarized in Figure 1 demonstrate that the positive correlation between bilayer free volume and the formation of MII is a general property of the MII conformation that is manifest in natural as well as synthetic membranes.

The effects of DHA phospholipid acyl chains and cholesterol on the early steps in G protein–coupled signaling were addressed in a series of experiments utilizing purified rhodopsin reconstituted into vesicles consisting of di22:6PC, 18:0,22:6PC, and 18:0,18:1PC with and without cholesterol (41). In the absence of G_t, K_{eq} followed the order di22:6PC > 18:0,22:6PC > 18:0,22:6PC + 30 mol% cholesterol > 18:0, 18:1PC > 18:0,18:1PC + 30 mol% cholesterol. This is consistent with previous findings (8,40) that reduced acyl chain polyunsaturation and the presence of cholesterol reduced the amount of MII formation. Membrane lipid composition and cholesterol also modulate the binding of MII to G_t (41). Increasing acyl chain unsaturation from 18:0,18:1PC to 18:0, 22:6PC resulted in a threefold enhancement in the $MII \cdot G_t$

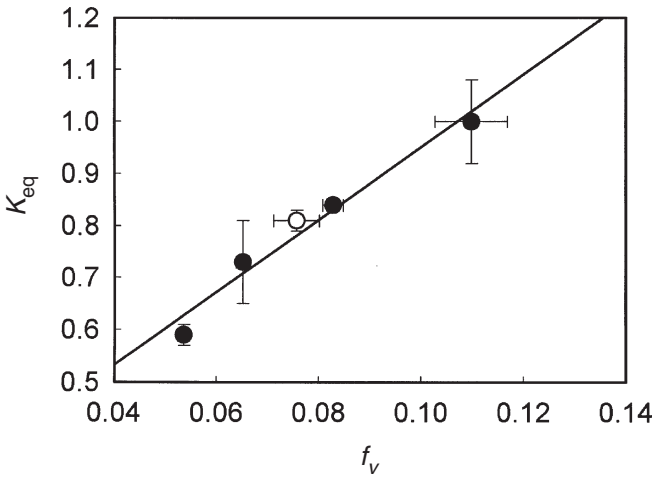

FIG. 1. Linear correlation of the metarhodopsin I (MI)-metarhodopsin II (MII) equilibrium constant, K_{eq}, and the phospholipid acyl chain packing free volume parameter, f_v, from bovine rod outer segment (ROS) disk samples with varying cholesterol concentrations at 37°C. The unfilled circle is the control disk at 15 mol% cholesterol, which received no methyl-β-cyclodextrin (MBCD) treatment. From left to right, the membrane cholesterol concentrations are 38, 30, 15, 12, and 4 mol% (see Fig. 6 in Ref. 30).

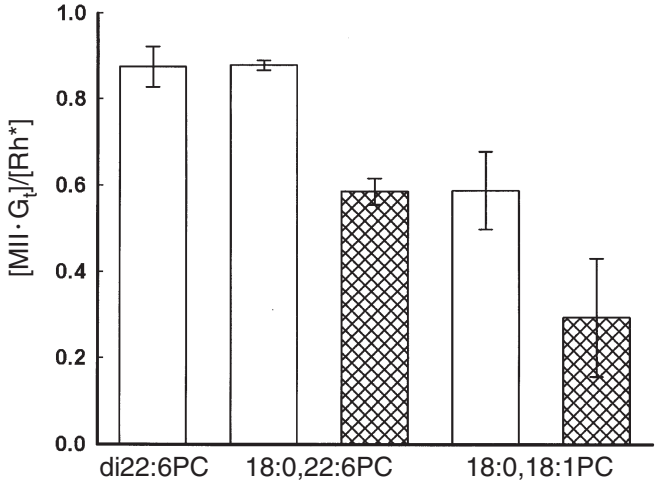

FIG. 2. Effect of 22:6 acyl chains and cholesterol on the efficiency of receptor-G protein coupling. The ratio [MII · G_t]/[Rh*] is the fraction of photoactivated rhodopsin that binds transducin (G_t) and thereby participates in signal transduction. Cross-hatched bars correspond to bilayers that include 30 mol% cholesterol. Value for 18:0,18:1PC/30 mol% cholesterol is significantly different from all others with $P < 0.01$, and values for both 18:0,22:6PC/30 mol% cholesterol and 18:0,18:1PC are significantly different from both di22:6PC and 18:0,22:6PC, $P < 0.005$ (see Fig. 5 in Ref. 41). Rh*, fraction of rhodopsin molecules that absorb a photon; for other abbreviation see Figure 1.

association constant, K_a. Further increase in unsaturation to di22:6PC resulted in a reduction in K_a relative to 18:0, 22:6PC. Cholesterol reduced K_a in both monounsaturated 18:0,18:1PC and polyunsaturated 18:0,22:6PC.

Combining the effects of membrane composition on receptor activation and receptor-G protein binding affinity reveals the net effect of membrane composition on the overall efficiency of formation of the receptor-G protein complex. Efficiency in this context refers to the fraction of rhodopsin molecules that absorb a photon, designated Rh*, and succeed in binding G_t. Thus, efficiency is denoted by the ratio [MII · G_t]/[Rh*]. In the rod cell, the ratio of rhodopsin to G_t is ~10:1 and only ~1 rhodopsin/100,000 absorbs a photon and is activated (see Ref. 42 for a review), with each rhodopsin activating several hundred G_t molecules to produce a visual response. Thus, the differences in the efficiency of receptor-G protein complex formation shown in Figure 2 are significant. Approximately 90% of the rhodopsin molecules that absorb a photon form a complex with G_t in 18:0, 22:6PC and di22:6PC vesicles, but only 60% succeed in binding G_t in 18:0,18:1PC vesicles. The presence of 30 mol% cholesterol resulted in only ~60 and 30% of photoactivated rhodopsin molecules forming a complex with G_t in 18:0,22:6PC and 18:0,18:1PC, respectively.

The time course of the formation of both MII and the MII · G complex after an activating flash was measured at physiologic temperature for rhodopsin in membranes consisting of 18:0,22:6PC, and 18:0,18:1PC with and without 30 mol% cholesterol (32). Complete analysis of the kinetic data acquired at 37°C showed that the time constants for MII, τ(MII), were in the order 18:0,22:6PC < 18:0,22:6PC/30 mol% cholesterol < 18:0,18:1PC < 18:0,18:1/30 mol% cholesterol, with values of 0.55 ± 0.06, 0.68 ± 0.07, 1.13 ± 0.1, and 1.83 ± 0.11 ms, respectively. Uncertainties are given as 1 SD, and all values of τ(MII) were significantly different from each other, $P < 0.03$. The time constant for MII · G_t complex formation, τ(MII · G_t), varied in the same order as τ(MII). The ratio τ(MII · G_t)/τ(MII) is an informative quantity with respect to understanding how membrane composition may alter the diffusion-dependent aspects of G protein–coupled signaling. Values of this ratio slightly >1 indicate that the receptor-G protein complex is formed nearly as rapidly as possible, whereas values significantly >1 indicate that the activated receptor must wait a period of time for a fruitful collision with G protein. Cholesterol has very little effect on the diffusion-dependent kinetics of MII · G_t formation in a 18:0,22:6PC bilayer (Fig. 3). This is consistent with a number of measurements that show that cholesterol has its smallest effect on the properties of bilayers containing DHA acyl chains (43). In contrast, in an 18:0,18:1PC bilayer, cholesterol increases the time required for MII formation by 50%, and quadruples the time required for MII · G_t formation, resulting in a value of τ(MII · G_t)/τ(MII) of nearly 3. In 18:0,22:6PC at 37°C, MII formation occurs in 0.55 ms, and MII and MII · G_t complex formation are nearly coincident. Because MII cannot react with G_t any more quickly than it is formed from MI, the rate of formation of the MII · G_t complex in 18:0,22:6PC appears to be maximal. In 18:0,22:6PC, the rate of MII · G_t complex formation, which is limited by lateral diffusion, is altered only slightly by the presence of 30 mol% cholesterol, further suggesting that 18:0,22:6PC has the optimal acyl chain composition for rapid formation of receptor-G protein complex among the PC examined in this study.

It is informative to assess directly the integrated response of the G protein–coupled signaling pathway by measuring the

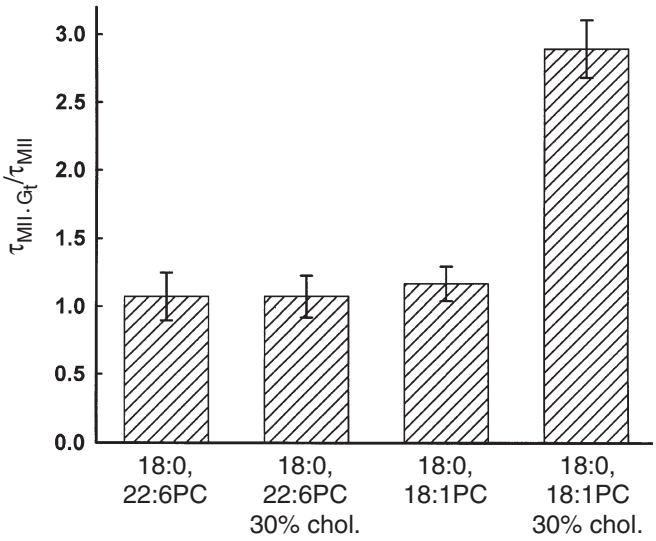

FIG. 3. Summary of the effects of phospholipid acyl chain composition and bilayer cholesterol content on the ratio of the time constants for MII and MII · G_t complex formation at 37°C. The processes that lead to formation of both the MII conformational state and the MII · G_t complex consist of multiple kinetic steps (32). The average time constants were used in the formulation presented here to facilitate comparison between the different membrane compositions of the overall time course of signal transduction. The value for 18:0,18:1/30 mol% cholesterol is significantly different from the other three values, $P < 0.001$ (recalculated from data in Fig. 5 of Ref. 32). For abbreviations see Figures 1 and 2.

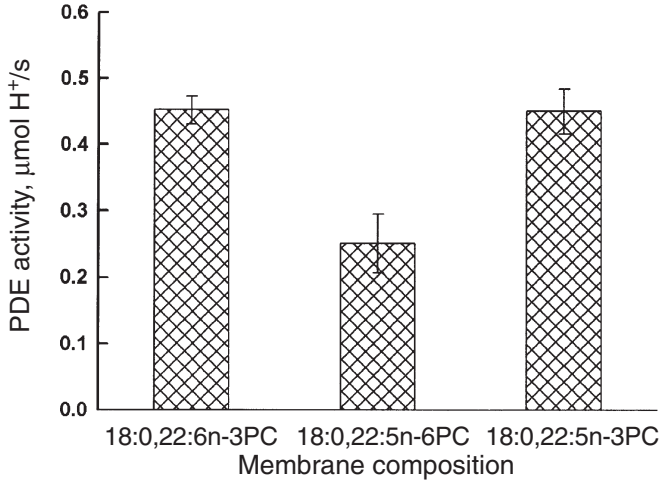

FIG. 4. Comparison of activities of the effector enzyme, phosphodiesterase (PDE), for rhodopsin, G_t, and PDE reconstituted in three membranes containing PC with a 22-carbon, polyunsaturated *sn*-2 acyl chain. The light intensity for this comparison was sufficient to bleach one rhodopsin per 20,000 rhodopsins, which is within the range of stimulus conditions associated with normal rod cell vision. 18:0,22:6n-3PC and 18:0,22:5n-3PC did not differ, but 18:0,22:5n-6 was significantly different from both of them, $P < 0.005$. For other abbreviation see Figure 2.

activity of the PDE. In visual signal transduction, PDE activity is highly amplified relative to the initial stimulus and would be expected to be sensitive to any alteration in functional efficiency of the receptor and/or G protein. For this reason, we examined PDE activity in large unilamellar vesicles containing rhodopsin, G_t, and PDE reconstituted in three different PC with small but significant variations in the composition of the polyunsaturated *sn*-2 acyl chain. The three PC all had an 18:0 acyl chain at the *sn*-1 position and DHA, DPA, or 22:5n-3 acyl chains at the *sn*-2 position. At a light stimulus level of 1 bleached rhodopsin/20,000 rhodopsins, the PDE activity in the two n-3-containing membranes was twice as high as in the DPA membrane, as shown in Figure 4.

DISCUSSION

The results presented here demonstrate that membrane lipid composition modulates several steps in G protein–coupled signal transduction. Increased membrane cholesterol and phospholipid acyl chain saturation both have inhibitory effects on this important form of signal transduction. These two compositional variables appear to modulate membrane protein function *via* the same physical mechanism, that is, by increasing phospholipid acyl chain packing order. Cholesterol-induced changes in the level of activated rhodopsin, MII, as measured by K_{eq}, and acyl chain packing, as measured by f_v, are linearly related over the entire range of cholesterol concentrations examined (Fig. 1). A linear correlation between changes in K_{eq} and f_v induced by changes in bilayer cholesterol was observed previously in studies of rhodopsin reconstituted in phospholipid membranes (39,40). In addition, temperature-induced changes in K_{eq} and f_v are linearly related for rhodopsin in ROS disk membranes and in reconstituted phospholipid vesicles (40,44). It is significant that the correlation shown in Figure 1 was obtained for measurements at 37°C, demonstrating that cholesterol causes correlated changes in acyl chain packing and rhodopsin activation at physiologic temperature. The MI to MII transition is accompanied by a 100 mL/mol volume expansion (45); thus, a reduction of membrane free volume would impose an energy barrier that would reduce the equilibrium concentration of MII. K_{eq} and f_v are linearly correlated over the entire range of membrane cholesterol concentration examined, and cholesterol had no observable structural perturbation on rhodopsin, leading us to conclude that cholesterol inhibits rhodopsin activation *via* an indirect effect of cholesterol on phospholipid acyl chain packing, i.e., by a free volume-mediated mechanism.

Previous studies showed that both decreased phospholipid acyl chain unsaturation and increased cholesterol concentration reduce the formation of MII *via* a mechanism linked to the specific packing properties of polyunsaturated acyl chains, DHA in particular, and the effect of cholesterol on these packing properties (40,41,44). The results presented here demonstrate that the functional ramifications of DHA acyl chains extend beyond the unimolecular transition from MI to MII to the rapid and efficient coupling of receptor and G protein, as well as the functional efficacy of the integrated signaling pathway as shown by the PDE effector enzyme activity. Current evidence indicates that G_t interacts with the three hydrophilic loops on the surface of rhodopsin, which means that the protein–protein interaction surfaces are external to the bilayer (46). The sensitivity of the MII · G_t binding

interaction to membrane composition (Figs. 2 and 3) demonstrates that protein–protein interactions, which occur on the hydrophilic surfaces of membrane proteins, are also affected by changes in membrane composition in the hydrophobic core of the membrane. This finding suggests for the first time that the protein–protein interactions, which occur on the hydrophilic surface of proteins, external to the membrane phospholipid bilayer, and are required in many signaling pathways, may be modulated by changes in the composition of the lipid hydrophobic core of the membrane.

The alterations in receptor conformation change, receptor-G protein binding strength and rate of receptor-G protein complex formation summarized in Figures 1–3 likely underlie the changes in PDE activity shown in Figure 4. This might reasonably be expected because the events measured in Figures 1–3 precede PDE activation in the G protein–mediated signaling cascade. The dependence of PDE activity on the presence of a polyunsaturated n-3 acyl chain at the *sn*-2 position (Fig. 4) clearly demonstrates that G protein–coupled signaling is exquisitely sensitive to phospholipid acyl chain unsaturation and double bond position. The reduced activity of PDE in 18:0,22:5n-6 PC compared with 18:0,22:6n-3 PC is a quantitative measure of the functional inequivalence of these two polyunsaturated phospholipid acyl chains. This comparison is crucial to our understanding of the biochemical basis for the effects of dietary n-3 fatty acid deficiency because such deficiency generally leads to the replacement of DHA with DPA (3).

To determine the effects of changes in membrane composition resulting from *in vivo* processes, we examined MII formation and PDE activity in ROS disk membranes purified from Long-Evans rats fed diets that were either adequate or deficient in n-3 fatty acids (Niu, S.-L., Mitchell, D.C., Lim, S.-Y., Wen, Z.-M., Kim, H.-Y., Salem, N., Jr., and Litman, B.J., unpublished data). The MI-MII equilibrium constant was ~15% higher in the membranes from the n-3 fatty acid–adequate rats, but the difference was not significant. However, the membranes from the adequate rats produced a level of PDE activity that was nearly fivefold higher than in membranes from the n-3 fatty acid–deficient rats, and the difference was significant ($P < 0.001$, paired *t*-test). A detailed analysis of the phospholipid species present in these two ROS disk membranes is underway, but a preliminary fatty acid analysis shows that ~80% of the DHA in the ROS disk membranes of the n-3 fatty acid–adequate rats was replaced with DPA in the n-3 fatty acid–deficient rats. The changes in MII formation and PDE activity in ROS obtained from rats raised on n-3 fatty acid–adequate or –deficient diets are essentially identical to the results obtained with reconstituted vesicle systems and isolated bovine ROS disk membranes. Together, these results form a body of information that provides an understanding at the molecular level of the changes in electroretinogram (ERG) associated with dietary n-3 fatty acid deficiency in animals and nonhuman primates.

The results presented here suggest that the delays and reduced amplitude in ERG responses observed in dietary n-3 deficiency (14,47–49) are due at least in part to reduced $MII \cdot G_t$ coupling efficiency and slower rate of $MII \cdot G_t$ formation when DHA phospholipid acyl chains are replaced by DPA. Biochemical analysis shows that the rates of G_t activation and PDE catalytic subunit activation are approximately equal (50). Assuming that the rates of G_t binding and G_t activation are similar, this means that a reduction in the rate of $MII \cdot G_t$ complex formation by 10% will delay the rod cell photoresponse by 5%, as calculated according to the model of Leskov *et al.* (50). The results presented here indicate that the effects of membrane composition on the rate and efficiency of receptor-G protein coupling, *via* lateral diffusion, and changes in the rate of MII formation would be sufficient to account for the delays in photoreceptor activity observed in dietary n-3 deficiency (14,47,48). Because of the similar signaling motif in other G protein–coupled signaling systems, the findings presented here should be generally applicable to other members in the G protein–coupled family, providing a molecular mechanism for the observed loss in cognitive skills (11), and odor (16) and spatial discrimination (17) observed in n-3 fatty acid deficiency.

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The Structure of DHA in Phospholipid Membranes

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ABSTRACT: Early experiments and molecular simulations of PUFA favored a rigid arrangement of double bonds in U-shaped or extended conformations such as angle-iron or helical. Although results of recent solid-state NMR measurements and molecular simulations have confirmed the existence of these structural motifs, they portray an image of DHA (22:6n-3) as a highly flexible molecule with rapid transitions between large numbers of conformers on the time scale from picoseconds to hundreds of nanoseconds. The low barriers to torsional rotation about C–C bonds that link the *cis*-locked double bonds with the methylene carbons between them are responsible for this unusual flexibility. Both the amplitude and frequency of motion increase toward the terminal methyl group of DHA.

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PUFA contain the repeating 1,4-pentadiene structural motif—two or more double bonds per chain separated by methylene groups (–CH=CH–CH_2–CH=CH–)—flanked by methylene groups on both sides [e.g. see Scheme 1 depicting 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (18:0–22:6n-3PC), a typical polyunsaturated phospholipid, with DHA at the *sn*-2 position of the glycerol]. Chain conformations can be characterized by dihedral bond angles (see Scheme 2 showing dihedral bond angles corresponding to conformations of lowest energy). The bond angles of lowest energy are well known: The dihedral angle between neighboring methylene groups are preferentially *gauche*$^+$, *gauche*$^-$, or *trans*; the double bonds in polyunsaturated chains are *cis* or *trans* (biosynthesis results almost exclusively in the *cis*-configuration); and the two C–C bonds of the methylene groups sandwiched between double bonds have lowest energy in the conformations *skew*$^+$ or *skew*$^-$ (see Scheme 2 for definitions) (1). However, the dihedral bond angles in hydrocarbon chains of biomembranes may deviate from these values because the lipid matrix is liquid crystalline: The bilayer structure is maintained while the order of hydrocarbon chains is liquid-like with rapid chain isomerization, rotational diffusive motions, and relatively unrestricted lateral diffusion. The strength of interactions between lipids as well as between lipids and membrane proteins is tightly linked to the structure of polyunsaturated chains.

Two competing views exist regarding the nature of perturbations caused by polyunsaturation. Until recently, many researchers ascribed the special role of polyunsaturated hydrocarbon chains to rigidity and bulkiness. Indeed, with six double bonds that are locked in a *cis*-orientation, DHA has fewer degrees of freedom than comparable saturated hydrocarbon chains. The existence of extended conformers of DHA such as angle-iron or helical was proposed (2–4). To the contrary, simulations by Rabinovich and Ripatti (5) and more recent experiments and simulations (6–11) emphasized various degrees of flexibility of the DHA chain. These two competing views of DHA are portrayed in this minireview.

PROPERTIES OF MEMBRANES COMPOSED OF POLYUNSATURATED LIPIDS

Early experiments studied the influence of polyunsaturated chains on global parameters of lipid packing in biomembranes. Monolayer experiments showed that introduction of one double bond into the *sn*-2 chain of PC greatly increased the molecular area (12,13). However, subsequent double bonds caused only minor additional increases. In particular, monolayers composed of lipids containing three or more *cis* double bonds varied little in area.

Although chain unsaturation is important for maintaining the fluid environment essential for the function of membrane proteins, polyunsaturated chains are not essential for the fluid state. Introduction of the first double bond lowers the transition temperature of typical lipids by about 50°C. The differences in phase transition temperatures from the introduction of additional double bonds are significantly smaller. There is even a small increase in phase transition temperatures for highly unsaturated chains (14,15).

Unsaturation greatly influences the order and motional properties of lipids in bilayers. Salem *et al.* (16) introduced the cholestane label into the sonicated PS fraction of neural lipids enriched in polyunsaturated DHA and found that polyunsaturation lowers order parameters in the hydrocarbon chain region. Extensive investigations were conducted with fluorescence labels. Stubbs *et al.* (17) conducted steady-state- and time-resolved measurements on 1,6-diphenyl-1,3,5-hexatriene (DPH)

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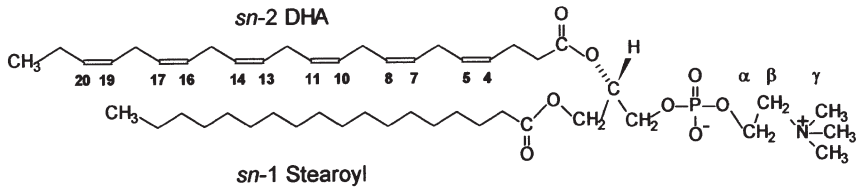
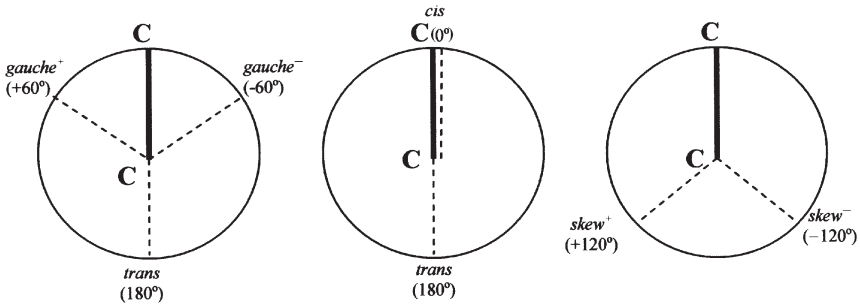
Abbreviations: 16:0–22:6n-3PC, 1-palmitoyl-2-docosahexanoyl-*sn*-glycero-3-phosphocholine; 18:0–22:6n-3PC, 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; DHA, docosahexaenoic acid (22:6n-3); DPH, 1,6-diphenyl-1,3,5-hexatriene.



SCHEME 1



SCHEME 2

in a series of mixed-chain polyunsaturated lipids. Results were interpreted in terms of a wobble-in-a-cone model. With increasing unsaturation, both the amplitude and the rate of wobble increased. Differences among the unsaturated PC were relatively small, suggesting that the introduction of the first double bond into one of the two hydrocarbon chains plays the most important role in lowering chain order. However, succeeding studies of DPH time-resolved fluorescence emission and decay of fluorescence anisotropy in polyunsaturated bilayers conducted in the laboratory of Litman (18,19) indicated lower chain order and increased chain dynamics in DHA-containing membranes. In particular, lipids with two DHA chains had a much lower orientational order compared with lipids that were polyunsaturated in the *sn*-2 chain only.

^{2}H NMR experiments on mixed-chain lipids with a saturated hydrocarbon chain in *sn*-1 revealed a decrease in *sn*-1 chain order with increasing unsaturation of the *sn*-2 chain. The decrease in chain order was largest in the second half of the chain near the terminal methyl group (20–23).

The experimental evidence pointed to a lower chain order and shorter correlation times of motion as consequences of polyunsaturation. However, these changes do not necessarily reflect behavior of the polyunsaturated chain itself. They could result from the incorporation of bulky PUFA that perturb packing of neighboring saturated hydrocarbon chains. Until recently, many investigators favored the latter interpretation (3,4,24–26). But a definitive answer could not be obtained without direct studies of conformations and motion of polyunsaturated lipids in membranes.

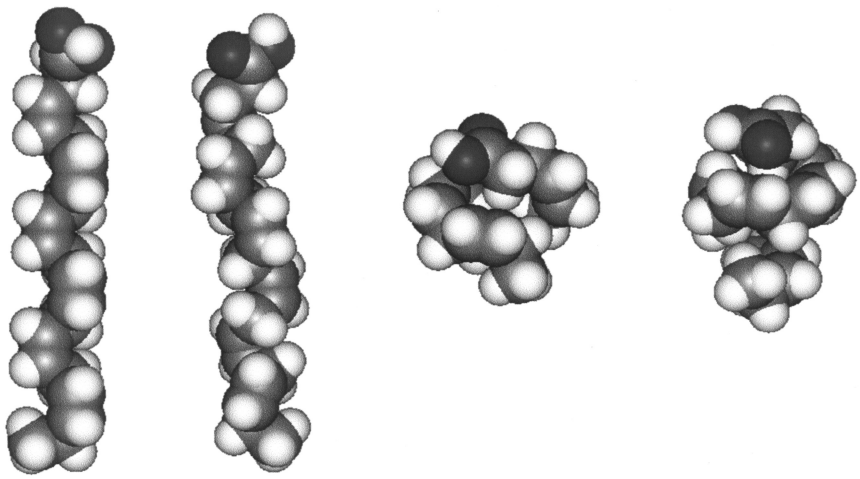
STRUCTURAL STUDIES ON POLYUNSATURATED HYDROCARBON CHAINS

Polyunsaturated chains are frequently shown as U-shaped structures. Although this choice for depicting polyunsaturated chains may stem merely from a matter of convenience when drawing such molecules, calculations on arachidonic acid indeed suggest that U-shaped structures are of low energy (27,28). Still other investigators proposed the existence of helical structures for all-*cis*-PUFA (24,29), and results of X-ray structural studies on crystals of arachidonic acid were interpreted as extended-chain conformations (30).

Applegate and Glomset (3,4,31) conducted very detailed molecular mechanics structural studies on mixed-chain polyunsaturated lipids in a lamellar arrangement. Their DHA conformations of lowest energy were extended conformations in which the six double bonds projected outward from the methylene axis in two nearly perpendicular planes to form an extend angle-iron shaped or helical structure [see Scheme 3; the left two conformations represent the angle-iron and helical conformers of Applegate and Glomset (4,32)].

Using a similar approach, Albrand and colleagues (33) predicted tightly back-folded helical conformations of DHA with 1.2 and 1.5 spirals to be most stable [see Scheme 3; the two conformers on the right represent the helical conformers reported by Albrand and colleagues (33)], but they did not rule out the existence of extended-helical conformations of DHA in phospholipids. *Note*: Coordinates of DHA chain conformations were kindly provided by K. Applegate and R. Dolmazon.

The low-energy conformers of DHA in the conformations above can be expressed as a sequence of the dihedral bond angles in polyunsaturated units with at least three *cis*-locked double bonds (=CH–CH_2–CH=CH–CH_2–CH=). The four dihedral angles that link methylene groups with double bonds adjust preferentially to the dihedral angles $skew^+$ or $skew^-$. The sequence (=$skew^{\pm}$ $skew^{\pm}$=$skew^-$ $skew^-$=) corresponds to an extended angle-iron conformation, (=$skew^{\pm}$$skew^{\pm}$=$skew^{\pm}$$skew^{\pm}$=) is extended helical, and (=$skew^{\pm}$ $skew^-$=$skew^{\pm}$ $skew^-$=) and (=$skew^{\pm}$ $skew^-$=$skew^-$ $skew^{\pm}$ =) result in back-folding of the chain in a hairpin-like fashion.



SCHEME 3

Although these configurations may represent some static energetic minima, they tell little about the true structure of PUFA in the dynamic environment of a liquid-crystalline matrix. The thermal energy in fluid hydrocarbon chains permits the bond angles to adopt values that deviate from the angles of lowest energy. Furthermore, dihedral bond angles could be in rapid exchange between the low-energy regions depicted in Scheme 2. The rate of exchange depends critically on the height of energy barriers between the energy minima. It is well known that the high potential barrier between the *cis* and *trans* isomers of double bonds effectively prevents transitions at physiological conditions. However, *gauche-trans* isomerization in segments of accumulated methylene groups and transitions between the $skew^+$ and $skew^-$ isomers of methylene groups between double bonds are rapid (8). Although these principal differences in rotational potential have been known for decades, the precise functional dependence of energy on dihedral angles, the differences in energy of chain conformers, and the pace of conformational transitions of FA are still a matter of investigation.

Additional complications arise from the incorporation of FA into aggregates of amphipathic molecules, such as lipid bilayers, that impart molecular order *via* intermolecular interactions. Indeed, FA conformation and conformational transitions are as much the result of inter- as of intramolecular interactions.

NMR STRUCTURAL STUDIES

Order parameter studies on monounsaturated oleic acid (18:1n-9) in the *sn*-2 position of PC were conducted in the laboratory of Seelig (34,35). The experiments established that the C–D order parameters of the olefin carbons C_9 and C_{10} are nonequivalent and much smaller than corresponding order parameters of saturated-chain segments. However, when the different geometries of methylene segments vs. double bonds are taken into account, the segmental fluctuations in the *sn*-1 and *sn*-2 chain were found to be identical. The authors concluded that the C=C bond vector is, on average, inclined at an angle of 7–8° to the bilayer normal.

Baenziger *et al.* (36,37) measured ^{2}H NMR order parameters of deuterated *cis,cis*-octadeca-6,9-dienoic acid (18:2n-9) that was specifically labeled with deuterium at carbons $C_{4–11}$, C_{14}, or C_{18}. The selective labeling enabled assignment of C–D order parameters to specific carbon atoms. The authors concluded that the chain adopts conformations that are consistent with rapid jumps between two low-energy conformations ($=skew^{\pm}\,skew^{\pm}=$ and $=skew^{\pm}\,skew^{-}=$). Although the authors observed motions around the C–C bonds between the two double bonds, they concluded that hydrocarbon chains with three or more double bonds likely cannot undergo the same jump motion and, consequently, will be highly ordered structures.

Rajamoorthi and Brown (38) conducted a study on PC with double bond-perdeuterated arachidonic acid (20:4n-6) in position *sn*-2. They observed up to six different order parameters for the eight C–D bonds, all of them significantly lower than order parameters in saturated chains. The authors concluded that the order of double bonds varies along the chain. Furthermore, deuterium spin-lattice relaxation measurements revealed the existence of a profile of motion along the polyunsaturated chain. An interpretation of order parameters in terms of specific chain conformers was not attempted.

Dratz and Deese (39) synthesized DHA (22:6n-3) with perdeuterated double bonds. The solid-state NMR spectrum suggested that C–D order parameters of all double bonds are low. Huber *et al.* (40) repeated this synthesis recently and compared the measured order parameters with results from molecular dynamics simulations. The most populated conformers corresponded to an extended arrangement of double bonds (helical and angle-iron). However, a significant concentration of bent-chain segments was found as well.

Saiz and Klein (41,42) conducted molecular dynamics simulations on 18:0–22:6n-3PC and found high concentrations of helical and angle-iron conformers in the polyunsaturated DHA chain. The calculations suggested larger fluctuations in molecular area of polyunsaturated chains compared to chains with less unsaturation.

NMR experiments on 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (16:0–22:6n-3PC) conducted by Everts and Davis (11) revealed a transient association between the choline headgroup and parts of the DHA chain, but not with the palmitate chain, suggesting higher DHA chain density near the interface. Furthermore, the authors measured correlation times of slow motion in the bilayer on the time scale of 10 μs. They concluded that methylene groups of both chains experience such motion, but correlation times of palmitate chain motion were significantly longer.

Our laboratory conducted ^{2}H, ^{13}C, and ^{1}H NMR experiments on mixed-chain 18:0–22:6n-3PC (7,8,43–48). We have been able to obtain an experimentally assigned C–H order parameter profile of DHA by taking advantage of the excellent resolution of ^{13}C resonances in magic angle spinning NMR experiments. Recently, we also conducted magic angle spinning experiments on entirely ^{2}H-labeled DHA (46). The order profile of the DHA chain is shown in Figure 1. The two C–H bonds at methylene carbon C_2 have nonequivalent order parameters, indicating motional restrictions of this chain segment. The order parameters of all double bonds, but also the order parameters of methylene groups between double bonds, are low. For the double bonds, this could be the result of a preferential orientation of the C–H bonds to the bilayer normal that is close to the "magic angle" of 54.7°. However, the exceptionally low order parameters of methylene groups between double bonds (C_6, C_9, C_{12}, C_{15}, C_{18}) suggest a significant contribution from rapid motion. The order parameters of the C–H bonds in double bonds 1–4 are nonequivalent, indicating that the double bond axis is, on average, inclined with respect to the bilayer normal.

The existence of rapid chain motion was confirmed by measurements of ^{13}C spin-lattice relaxation times (see Fig. 2) (7,8). Motions with correlation times of the order of 1 ns shorten spin-lattice relaxation times the most. Relaxation times increase when motion is slower or faster than this. By measuring the dependence of relaxation times on magnetic field strength, we determined that the increase in relaxation times toward the terminal methyl group is the result of motion with correlation times faster than 1 ns. Figure 2 reveals that motion is slowest near the carbonyl group where the chain is affixed to the lipid glycerol, the most rigid section of a phospholipid. Motional freedom increases stepwise from double bond to double bond, suggesting existence of very flexible hinges between olefin carbons. The carbons near the terminal methyl group are moving with motional correlation times in the range of 10 ps. In particular, the last double bond (number 6 in Fig. 2) has long spin-lattice relaxation times and a low order. Furthermore, the methylene group between double bond numbers 5 and 6 has zero-order parameters, indicating exceptional flexibility of the tail section of DHA near the terminal methyl group.

The key to understanding behavior of PUFA lies in the rotational potentials about dihedral angles along the chain. This remarkable flexibility profile of DHA motivated our collaborators MacKerell and Feller (8) to conduct quantum mechanical calculations on model compounds to obtain optimized potentials of mean force for rotation about the dihedral angles between double bonds and molecular dynamics simulations to calculate motional behavior of DHA chains in bilayers (Fig. 3).

The calculations revealed exceptionally low energy potentials for rotations about dihedral angles between double bonds. The potentials were lowest at dihedral angles near ±100°. However, the potential was essentially flat, covering

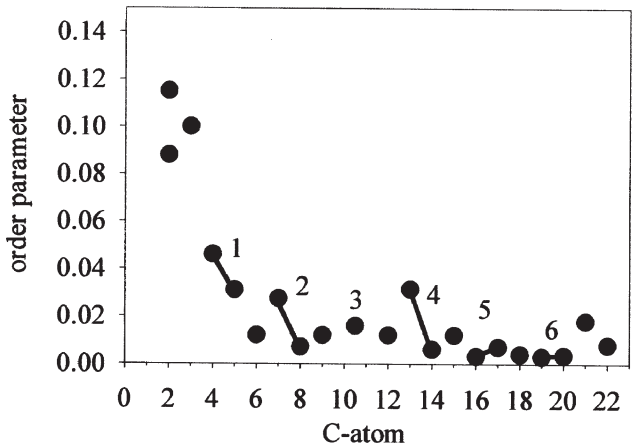


FIG. 1. Order parameter profile of a docosahexaenoic hydrocarbon chain in a lipid bilayer. Carbon atoms are numbered from 1–22 beginning at the carbonyl group. The location of the six double bonds is indicated with bars and numbers. The resonances of olefinic carbons C_{10} and C_{11} are superimposed. The corresponding order parameter in this figure is an averaged value.

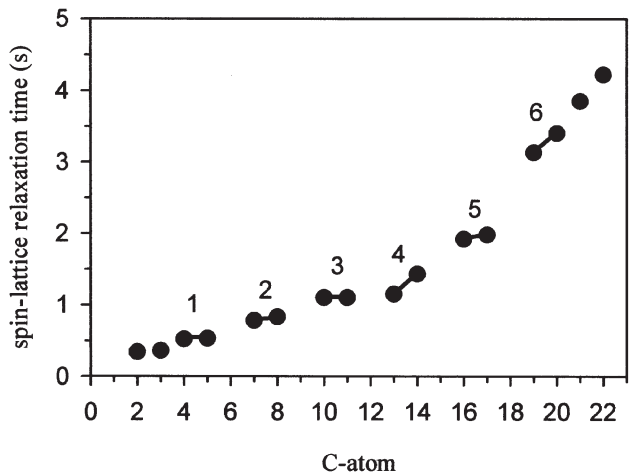


FIG. 2. ^{13}C spin-lattice relaxation times of the docosahexaenoyl hydrocarbon chain in a lipid bilayer. The relaxation times increase stepwise from double bond to double bond.

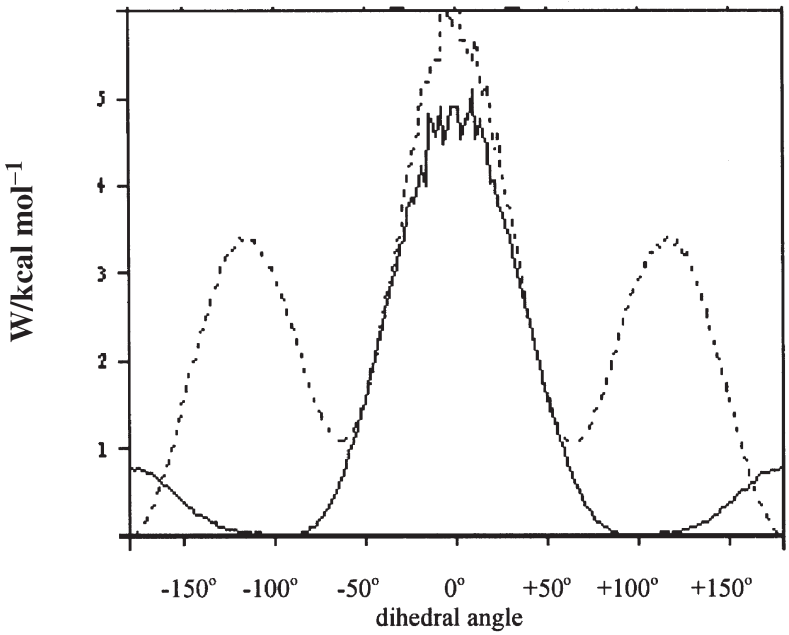


FIG. 3. Potential of mean force for rotations about dihedral angles. The solid line is the potential for rotations about bonds between the methylene carbon and the olefinic carbons on either side of it. The dashed line is the potential between accumulated methylene groups. Reprinted in part from Reference 8. Copyright 2002 American Chemical Society.

an angular range of ±30° that included the orientations *skew*$^{\pm}$ (±120°). The potential rose to less than one kcal/mol at 180°. Thermal motions within the DHA chain in the fluid phase enable a barrier of such low height to be passed with ease. Instead of viewing these dihedral bond angles as locked into a *skew*$^{\pm}$ orientation, they should be viewed as very wide regions of permitted dihedral angles centered at values *skew*$^{\pm}$. However, a significant barrier, centered at 0°, prevents full circular motion. Furthermore, steric hindrance within the polyunsaturated chains enforces a correlation between the bond angles that link the methylene groups with double bonds. This is easily contemplated by mechanically moving a calotte model of DHA.

The potential of mean force for rotation about C–C bonds between accumulated methylene groups differs significantly from the one above. Not only are the angles of lowest potential (*gauche*$^{+}$, *gauche*$^{-}$, and *trans*) very different from *skew*$^{+}$ and *skew*$^{-}$, the height of potential barriers between the various dihedral angles is different as well. For multiple methylene groups, dihedral bond angles have the lowest energy for the *trans* (180°) orientation. Consequently, they are populated the most. The energy barriers between *trans* and *gauche*$^{\pm}$ are 3 kcal/mol, much higher than the barrier between *skew*$^{\pm}$.

Considering the differences in height of potential barriers, it is not surprising that the methylene groups between double bonds act as flexible hinges of the DHA chain. Indeed, the analysis of molecular dynamics simulations revealed that the probability distribution function for the angle between the C–H vector of methylene groups between double bonds and the bilayer normal is a constant, confirming that the low order parameters measured in experiments are the result of random motion (8).

The molecular dynamics simulations enabled us to evaluate the correlation times of chain motion quantitatively. The correctness of the calculated correlation times was confirmed by comparison with NMR results. The correlation functions of ^{1}H–^{13}C bond vector motion, after some mathematical conversion, yielded NMR spin-lattice relaxation times of chain carbon atoms. The quantitative agreement between experiment and theory was excellent, confirming that the molecular simulations correctly reflect the fast motion of the DHA chain (7,8). Scheme 4 shows the location of the 10 very flexible C–C bonds in the DHA chain.

SCHEME 4

At ambient temperature the polyunsaturated DHA chain has an average length of about 14 Å and occupies a lateral area of about 36 $Å^2$ (43). The DHA chains in membranes are, on average, much shorter than DHA chains in extended conformations such as angle-iron or helical. Therefore, they do bend, tilt, and back-fold, increasing lateral area per molecule. Polyunsaturated chains, when paired with saturated chains as in 18:0–22:6n-3PC, undergo a disproportionally large fractional change of area when exposed to lateral tension (43,49,50). This confirms their exceptional flexibility. The lower-order parameters and shorter motional correlation times of labels in biomembranes rich in polyunsaturated lipids appear to be the consequence of DHA flexibility itself rather than the result of perturbations caused by DHA rigidity and bulkiness.

In conclusion, the results of recent NMR experiments and simulations portray PUFA as molecules with exceptional flexibility. It is apparent that polyunsaturated chains impart a significant degree of flexibility to bilayers. Perhaps one of the functions of polyunsaturated chains is to provide a flexible environment for integral receptor proteins such as rhodopsin that undergo structural transitions during activation. Dratz and Holte (51) proposed such a role for DHA in the "molecular spring" model. We recently obtained evidence that DHA in 18:0–22:6n-3PC has a distinct distribution of chain densities with the polyunsaturated DHA chain exhibiting higher density near the water interface, in contrast to saturated chains concentrating their density in the bilayer center (7). It has been suggested that DHA may alter the lateral pressure profile across lipid bilayers, which in turn may have an influence on conformational transitions of integral membrane proteins (50,52–54).

On the other hand, polyunsaturated molecules such as DHA may act as ligands that bind to receptor proteins. For example, there is evidence that DHA is a ligand for the retinoid X-receptor in mouse brain (55). The productive conformation of arachidonic acid bound to prostaglandin synthase (56) and the conformation of DHA in human brain FA-binding protein (57) were determined by X-ray diffraction on protein crystals. The crystal structures revealed the existence of U-shaped or extended L-shaped conformations of the PUFA. Since the polyunsaturated chains rapidly convert between a large number of conformers when located in the lipid matrix, the conformation in the protein-bound state must be dictated by interactions with the binding pocket. Structural motifs such as angle-iron, helical, or hairpin combined with chain flexibility may result in a particularly tight fit to protein surfaces. This cohesion, combined with pointed interactions of the π-electrons of polyunsaturated chains, may result in sufficient specificity to distinguish between FA. The exceptional flexibility of polyunsaturated chains could be a key property for entering into the binding pockets of proteins.

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Effects of Docosapentaenoic Acid on Neuronal Apoptosis

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ABSTRACT: We previously established that n-3 FA status in membrane phospholipids influences the biosynthesis and accumulation of PS in neuronal tissues. We also demonstrated that neuronal apoptosis under adverse conditions is prevented by DHA enrichment in a PS-dependent manner. In this study, we examined the effect of a structural analog of DHA, docosapentaenoic acid (22:5n-6, DPA), which accumulates in neuronal membranes during n-3 FA deficiency. We observed that enrichment of neuronal cells with DPA increased the total PS content in comparison to nonenriched control. However, the increase was significantly less than that observed in DHA-enriched cells, primarily due to the fact that the 18:0,22:5n-6 species was not accumulated as effectively as 18:0,22:6n-3 in PS. As was the case with DHA, DPA enrichment also protected against cell death induced by staurosporine treatment in Neuro 2A cells, but to a lesser extent. These data indicate that provision of DPA in place of DHA is sufficient neither for fully supporting PS accumulation nor for cell survival. The *in vitro* interaction between Raf-1 and membrane was affected not only by the PS content but also by the fatty acyl composition in PS. The reduction of PS concentration as well as the substitution of 18:0,22:6 with 16:0,18:1 in the liposome considerably reduced the interaction with Raf-1. These data suggest that depletion of DHA from neuronal tissues may have a compounding effect on Raf-1 translocation in growth factor signaling. The fact that DPA cannot fully support the protective role played by DHA may provide a basis for the adverse effect of n-3 FA deficiency on neuronal development and function.

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Neuronal membranes are highly enriched with long-chain PUFA, particularly DHA (22:6n-3) (1,2). It is widely accepted that DHA is essential for proper neuronal development and function (3–6). Under n-3 FA-deficient conditions, DHA is replaced by docosapentaenoic acid (DPA, 22:5n-6), which is usually a minor component in neuronal membranes (7).

We and others have established that DHA positively modulates the biosynthesis and accumulation of the major anionic phospholipid, PS, in neuronal membranes (8–10). The importance of PS in various signaling events supporting cellular functions, such as activation of protein kinase C and Raf-1 kinase in particular, is well established (11–13). DHA has been shown to exert a protective effect on apoptosis of developing rat retinal photoreceptors (14) and sphingosine-induced apoptosis in HL-60 cells (15). We also found that DHA enrichment prevents apoptotic cell death induced by serum starvation (16,17) or staurosporine (ST) treatment (18). These antiapoptotic effects were demonstrated only in cases where cells were allowed to enrich DHA, suggesting that the observed effect may be mediated through membrane events. When the cells were enriched with DHA under the condition where PS accumulation is inhibited, the protective effect disappeared, suggesting that PS is an important component for the observed protective effect of DHA (16,18). During n-3 FA deficiency, where DHA is replaced by DPA, significant decreases in the PS content were observed selectively in neuronal tissues (10,19). In the present study we examined the effect of DHA and DPA on the survival of cells in relation to their capacity to modify membrane phospholipids. We have found that DPA is not as effective as DHA in accumulating PS or preventing ST-induced apoptosis. The Raf-1/membrane interaction, which is dependent on PS concentration, also is influenced by the presence of DHA in the membrane. The provision of DPA in place of DHA may not fully support the growth factor signaling that leads to the protective function of DHA.

MATERIALS AND METHODS

Chemicals. ST was purchased from Calbiochem (La Jolla, CA); DHA was from Nu-Chek-Prep (Elysian, MN); DPA was a generous gift from Omegatech (currently incorporated into Martek, Columbia, MD) and urea-crystallized by Nu-Chek-Prep. (±) α-Tocopherol and methylthiazoletetrazolium-based cell growth determination kit were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture and DHA enrichment. Neuro 2A (mouse neuroblastoma) cells were purchased from American Type Culture Collection (Manasas, VA), grown and maintained in DMEM (Biofluids, Gaithersburg, MD) with 5% FBS (Life Technologies, Gaithersburg, MD). Cells (2×10^5) were seeded in six-well plates for caspase-3 activity and phospholipid analysis. After 24 h, cells were supplemented with 20 μM DHA, which was coupled to 1% FBS in the presence of 40 μM vitamin E. FA preparation was performed in the argon box to prevent oxidation.

Caspase-3 activity measurement. At the end of FA supplementation, control and enriched cells were washed and exposed

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Abbreviations: 16:0,18:1-PS, 1-palmitoyl-2-oleyl-3-glycerophosphoserine; 18:0,22:5-PS, 1-stearoyl-2-docosapentaenoyl-3-glycerophosphoserine; 18:0, 22:6-PS, 1-stearoyl-2-docosahexaenoyl-3-glycerophosphoserine; 22:5n-6, docosapentaenoic acid (DPA), RU, resonance unit; ST, staurosporine.

to ST (100 nM) in 1% FBS for 5 h to induce apoptosis. At the end of the treatment, cells, including floating cells, were harvested and lysed by using lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA), and caspase-3 activity was measured by using an assay kit (Biomol, Plymouth Meeting, PA) according to the manufacturer's protocol. In a 96-well plate, cell lysates containing 15 μg of protein were added to 200 μM final concentration of N-acetyl-Asp-Glu-Val-Asp *p*-nitro-anilide substrate in 50 μL assay volume. After incubation for 2 h at 37°C, the absorbance was measured at 405 nm (SpectraMax Plus; Molecular Devices, Sunnyvale, CA).

Analysis of Raf-1 and membrane interaction. BSA-free anti-Raf-1 antibody (Transduction Laboratories, Lexington, KY) was immobilized on a CM5 sensor chip (Biacore, Uppsala, Sweden) using Biacore X *via* amine cross-linking as described earlier (16). Raf-1 was captured on the chip of the experimental cell in the range of 15–20 resonance units (RU) by passing Neuro 2A cell lysate collected in radioimmune precipitation assay buffer (1× PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS) containing 100 μg/mL phenylmethylsulfonyl fluoride (PMSF) over this cell. Unilamellar vesicles containing varying proportions of 16:0,18:1 PC, PE, and PS (Avanti Polar Lipids, Alabaster, AL) were prepared by extrusion through a 0.1 μm polycarbonate filter as described earlier (16). The PE proportion was held constant at 50%. These lipid vesicles were injected into the flow cell, and the interaction with the Raf-1 captured on the sensor chip surface was monitored.

Phospholipid molecular species analysis. Phospholipids were extracted according to Bligh and Dyer (20), and PS molecular species were analyzed using RP-HPLC/EI-MS with a C_{18} column (150 × 20 mm, 5 μm; Phenomenex, Torrance, CA) as described previously (10,19,21). Separation was accomplished using a mobile phase containing water/0.5% ammonium hydroxide in methanol/hexane, changing from 12:88:0 to 0:88:12 in 17 min after holding the initial composition for 3 min at a flow rate of 0.4 mL/min. Separated individual PS species were detected using an Agilent HPLC-MS Series 1100 MSD instrument. The capillary voltage was set at 4000 V, and the exit voltage was set at 200 V. Quantification was based on the area ratio calculated against the deuterium-labeled internal standard of the same phospholipid class.

RESULTS

When Neuro 2A cells were enriched with 20 μM DHA or DPA for 24 h, total PS content increased significantly, although the extent of the increase differed between these two FA. The total PS level after DPA enrichment was approximately 75% of the PS level in DHA-enriched cells (Fig. 1). As 18:0,22:6 species increased most prominently in PS by DHA enrichment, 18:0,22:5-PS (1-stearoyl-2-docosapentaenoyl-3-glycerophosphoserine) was the major PS species in DPA-enriched cells. However, 18:0,22:5-PS was not accumulated as efficiently as 18:0,22:6 species, contributing to the

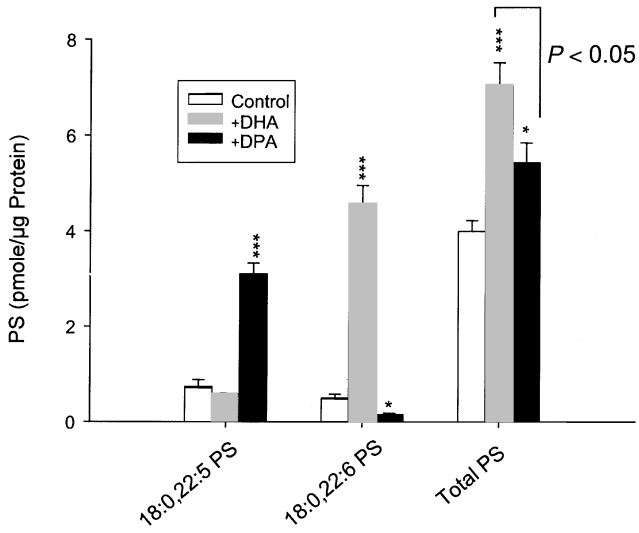

FIG. 1. Effect of DHA or docosapentaenoic acid (DPA) enrichment on PS contents in Neuro 2A cells determined by RP-HPLC/EI-MS. Significant increases of the total PS content are indicated with DHA or DPA enrichment primarily due to the accumulation of 18:0,22:6- or 18:0,22:5-PS species. The statistical analysis was performed against the nonenriched control group according to Student's *t*-test. The difference in the total PS level between DHA- and DPA-enriched groups is statistically significant ($P < 0.05$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

fact that the total PS content in DPA-enriched cells was lower than in DHA-enriched cells. This result is comparable to the previous findings from *in vivo* feeding studies using n-3 FA-deficient diets (10,17). Significant decreases in the PS content (by 20–40%) occurred in neuronal tissues, including subcellular fractions of brain cortex, hippocampus, and olfactory bulb, despite the reciprocal replacement of DHA by DPA at the total FA level (Fig. 2). The data from both cell and tissue samples suggest that DPA provision in place of DHA is not sufficient to maintain PS levels normally found in neuronal membranes.

We previously demonstrated that DHA enrichment in cell membranes partially prevents apoptotic cell death and that this effect is dependent on DHA's ability to promote PS accumulation (16,18). Since DPA is not as effective as DHA in increasing PS, we examined whether the extent of protection by DPA enrichment is compromised accordingly. When apoptotic cell death was induced by 100 nM ST treatment for 5 h as described earlier (18), DHA-enriched cells showed 40% reduction in caspase-3 activity in comparison to nonenriched control. DPA-enriched cells also showed protection; however, the caspase-3 activity decreased by only 24% (Fig. 3). The observed diminished protection is consistent with the notion that the protective effect is PS-dependent, as DPA is less effective in accumulating PS in comparison to DHA.

The PS concentration in neuronal membranes following DHA enrichment is primarily due to the increase of 18:0,22:6 species. We have shown that translocation of Raf-1 kinase, an important upstream event for transmitting growth-factor

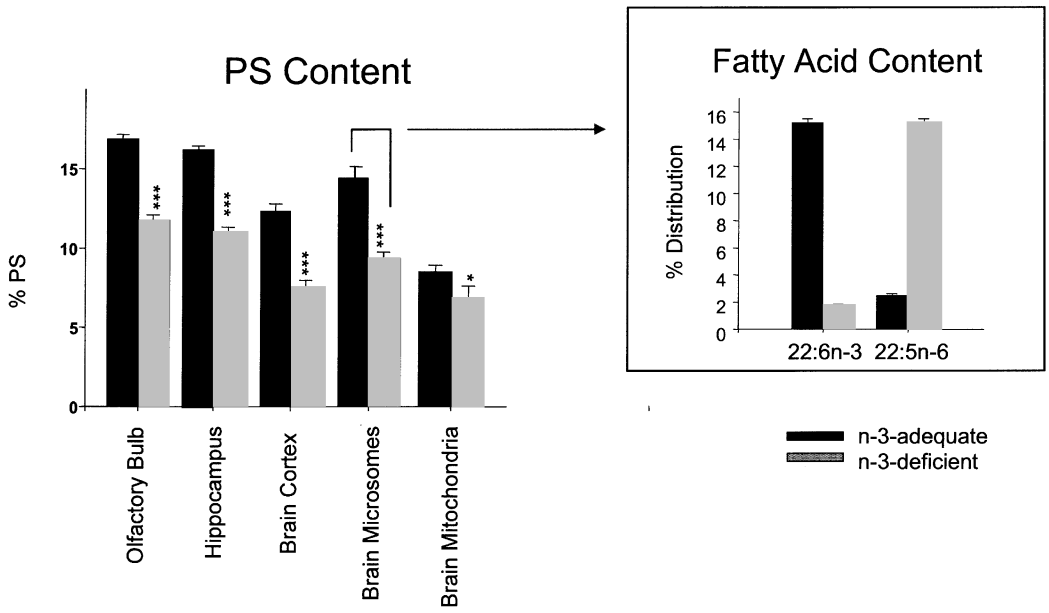

FIG. 2. Effect of n-3 FA deficiency on PS contents in rat neuronal tissues (from 10,19). Under deficiency, 20–40% reduction of PS is indicated. The inset of the figure shows the reciprocal replacement of DHA by DPA at the total FA level in brain cortex microsomes after n-3 FA-deficient diet. The statistical analysis was performed against the n-3 adequate control group by Student's *t*-test. *, $P < 0.05$; ***, $P < 0.001$. For abbreviation see Figure 1.

signaling, is PS-concentration dependent by *in vitro* biomolecular interaction analysis (16). Since n-3 deficiency not only decreases PS but also dramatically decreases 22:6n-3 content in neuronal membranes, we tested the influence of the fatty acyl compositional change on the Raf-1/membrane interaction. *In vitro* biomolecular interaction between Raf-1 and membrane was examined using liposomes containing 16:0,18:1 PS/PE/PC species and compared with the data observed for 18:0,22:6 liposomes. The interaction of Raf-1 with 16:0,18:1 liposomes also showed PS concentration dependence (Fig. 4). Liposomes containing 0–10% PS did not interact at all with Raf-1. The RU, which represent the extent of the interaction, were 5, 32, and 50 for the liposomes containing 20, 40, and 50% PS, respectively. At the corresponding PS proportions and under similar experimental settings, the liposomes containing 18:0,22:6 species interacted with RU of 10, 200, and 160, respectively (16). Considerably lower responses observed for 16:0,18:1 species indicated that the extent of Raf-1-liposome interaction also was affected by the presence of specific polyunsaturated fatty acyl chains.

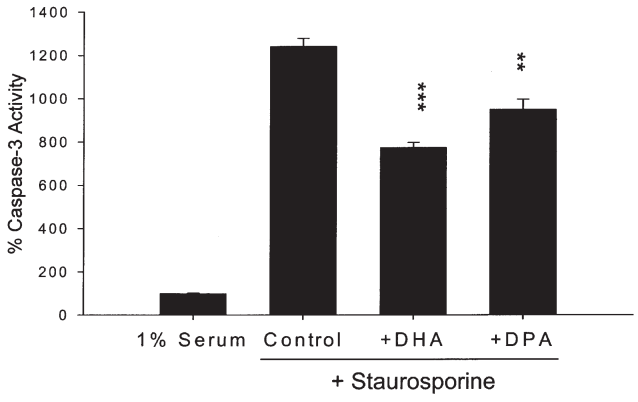

FIG. 3. The effect of DHA or DPA enrichment on apoptotic cell death induced by treating Neuro 2A cells with 100 nM staurosporine for 5 h. Both FA showed protection although DHA was more effective in preventing apoptosis. The comparison was made against staurosporine-treated, nonenriched control by Student's *t*-test. **, $P < 0.01$; ***, $P < 0.001$. For abbreviation see Figure 1.

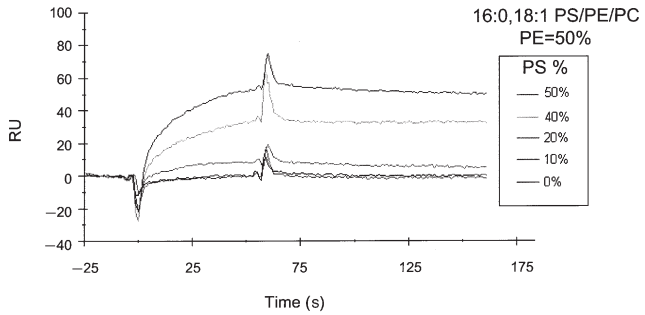

FIG. 4. The effect of the PS composition on the Raf-1/membrane interaction. Unilamellar vesicles consisting of 16:0,18:1-PS/PE/PC in the ratio ranging from 0:50:50 to 50:50:0 interacted with Raf-1 captured on the sensor surface. The RU (resonance unit) represents the extent of interaction. A response of 1 RU corresponds to a change in surface concentration on the sensor chip of approximately 1 pg/mm^2. The interaction between 16:0,18:1 liposomes and Raf-1 captured on the sensor chip surface through immobilized anti-Raf-1 antibody was PS-dependent.

DISCUSSION

During n-3 FA deficiency, DPA, the counterpart of DHA in the n-6 FA family, accumulates to replace DHA (7). Under normal conditions, chain elongation and desaturation of linoleic acid (18:2n-6) up to 22:5n-6 FA do not generally occur, as reflected by the fact that arachidonic acid (20:4n-6) is the most common long-chain n-6 PUFA found in animal tissues. Replacement of DHA with structurally similar DPA appears particularly prominent in neuronal membranes where DHA is highly enriched, suggesting the need for a long-chain highly unsaturated FA to maintain cellular function in the neuronal system (22). Although reciprocal replacement of DHA by DPA occurs at the total FA level, the phospholipid profile seems to be differentially affected by depleting n-3 FA from the diet (10,19). The significant reduction of PS during n-3 FA deficiency (Fig. 2) suggests that the phospholipid biosynthetic pathways maintaining the PS profile are affected by the absence of DHA in the neuronal tissues (9,23). Our present data also indicate that neuronal cells enriched with DPA accumulate less PS in comparison with DHA-enriched cells, consistently supporting an effective role of DHA in the accumulation of PS in neuronal membranes.

DHA's prevention of apoptotic cell death induced by serum starvation or ST treatment is mediated at least in part through positively modulating PS accumulation (16,18). Since DPA enrichment also increases the PS content, it is expected that DPA enrichment also exerts an anti-apoptotic effect. Indeed, we observed in this study that DPA-enriched cells were resistant to apoptotic cell death in comparison to nonenriched controls, although to a lesser degree than in DHA-enriched cells. Considering the notion that PS accumulation is important for the protective effect, it is anticipated that DPA exerts less protection, as DHA is most effective in promoting PS accumulation.

Membrane translocation is an important step in the activation of Raf-1 kinase (12,13), which transmits signaling of various growth factors. Although the proportion of PS in membranes is a critical factor influencing Raf-1/membrane interaction (16), the presence of the 22:6n-3 fatty acyl chain in the membrane also may make an important contribution to Raf-1 translocation. Our current data support the proposition, as the Raf-1 membrane interaction was considerably diminished when membranes were made with 16:0,18:1 species in comparison to 18:0,22:6 species. To relate this effect to the n-3 FA deficiency condition, direct comparison should be made between 18:0,22:6 and 18:0,22:5 species when 18:0,22:5 phospholipid species become available. Nevertheless, it is suggested that Raf-1 translocation is facilitated by DHA, not only because of the substantial increase of PS, but also because of the specific increase of DHA in neuronal membranes. The loss of PS and DHA species in neuronal membranes resulting from n-3 FA deficiency may have compounding adverse effects on Raf-1 activation. Considering that Raf-1 activation is an important upstream event in growth factor signaling leading to proliferation, differentiation, and cell survival, compromised Raf-1 translocation due to the lack of DHA may have a significant impact on neuronal development and function. In conclusion, in these experiments on rodent neuronal cells, DPA provision is not sufficient to sustain neuronal survival at the level supported by DHA, perhaps explaining some of the functional deficits associated with n-3 FA deficiency.

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Increased Blood Pressure Later in Life May Be Associated with Perinatal n-3 Fatty Acid Deficiency

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ABSTRACT: Hypertension is a major risk factor for cardiovascular and cerebrovascular disease. Previous work in both animals and humans with high blood pressure has demonstrated the antihypertensive effects of n-3 polyunsaturated fatty acids (PUFA), although it is not known whether these nutrients are effective in preventing hypertension. The predominant n-3 PUFA in the mammalian nervous system, docosahexaenoic acid (DHA), is deposited into synaptic membranes at a high rate during the perinatal period, and recent observations indicate that the perinatal environment is important for the normal development of blood pressure control. This study investigated the importance of perinatal n-3 PUFA supply in the control of blood pressure in adult Sprague-Dawley rats. Pregnant rat dams were fed semisynthetic diets that were either deficient in (DEF) or supplemented with (CON) n-3 PUFA. Offspring were fed the same diets as their mothers until 9 wk; then, half of the rats from each group were crossed over to the opposite diet, creating four groups, i.e., CON-CON; CON-DEF; DEF-DEF, DEF-CON. Mean arterial blood pressures (MAP) were measured directly, at 33 wk of age, by cannulation of the femoral artery. The phospholipid fatty acid profile of the hypothalamic region was determined by capillary gas-liquid chromatography. The tissue phospholipid fatty acid profile reflected the diet that the rats were consuming at the time of testing. Both groups receiving DEF after 9 wk of age (i.e., DEF-DEF and CON-DEF) had similar profiles with a reduction in DHA levels of 30%, compared with rats receiving CON (i.e., CON-CON and DEF-CON). DEF-DEF rats had significantly raised MAP compared with all other groups, with differences as great as 17 mm Hg. DEF-CON rats had raised MAP compared with CON-CON rats, and DEF-DEF rats had higher MAP than CON-DEF rats, despite the fact that their respective fatty acid profiles were not different. These findings indicate that inadequate levels of DHA in the perinatal period are associated with altered blood pressure control in later life. The way in which these long-term effects are produced remains to be elucidated.

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Hypertension is a major risk factor for cardiovascular disease (CVD); in the United States in 2000, it was responsible for ~1 million deaths (i.e., 36% of all mortality) (1,2). CVD is expected to cost $329.2 billion in the United States in 2002 (3).

Precise control of blood pressure, which is essential for the maintenance and regulation of tissue perfusion in response to demand, involves a complex interaction among many organs, including the kidney, vasculature, heart, and brain. Regulation of fluid and electrolyte balance and, hence, blood volume, is performed by the kidney, which enacts a wide range of humoral and neural afferent signals when homeostasis is perturbed. Many of these signals act through the central nervous system to activate efferent autonomic neural pathways and other humoral modulators of fluid balance, such as vasopressin, and the cardiovascular system. Neural control of blood pressure is orchestrated by the hypothalamus, which communicates with preganglionic sympathetic and parasympathetic fibers, both directly and indirectly, *via* the vasomotor center, embedded within the reticular formation of the medulla and pons (4). Circumventricular organs (CVO), which lack a blood brain barrier, such as the organum vasculosum of the lamina terminalis and subfornical organ, are sensitive to changes in plasma osmolality and circulating hormones. Information from the CVO is integrated by the hypothalamus and other brain areas, which initiate the processes responsible for restoration of fluid balance (5). This is of key importance in the maintenance of blood pressure. Much work has attempted to understand these complex homeostatic processes, but the mechanism by which the set-point of blood pressure is determined remains unknown. Presumably, it is a disruption of this mechanism that leads to essential hypertension.

The predominant n-3 polyunsaturated fatty acid (PUFA) in the brain is docosahexaenoic acid (DHA). DHA is a long-chain metabolite of the essential fatty acid, α-linolenic acid (ALA). DHA comprises ~15% of the phospholipid fatty acids within the hypothalamus (6), and up to 30% of those within the cortex (7). DHA is transferred from maternal stores *via* the placenta (and breast milk) (8) and is rapidly concentrated in synapses during early perinatal life (9). DHA has been demonstrated to play an important role in retinal function (10–12), visual acuity (13,14), olfaction (15), and fluid balance (16,17). Supplementation with n-3 PUFA has also been shown to prevent an increase in systolic blood pressure in spontaneously hypertensive rats (SHR) (18).

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Abbreviations: ALA, (18:3n-3, α-linolenic acid); CON, n-3–sufficient diet; CVD, cardiovascular disease; CVO, circumventricular organ; DEF, n-3–deficient diet; DHA, (22:6n-3, docosahexaenoic acid); MAP, mean arterial pressure; ΔMAP, change in mean arterial pressure; PUFA, polyunsaturated fatty acid; SHR, spontaneously hypertensive rat.

Numerous studies in humans (19–22) and animals (6,18,23,24) have demonstrated an effect of n-3 PUFA on blood pressure, such that high dietary intakes of n-3 PUFA are associated with lower blood pressure, especially in those with existing hypertension. Recently, the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico Prevenzione study of 11,324 postmyocardial infarction survivors demonstrated that dietary supplementation with 1 g/d of n-3 PUFA resulted in a 13% reduction in mortality at 12 mon (25). Further evidence for a dietary effect lies in the observation that Japanese immigrants in Brazil assume the same rates of hypertension as their fellow Brazilians (26). Similarly, the Honolulu Heart Study was one of the first studies to demonstrate that adoption of the indigenous diet leads to important changes in fatty acid profile, blood pressure, and risk for cardiovascular events such as coronary heart disease (27).

Despite the number of studies that have investigated the antihypertensive effects of n-3 PUFA, little or no work has addressed whether early administration of n-3 PUFA may reduce, or even prevent the onset of hypertension. Development of blood pressure control appears to be vulnerable in early life; several studies have associated perinatal factors such as low birth weight (28), low protein intake (29), and maternal undernutrition (30–32) with elevated blood pressure in later life. It follows that nutrients such as n-3 PUFA, which are rapidly accumulated by neurons during the perinatal period, may be important for the subsequent development of blood pressure control. This study utilized a double crossover study design of dietary manipulation over the perinatal period to assess the effect of early n-3 PUFA supply on blood pressure in later life.

METHODS

Animals and diets. All procedures involving animals were conducted in accordance with local Institutional Ethics Committees. Female Sprague-Dawley rats were mated and fed a diet either deficient in (DEF) or supplemented with (CON) n-3 PUFA for the duration of pregnancy and up to the time of weaning. Offspring were fed the same diets as their mothers.

The diets (Table 1) were semisynthetic mixtures, supplemented with either safflower oil or canola oil, corresponding to the DEF and CON diets, respectively (12). The CON diet provided adequate amounts of ALA, and has previously been shown to promote normal brain DHA levels (33). Diets and water were consumed *ad libitum*. Room temperature was maintained at 21°C.

Pups ($n = 40$) were weaned at 3 wk (DEF, $n = 20$; CON, $n = 20$) into group boxes ($n \cong 2$/box) and fed the same diet as their dams (diet 1) for a further 5 wk. Then, the pups were weighed (i.e., at 8 wk) and transferred to individual cages. The rats used in this study represent the offspring from 12 litters (6 litters/diet). Use of offspring from several litters enabled weight and sex matching before (pseudorandom) assignment to the post-crossover diet group, and reduced the effect of intrafamilial bias.

TABLE 1
Diet Compositions Including Assayed Fatty Acid Composition[a]

Component	(g/kg)	
Casein, vitamin-free	300	
Carbohydrate	370	
Cornstarch	200	
Sucrose	100	
Glucose	70	
Cellulose	100	
Kaolin	30	
Mineral mix	68	
Vitamin mix	27	
DL-Methionine	2	
L-Arginine	3	
Supplementary oil	100	
Fatty acid[a]	CON	DEF
14:0	0.38	0.41
16:0	5.15	7.30
16:1	0.28	0.14
18:0	2.70	2.63
18:1	58.93	15.04
18:2n-6	20.55	71.99
18:3n-3	8.19	1.01
20:0	1.01	0.32
20:1	1.47	0.08
22:1	0.38	—
Total n-6	20.55	71.99
Total n-3	8.19	1.01
n-6/n-3	2.51	71.27

[a]CON, n-3–sufficient diet; DEF, n-3–deficient diet.

After 1 wk of habituation to the cages (i.e., at 9 wk of age), half of the CON rats were crossed to the deficient diet (CON-DEF), and half of the DEF rats were crossed to the control diet (DEF-CON). The remaining rats from either group maintained their initial diets (CON-CON and DEF-DEF). All rats consumed the second diet for the remainder of the study. There were equal numbers of males and females in each of the groups.

Neural fatty acid equilibration. On the basis of previous work (34) and a recent pilot study, we determined that the delay for crossed-over rats to assume stable neural phospholipid fatty acid levels (e.g., CON-DEF ≈ DEF-DEF) was ~25 wk. In addition, samples were taken from nonstudy rats, fed the same diets, to ensure that physiologic assessments followed stabilization of the brain fatty acid levels, after the switch of diets.

Blood pressure. At 33 wk of age, rats were surgically prepared, under general anesthesia (equithesin, 3 mL/kg, intraperitoneal), with a cannula (0.5 mm i.d., 0.8 mm o.d., Dural Plastics, Sydney, Australia) inserted into a femoral artery and brought to the surface at the back of the neck. The cannula was filled with sterile isotonic saline containing 50–200 U/mL heparin. Mean arterial pressure (MAP) was measured in conscious, unrestrained rats over 30–45 min on two separate occasions (i.e., wk 33 and 34) with a 4- to 6-d recovery period in between. The blood pressure was measured directly *via* a J RACK pressure amplifier (Biosignals, Melbourne, Australia) using Cobe disposable transducers and displayed

on a thermal arraycorder (Graphtec WR 7700, Sydney, Australia).

Fatty acid analysis. After the conclusion of physiologic recordings, deeply anesthetized rats were killed by CO_2 asphyxiation. Whole brains were removed, frozen in liquid nitrogen, and stored at −70°C, before sectioning. An area approximating the hypothalamic region was obtained by dissecting coronally from the level of the optic chiasm to the closure of the third cerebral ventricle. The cortices were removed. Lipids from the hypothalamic region were extracted from the tissue into 5 mL of chloroform/methanol 1:1 (containing 10 mg/L of butylated hydroxytoluene as antioxidant), and the phospholipids were separated from the neutral lipids by thin-layer chromatography. The methyl esters of the phospholipid fatty acids were formed by a saponification step using KOH followed by transesterification in BF_3 in methanol; the fatty acid methyl esters were separated by capillary gas–liquid chromatography (Shimadzu GC 15A, Melbourne, Australia) using a 50 m × 0.32 mm (i.d.) fused silica-bonded phase column (BPX70; SGE, Melbourne, Australia). The column oven was programmed from 125°C for 3 min rising to 220°C at 8°C/min with helium (flow rate of 43 cm^3/s) acting as the carrier gas. Fatty acids were identified by comparison against a calibrated standard (mixtures of fatty acid methyl esters), and the results were calculated using response factors derived from chromatographing standards of known composition (Nu-Chek-Prep, Elysian, MN).

Statistical analysis. A two-way ANOVA (diet 1 × diet 2) and *post-hoc* Tukey's test ($\alpha = 0.05$; Statistica, Statsoft) were used for comparisons between treatment (diet 1 × diet 2) groups and fatty acid composition. For analysis of blood pressure recordings, a three-way ANOVA (3 factor, mixed design: repeated measures on 1 factor (time); diet 1 × diet 2 × day of treatment and *post-hoc* Tukey's test ($\alpha = 0.05$; Statistica, Statsoft) was used for comparisons between treatment (diet) groups. All ANOVA used a Huynh–Feldt correction to safeguard against Type II errors of nested designs.

RESULTS

Animals. The rats used in this study appeared healthy in all respects. No differences in general appearance or activity were observed. However, rats in both groups were obese [males: 703 ± 45 g (range 629–795 g; median 702 g), females: 405 ± 36 g (range 350–491 g; median 412 g)] compared with rats of this species raised on standard chow diets, which have only one-third the lipid content. There were no significant differences in body weight between groups.

Fatty acid analysis. The results of the fatty acid analysis are provided in Table 2. Together, the saturates and monounsaturates comprised ~60% of the phospholipid fatty acids in the hypothalamus. There were no differences in the amounts of monounsaturated fatty acids, nor the majority of saturates. There were no differences between groups receiving the same

TABLE 2
Hypothalamic Phospholipid Fatty Acid Composition of Rats Fed Diets That Differed in Fatty Acid Composition[a,b]

Fatty acid	DEF-DEF	CON-CON (g fatty acid/100 g phospholipid fatty acid)	CON-DEF (g fatty acid/100 g phospholipid fatty acid)	DEF-CON
16:0	16.03 ± 1.50	15.50 ± 0.70	16.19 ± 0.99	15.14 ± 1.38
18:0	17.43 ± 0.61	18.18 ± 0.61	18.54 ± 1.58	18.14 ± 0.60
20:0	0.30 ± 0.01	0.27 ± 0.02[e]	0.33 ± 0.01[d]	0.31 ± 0.03
22:0	0.29 ± 0.01[d]	0.27 ± 0.03[d]	0.31 ± 0.02	0.35 ± 0.05[c,d]
24:0	0.31 ± 0.03	0.27 ± 0.04[d]	0.29 ± 0.02	0.37 ± 0.08[d]
Total saturated	34.36 ± 0.23	34.49 ± 0.18	35.66 ± 0.28	34.31 ± 0.28
18:1	24.60 ± 0.53	20.96 ± 12.21	24.49 ± 1.39	25.35 ± 1.02
20:1	2.79 ± 0.30	2.85 ± 0.28	2.84 ± 0.10	2.96 ± 0.20
24:1	0.32 ± 0.03	0.29 ± 0.03	0.28 ± 0.03	0.38 ± 0.13
Total monounsaturated	27.71 ± 0.08[d]	24.10 ± 0.71[c,e,f]	27.61 ± 0.11[d]	28.69 ± 0.13[d]
18:2n-6	0.77 ± 0.11[d,f]	0.45 ± 0.04[c,e]	0.85 ± 0.17[d,f]	0.47 ± 0.06[c,e]
20:3n-6	0.37 ± 0.03	0.35 ± 0.04	0.37 ± 0.04	0.34 ± 0.02
20:4n-6	9.29 ± 0.36[d,f]	8.52 ± 0.19[c]	9.11 ± 0.40[f]	8.29 ± 0.40[c,e]
22:4n-6	4.49 ± 0.27[d,f]	3.40 ± 0.12[c,e]	4.17 ± 0.45	3.74 ± 0.40[c,e]
22:5n-6	2.68 ± 0.47[d,e,f]	0.15 ± 0.02[c,e]	1.72 ± 0.27[c,d,f]	0.19 ± 0.04[c,e]
Total n-6	17.59 ± 0.47[d,f]	12.86 ± 0.27[c,e]	16.22 ± 0.87[d,f]	13.03 ± 0.72[c,e]
20:5n-3	0.31 ± 0.03	0.32 ± 0.04	0.33 ± 0.02	0.36 ± 0.04
22:5n-3	0.20 ± 0.04[d,f]	0.47 ± 0.04[c,e]	0.23 ± 0.02[d,f]	0.46 ± 0.07[c,e]
22:6n-3	10.76 ± 1.24[d,f]	12.91 ± 0.78[c,e]	10.87 ± 1.14[d,f]	13.71 ± 1.25[c,e]
Total n-3	11.27 ± 1.3[d,f]	13.69 ± 0.78[c,e]	11.43 ± 1.13[d,f]	14.52 ± 1.32[c,e]
22:5n-6/22:6n-3	0.25 ± 0.06[d,e,f]	0.01 ± 0.00[c,e]	0.16 ± 0.03[c,d,f]	0.01 ± 0.00[c,e]
22:5n-6+22:6n-3	13.43 ± 0.88	13.06 ± 0.76	12.59 ± 1.23	13.90 ± 1.29
n-6/n-3	1.58 ± 0.16[d,f]	0.94 ± 0.04[c,e]	1.43 ± 0.11[d,f]	0.90 ± 0.05[c,e]
n-6 + n-3	28.86 ± 1.45	26.55 ± 1.00	27.66 ± 1.78	27.55 ± 1.98

[a]Values are means ± SD.
[b]CON, n-3–sufficient diet; DEF, n-3–deficient diet.
[c–f]Significantly different from other groups (as labeled in columns; $P < 0.05$).

diet immediately before tissue analysis (i.e., CON-CON ≈ DEF-CON and DEF-DEF ≈ CON-DEF), for any of the PUFA, with the exception of 22:5n-6, which was highest in DEF-DEF rats. Rats fed CON after week 9 had average DHA levels that were 23% higher than those receiving DEF ($P < 0.05$). Conversely, the levels of 22:5n-6 at the end of the study were 13 times higher in rats fed DEF compared with those fed CON. There was a small, but significant ($P < 0.05$), change in arachidonic acid between those fed DEF after crossover (9.20 ± 0.38% total phospholipids fatty acids) compared with those fed CON (8.41 ± 0.28). Although the ratio of n-6/n-3 was different, the combined n-6 + n-3 values were not different for the two groups.

Blood pressure. No differences between the two blood pressure measurements (i.e., 4–6 d apart) were found. Hence, all reported values for MAP represent the average of two measurements for each rat. Group results for MAP measurement are shown in Figure 1. DEF-DEF rats had a significantly higher MAP than rats in all other groups ($P < 0.05$); the differences were as great as 17 mm Hg. Significantly, those rats fed the deficient diet and crossed to the control diet had a higher MAP (mm Hg) than did those fed and maintained on the control diet (DEF-CON: 110.6 ± 2.7; CON-CON: 103.5 ± 3.1; $P < 0.05$). Therefore, despite restoration of the neural fatty acid profile, repletion with the control diet was unable to nullify the hypertensive effects of perinatal n-3 PUFA deficiency. Conversely, the supply of n-3 PUFA early in life appeared to protect against the development of hypertension associated with n-3 PUFA deficiency. This is supported by the observation that CON-DEF rats had significantly ($P < 0.05$) lower blood pressures (108.4 ± 3.5 mm Hg) than DEF-DEF rats (120.3 ± 3.8 mm Hg), despite having neural PUFA profiles that were not significantly different.

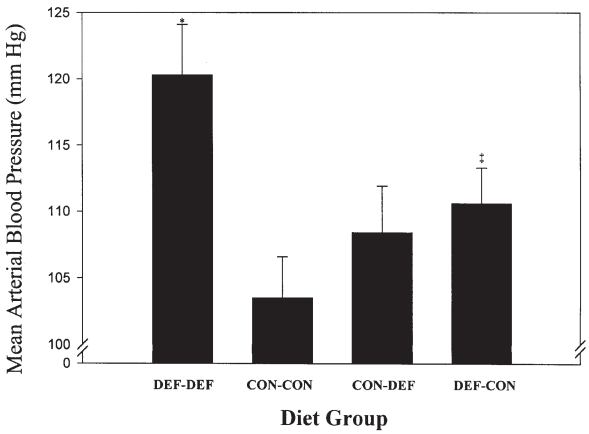

FIG. 1. Effect of dietary fatty acid supply on mean arterial blood pressure. Groups are labeled according to the diets consumed before and after 9 wk of age. Diets were semisynthetic premixtures, either supplemented (CON) or deficient (DEF) in n-3 polyunsaturated fatty acids (see Table 1 for details). *Significantly higher than all other groups ($P < 0.05$); ‡significantly higher than CON-CON ($P < 0.05$).

DISCUSSION

Reports of the effects of dietary n-3 fatty acids on blood pressure are equivocal, although the majority of published studies (see, for example, Refs. 22 and 35) indicate that dietary supplementation with long-chain n-3 PUFA is effective in reducing blood pressure in hypertensive adult humans and in animal models of hypertension (23,24). Indeed, meta-analyses (36–38) have indicated that n-3 supplementation is associated with a small but significant reduction in blood pressure, particularly in older, hypertensive subjects.

As indicated by the data in Table 2, the double crossover design generated two pairs of experimental groups that, effectively, differed only in the supply of fatty acids early in life. From this, it was demonstrated that dietary n-3 fatty acids, supplied in the early developmental period, affected blood pressure later in life. Given that Sprague-Dawley rats possess little predilection for hypertension, these changes are highly significant because despite having the same neural fatty acid profile at the time of measurement, significant differences in blood pressure were found between DEF-DEF and CON-DEF (ΔMAP: 12 mm Hg; $P < 0.05$), and DEF-CON and CON-CON (ΔMAP: 7 mm Hg; $P < 0.05$) groups. The presence of physiologic alterations in adult rats deprived of n-3 fatty acids in the perinatal period suggests the existence of a critical period for accretion of these nutrients in the nervous system.

Our results are consistent with recent investigations that demonstrate the effect of both intrauterine (30) and early postnatal factors (39) on adult neural and cardiovascular function. In particular, perinatal protein supply (32) and sodium intake (40) have been shown to influence blood pressure in adult rats. Furthermore, the studies performed by Langley-Evans and co-workers (32) suggest that the nature of fat present in the diet can modulate the effects of protein supply on blood pressure.

Recently, it was found (17) that body fluid and sodium regulation in adult rats were altered by dietary fatty acid manipulation in the perinatal period. Given the link between mechanisms that regulate sodium intake, water intake, and blood pressure, and the direct influence of salt intake on blood pressure (41,42), it follows that anomalies in sodium regulatory mechanisms may lead to alterations in fluid and sodium intake and, accordingly, blood pressure. Indeed, approximately half of those with essential hypertension are classified as sodium sensitive (43). Dysregulation of the renin-angiotensin-aldosterone system, either centrally or within the kidney, can lead to sodium-sensitive hypertension, in which pressure-natriuresis is reduced and higher arterial pressures ensue to maintain sodium balance (44).

In light of the many factors known to affect blood pressure, the exact mechanisms of these early developmental changes are unclear. However, it is possible that early changes in membrane characteristics permanently alter the function of receptor systems involved in blood pressure regulation. Indeed, n-3 PUFA have been demonstrated to play an important

role in other neural membrane-based receptor systems, such as phototransduction in the retina (12). Notably, the receptors for angiotensin II and photic stimulation of the retina (e.g., rhodopsin) share a common morphology. They are both G-protein–coupled receptors belonging to the 7-transmembrane domain superfamily. Furthermore, recent work by Kitajka *et al*. (45) demonstrated that n-3 PUFA manipulation alters the expression of many genes, with roles that vary from energy metabolism (e.g., ATP synthase) to neurotransmission (e.g., vasopressin V1b receptor, somatostatin).

Although the mechanism of their action remains undefined, the supply of n-3 PUFA early in life appears to be important for normal neural function and development, such that adequate intake of n-3 PUFA in the perinatal period may reduce the risk of hypertension later in life.

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Pathophysiologic Role of Redox Status in Blood Platelet Activation. Influence of Docosahexaenoic Acid

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ABSTRACT: Decrease of platelet glutathione peroxidase activity results in increased life span of lipid hydroperoxides, especially the 12-lipoxygenase product of arachidonic acid, 12-HpETE. Phospholipase A_2 activity is subsequently enhanced with the release of arachidonic acid, which results in higher thromboxane formation and platelet function. Docosahexaenoic acid may either potentiate platelet lipid peroxidation or lower it when used at high or low concentrations, respectively. In the case of slowing down lipid peroxidation, docosahexaenoic acid was specifically incorporated in plasmalogen ethanolamine phospholipids. This could have a relevant pathophysiologic role in atherothrombosis.

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The implication of blood platelets in atherothrombogenesis has been well documented, with a clear relationship between a propensity to aggregate and secrete their granule content and the vascular risk (1). Such platelet activation occurs in several ways, but a prominent path relates to the arachidonic acid (AA) cascade. Briefly, AA is released from membrane phospholipids in response to various agonists such as collagen and thrombin, which is the limiting step in the generation of proaggregatory prostanoids, especially thromboxane (Tx) A_2. In parallel, AA is oxygenated by 12-lipoxygenase to provide its 12-hydroperoxy derivative, 12-hydroperoxyeicosatetraenoic acid (12-HpETE), further reduced into 12-hydroxyeicosatetraenoic acid (12-HETE) by glutathione peroxidase (GPx), a pathway that is quantitatively important but not completely recognized for its biological relevance (for a review, see Ref. 2). Evidence will be given below in favor of its pathophysiologic role. On the other hand, great emphasis has been placed on polyunsaturated fatty acids (PUFA) of the n-3 family because they have been reported to reduce the cardiovascular risk and AA-dependent platelet hyperactivity (3). Among the n-3 PUFA, EPA and DHA have been credited with having the most beneficial effects. It has been assumed for a long time that EPA plays a major role in this regard as an analog of AA because it is able to compete with the AA cascade leading to TxA_2. We also bring evidence for a specific effect of DHA on this cascade. In particular, the role of DHA on blood platelet redox status is reported in relation to its metabolism.

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Abbreviations: AA, arachidonic acid; $cPLA_2$, cytosolic form of phospholipase A_2; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPx, glutathione peroxidase; 12-HpETE, 12-hydroperoxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acids; Tx, thromboxane.

PEROXIDE TONE AND PLATELET FUNCTION: ROLE OF THE LIPOXYGENASE PATHWAY

Human platelet functions have been reported to be enhanced in populations subject to cardiovascular risk, such as diabetics (4–6) and elderly people (7). In those platelets, an exacerbation of AA oxygenation has been described. Specifically, higher TxA_2 formation (5–7) could explain at least part of the platelet reactivity, but the mechanisms involved are not completely understood. In diabetic platelets, increased phospholipase A_2 activity could be responsible for the higher TxA_2 formation (6). On the other hand, greater cyclooxygenase activity has been shown in platelets from elderly people (7). Interestingly, in both situations, the whole platelet GPx activity was found to be significantly decreased (8,9). Considering the GPx-1 isoform, the main enzyme capable of degrading fatty acid hydroperoxides (10), it appears that its lower activity is not of the same origin in the two populations. In diabetic platelets, the decreased activity could be due to abnormal glycation of the protein, which then becomes less active (11). In platelets from the elderly, the decreased enzyme activity correlates well with its quantity (12). This could putatively relate to lower selenium content because this element has been found to be significantly diminished in plasma from these subjects (7). Another GPx isoform, GPx-4, also called phospholipid hydroperoxide GPx, has been described in platelets (13). However, its activity appears to be 50-fold lower than that of GPx-1 (13), which makes it unlikely that the decreased activity observed in platelets from elderly people could be attributed to GPx-4 alteration.

From these data, we hypothesized that low GPx-1 activity would lead to an increased life span for its main substrate, 12-HpETE, which might accelerate the AA cascade. Indeed, 12-HpETE has been shown to stimulate its own formation by activating 12-lipoxygenase (14,15), and peroxides in general are known to enhance cyclooxygenase activity, at least when investigated with the purified enzyme (16). However, relatively

high concentrations of 12-HpETE have been described as an inhibitor of Tx synthase (17). We then investigated a range of nM/μM concentrations of this hydroperoxide upon the AA cascade in normal human platelets. We first found that 12-HpETE, with an optimal concentration of 1 μM, potentiates AA-induced platelet aggregation by stimulating prostaglandin H synthase (18). Even lower concentrations (100 nM) are able to activate the cytosolic form of phospholipase A_2 ($cPLA_2$), the main enzyme responsible for releasing endogenous AA from membrane phospholipids (19), inducing its phosphorylation and translocation from the platelet cytosol to membranes (20). This was accompanied by the release of AA from specific phosphatidylcholine (PC) and phosphatidylethanolamine (PE) subclasses (20). More recent work has shown that the 12-HpETE-dependent activation of $cPLA_2$ occurs *via* stimulation of p38 mitogen-activated protein kinase phosphorylation (Coulon, L., Calzada, C., Moulin, P., Véricel, E., and Lagarde, M., unpublished data). This evidence for an exacerbation of AA metabolism by 12-HpETE in platelets points out the pathophysiologic consequences of lower activity of platelet GPx, especially GPx-1, because this will raise the intracellular concentrations of 12-HpETE into the pathophysiologic range.

EFFECT OF DHA ON THE PLATELET REDOX STATUS

Among n-3 PUFA, DHA contributes to the inhibition of platelet functions when it is incorporated into membrane phospholipids. It differs from EPA in the mechanism of inhibition in several ways, especially as a potent inhibitor of TxA_2-induced aggregation, acting at the receptor level, whereas EPA is almost inactive in this regard (21). However, to exert such an action, as well as to inhibit the AA cascade as EPA does, DHA must be used at relatively high concentrations. A side effect of using high concentrations of n-3 fatty acids is increased lipid peroxidation both *in vivo* (22,23) and *in vitro* (24,25). Such an adverse effect might be especially deleterious in populations that already suffer from having lower antioxidant potential, such as diabetics and elderly people. For the latter group, who have increased platelet functions associated with exacerbated AA oxygenation and lower vitamin E and GPx (7,9,12), we investigated whether it would be possible to maintain the beneficial effect of n-3 long-chain PUFA (mainly DHA) without inducing oxidative stress. For that purpose, elderly people were selected for supplementation with 180 mg/d (150 mg DHA + 30 mg EPA) in a double-blind, randomized study with sunflower oil as a placebo. Interestingly, such low supplementation decreased the lipid peroxidation and increased the vitamin E level (both α- and γ-tocopherols) in platelets from the DHA group, whereas no modifications were observed in the placebo group (26). Similar results were obtained earlier with low-EPA supplementation in the form of pure 2-eicosapentaenoyl/1,3-dioctanoyl-glycerol (27). In that case, we failed to find any accumulation of EPA in any platelet lipid pool, but after the low DHA supplementation, a significant increase (+26 %) in the DHA proportion could be seen in PE only. This prompted us to investigate whether it would be possible to relate the antioxidant and prooxidant potential of DHA in platelets to a specific metabolic fate in the phospholipid subclasses. To do this, normal human platelets were incubated with low, high, and intermediate concentrations of DHA (DHA/albumin ratio of 0.01, 1, and 0.1, respectively) overnight to reach the steady state for DHA distribution within the platelet lipids. As a result, compared with control (no DHA added), the highest concentration induced higher formation of malondialdehyde and 12-HETE, taken as markers of peroxidation, in agreement with previous results (24,25). In contrast, the lowest concentration of DHA significantly lowered both peroxidation markers, whereas the intermediate concentration had no effect. Interestingly, the distribution of DHA within phospholipid subclasses was different according to the concentrations used. For the highest and intermediate concentrations, DHA accumulated similarly in diacyl-glycerophosphocholine (diacyl-GPC) and diacyl-glycerophosphoethanolamine (diacyl-GPE) but was lower in alkenylacyl-GPE (plasmalogen PE); however, plasmalogen PE was the only significant pool in which DHA accumulated when used at the lowest concentration (28). This suggests that the specific accumulation in the latter phospholipid subclass might be associated with the antioxidant activity observed under those conditions. We might then speculate that the strong accumulation of DHA in diacyl species of PC and PE would instead be related more to prooxidant effects, thus making the antioxidant one associated with the incorporation into plasmalogen PE. Interestingly, the latter subclass has been reported to exhibit antioxidant activities (29,30). Whether the incorporation of DHA into this phospholipid subclass affects its antioxidant properties remains to be determined. It is difficult to compare EPA with DHA with respect to antioxidant activity. It indeed appeared to lower platelet lipid peroxidation when administered at a low dosage to elderly people in normalizing platelet α- and γ-tocopherol contents (27). However, no data are available on its possible antioxidant mechanism, although it also shows some propensity to accumulate into plasmalogen PE, either *in vivo* (31) or after *in vitro* activation (32).

CONCLUSION

Overall, these studies indicate that the peroxide tone of platelets is associated with their hyperactivity, which appears to have pathophysiologic relevance. The increased life span of 12-HpETE, as a result of low GPx activity, could play a crucial role in exacerbating the AA cascade, thus leading to pro-aggregatory prostanoids. The n-3 PUFA, especially EPA and DHA, may slow down this process but may play an ambiguous role considering that although high concentrations antagonize AA metabolism and promote lipid peroxidation, low concentrations may decrease it instead. The use of low dosages might be of great benefit in slowing down the AA

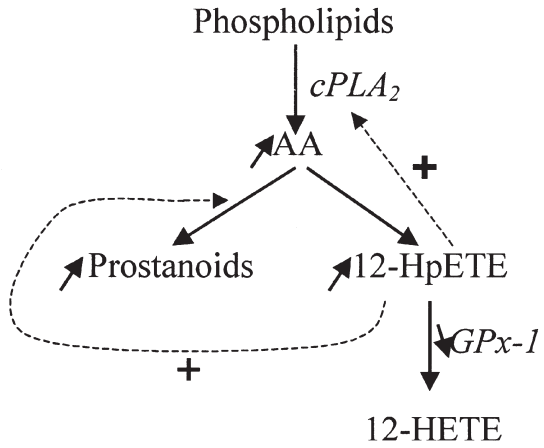

FIG. 1. Low activity of cytosolic glutathione peroxidase (GPx-1) will increase the life span of 12-HpETE, which in turn stimulates the release of endogenous arachidonic acid (AA), through activation of the cytosolic form of phospholipase A_2 (cPLA$_2$) and its cyclooxygenation into pro-aggregatory prostanoids. In this framework, DHA/EPA at high concentrations will enhance the peroxide tone, whereas low concentrations will lower it *via* a mechanism that remains to be established. 12-HpETE, 12-hydroperoxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid.

cascade through lowering the platelet peroxide tone. Figure 1 summarizes the conclusion schematically.

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Effect of the Δ^6-Desaturase Inhibitor SC-26196 on PUFA Metabolism in Human Cells

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ABSTRACT: The objective of this study was to determine the effect of 2,2-diphenyl-5-(4-{[(1*E*)-pyridin-3-yl-methylidene]-amino}piperazin-1-yl)pentanenitrile (SC-26196), a Δ^6-desaturase inhibitor, on PUFA metabolism in human cells. SC-26196 inhibited the desaturation of 2 μM [1-^{14}C]18:2n-6 by 87–95% in cultured human skin fibroblasts, coronary artery smooth muscle cells, and astrocytes. By contrast, SC-26196 did not affect the conversion of [1-^{14}C]20:3n-6 to 20:4 in the fibroblasts, demonstrating that it is selective for Δ^6-desaturase. The IC_{50} values for inhibition of the desaturation of 2 μM [1-^{14}C]18:3n-3 and [3-^{14}C]24:5n-3 in the fibroblasts, 0.2–0.4 μM, were similar to those for the inhibition of [1-^{14}C]18:2n-6 desaturation, and the rates of recovery of [1-^{14}C]18:2n-6 and [3-^{14}C]24:5n-3 desaturation after removal of SC-26196 from the culture medium also were similar. SC-26196 reduced the conversion of [3-^{14}C]22:5n-3 and [3-^{14}C]24:5n-3 to DHA by 75 and 84%, respectively, but it had no effect on the retroconversion of [3-^{14}C]24:6n-3 to DHA. These results demonstrate that SC-26196 effectively inhibits the desaturation of 18- and 24-carbon PUFA and, therefore, decreases the synthesis of arachidonic acid, EPA, and DHA in human cells. Furthermore, they provide additional evidence that the conversion of 22:5n-3 to DHA involves Δ^6-desaturation.

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FA Δ^6-desaturation is a key reaction in mammalian PUFA metabolism (1,2). It is the rate-limiting step in the conversion of linoleic acid (18:2n-6) to arachidonic acid (20:4n-6), the n-6 PUFA necessary for eicosanoid biosynthesis (1). Δ^6-Desaturation is also the initial reaction in the conversion of α-linolenic acid (18:3n-3) to EPA (20:5n-3), the n-3 analog of arachidonic acid (1,2). In addition, Δ^6-desaturation is required for the conversion of tetracosapentaenoic acid (24:5n-3) to DHA (3,4), the n-3 PUFA required for normal development and function of the central nervous system (5). Human and rat Δ^6-desaturases have been cloned (6,7), and the human gene, designated FA desaturase-2 (FADS2), has been localized to a 1.4-mb region of human chromosome 11q12-q13.1 (8). The FA Δ^5-desaturase, a second desaturase necessary for the production of arachidonic acid and EPA from their respective PUFA precursors (1,2), also has been cloned (9). This gene, designated FA desaturase-1 (FADS1), is present in the same 1.4-mb segment of human chromosome 11q12-q13.1 and is located 11.3 kb distant from FADS2 (8).

Numerous potent and selective inhibitors of mammalian Δ^6- and Δ^5-desaturases have been developed recently (10,11). One of these compounds, 2,2-diphenyl-5-(4-{[(1*E*)-pyridin-3-yl-methylidene]amino}piperazin-1-yl)pentanenitrile (SC-26196), inhibits the Δ^6-desaturation of linoleic acid in isolated rat liver microsomes with an IC_{50} of 0.2 μM (11). The IC_{50} of SC-26196 for Δ^5- and Δ^9-desaturation in these microsomes is >200 μM, indicating that the inhibitor is highly selective for Δ^6-desaturation. SC-26196 also inhibited the desaturation of linoleic acid when it was administered to mice. This produced an anti-inflammatory effect, presumably because eicosanoid production was reduced due to the lower availability of arachidonic acid (10,11).

Because the rodent studies indicated that SC-26196 is potentially useful as an anti-inflammatory agent, we wished to determine whether this compound is a potent and selective Δ^6-desaturase inhibitor in human tissues. We also investigated whether SC-26196 is effective against n-3 PUFA, including 24:5n-3, which is thought to be an intermediate in the conversion of 22:5n-3 to DHA (3,4). Human skin fibroblasts (HSF) were utilized for most of the studies because previous work demonstrated that these cells readily desaturate 18- and 24-carbon n-3 and n-6 PUFA substrates (12,13).

MATERIALS AND METHODS

Cell culture. Eagle's minimum essential medium (MEM) was modified by the addition of L-glutamine, gentamicin (Life Technologies, Grand Island, NY), nonessential amino acids, HEPES, and basal medium Eagle (BME) vitamins (Sigma, St. Louis, MO) (13). Normal HSF were obtained from stock cultures maintained by the University of Iowa Cardiovascular Center Tissue Culture Laboratory (Iowa City, IA) and were grown in 75 cm^2 vented flasks at 37°C in the modified Eagle's MEM containing 10% FBS (HyClone, Logan, UT) (13). Human coronary artery smooth muscle cells (CC-2576) were obtained from Clonetics (BioWhittaker, Walkersville, MD) and grown in Clonetics smooth muscle cell medium (CC-3182) supplemented with 5% FBS. Human astrocytes also

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Abbreviations: AM, acetoxymethyl ester; BME, basal medium Eagle; FADS, fatty acid desaturase; HSF, human skin fibroblasts; MEM, minimum essential medium; SC-26196, 2,2-diphenyl-5-(4-{[(1*E*)-pyridin-3-yl-methylidene]amino}piperazin-1-yl)pentanenitrile.

were obtained from Clonetics (CC-2567) and were grown in Clonetics astrocyte medium (CC-3186) supplemented with 5% FBS. Cell viability was determined using the commercially available calcein acetoxymethyl ester (AM) reagent obtained from Molecular Probes (Eugene, OR) (14,15).

Incubation and analysis. HSF from stock cultures were suspended by incubation with trypsin-EDTA solution (Sigma) and seeded in 10 cm^2 wells for each experiment (16). The HSF were incubated with modified Eagle's MEM containing 10% FBS in humidified 5% CO_2 chambers at 37°C until they reached 85–90% confluence. The cultures were then incubated for 3 h at 37°C with modified Eagle's MEM containing 2% FBS and either SC-26196, a Δ^6-desaturase inhibitor kindly provided by the Pharmacia Corporation (10), dissolved in DMSO, or an equivalent amount of DMSO alone. In one experiment, SC-26196 was replaced by anthranilicanilide, a Δ^5-desaturase inhibitor also provided by Pharmacia Corporation (11), dissolved in DMSO. Radiolabeled FA was added, and the incubation was continued for 24 h unless indicated otherwise. [1-^{14}C]18:2n-6, [1-^{14}C]18:3n-3, [1-^{14}C]γ-linolenic acid (18:3n-6), and [1-^{14}C]dihomo-γ-linolenic acid (20:3n-6) were purchased from American Radiolabeled Chemicals (St. Louis, MO), and the corresponding unlabeled FA were obtained from Cayman Chemical (Ann Arbor, MI). [3-^{14}C]Docosapentaenoic acid (22:5n-3 and 22:5n-6), [3-^{14}C]tetracosapentaenoic acid (24:5n-3), [3-^{14}C]tetracosahexaenoic acid (24:6n-3), and the corresponding unlabeled FA were generously provided by Dr. Howard Sprecher, Department of Medical Biochemistry, Ohio State University (Columbus, OH).

The human coronary artery smooth muscle cells and astrocytes were treated similarly and tested when the cultures were 90% confluent. These cultures were incubated with 2 μM [1-^{14}C]18:2 in the respective Clonetics medium containing 5% FBS. At the end of the incubation, the medium was removed and centrifuged at 1000 × *g* to sediment any cell debris. The supernatant solution was removed, acidified to pH 4 with formic acid, and extracted twice with 4 vol of ethyl acetate. An aliquot of the combined ethyl acetate extracts was dried under N_2, dissolved in scintillator solution, and assayed for radioactivity in a liquid scintillation spectrometer (16). Quenching was monitored with the external standard.

The cell monolayer was washed with ice-cold Dulbecco's PBS solution immediately after the medium was removed. The washed cells then were scraped with a rubber policeman into 2 mL ice-cold methanol containing 1% acetic acid; after 4 mL chloroform and 1.5 mL acidified NaCl were added, the chloroform phase was isolated (16). After the solvent was evaporated under a stream of N_2, the lipid residue was suspended in 0.5 mL of a 2:1 mixture of chloroform and methanol, and the radioactivity contained in an aliquot of this mixture was measured by liquid scintillation spectrometry (16).

Chromatography. The radioactive FA contained in the medium and cell lipid extracts were separated by HPLC (17,18). Aliquots of the medium and cell lipid extract containing 20,000–50,000 dpm were dried under N_2, transesterified with 12% BF_3 in methanol at 95°C for 45 min, and extracted with *n*-heptane. The FAME were then separated on an Alltech (Deerfield, IL) 3-μm Adsorbosphere 4.6 × 150 mm reversed-phase C18 column (18), using a Gilson (Middleton, WI) dual pump gradient HPLC system equipped with an automatic sample injector (17). The solvent consisted of acetonitrile and water adjusted to pH 4.0 with formic acid. For most separations, the gradient began at 76% acetonitrile and was increased stepwise over 10 min to 86% acetonitrile. It remained at 86% acetonitrile for 25 min and was then increased over 2 min to 100% acetonitrile, where it was maintained for an additional 23 min. The flow rate was 0.7 mL/min. To separate 18:3n-3 from 22:6n-3, the initial 76% acetonitrile concentration was maintained for 45 min, increased over 1 min to 100% acetonitrile, and then maintained for 14 min (12). The effluent was mixed with 2.1 mL of BudgetSolve liquid scintillation solution (RPI, Mt. Prospect, IL), and the radioactivity was assayed by passing the mixture through an in-line flow scintillation detector (IN/US Systems, Tampa, FL). Radiolabeled FAME standards were included with each set of chromatograms.

RESULTS

Effect of SC-26196 on 18:2n-6 metabolism. To evaluate the effectiveness of SC-26196 in human cells, we initially investigated its ability to inhibit the Δ^6-desaturation of 18:2n-6 (1,2). Control cultures and those treated with 2 μM SC-26196 were incubated for 24 h in a medium containing 2 μM [1-^{14}C]18:2n-6. The presence of SC-26196 did not affect the total amount of radiolabeled FA taken up by the cells. However, as shown by the HPLC analyses in Figure 1, major differences were observed in the amounts of radiolabeled products that were formed. In the control HSF cultures, 49% of the cell lipid radioactivity was contained in two main products, 20:3 and 20:4 (Fig. 1A). By contrast, 20:3 and 20:4 accounted for only 3.5% of the radioactivity incorporated into the cell lipids when 2 μM SC-26196 was present, a 93% reduction, and 88% remained as 18:2 (Fig. 1B). Unmodified 18:2 was the main radiolabeled FA contained in the medium after the 24-h incubation in both sets of HSF cultures. In the control cultures, 20:3 accounted for 4.9% of the radioactivity in the medium, but no radiolabeled 20:3 or other Δ^6-desaturation products were detected in the medium when SC-26196 was present (data not shown).

To determine the extent to which SC-26196 was effective in other human cells, we tested its ability to inhibit the desaturation of 2 μM [1-^{14}C]18:2n-6 in the human coronary artery smooth muscle and astrocyte cultures. Substantial amounts of radiolabeled 20:3 and 20:4 and a small amount of 22:4 were present in the smooth muscle cell lipids after incubation for 24 h (Fig.1C). The formation of these products was almost completely inhibited when 2 μM SC-26196 was present in the incubation medium (Fig. 1D). Similarly, substantial amounts of radiolabeled 20:3, 20:4, and 22:4 were present in the human astrocytes after a 24-h incubation with [1-^{14}C]18:2n-6

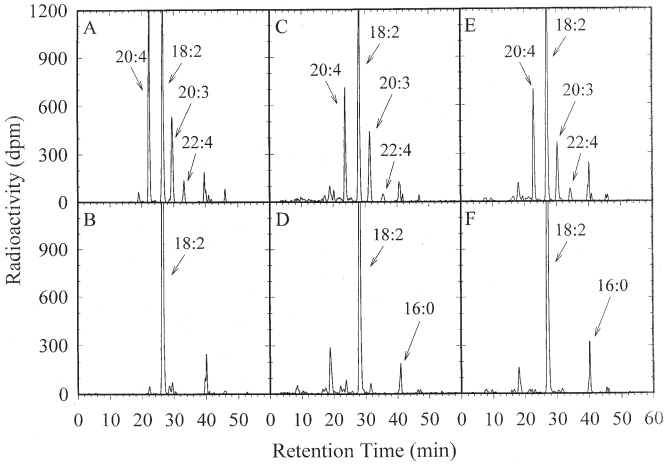

FIG. 1. Effect of SC-26196 on the metabolism of linoleic acid. Experimental cultures were incubated initially for 3 h with 2 μM SC-26196 dissolved in DMSO at 37°C, and control cultures were incubated with an equivalent amount of DMSO alone. At the end of the 3-h incubation, 2 μM [1-^{14}C]18:2n-6 was added, and the incubation was continued for 24 h at 37°C. SC-26196 remained in the medium of the experimental cultures during the incubation with the [1-^{14}C]18:2n-6. After the cells were washed, the lipids were extracted and methylated, and the radiolabeled FAME were assayed by RP-HPLC with an on-line flow scintillation detector. The HPLC tracings shown are from: (A) a control HSF culture, and (B) an HSF culture treated with SC-26196; (C) a control human coronary artery smooth muscle culture, and (D) a smooth muscle cell culture treated with 2 μM SC-26196; (E) a control human astrocyte culture, and (F) an astrocyte culture treated with 2 μM SC-26196. The HSF were incubated in a medium containing modified Eagle's MEM and 2% FBS, and the smooth muscle and astrocyte cultures were incubated in their respective Clonetics media containing 5% FBS. Abbreviations: SC-26196, 2,2-diphenyl-5-(4-{[(1*E*)-pyridin-3-yl-methylidene]amino}piperazin-1-yl)pentanenitrile; HSF, human skin fibroblasts; MEM, minimum essential medium.

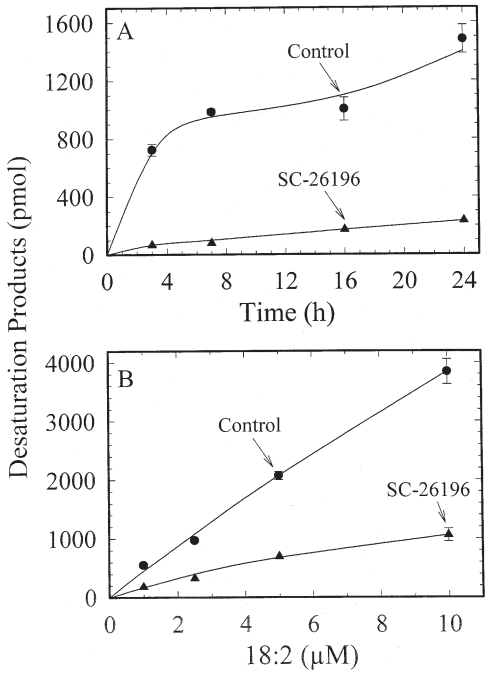

FIG. 2. Effect of time of incubation and FA concentration on the inhibition of linoleic acid desaturation produced by SC-26196 in HSF. The experimental design, analysis, number of replicates, and reproducibility were as described in Figure 1. In A, the culture medium contained 2 μM SC-26196, and the time of incubation after addition of 2 μM [1-^{14}C]18:2n-6 varied from 3 to 25 h; in B, the cultures were incubated with 1 μM SC-26196 and 1–10 μM [1-^{14}C]18:2n-6. Each data point represents the sum of the radiolabeled desaturation products contained in the hydrolyzed cell lipid extract as determined by HPLC, and the pmol values were calculated using the specific activity of the [1-^{14}C]18:2n-6 added to the culture medium. Standard error bars are shown where they are larger than the size of the data point. See Figure 1 for abbreviations.

(Fig. 1E), and the formation of these products also was almost completely inhibited by 2 μM SC-26196 (Fig. 1F). The vascular smooth muscle cells and astrocytes released a small amount of radiolabeled 20:3 and 20:4 into the culture medium, but this did not occur when SC-26196 was present in the incubation medium (data not shown). These results demonstrate that the effectiveness of SC-26196 in human cells is not limited to skin fibroblasts and suggest that the inhibitor may have a wide spectrum of action in human tissues.

The effect of time of incubation and FA concentration on the inhibition produced by SC-26196 in the HSF is shown in Figure 2. Each point represents the total amount of desaturation products detected by HPLC analysis of the hydrolyzed cell lipid extract. In the time-dependent study (Fig. 2A), the medium contained 2 μM SC-26196 and 2 μM [1-^{14}C]18:2n-6. A substantial reduction in the formation of desaturation products occurred within 3 h, the earliest time tested. More desaturation products were formed as the incubation progressed, but the percentage reduction produced by SC-26196 relative to the control cultures was similar throughout the 24-h period.

To determine whether the inhibition could be overcome by raising the FA concentration, the HSF were incubated for 24 h with 1 μM SC-26196 and increasing amounts of [1-^{14}C]18:2n-6. Although the total amount of desaturation products increased as the FA concentration was raised from 1 to 10 μM, the cultures incubated with SC-26196 converted substantially less of the 18:2 to desaturation products than the corresponding control cultures at each FA concentration (Fig. 2B). Therefore, a 10-fold excess of 18:2 was not sufficient to overcome the inhibitory effect of 1 μM SC-26196.

In additional experiments, the effect of SC-26196 on HSF viability was assessed to determine whether the decrease in the formation of Δ^6-desaturase products might be due to cytotoxicity. The calcein AM assay was used as an index of cell viability (14,15). Compared with control HSF cultures incubated for 24 h, those incubated under the same conditions with 2.5 and 10 μM SC-26196 exhibited a 5.1 ± 0.7 and 6.6 ± 0.9% reduction in viability, respectively (mean ± SEM, n = 8). Thus, the decrease in formation of 18:2n-6 desaturation products far exceeded the small reduction in cell viability produced by the inhibitor.

Selectivity of SC-26196 for Δ^6-desaturase. The conversion of 18:2n-6 to 20:4 requires two additional reactions after the initial Δ^6-desaturation, chain elongation, and Δ^5-desaturation

(1,2). It was not possible to determine whether SC-26196 had any effect on these reactions when the cells were incubated with [1-^{14}C]18:2n-6 because conversion to these products was almost completely eliminated. Therefore, we tested the effect of the inhibitor directly on the utilization of radiolabeled 18:3n-6 and 20:3n-6 in HSF.

The HSF cultures incubated for 24 h with 2 μM [1-^{14}C]-18:3n-6 converted 78% of the radioactivity incorporated into the cell lipids to 20-carbon products. In the corresponding incubation with 2 μM SC-26196, the 20-carbon products contained 82% of the incorporated radioactivity, indicating that the inhibitor did not affect the chain-elongation reaction (data not shown).

SC-26196 did not inhibit the conversion of 20:3n-6 to 20:4 (Fig. 3). Control HSF incubated for 24 h with 2 μM [1-^{14}C]-20:3n-6 converted 33% of the radioactivity present in the cell lipids to 20:4n-6 (Fig. 3A), and those treated with SC-26196 converted 35% (Fig. 3B). To be certain that this Δ^5-desaturation reaction was susceptible to inhibition in HSF, we tested the effect of anthranilicanilide on the conversion of 20:3n-6 to 20:4. Anthranilicanilide is a selective Δ^5-desaturase inhibitor that has an IC_{50} of 0.04 μM in rat liver microsomes (10,11). The addition of 2 μM anthranilicanilide reduced the conversion of 2 μM [1-^{14}C]20:3n-6 to 20:4 to 2.7% (Fig. 3C), a decrease of >90% compared with the control cultures. Thus, like Δ^6-desaturation, the Δ^5-desaturation reaction is susceptible to inhibition in HSF, but it is not affected by SC-26196. Taken together, these results demonstrate that SC-26196 reduces the conversion of 18:2n-6 to 20:4 in HSF by inhibiting the Δ^6-desaturase reaction.

Effect of SC-26196 on n-3 PUFA metabolism. To determine whether SC-26196 was effective against n-3 PUFA substrates, we investigated its effect on the metabolism of 18:3n-3 in HSF. The results are shown in Figure 4. When control HSF were incubated for 24 h with 2 μM [1-^{14}C]18:3n-3, 57% of the cell lipid radioactivity was present in three products, 20:5, 22:5, and 22:6 (Fig. 4A). The conversion of 18:3n-3 to these products requires Δ^6-desaturation (1,2). The addition of 2 μM SC-26196 reduced the amount of [1-^{14}C]18:3n-3 radioactivity converted to these products to 12%, an 80% reduction, whereas the amounts remaining in 18:3 and converted to its chain-elongated product, 20:3, increased threefold (Fig. 4B).

The effect of SC-26196 on 22:5n-3 metabolism also was investigated. The main radiolabeled products synthesized from 2 μM [3-^{14}C]22:5n-3 by the control HSF were 20:5 and 22:6, accounting for 22 and 20%, respectively, of the radioactivity contained in the cell lipids (Fig. 4C). When 2 μM SC-26196 was added, only 5% of the cell lipid radioactivity was converted to 22:6, a 75% reduction. By contrast, the amount retroconverted to 20:5 increased to 27% (Fig. 4D).

To determine whether the reduction in 22:6 was due to inhibition of the Δ^6-desaturation of the 24-carbon intermediate that forms when n-3 PUFA precursors are converted to DHA (1–4), we investigated the effect of SC-26196 on the metabolism of 24:5n-3. The HPLC results are shown in Figure 5.

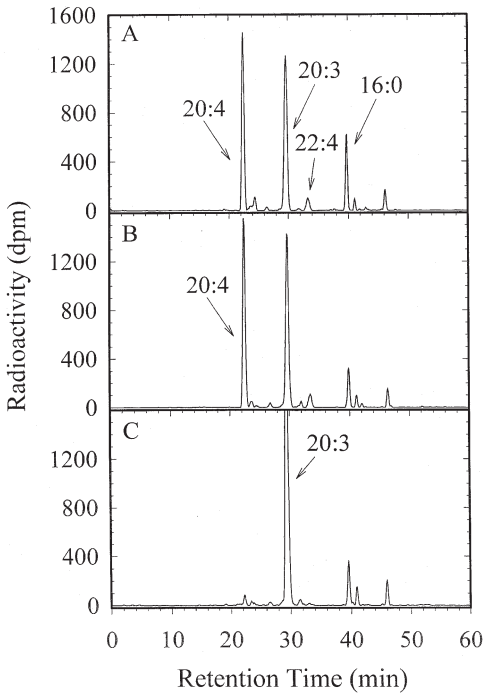

FIG. 3. Effect of Δ^6- and Δ^5-desaturase inhibitors on the metabolism of dihomo-γ-linolenic acid. The experimental design was as described in Figure 1, except that the HSF were incubated with 2 μM [1-^{14}C]-20:3n-6. The HPLC tracings shown are from cultures incubated with: (A) 20:3n-6 alone; (B) 20:3n-6 plus 2 μM SC-26196; and (C) 20:3n-6 plus 2 μM anthranilicanilide (CP-74006). Single tracings are shown, but similar chromatograms were obtained from two additional cultures in each case. See Figure 1 for abbreviation.

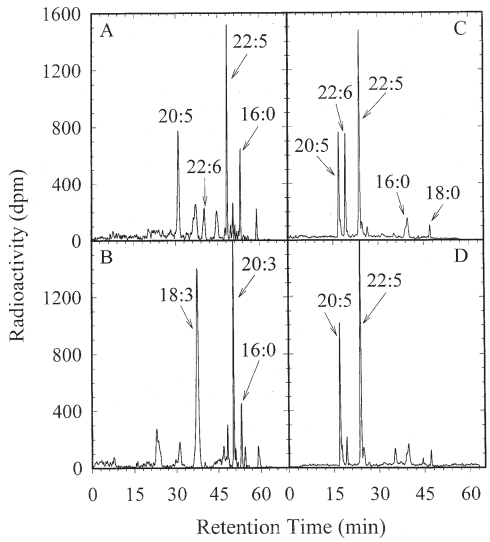

FIG. 4. Effect of SC-26196 on the metabolism of α-linolenic and docosapentaenoic acids in HSF. The experimental design and analysis were as described in Figure 1, except that the cells were incubated with: (A) 2 μM [1-^{14}C]18:3n-3; (B) 2 μM [1-^{14}C]18:3n-3 plus 2 μM SC-26196; (C) 2 μM [3-^{14}C]22:5n-3; and (D) 2 μM [3-^{14}C]22:5n-3 plus 2 μM SC-26196. Single tracings are shown, but similar chromatograms were obtained from two additional cultures in each case. See Figure 1 for abbreviations.

When the HSF were incubated for 24 h with 2 μM [3-^{14}C]-24:5n-3, 55% of the radioactivity incorporated into the cell lipids was converted to 22:6 (Fig. 5A). When 2 μM SC-26196 was added, only 9% of the 24:5n-3 was converted to 22:6, an 84% decrease (Fig. 5B). To determine whether the inhibitory effect occurred on the desaturation of 24:5n-3 or the retroconversion of the resulting product, we also investigated the effect of SC-26196 on the conversion of 24:6n-3 to 22:6. Control cultures incubated for 24 h with 2 μM [3-^{14}C]24:6n-3 converted 68% of the radioactivity incorporated into the cell lipids to 22:6 (Fig. 5C), whereas 64% of the incorporated radioactivity was converted to 22:6 by the corresponding cultures containing 2 μM SC-26196 (Fig. 5D). Taken together, these results indicate that SC-26196 reduces the conversion of 22:5n-3 to 22:6 by inhibiting the Δ^6-desaturation of 24:5n-3, not the retroconversion of 24:6.

Effect of SC-26196 concentration. The effectiveness of SC-26196 in inhibiting the desaturation of the 18- and 24-carbon PUFA substrates was compared, and the results are shown in Figure 6. The HSF cultures were incubated with 2 μM [1-^{14}C]18:3n-3 (Fig. 6A) and 2 μM [3-^{14}C]24:5n-3 (Fig. 6B) in the presence of increasing concentrations of SC-26196. Each point represents the sum of the Δ^6-desaturated FA products contained in the HSF, as determined by HPLC analysis of the cell lipid extract. More inhibition occurred as the SC-26196 concentration increased, and the calculated IC_{50} values were 0.4 and 0.2 μM for the incubations with 18:3n-3 and 24:5n-3, respectively. In a similar experiment with 2 μM [1-^{14}C]18:2n-6, the calculated IC_{50} value was 0.1 μM (data not shown). These findings indicate that the effectiveness of SC-26196 in HSF is approximately similar for 18- and 24-carbon PUFA substrates.

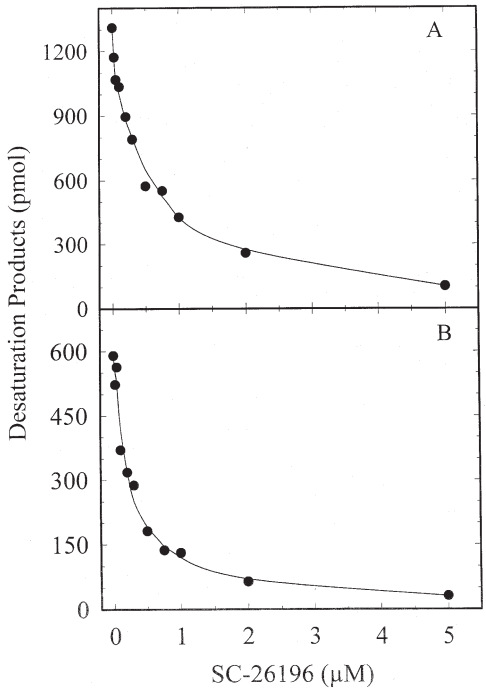

FIG. 6. Effect of SC-26196 concentration on the formation of radiolabeled FA desaturation products from α-linolenic and tetracosapentaenoic acids in HSF. The experimental design and analysis were as described in Figure 1, except that the SC-26196 concentration varied from 25 nM to 5 μM. The HSF were incubated with: (A) 2 μM [1-^{14}C]-18:3n-3; or (B) 2 μM [3-^{14}C]24:5n-3. Each data point represents the sum of the radiolabeled desaturation products in the hydrolyzed and methylated cell lipid extract determined by HPLC analysis. The pmol values were calculated from the specific activity of the radiolabeled FA added to the culture medium. See Figure 1 for abbreviations.

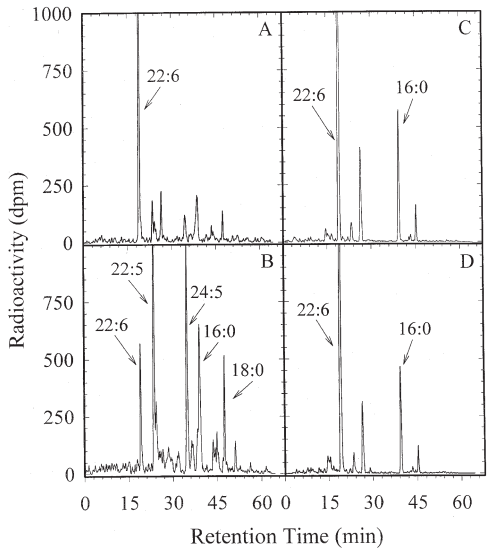

FIG. 5. Effect of SC-26196 on the metabolism of tetracosapentaenoic and tetracosahexaenoic acids in HSF. The experimental design and analysis were as described in Figure 1, except that the cells were incubated with: (A) 2 μM [3-^{14}C]24:5n-3; (B) 2 μM [3-^{14}C]24:5n-3 plus 2 μM SC-26196; (C) 2 μM [3-^{14}C]24:6n-3; and (D) 2 μM [3-^{14}C]24:6n-3 plus 2 μM SC-26196. Single tracings are shown, but similar chromatograms were obtained from two additional cultures in each case. See Figure 1 for abbreviations.

Recovery of Δ^6-desaturation capacity. Additional studies were done to assess the recovery of Δ^6-desaturation after exposure of the HSF to SC-26196. The cultures were incubated with a medium containing 2 μM SC-26196 for 3 h; after the inhibitor was removed, the incubation was continued for varying times in fresh medium containing 2% FBS. The Δ^6-desaturation activity was measured by adding radiolabeled FA at the end of the recovery period; each point in Figure 7 represents the sum of the radiolabeled Δ^6-desaturation products contained in the cell lipid extract as determined by HPLC. Two separate experiments were done, one with [1-^{14}C]18:2n-6 in which recovery was measured over a 24-h period after removal of the inhibitor, and the other with [3-^{14}C]24:5n-3 in which the recovery period was extended to 48 h. In both cases, a progressive increase in the formation of Δ^6-desaturation products occurred during the recovery period. When 2 μM [1-^{14}C]18:2n-6 served as the tracer, the formation of Δ^6-desaturation products by the HSF that had been exposed to SC-26196 was 65% as much at the end of the 24-h recovery period as in corresponding control cultures (Fig. 7A). When 2 μM [3-^{14}C]24:5n-3 served as the tracer, the recovery reached 75% of the control value at the end of the

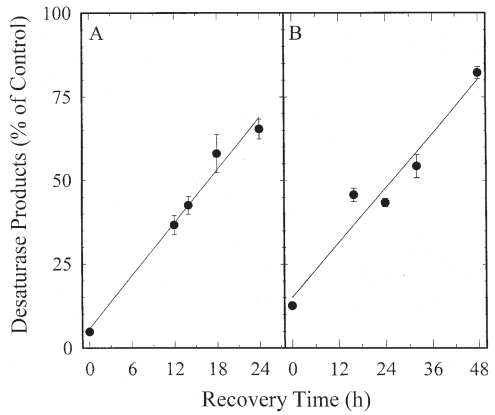

FIG. 7. Recovery of Δ^6-desaturation activity in HSF after removal of SC-26196 from the incubation medium. The data points indicate the percentage of desaturation products formed at various recovery times, relative to the amount formed by the control cultures that were not exposed to SC-26196. In A, the tracer used to measure Δ^6-desaturation was 2 μM [1-^{14}C]18:2n-6 and recovery was followed for 24 h; in B, the tracer used was 2 μM [3-^{14}C]24:5n-3 and recovery was followed for 48 h. The experimental design was the same in both cases. Three cultures that had not been exposed to the inhibitor were incubated for 24 h with the radiolabeled FA, and the desaturation products were determined by HPLC analysis. These results were taken as the control (100%) value. Three additional cultures were incubated with 2 μM SC-26196, and the radiolabeled FA was added to these cultures immediately, without any period of recovery. The desaturation products formed by these cultures were calculated as a percentage of the control values and are the points shown at "0" recovery time on the *x*-axis. The remaining cultures were incubated for 3 h with 2 μM SC-26196. After the medium containing the inhibitor was removed, sets of three cultures were incubated in fresh medium containing 2% FBS for each of the recovery times shown on the *x*-axis. At the end of the recovery period for each set of cultures, 2 μM radiolabeled FA was added and the incubation continued for 24 h. The sum of the radiolabeled desaturation products formed during this 24-h incubation was determined by HPLC, and the values are presented as a percentage of the control value. Each point is the mean of values obtained from three separate cultures, and the error bars indicate the SEM. See Figure 1 for abbreviations.

48-h recovery period (Fig. 7B). Thus, the HSF recovered the capacity to perform Δ^6-desaturation after removal of the inhibitor, but the recovery occurred slowly.

DISCUSSION

Obukowicz *et al*. (10) showed that SC-26196 is a potent and selective inhibitor of linoleic acid Δ^6-desaturation in rat liver microsomes and that it inhibits the conversion of linoleic acid to arachidonic acid in the liver when it is administered to mice. The present results extend these findings to human cells and provide additional information about the properties and effectiveness of the inhibitor. They demonstrate that SC-26196 does not inhibit the conversion of dihomo-γ-linolenic acid to arachidonic acid, a reaction mediated byΔ^5-desaturase. This indicates that SC-26196 is a selective inhibitor of Δ^6-desaturation in intact human cells, a finding that is consistent with the results in isolated rat liver microsomes (10). The present data show that SC-26196 also is effective against α-linolenic acid, the n-3 analog of linoleic acid, and that it inhibits the Δ^6-desaturation of the 24-carbon intermediate formed in the conversion of docosapentaenoic acid to DHA (3,4). Therefore, in addition to inhibiting arachidonic acid formation, SC-26196 will decrease the synthesis of EPA and DHA from n-3 PUFA precursors. The fact that SC-26196 is effective in three different kinds of human cells suggests that it most likely has a wide spectrum of action in human tissues.

The studies of Obukowicz *et al*. (10) demonstrated that SC-26196 had a direct inhibitory effect on the Δ^6-desaturase in isolated rat liver microsomes. We obtained IC_{50} values in HSF in the same range as those reported for SC-26196 in the incubations with microsomes, suggesting that a similar inhibitory process occurred in the intact cells. The slow recovery of Δ^6-desaturation after removal of SC-26196 from the culture medium suggests that the inhibitor either binds irreversibly to the enzyme or dissociates very slowly after it binds. Alternatively, the inhibitor may be resistant to inactivation or excretion by HSF and, as a result, remains active in the cells for a prolonged period even though it is no longer available in the extracellular fluid.

The finding that SC-26196 reduced the desaturation of α-linolenic acid to almost the same extent as linoleic acid is consistent with a large body of evidence indicating that the same Δ^6-desaturase acts on both of these 18-carbon PUFA (1,6). On the basis of previous studies with rat liver homogenates, it was concluded that the same enzyme catalyzes the Δ^6-desaturation of the 24-carbon intermediates formed in PUFA metabolism (19,20). In agreement with this conclusion, we observed previously that the desaturation of the 18- and 24-carbon PUFA was reduced to the same extent in mutant HSF deficient in Δ^6-desaturase (13). The present finding that SC-26196 inhibits the Δ^6-desaturation of 24:5n-3 in HSF with an IC_{50} value in the same range as those for linoleic and α-linolenic acids also suggests that the 18- and 24-carbon PUFA substrates are acted on by a single Δ^6-desaturase. Similarly, the observation that the recovery of Δ^6-desaturation after removal of the inhibitor occurred at roughly similar rates when either [1-^{14}C]18:2n-6 or [3-^{14}C]24:5n-3 was used as the tracer provides further support for the conclusion that the same enzyme acts on the 18- and 24-carbon PUFA substrates.

In contrast to these results, data obtained with two human malignant cell lines have been interpreted to indicate that separate Δ^6-desaturases act on the 18- and 24-carbon PUFA (21). Molecular evidence also suggests the possibility that there may be two human Δ^6-desaturases. A third PUFA desaturase gene designated FA desaturase-3 (FADS3) is present in the 1.4-mb region of human chromosome 11q12-q13.1, which contains the Δ^6- and Δ^5-desaturase genes (8). The possibility that FADS3 is a second Δ^6-desaturase gene was discounted initially because the coding sequence has only 60–70% homology with FADS2 (8). However, if the FADS3 gene product is selective for 24-carbon PUFA substrates, this difference might be explained by the considerable difference in size between the 18- and 24-carbon PUFA. Although the present results do not exclude the possibility of separate Δ^6-desaturases

for 18- and 24-carbon PUFA, the fact that the SC-26196 IC_{50} values obtained for the 18- and 24-carbon PUFA are so similar makes it unlikely because the two enzymes would have to interact almost identically with the inhibitor.

The fact that SC-26196 inhibits the synthesis of EPA and DHA, the biologically active members of the n-3 PUFA series, is unlikely to cause any serious problems in mammalian organisms. Although EPA can be converted to eicosanoids that have anti-inflammatory and antithrombotic properties (22,23), very little EPA is normally present in the tissues and it likely is not biologically essential (24). On the other hand, DHA is necessary for normal development and function of the central nervous system (3,25–33). Some DHA is synthesized from n-3 PUFA precursors in the liver (34–37), microvascular endothelium (38), and astrocytes (39,40). However, these sources may not be essential because mammalian cells readily incorporate DHA when it is available in the extracellular fluid (41–43). Similarly, the brain can effectively utilize DHA present in the diet (44–46).

In conclusion, the present results demonstrate that SC-26196 is a potent, selective, and long-acting Δ^6-desaturase inhibitor that is effective against 18- and 24-carbon PUFA substrates in human cells. In addition to suppressing the conversion of linoleic acid to arachidonic acid, SC-26196 inhibits the conversion of n-3 PUFA precursors to EPA and DHA. The similarity in IC_{50} values for these substrates is consistent with data indicating that a single enzyme catalyzes the Δ^6-desaturation of 18- and 24-carbon PUFA (13,20). Furthermore, the fact that this selective Δ^6-desaturase inhibitor considerably reduces the conversion of docosapentaenoic acid to DHA provides additional support for the mechanism proposed by Sprecher and co-workers that the Δ^4-double bond in DHA is formed by the Δ^6-desaturation of a 24-carbon intermediate (1–4).

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Why Is Carbon from Some Polyunsaturates Extensively Recycled into Lipid Synthesis?

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ABSTRACT: We summarize here the evidence indicating that carbon from α-linolenate and linoleate is readily recycled into newly synthesized lipids. This pathway consumes the majority of these fatty acids that is not β-oxidized as a fuel. Docosahexaenoate undergoes less β-oxidation and carbon recycling than do α-linolenate or linoleate, but is it still actively metabolized by this pathway? Among polyunsaturates, arachidonate appears to undergo the least β-oxidation and carbon recycling, an observation that may help account for the resistance of brain membranes to loss of arachidonate during dietary deficiency of n-6 polyunsaturates. Preliminary evidence suggests that *de novo* lipid synthesis consumes carbon from α-linolenate and linoleate in preference to palmitate, but this merits systematic study. Active β-oxidation and carbon recycling of 18-carbon polyunsaturates does not diminish the importance of being able to convert α-linolenate and linoleate to long-chain polyunsaturates but suggests that a broad perspective is required in studying the metabolism of polyunsaturates in general and α-linolenate and linoleate in particular.

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Evidence is emerging from several laboratories that β-oxidation normally consumes the majority of linoleate (LA; 18:2n-6) and α-linolenate (ALA; 18:3n-3) intake. This evidence takes several forms. Tracer and whole-body fatty acid balance studies show that β-oxidation of LA and ALA normally accounts for 65–85% of their intake, a figure that can rise to >100% during energy deficit. Among the common dietary fatty acids, ALA is a preferred substrate for β-oxidation in isolated mitochondria and for ketogenesis in isolated hepatocytes. Because ketones are not only important fuels but also the main substrates for lipid synthesis in neonates, β-oxidation of LA and ALA at this stage in life appears to have two significant end points, i.e., (i) use as a fuel, and (ii) use as ketogenic substrates for lipid synthesis. This evidence has been reviewed in detail elsewhere (1,2). The highlights of those reviews, including more recent publications and some recent unpublished results from our laboratory, are described here.

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Abbreviations: ALA, α-linolenate; DHA, docosahexaenoate; HMG, 3-hydroxy-3-methylglutaryl; LA, linoleate; LC-PUFA, long-chain polyunsaturated fatty acids.

The question is no longer: Are LA and ALA metabolized to a significant extent *via* a "polyunsaturated fatty acid (PUFA)-independent" route? Rather, it is: *Why* are LA and ALA extensively "carbon recycled" into lipid synthesis, especially in neonates? Compared with other common fatty acids, which are also possible substrates for ketogenesis, does the apparently preferred use of LA and ALA confer any advantages or benefits, or does it have a negative effect on the synthesis of long-chain polyunsaturates (LC-PUFA)? Ultimately, can we learn anything about PUFA metabolism by further evaluating this substantial "PUFA-independent" pathway of LA and ALA metabolism?

TISSUE DISTRIBUTION OF POLYUNSATURATES

Until about 10 yr ago, relatively little was known about the whole-body utilization of PUFA such as ALA or LA. Rather, most studies focused on developing an understanding of how ALA and LA fulfill their important roles in metabolism *via* conversion to LC-PUFA. Intuitively, one can estimate that because tissue levels of LA or ALA change little in adult animals or humans of stable body weight, most of the LA or ALA consumed must be β-oxidized as a fuel. Less would be β-oxidized in rapidly growing young animals or, presumably, during pregnancy and lactation. We were surprised to learn that, gram for gram, PUFA were considered to contribute more to whole-body fatty acid oxidation in humans than saturates, i.e., PUFA were more easily β-oxidized than saturates (3). This prompted our studies addressing directly the utilization of PUFA using whole-body fatty acid balance methodology (4,5). We have continued with these studies, and others (6–8) are using similar quantitative methods to investigate broader aspects of LA and ALA metabolism.

It is becoming clear from work with rodent models that when LA and ALA are consumed at about the recommended intake, they normally *accumulate* primarily in skin, muscle, and adipose tissue (5,7). Accumulation is not the same as tissue concentration. In rats, tissue concentration of LA is higher in many organs, particularly liver, than it is in adipose tissue or skin, but its accumulation, measured as *change in tissue content over time* [*mg/(organ·unit time)*] favors skin, adipose tissue, and skeletal muscle. Given that adipose tissue is primarily a fuel store, and that skeletal muscle is a major energy-

consuming tissue, these observations indirectly tell us that most ALA and LA is β-oxidized.

β-OXIDATION OF POLYUNSATURATES

In addition to *in vitro* methods, two *in vivo* approaches have been used to study β-oxidation of PUFA, i.e., (i) whole-body fatty acid oxidation relative to intake, and (ii) tracer oxidation. Each of these *in vivo* approaches has advantages and disadvantages. The whole-body method requires quantitative measurement of fatty acid intake, accumulation in the entire body, and excretion. Its primary usefulness is to study β-oxidation or whole-body disappearance of LA and ALA because neither LA nor ALA is synthesized by animals unless their 16-carbon analogs are present in the diet. Hence, unlike for other fatty acids, their synthesis normally does not confound the whole-body balance data. Thus, fatty acid disappearance = intake – (excretion + accumulation). If no LC-PUFA are present in the diet, the net percentage conversion of LA or ALA to their respective LC-PUFA is easily determined because the rise in body LC-PUFA over the study period comes only from stored or consumed LA or ALA. Only *net* LC-PUFA synthesis can be determined because LC-PUFA themselves also undergo some β-oxidation, which cannot be distinguished from ALA oxidation. In addition to being able to measure whole-body conversion of LA or ALA to LC-PUFA, the whole-body fatty acid balance method has the unique advantage of being able to demonstrate when oxidation *exceeds* intake, which occurs during energy deficit (4,9). No other method can provide this information.

However, the proportion of LA or ALA oxidation lost *via* β-oxidation of LC-PUFA cannot easily be determined using the whole-body fatty acid balance method. Also, although LA or ALA "disappearance" indicates that these two fatty acids are no longer present as such in the body, this method does not tell us what proportion has been completely metabolized to CO_2 or whether their carbon is still present in the body in a "non-PUFA" form, e.g., in lipids or other molecules synthesized *de novo*. That information is uniquely available using a tracer.

^{13}C or ^{14}C is the most appropriate tracer with which to measure LA or ALA metabolism *in vivo*. The necessary equipment for ^{13}C analysis (isotope ratio mass spectrometer) is expensive and requires continuous technical support. The breath samples required to measure oxidation of labeled LA or ALA by isotope ratio mass spectrometry are much easier to collect and analyze than are quantitative tissue lipid extractions. In any event, the results obtained for oxidation of ^{13}C- or ^{14}C-LA are similar to those of whole-body fatty acid balance (5,10).

Whether done *in vitro* or *in vivo*, studies on β-oxidation of LA or ALA are in good agreement regarding the rank order of their oxidation compared with other common dietary long-chain fatty acids (Table 1). Only the *in vivo* studies give a whole-body perspective. Whether the model is the rat or the human, the data are similar, i.e., LA and ALA oxidation is normally 65–85% of intake, which exceeds that of all other common dietary fatty acids with the exception of oleate (18:1n-9). In most of these studies, the rate of ALA oxidation exceeded that of LA by 25–50%. Taken collectively, these various approaches have, to date, left no doubt that β-oxidation is *always* the predominant route of LA and ALA utilization in the body. They have also shown that β-oxidation of LA or ALA during energy deficit (fasting, long-term weight loss) or zinc deficiency can exceed their respective intake by a factor of at least 2 in both humans and rats (4,9,11), making tissue loss of LA or ALA under these conditions faster than *via* their elimination from the diet (traditional deficiency studies).

TABLE 1
Overview of Studies Reporting β-Oxidation of Common Dietary Fatty Acids[a,b]

Reference	SA	PA	OA	EA	LA	ALA
Humans, *in vivo* (45)	70	87	106	127	100	146
Humans, *in vivo* (46)	30	—	160	—	100	—
Humans, *in vivo* (47)	—	—	122	143	100	169
Rat, *in vivo* (48)	—	50	—	—	100	—
Rat, *in vivo* (10)	49	63	116	—	100	135
Rat, *in vivo* (49)	21	53	—	100	—	—
Rat, liver mitochondria (50)	—	—	85	—	100	140
Rat, liver mitochondria (51)[c]	29	75	54	—	100	157
Rat, liver mitochondria (52)	4	7	15	—	100	—
Rat, perfused heart (53)	—	94	93	72	100	113
Rat serum, liver and brain (54)[d]	—	109	—	—	100	333
Catfish, *in vivo* (55)	—	—	—	—	100	161
Mean	34	67	94	114	100	191

[a]Each study used ^{14}C- or ^{13}C-labeled fatty acids and all reported data for linoleic acid and at least one of the other fatty acids shown. Because different species, fatty acids, or methodology were used, the data within each study were normalized to values obtained for linoleic acid (100%).
[b]Abbreviations: SA, stearic acid; PA, palmitic acid; OA, oleic acid; EA, elaidic acid; LA, linoleic acid; ALA, α-linolenic acid; —, not reported.
[c]Oxidation of eicosapentaenoic acid was equivalent to that of palmitic acid.
[d]Serum, liver, and brain values were pooled; data were reported as water-soluble ^{14}C.

KETOGENESIS FROM LA AND ALA

Were it not for the pioneering observations in 1975 on the appearance of ^{14}C from LA and ALA in brain palmitate, oleate, and cholesterol [as well as in the expected products, arachidonate (20:4n-6) and docosahexaenoate (DHA; 22:6n-3), respectively] in suckling rats (12–14), we might still be directing our attention concerning β-oxidation of PUFA only toward their use as fuels. However, those reports and others that followed (15–17) using the ^{14}C and ^{13}C methodology made it abundantly clear that a significant component of the carbon from LA and ALA that was not completely β-oxidized was recovered in lipids synthesized *de novo*.

Carbon from an oxidized fatty acid is incorporated into newly synthesized cholesterol or fatty acids *via* acetyl CoA or *via* the ketone body, acetoacetate (18). Labeled carbon in acetyl CoA that comes from an oxidized fatty acid can leave the mitochondrion as citrate. Citrate can be converted back to acetyl CoA in the cytosol where it is a substrate for fatty acid

synthesis. Labeled acetyl CoA can also be converted to acetoacetyl CoA in the cytosol and then to 3-hydroxy-3-methylglutaryl (HMG)-CoA, mevalonate, and cholesterol. Mitochondrial acetoacetate is also metabolized to its more stable and more commonly measured analog, β-hydroxybutyrate, which must be converted back to acetoacetate before being used in lipid synthesis. Acetoacetate can also be decarboxylated to acetone, which can potentially donate carbon to glucose synthesis but does not appear to be a lipid substrate. It is noteworthy that mitochondrial HMG appears dedicated to ketone body synthesis, whereas this substrate in the cytosol is used in cholesterol synthesis.

Two experimental approaches demonstrate that LA and ALA are ketogenic. First, isolated rat hepatocytes incubated with different fatty acid substrates show that LA and ALA are more ketogenic than oleate (19). The ketogenic capacity of these rat hepatocytes was highest early in the suckling period, suggesting that ketogenesis from PUFA may be more important during suckling than later in life. Second, we reported that perchloric acid extracts of gut, brain, and liver (all of which contain ketone bodies) are labeled 3–24 h after dosing with ^{13}C-ALA (20). These studies provide indirect proof that carbon recycling of LA or ALA into lipids synthesized *de novo* can proceed *via* ketone bodies; they do not establish whether acetate or ketone bodies are, in fact, the predominant intermediate in these experiments. In my view, it is not particularly important to prove whether acetate or acetoacetate is the predominant intermediate between LA or ALA and lipid synthesis. However, knowing that ketone bodies are probably intermediates in carbon recycling from LA or ALA gives us a blood metabolite to monitor and a mechanism that accounts for the recycling of the PUFA carbon skeleton into lipids synthesized *de novo*.

CARBON RECYCLING VS. CONVERSION TO LC-PUFA

Given the importance of and widespread interest in measuring the conversion of LA or ALA to their respective LC-PUFA, a logical point of reference is to compare carbon recycling of LA or ALA to their conversion to LC-PUFA. This gives us a quantitative indication of the relative utilization of LA or ALA in these "competing" pathways, but by no means tells us about the relative *importance* of LA or ALA being utilized in one or the other pathway. A few studies provide data on the comparative utilization of ALA or LA for LC-PUFA or *de novo* lipid synthesis in species ranging from the rat, to rhesus, to catfish. This comparison has most commonly been reported for brain where it is clear that in the fetal or early postnatal period, carbon recycling from ALA consumes 5–8 times the carbon that goes into brain DHA synthesis (Table 2). Much more carbon recycling seems to occur in neonatal rat lung but, except in catfish, less seems to occur in liver (Tables 2,3).

The relative degree of carbon recycling also depends on metabolites being compared and the units of measure. DHA is considered the most significant n-3 LC-PUFA especially in the brain, and labeled DHA is more commonly measured than the intermediate LC-PUFA, EPA and n-3-docosapentaenoic acid (DPA). However, DHA is also the least labeled if ALA is the labeled precursor. Conversely, tracer appearing in cholesterol, palmitate, stearate, or oleate is relatively easy to detect and accounts for most of the newly synthesized lipid. Data for tracer metabolism are usually given as a percentage of dose, or units of tracer/organ, or per gram of organ weight. In Table 3, we show both mass of tracer/organ (ng ^{13}C/lung) and mass of tracer/unit of tracee (ng ^{13}C/mg individual lung lipid). The latter is equivalent to specific activity, which corrects for differences in the pool size of the lipids incorporating the tracer in question. Because the individual pools of non-PUFA lipids into which PUFA carbon can be recycled are generally much larger than the n-3 or n-6 LC-PUFA, using "specific activity" units may reduce bias in determining the relative use of LA or ALA carbon in these two pathways.

TABLE 2
Summary of Various Studies Reporting Incorporation of Labeled Carbon from ALA into DHA vs. Products of *de novo* Lipogenesis

Reference	Tissue	Species	Time[a]	DNL/DHA[b]
12	Brain	Rat	48 h	8.3
13,14	Brain	Rat	48 h	5.0
55	Liver	Catfish[c]	4 h	11.8[d]
16[e]	Brain	Rhesus	5 d	5.6
	Liver	Rhesus	5 d	1.4
	Retina	Rhesus	5 d	1.5
19	Brain	Rat	48 h	7.1
	Liver	Rat	48 h	1.1

[a]Time after dosing.
[b]Products of *de novo* lipid (DNL) synthesis (fatty acids and cholesterol).
[c]Two catfish species were reported but the data are combined here.
[d]Carbon recycling from ^{14}C linoleate into products of *de novo* lipid synthesis compared with arachidonate gave a similar result as for carbon recycling from ^{14}C-α-linolenate.
[e]Data for carbon recycling into cholesterol were not reported; only data for carbon recycling into saturated and monounsaturated fatty acids appear. In this table, only this report (16) compared 18-carbon polyunsaturated fatty acid (PUFA) recycling vs. incorporation into long-chain (LC)-PUFA in the intentional absence of dietary LC-PUFA.

PROBING CARBON RECYCLING OF POLYUNSATURATES

There are some obvious variables to probe in determining the relative biological significance of carbon recycling from LA or ALA. These include the effects of (i) age, (ii) the presence or absence of LC-PUFA, and (iii) high or low intakes of LA or ALA. In addition, carbon recycling between different long-chain fatty acids should be compared, as should the end products, i.e., fatty acids vs. cholesterol. We are unaware of a systematic comparison of carbon recycling at different ages but it has been reported in fetal, neonatal, adult nonpregnant, and pregnant animals (11–17). Hence, it appears to be an integral feature of PUFA metabolism throughout the life cycle.

Three similar studies demonstrating significant carbon recycling from ALA have been done in suckling rats, i.e., in the presence of continuous intake of LC-PUFA from milk. The presence of LC-PUFA such as DHA appears to inflate the amount of carbon recycling from ALA because there is no need to make DHA

TABLE 3
^{13}C Incorporation into Palmitate, Oleate, Total Sterols, and n-3 Polyunsaturate of Suckling Rat Lung[a]

	3 h	12 h	48 h	8 d
	(ng ^{13}C/lung)[b]			
Palmitate	172 ± 6	372 ± 97	263 ± 14	46 ± 2
Oleate	9 ± 0.3	9 ± 6	16 ± 2	13 ± 1
Total sterols	214 ± 11	376 ± 50	636 ± 35	350 ± 7
α-Linolenate	7797 ± 942	8762 ± 2090	2391 ± 28	503 ± 53
Eicosapentaenoate	114 ± 43	67 ± 20	150 ± 10	14 ± 0.2
Docosapentaenoate	20 ± 0.2	38 ± 16	159 ± 7	46 ± 1
Docosahexaenoate	2 ± 0.2	5 ± 0.3	49 ± 0.3	30 ± 0.2
	(ng ^{13}C/mg lipid)[c]			
Sum of saturates and monounsaturates	660 ± 23	1220 ± 318	400 ± 21	70 ± 3
Total sterols	260 ± 50	450 ± 60	180 ± 10	30 ± 1
Docosahexaenoate	20 ± 2	50 ± 3	180 ± 10	30 ± 2

[a]After an oral dose of 3.5 mg of [U-^{13}C]-α-linolenate was given to 6-d-old rats, the incorporation was monitored from 3 h to 8 d postdose.
[b]Total lung mass (mean ± SD, n = 4/group).
[c]Specific enrichment (equivalent to specific activity); lipid = palmitate + stearate + oleate for sum of saturates and monounsaturates, cholesterol + desmosterol for total sterols, or docosahexaenoate itself.

from ALA; therefore, ALA carbon appears more readily in alternative pathways (12–14,20). However, in the absence of DHA intake, neonatal baboons recycle about five times more ALA carbon into brain fatty acids synthesized *de novo* than they put into brain DHA (17; Table 2). Hence, carbon recycling of ALA still occurs when DHA is absent, i.e., this pathway of ALA metabolism does not depend on the presence of DHA.

A direct comparison of ALA recycling in the presence or absence of DHA has been done *in vitro* using cultured rat astrocytes (21). The presence of DHA in that model *increased* ^{14}C-ALA recycling into palmitate, stearate, and oleate. Unfortunately, these data were not precisely quantified, making it difficult to say how much of an increase actually occurred. It is predictable that the presence of DHA would increase carbon recycling of ALA. However, this *in vitro* report (21) confirms the *in vivo* report (17) that the absence of DHA does not prevent significant carbon recycling of ALA into newly synthesized brain fatty acids. This demonstrates that carbon recycling from ALA into *de novo* lipid synthesis is an integral feature of ALA metabolism irrespective of concurrent DHA intake or availability. To our knowledge, the comparable assessment of the effect of arachidonate on LA recycling has not yet been done.

It is also important to know whether excess amounts of LA or ALA are the reason for carbon recycling of these fatty acids. We approached this by examining carbon recycling of LA during extreme LA deficiency (22,23). The rationale was that if carbon recycling of LA was a function of excess LA in tissues, this pathway should disappear when tissue LA levels are markedly below "normal" levels, i.e., during extreme LA deficiency. We found that ^{14}C from ^{14}C-LA actually appeared in sterols and in saturated or monounsaturated fatty acids in *increased* amounts in LA-deficient rats, partly as a result of a decrease in the amount of ^{14}C-LA being completely oxidized to $^{14}CO_2$. Hence, carbon recycling seems to be an obligatory route of LA metabolism in rats because it occurs even during extreme and prolonged LA deficiency.

There are few studies of carbon recycling of fatty acids other than LA or ALA. Nevertheless, this is an important comparison to gain a perspective on its relative importance for these two fatty acids because of our dependence on diet as their exclusive source (rather than some endogenous synthesis for fatty acids like for palmitate). Among fatty acids other than LA or ALA, and perhaps most interestingly, some DHA is clearly carbon recycled into newly synthesized brain fatty acids in neonatal primates (17). DHA was not as extensively recycled as ALA, but DHA recycling was similar to that of LA. In a rat model using ^{14}C recovered in water-soluble products as an index of carbon recycling, DHA was equally or more extensively recycled than LA or palmitate (15).

Carbon recycling of the other main LC-PUFA, arachidonic acid, has been reported not to occur in neonatal baboons (24). However, in young pigs, we observed low amounts of arachidonic acid recycling into palmitate (Table 4). It is too early to be certain, but these two reports in different species and under different rearing conditions make the provocative suggestion that there is significantly less carbon recycling of arachidonate than of DHA. Combined with preliminary data suggesting that low amounts of palmitate are recycled into brain cholesterol in suckling piglets (Cunnane, S., and Ryan, M.A., unpublished data), these results collectively suggest that the rank order of carbon recycling of long-chain fatty acids favors ALA over LA, DHA, or palmitate, and that AA recycling is the lowest of the fatty acids studied so far. This is a preliminary assessment and awaits a more complete comparison of all of these fatty acids in the same model. Nevertheless, lower carbon recycling of AA than DHA gives us a rationale for the following observations: (i) The lack of AA in an infant formula does not reduce infant brain AA relative to that in breast-fed infants, but a similar lack of DHA does reduce infant brain DHA (25,26); and (ii) the rat brain appears to retain more AA during extreme LA deficiency than it retains DHA during extreme ALA deficiency (23,27). These studies arenot directly comparable (infants vs. rats; one genera-

TABLE 4
^{13}C Enrichment in Liver and Brain Total Lipids 24 h After an Oral Dose of ^{13}C-Arachidonate Given to Young Pigs[a]

		ng ^{13}C/g tissue	^{13}C/mg fatty acid
16:0	Liver	24 ± 19	7 ± 4
	Brain	ND	ND
20:3n-6	Liver	1 ± 1	11 ± 6
	Brain	<1	3 ± 3
20:4n-6	Liver	145 ± 86	42 ± 14
	Brain	44 ± 18	15 ± 6
22:4n-6	Liver	2 ± 2	13 ± 7
	Brain	6 ± 6	5 ± 4
22:5n-6	Liver	2 ± 1	40 ± 35
	Brain	5 ± 4	6 ± 6

[a]Data are presented as means ± SD, $n = 6$. Each pig was administered orally 24.6 mg of [1-^{13}C]arachidonate emulsified in 5 mL of milk formula. The pigs were killed 24 h later and total lipid extracts of brain and liver were prepared. ^{13}C analysis was done by GC combustion isotope ratio MS. ^{13}C incorporation into liver palmitate is the evidence for carbon recycling from ^{13}C-arachidonate. The amount of carbon recycling relative to the conversion of ^{13}C-arachidonate to 22-carbon PUFA depends on the units chosen. ND, not determined.

tion of LA deficiency vs. three generations of ALA deficiency) but they are consistent with the more avid retention of AA compared with other brain PUFA, something that we suggest may depend in part on lower carbon recycling of AA.

The foregoing makes the case that β-oxidation and some amount of carbon recycling is an integral, indeed obligatory, component of LA and ALA metabolism in various animal models. Most, but not all, of the reports on carbon recycling of LA or ALA have used pregnant, fetal, or neonatal animals. The main exception is that of carbon recycling from ^{14}C-LA in extreme LA deficiency, in which the rats were 400- to 500-g adults (22,23). We are unaware of reports in which this question has been addressed in studies with infant or adult humans. Nevertheless, carbon recycling seems to be observed whenever the experimental design allows for it to be detected, reinforcing our impression of its widespread occurrence.

WHY ARE POLYUNSATURATES EXTENSIVELY β-OXIDIZED?

Given that PUFA are extensively β-oxidized and that carbon recycling occurs to a greater or lesser extent in all mammalian species and at all ages, it is reasonable to ask why this is so. Other essential nutrients such as indispensable amino acids are also oxidized and presumably must therefore also undergo some amount of carbon recycling. The carbon from other important molecules such as glucose has innumerable metabolic destinations. Hence, the carbon from many nutrients is widely distributed in other metabolites. For several reasons, we are not convinced that simply "participating in intermediary metabolism" is all there is to β-oxidation and carbon recycling of PUFA.

First, LA and ALA are clearly the most easily β-oxidized of the common dietary long-chain fatty acids (Table 1). If LA and ALA were simply participating in metabolism, we would not expect this hierarchy, especially because removal of their double bonds before cleaving off two carbons during their β-oxidation requires more enzyme-dependent steps than for oxidizing a saturated fatty acid. In theory, this should make their β-oxidation slower, not faster, than that of saturated fatty acids such as palmitate.

Second, both LA and ALA have longer-chain fatty acid products (AA and DHA, respectively) that are more important in membranes than either LA or ALA themselves. Nevertheless, LA and ALA compete with LC-PUFA for incorporation into the same membrane phospholipids. Because absorption from the gut does not particularly favor one PUFA over another, one way to make sure that LA and ALA do not compete excessively with AA or DHA for incorporation into membrane phospholipids would be to have a safe and effective way of getting rid of them, i.e., relatively rapid β-oxidation.

Third, LA, but more especially ALA, is quite easily peroxidized, at least in *in vitro* systems. Depending on the amount of PUFA consumed relative to the growth rate, neonates could have low antioxidant reserves; thus, active disposal of LA or ALA through β-oxidation could effectively reduce this potential risk of free radical toxicity. Such a problem (and solution) might explain more rapid β-oxidation but would not really explain why extensive carbon recycling of LA and ALA would also occur.

Fourth, rapid fatty acid β-oxidation leads to ketone body production, and LA and ALA are moderately ketogenic relative to other long-chain fatty acids (19). For reasons that are unknown, newborn infants are normally in a state of chronic, mild ketonemia (28). Ketone bodies are implicated in reducing the risk of seizure (see Ref. 29 for a review), to which infants are especially susceptible. Ketone bodies may be essential brain fuels in the fetal and neonatal period in many species including humans (30–32) and are the preferred substrates for brain lipid synthesis (33–37). Our data show that within 48 h of dosing in suckling rats, β-oxidation consumes >80% of ^{13}C-ALA (Fig. 1), and carbon recycling into brain cholesterol consumes 10–13 times more carbon from ALA than does DHA (Fig. 2). Hence, at least in neonates, β-oxidation of ALA is an efficient means of generating ketone bodies needed in other aspects of brain growth.

WHY EXTENSIVELY RECYCLE POLYUNSATURATES INTO NEWLY SYNTHESIZED LIPIDS?

The teleological question remains: Why recycle PUFA into newly synthesized lipids? Why oxidize *any* fatty acid to synthesize another one, or to synthesize cholesterol? We suggest three possible reasons.

First is the unique problem of brain lipid synthesis. The main brain lipid, cholesterol, is important for neuronal membrane structure, synaptogenesis (38), and normal expression of embryonic patterning proteins such as in hedgehogs (39). Several reports show that the mammalian brain probably imports little or no cholesterol (40–42). The same appears to be true for saturates and monounsaturates (43), though it is more controversial than for cholesterol. The problem is that the brain has a relatively

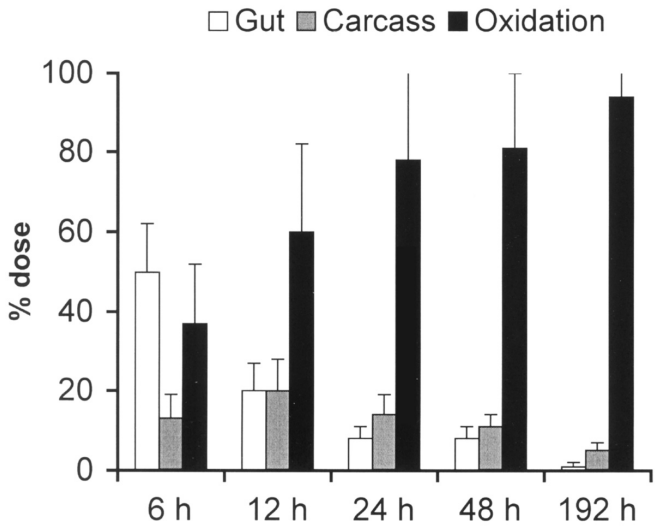

FIG. 1. Gut and carcass (whole body – gut) uptake compared with β-oxidation of a 3.5-mg oral dose of [U-^{13}C]α-linolenate in 6-d-old suckling rats. The rat pups remained with the dam throughout the study. Different rat pups were killed at the time points indicated (n = 4 ^{13}C tracer-dosed and saline-injected controls/time point). Time is given in hours after dosing. Total lipids in gut (including contents) and remaining carcass were extracted into chloroform/methanol, and ^{13}C was measured by isotope ratio MS. Maximal ^{13}C enrichment in carcass total lipids was 12 h postdose. Within 192 h of dosing (8 d), 94% of the tracer was no longer detectable in gut or carcass lipids, i.e., it was completely β-oxidized.

high amount of cholesterol, accumulates it rapidly, and must, apparently, synthesize it all endogenously. The appropriate enzymes are present in the brain so this is not a problem biochemically, but a water-soluble substrate is still needed. Glucose is the obvious candidate but, in fact, is a distant second candidate to ketone bodies as the carbon donor to brain lipid synthesis, at least in neonates (33–37). Hence, ALA and LA function as indirect substrates for *all* of the brain's lipid components (LC-PUFA, saturates, monounsaturates, cholesterol), not just LC-PUFA.

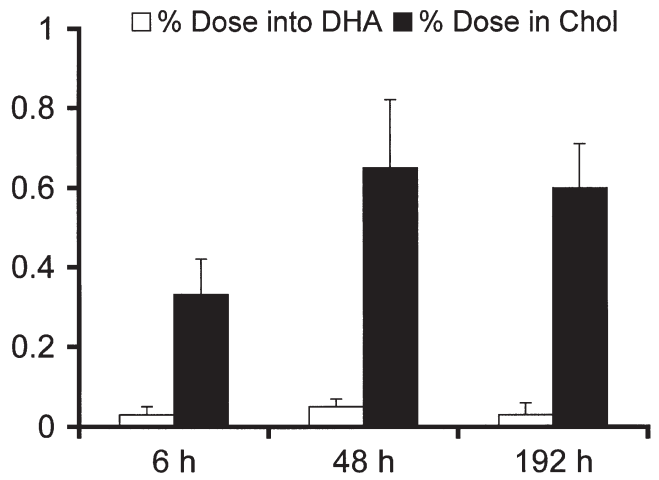

FIG. 2. Quantitative comparison (% dose) of ^{13}C appearing in whole-body cholesterol vs. whole-body docosahexaenoate (DHA) in 6-d-old suckling rats orally dosed with 3.5 mg of [U-^{13}C]α-linolenate. ^{13}C enrichment in cholesterol exceeded that in DHA by 10- to 13-fold at the time points shown. Experimental details are given in the legend to Figure 1.

Second is the important problem of balancing membrane lipid composition. Lipids vary widely in their ability to "rigidify" (stiffen) or "fluidize" (soften) membranes. PUFA tend to fluidize, whereas cholesterol tends to rigidify membranes, especially in model systems. These differences arise from the amount of intrinsic motion within the lipid molecule, with saturated fatty acids and cholesterol having less motion than PUFA. Lipid-rich microdomains (rafts), which are more or less concentrated in cholesterol, therefore affect the conformation of membrane proteins, receptors, and ion channel activity. Despite these opposite tendencies to disrupt membranes, diets that provide more or less PUFA have relatively little effect on membrane-bound enzymes. In part, this must be due to the fact that raising the PUFA content of a membrane also tends to raise its cholesterol content. Hence, the membrane compensates for a fluidizing influence of added PUFA by incorporating more cholesterol.

This capacity to minimize the disruptive effect of changing only one membrane component at a time is particularly important in the brain where there would seem to be relatively little margin for error in membrane composition to sustain normal neuronal function. Indeed, the brain appears to be the organ with the most resistance to changing membrane PUFA or cholesterol content during manipulation of dietary PUFA or cholesterol. This resistance is probably achieved by exerting nearly total control of membrane lipid composition *via* endogenous lipid synthesis. Nevertheless, markedly raising dietary DHA will raise brain DHA in young rats, just as markedly decreasing dietary n-3 PUFA will severely deplete brain DHA. Interestingly, raising dietary DHA from 0 to 1.6 weight % of the diet raises brain DHA three- to fivefold and, concomitantly, raises the brain cholesterol/phospholipid ratio by as much as 60% (44; Table 5). The increase in the brain cholesterol/phospholipid ratio no doubt occurs because of the rise in brain DHA and helps dampen perturbations in membrane function and neurotransmitter release that would occur if there were no compensation for the change in dietary DHA. The added cholesterol is synthesized endogenously, mostly from ketone bodies. PUFA such as ALA contribute carbon to a greater or lesser extent to this process. This is one example of why it would be advantageous for ALA to simultaneously contribute carbon to both DHA and cholesterol synthesis.

Third, there is a rapid demand for lipids at critical times in development, e.g., neonatal lung surfactant. ^{13}C-ALA is readily incorporated into suckling rat lung cholesterol and palmitate at a ratio exceeding that going into lung DHA of 3- to 200-fold depending on the units used (Table 3). Thus, a process such as carbon recycling may take advantage of available substrates to make certain products, e.g., using ALA carbon to make palmitate-rich lung surfactant, that are in high demand during key periods in development.

In conclusion, β-oxidation and carbon recycling are quantitatively dominant pathways of LA and ALA metabolism, especially during early development. Hence, they are integral to the utilization of these two 18-carbon PUFA. We have speculated

TABLE 5
Reduction in the Concentration of DHA and Cholesterol in Various Rat Brain Regions During Chronic, Multigenerational Deficiency of n-3 PUFA[a,b,c]

	Frontal cortex	Striatum	Hippocampus	Cerebellum
DHA in phosphatidylcholine				
CTL	3.5	3.0	3.0	5.0
DEF	0.7	0.8	0.5	1.1
DEF/CTL (%)	20.0	26.7	16.7	22.0
DHA in phosphatidylethanolamine				
CTL	20.9	22.0	21.7	20.7
DEF	3.9	4.8	4.1	5.5
DEF/CTL (%)	18.7	21.8	18.9	27.4
Cholesterol (μmol/100 g brain)				
CTL	5.15	13.21	12.97	4.59
DEF	4.47	8.46	9.84	3.84
DEF/CTL (%)	86.8	64.0	77.1	83.7

[a]*Source:* Reference 44.
[b]Abbreviations: CTL, control; DEF, chronic, multigenerational n-3 PUFA deficient; for other abbreviation see Table 2.
[c]DHA data are given as % composition.

on why this is so but, as yet, little hard evidence exists to support these speculations. Our reasoning is that there are limits to the optimal range for 18-carbon PUFA and LC-PUFA in membranes, beyond which membrane function is likely to be impaired. Hence, although it is desirable to be able to make DHA from ALA in the event that DHA is absent from the diet, it is also desirable to limit this conversion and have a means of disposing of excess ALA. Lipids such as cholesterol also have important roles, particularly in the brain, yet brain cholesterol synthesis appears largely if not completely restricted to the brain. Hence, again, it is desirable to maintain PUFA and cholesterol at a suitable ratio in brain membranes. We therefore interpret the biological utility of carbon recycling of 18-carbon PUFA, especially ALA, to be related to sustaining an optimal membrane content of lipids including not only LC-PUFA such as DHA but also cholesterol and saturated and monounsaturated fatty acids.

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Kinetic Analysis of the Selectivity of Acylcarnitine Synthesis in Rat Mitochondria

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ABSTRACT: Mitochondrial acylcarnitine synthesis is an obligatory step in the transport of cytosolic long-chain FA into the mitochondria. It is an important control point in the partitioning of cytosolic fatty acids to synthetic pathways or to mitochondrial β-oxidation. Mitochondrial carnitine palmitoyltransferase I (CPT I; EC 2.3.1.21) is the enzyme that catalyzes the transformation of long-chain fatty acylCoA esters to acylcarnitine. Additionally, the isoform of acylCoA synthetase (EC 6.2.1.3) found in mitochondria, which is in close proximity to CPT I on the outer membrane, may act in concert with CPT I to form acylcarnitines from cytosolic nonesterified FA (NEFA). The mitochondrial acylcarnitine synthesis pathway is exposed to multiple fatty acid substrates present simultaneously in the cell milieu, with each fatty acid present at varying pool sizes. The selectivity of this pathway for any particular fatty acid substrate under conditions of multisubstrate availability has not yet been tested experimentally. Our objective was to develop mathematical equations that make use of kinetic constants derived from single-substrate experiments to predict the selectivity of the acylcarnitine synthesis pathway under conditions in which two or more substrates are present simultaneously. In addition, the derived equations must be verifiable by experiment. Our approach was to begin with a Michaelis–Menten model that describes the initial rates of an enzyme system acting on multiple and mutually competitive substrates. From this, we derived equations expressing ratios of reaction rates and fractional turnover rates for pairs of substrates. The derived equations do not require assumptions concerning the degree of enzyme saturation. Using rat mitochondrial preparations and the NEFA substrate pairs, linolenic-oleic acids and palmitic-linoleic acids, we showed that the shape of the experimentally derived data on acylcarnitine synthesis fits the predictions of the derived model equations. We further validated the derived equations by showing that their predictions calculated from previously published kinetic constants were consistent with data from actual experiments. Thus, we are able to conclude that with respect to acylcarnitine synthesis, the fractional turnover rate of the linolenic acid pool would always be 2.9-fold faster than that of the oleate pool regardless of the pool size of either fatty acid. Similarly, the fractional turnover rate of the palmitate pool would always be 1.8-fold faster than that of the linoleate pool regardless of pool size. We extended our kinetic model to more than two mutually competitive substrates. Using previously published rate constants for eight physiologically relevant fatty acids, the derived model predicts that regardless of pool size of any of the fatty acids, the linolenate pool, whether as NEFA or as a CoA ester, would always have the highest fractional turnover rate with respect to acylcarnitine synthesis. Conversely, the stearate pool whether as NEFA or as CoA ester will have the lowest fractional turnover rate relative to all the other fatty acids.

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It has been repeatedly observed that α-linolenic acid (LN) relative to linoleic acid (LA) is underrepresented in various tissues and organs in the body regardless of the ratios of these two essential FA in the diet (1). Results *in vitro* suggest that this phenomenon is not due to selectivity of esterification pathways for LA over LN (2), nor to extensive metabolic conversion of LN to very long-chain PUFA (3); rather, it is due to the rapid β-oxidation of LN relative to other fatty acids (4–6). The rate of mitochondrial β-oxidation of fatty acids in general is increased by dietary LN, an effect likely to be the result of diet-mediated induction of the oxidative pathway enzymes (7). However, fine control of hepatic mitochondrial β-oxidation is exerted through carnitine palmitoyltransferase I (CPT I; EC 2.3.1.21) regardless of the nutritional and metabolic state of the animal (8).

CPT I regulates the β-oxidation of fatty acids through kinetic control of the conversion of CoA esters of fatty acids to acylcarnitines, an obligatory step in the transport of long-chain fatty acids from the cytosol into the mitochondria. Additionally, recent reports suggest that the isoform of acylCoA synthetase (EC 6.2.1.3), ACS5, found in the outer leaflet of mitochondrial membranes may channel cytosolic nonesterified fatty acids (NEFA) directly to CPT I for acylcarnitine synthesis (9), possibly through proximity to CPT I (10). These two enzymes acting in sequence form a pathway for the transformation of cytosolic NEFA to acylcarnitines. We previously showed that CPT I exhibits the fastest maximum turnover capacity (V_{max}) with LN as substrate relative to other fatty acids, whether the substrates are presented as NEFA or as acylCoA esters (11). The selectivity of CPT I for LN has since been confirmed by others (7,12). The central role of CPT I in the rate control of β-oxidation (8) and its presumed preferentiality for LN in a mixture of substrates provide an enzymatic rationale for the underrepresentation of LN in the body, i.e., the channeling of this fatty acid into β-oxidation in preference to esterification and metabolic conversion to higher derivatives. However, the notion that LN is a preferred substrate for acylcarnitine synthesis even in the presence of

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Abbreviations: ACS5, isoform of acyl CoA synthetase (EC 6.2.1.3); ARA, arachidonic acid; CPT I, carnitine palmitoyltransferase I; LA, linoleic acid; LN, α-linolenic acid; NEFA, nonesterified FA; OL, oleic acid; PA, palmitic acid; ST, stearic acid.

other fatty acid substrates has not yet been tested by experiment. This is a physiologically relevant question in view of the hypothesis that LN is an important source, through β-oxidation, of carbon units for brain metabolism (13) and in view of the fact that intracellular pool sizes of the different fatty acids can be easily manipulated through dietary means. We approached the problem by measuring initial rates of acylcarnitine synthesis in isolated mitochondria using pairs of NEFA substrates differentially labeled and present simultaneously in the assay mixture.

MATERIALS AND METHODS

Preparation of mitochondria and assay of acylcarnitine synthesis. The preparation of rat liver mitochondria from 250-g, fed, male Sprague–Dawley rats and CPT I assays were conducted as described previously (11). L-[^{3}H-*methyl*]-carnitine and unlabeled NEFA were used to assay rates of total acylcarnitine synthesis. Unlabeled carnitine, [^{3}H]-labeled oleic acid (OL; 15,000 dpm/nmol), [1-^{14}C]-labeled LN (4000 dpm/nmol), [^{3}H]-labeled palmitic acid (PA; 15,000 dpm/nmol), and [1-^{14}C]-labeled LA (12,000 dpm/nmol) were used to determine individual rates of acylcarnitine synthesis, based on the appearance of the corresponding radiolabeled acylcarnitines. Labeled carnitine was from Amersham (Oakville, Canada) and labeled fatty acids were from Dupont (Mississauga, Canada). Neat fatty acids were from Nu-Chek-Prep (Elysian, MN). In order to separate radioactive NEFA substrate from the radioactive acylcarnitine products, the *n*-butanol extracts of the reaction mixtures were mixed with an equal volume of $CHCl_3/CH_3OH$ (2:1, vol/vol), and the resulting azeotropic mixture of solvents removed under a stream of N_2. The residue was dissolved in $CHCl_3/CH_3OH/H_2O$ (2:1:0.8, by vol) and applied to precoated Whatman-type KL6D (Chromatographic Specialities, Brockville, Canada) silica-gel thin-layer plates. The plates were developed with $CHCl_3/CH_3OH/H_2O$/acetic acid (6:4:0.75:0.25, by vol) to separate the fatty acylcarnitines from excess NEFA, fatty acylCoA intermediates, and PC. The region corresponding to fatty acylcarnitines, visualized with I_2 vapors, was scraped and counted in a Packard (Downers Grove, IL) scintillation counter using double-label routines to calculate [^{3}H] and [^{14}C] dpm.

BSA binding of FA binary mixtures. Concentrations of unbound NEFA in equilibrium with the BSA carrier were determined using fatty acid-BSA binding isotherms. OL/LN total (bound + unbound) molar ratios ranging from 0.2 to 2.2 were tested using [^{3}H]OL and [1-^{14}C]LN. This range covered the OL/LN ratios studied in the acylcarnitine assays. Similarly, we determined the fatty acid-BSA binding isotherms for PA/LA. We used the heptane-partition method (14) to determine the equilibrium between unbound and BSA-bound NEFA under conditions identical to that used in the CPT I assays. The molar ratios of each of the NEFA to BSA ranged from 0.1 to 1.2.

Miscellaneous procedures. The concentrations of all NEFA preparations were determined quantitatively by GC using heptadecanoic acid as an internal standard as described previously (11). Protein was measured according to the procedure of Lowry *et al.* (15).

Mathematical derivations. The total initial velocity ν_T of an enzyme acting on two substrates *A* and *B* is equal to the sum of the individual initial velocities for each substrate, ν_A and ν_B.

$$\nu_T = \nu_A + \nu_B \quad [1]$$

For an enzyme showing hyperbolic kinetics toward two mutually competitive substrates, ν_T expands to

$$\nu_T = \frac{V_A \cdot [A]}{[A] + K_A\left(1 + \frac{[B]}{K_B}\right)} + \frac{V_B \cdot [B]}{[B] + K_B\left(1 + \frac{[A]}{K_A}\right)} \quad [2]$$

where *V* and *K* represent the maximal velocities and the saturability tendencies, respectively, with respect to substrates *A* and *B*. The denominators of the right side of Equation 2 reflect the mutual direct competition between the two substrates *A* and *B* for the same enzyme. It is to be noted that at saturating conditions, neither substrate necessarily attains its maximum velocity *V*, as can be ascertained from the nature of the polynomials in both denominators. Thus, the usual simplification of the Michaelis–Menten formula at saturating conditions for a single substrate cannot be applied to Equation 2.

Another equation that is verifiable by experiment can be derived by finding the ratio of the individual initial velocities ν_A and ν_B (first and second terms on the right side of Eq. 2) which is

$$\frac{\nu_A}{\nu_B} = \frac{V_A/K_A}{V_B/K_B} \cdot \frac{[A]}{[B]} \quad [3]$$

Equation 3 predicts that the ratio of the velocities of reaction for substrates *A* and *B* will be a linear function of the ratio of the corresponding substrate concentrations.

RESULTS

The BSA-binding isotherms of PA, OL, LA, and LN remained the same whether they were measured for each fatty acid singly, or for each fatty acid in the presence of a second fatty acid (data not shown). Furthermore, the molar ratio of unbound OL and LN, and that of unbound PA and LA were linearly correlated with their corresponding molar ratios of total (bound + unbound) NEFA (Fig. 1). This means that under our conditions, the individual fatty acid to BSA binding equilibria are independent of each other and that molar ratios of unbound fatty acids can be interpolated from molar ratios of total amounts of fatty acids in the assay mixtures. We also showed in the past that the dissociation rates of NEFA from BSA exceed the catalytic rate of acylcarnitine synthesis by 50–300 orders of magnitude and therefore have insignificant effects on rates of reaction (11).

The data in Figure 2 confirm the prediction of Equation 3 that the ratio of the initial velocities for two substrates pres-

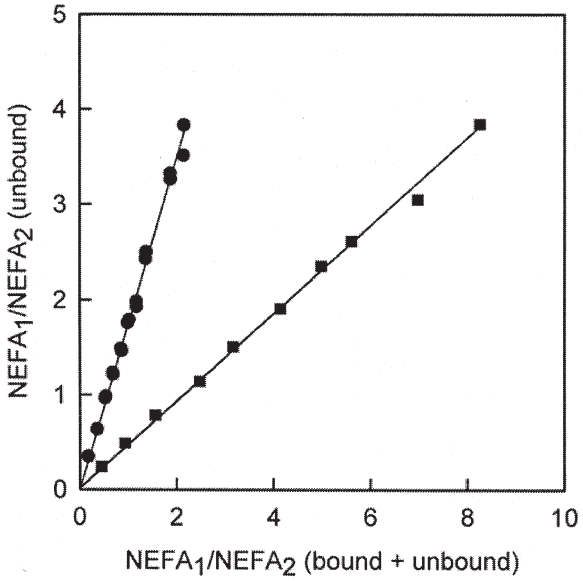

FIG. 1. Linear correlation between the ratios of total nonesterified FA (NEFA)1 and NEFA2 (unbound + BSA-bound) to the corresponding ratios of the unbound FA. The concentrations of the unbound FA used to calculate points on the *y*-axis are values in equilibrium with the corresponding BSA-bound FA. Circles represent oleic acid (OL)–α-linolenic acid (LN) pairs, and squares represent palmitic acid (PA)–linoleic acid (LA) pairs.

ent simultaneously is a linear function of the ratio of the corresponding substrate concentrations. The regression coefficients r^2 for the PA/LA and OL/LN NEFA pairs were 0.97

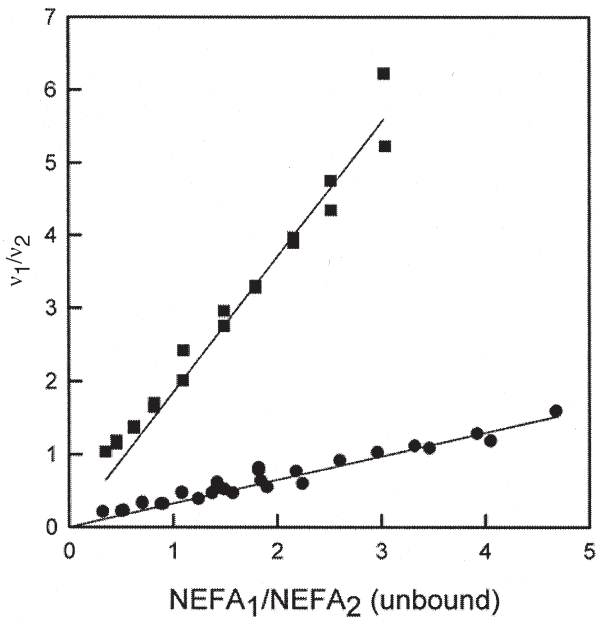

FIG. 2. Linear correlation between ratios of carnitine palmitoyl transferase (CPT) I initial velocities v_1 and v_2 and ratios of the concentrations of the corresponding unbound FFA substrates nonesterified fatty acids (NEFA)1 and NEFA2. The mutually competitive substrates NEFA1 and NEFA2 are present concurrently in the enzyme reaction. Circles represent OL–LN pairs and the subscripts 1 and 2 refer to OL and LN, respectively. Squares represent PA–LA pairs and the subscripts 1 and 2 refer to PA and LA, respectively. For other abbreviations see Figure 1.

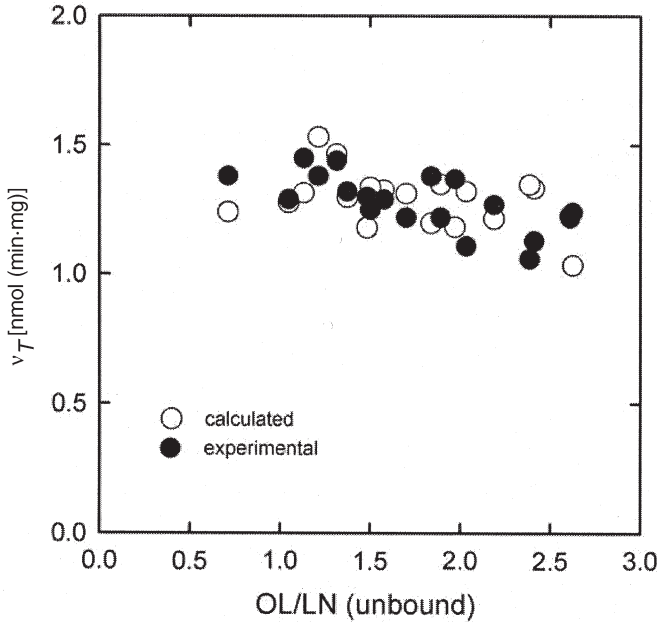

FIG. 3. Verification by experiment of the predicted total velocity v_T calculated from 16 previously published single-substrate kinetic constants. The assumptions are as follows: the initial velocities due to OL and LN are additive; OL and LN are direct and mutually competitive inhibitors. Kinetic constants for NEFA published in Reference 11 were used for the calculated values of v_T. For other abbreviations see Figures 1 and 2.

and 0.93, respectively. The calculated slopes were 1.8 ± 0.04 (n = 24) and 0.34 ± 0.01 (n = 19) for the PA/LA and OL/LN pairs, respectively. These values indicate that acylcarnitine synthesis is selective for PA over LA (slope > 1) and for LN over OL (slope < 1). As further confirmation of the validity of the derived equations, previously published kinetic constants (11) from single-substrate experiments were used to calculate the slope term of Equation 3, and hence to predict the selectivity of acylcarnitine for PA over LA, and for OL over LN. The predicted values were 1.4 and 0.39 for PA/LA and OL/LN NEFA, respectively, values that compare well with data from the actual experiments.

Figure 3 is a further confirmation of the applicability of Equations 1–3 on mitochondrial acylcarnitine synthesis. First, using previously published kinetic constants derived from single-substrate experiments (11), Equation 2 was used to predict total (v_T) initial velocities when two substrates, OL and LN, are present. Next, total initial velocities for these two combined substrates were determined in actual experiments, using the acylation of labeled carnitine as a measure of total acylcarnitine synthesis. Figure 3 shows that both sets of data, theoretical and experimental, are superimposed, indicating that the kinetics of acylcarnitine synthesis followed the predictions of Equations 1–3.

DISCUSSION

Our main objective was to develop a mathematical procedure that could predict the selectivity of the acylcarnitine synthe-

sis toward different fatty acid substrates when they are present simultaneously, by using previously published kinetic constants obtained experimentally from single-substrate experiments. It was not our intent to generate more *V* and *K* values for CPT I, for which many have already appeared in the literature. We also asked the question whether mitochondrial acylcarnitine synthesis exhibits preferentiality toward LN regardless of the concentrations of competing substrates present simultaneously.

There are two possible approaches to this question of multisubstrate kinetics; one uses integrated rate equations (16,17) and the other, the method of initial rates. Although the method of integrated rate equations presents some advantages over the other, it requires that the reaction be allowed to proceed to near equilibrium, and therefore that the enzyme or enzymes remain stable throughout the relatively long assay period. Because there are no indications in the literature concerning the stability of mitochondrial acylCoA synthetase or of CPT I over long assay periods, we chose the alternative method of initial rates to study the selectivity of acylcarnitine synthesis. In the method of initial rates, conversion of radiotracers is practically the only reliable way to measure enzyme activity, and this effectively limits the experiments to two fatty acid substrates at a time, each one labeled with either ^{3}H or with ^{14}C. Based on previous studies (11), the NEFA pair OL and LN was tested because these fatty acids have similar *K* but dissimilar *V*. Additionally, the NEFA pair PA and LA was tested because they have similar *V* but dissimilar *K*.

The slopes of the lines in Figure 2 correspond to the constant in Equation 3, that is, $(V_A/K_A)/(V_B/K_B)$. The slope is a proportionality constant, relating the dependent variable, which is the ratio of initial rate velocities for the substrate pair, to the independent variable, which is the corresponding ratio of substrate concentrations. As shown in Equation 3, this proportionality constant is independent of concentration, whether saturating or nonsaturating, and therefore applicable to all possible concentrations of each of the substrates. The significance of this prediction is more readily apparent when Equation 3 is rearranged to show ratios of fractional turnover rates:

$$\frac{(\nu_A/A)}{(\nu_B/B)} = \frac{V_A/K_A}{V_B/K_B} \qquad [4]$$

The fractional turnover rate of a given substrate is a measure of the rate of flux of its pool. It is equal to the reaction rate divided by the substrate pool size and has dimensions of reciprocal time. In the case of the OL/LN NEFA pair in which the ratio of the constants at the right-hand side of Equation 4 was determined by experiment to be 0.34, the fractional turnover rate of the OL pool due to acylcarnitine synthesis will always be 2.9-fold slower than that of the LN pool regardless of the OL or LN pool size. Similarly, for the PA/LA NEFA pair in which the ratio of the constants was determined to be 1.8, the fractional rate of disappearance of the PA pool due to acylcarnitine synthesis will always be 1.8-fold faster than that of the LA pool, regardless of pool size for either fatty acid.

Because the substrates we used in the experiments were NEFA, we must address the issue of the kinetic behavior of a pathway involving two enzymes in sequence, namely, mitochondrial acylCoA synthetase followed by CPT I. First, it can be shown mathematically that two Michaelis–Menten enzymes in sequence behave qualitatively as a single Michaelis–Menten enzyme, on the condition that the flux achieves a steady state. The observation that our experimental data fit the predictions of Equations 1–3 confirms the assumption that acylcarnitine synthesis from NEFA follows hyperbolic kinetics. It is true that the data permit assignment of selectivity to the acylcarnitine synthesis pathway only as a whole and not to the individual enzymes. However, we showed previously that the relative magnitudes of *V* and *K* of mitochondrial acylcarnitine synthesis for a range of different NEFA substrates are similar qualitatively to those obtained using the corresponding purified CoA esters as substrates (11). Recent reports suggest that mitochondrial acylCoA synthetase and CPT I are physically (10) and metabolically (9) linked. Of the five known isoforms of acylCoA synthetase, only one, ACS5, is found on the mitochondrial outer membrane and is believed to channel cytosolic NEFA specifically to CPT I (9), perhaps through proximity (10). As of now, there are no reports indicating that ACS5 exhibits selectivity for any particular fatty acid. Taken together, it would appear that CPT I and not mitochondrial acylCoA synthetase would be the primary agent that gives rise to selectivity in acylcarnitine synthesis. However, the particularities of the five known isoforms of acylCoA synthetase have not yet been fully elucidated, and there are indications that isoforms other than mitochondrial ACS5 may contribute acylCoA esters destined for CPT I into the mitochondria (9). More studies are warranted in this area.

It would be useful to extend the system reported here from binary substrates to the whole spectrum of fatty acids simultaneously available to mitochondrial CPT I *in vivo*. Equation 2 is easily extended to the *n*th substrate

$$\nu_T = \frac{V_A \cdot [A]}{[A] + K_A\left(1 + \frac{[B]}{K_B} + \cdots + \frac{[n]}{K_n}\right)} + \frac{V_B \cdot [B]}{[B] + K_B\left(1 + \frac{[A]}{K_A} + \cdots + \frac{[n]}{K_n}\right)} + \cdots + \frac{V_n \cdot [n]}{[n] + K_n\left(1 + \frac{[A]}{K_A} + \frac{[B]}{K_B} + \cdots + \frac{[(n-1)]}{K_{(n-1)}}\right)} \qquad [5]$$

As in the derivation of Equations 3 and 4, the ratios of the fractional turnover rates of a given substrate *m* to any other substrate *n* in a multiple substrate system in Equation 5 can be shown to be

$$\frac{(\nu_m/[m])}{(\nu_n/[n])} = \frac{V_m/K_m}{V_n/K_n} \qquad [6]$$

Like Equations 3 and 4, the applicability of Equation 6 does not depend on substrate concentrations, whether the system is saturated or nonsaturated. However, it was not possible to find a mathematical expression for the fractional turnover rate of a given substrate as a function of all of the other variables in Equation 5. To predict and compare the relative fractional

TABLE 1
Comparison of Carnitine Palmitoyl Transferase (CPT) I Fractional Turnover Rates of Different Fatty Acid Substrates[a,b]

Palmitate	Stearate	Oleate	Linoleate	Linolenate	Arachidonate	EPA	DHA
0.93 (LN)	0.084 (LN)	0.39 (LN)	0.68 (LN)	1 (LN)	0.27 (LN)	0.32 (LN)	0.29 (LN)
1 (PA)	0.091 (PA)	0.42 (PA)	0.74 (PA)	1.1 (PA)	0.29 (PA)	0.35 (PA)	0.31 (PA)
1.4 (LA)	0.12 (LA)	0.57 (LA)	1 (LA)	1.5 (LA)	0.39 (LA)	0.48 (LA)	0.42 (LA)
2.4 (OL)	0.22 (OL)	1 (OL)	1.8 (OL)	2.6 (OL)	0.69 (OL)	0.83 (OL)	0.74 (OL)
2.9 (EPA)	0.26 (EPA)	1.2 (EPA)	2.1 (EPA)	3.1 (EPA)	0.82 (EPA)	1 (EPA)	0.89 (EPA)
3.2 (DHA)	0.29 (DHA)	1.4 (DHA)	2.4 (DHA)	3.5 (DHA)	0.93 (DHA)	1.1 (DHA)	1 (DHA)
3.5 (ARA)	0.32 (ARA)	1.5 (ARA)	2.6 (ARA)	3.8 (ARA)	1 (ARA)	1.2 (ARA)	1.1 (AR)
11 (ST)	1 (ST)	4.6 (ST)	8.1 (ST)	12 (ST)	3.2 (ST)	3.8 (ST)	3.4 (ST)

[a]Values represent the magnitude of the fractional turnover rates of the column fatty acids relative to those of the fatty acids in parenthesis, calculated from kinetic constants published previously (Ref. 11) using the right-hand side of Equation 6, $(V_m/K_m)/(V_n/K_n)$.
[b]Abbreviations: LN, α-linolenic acid; PA, palmitic acid; LA, linoleic acid; ARA, arachidonic acid; ST, stearic acid.

turnover rates of fatty acids of physiologic interest other than those presented here, we used previously published kinetic constants *V* and *K* for NEFA (11) in Equation 6 to generate values for all possible pairings of eight fatty acids reported. The results are shown in Table 1. Each value in Table 1 is the ratio of the fractional turnover rate of the group fatty acid (column heading) relative to the row fatty acid (in parentheses). Each column is arranged according to increasing value of this ratio. Organized in this manner, Table 1 shows that each column group consistently yields the same relative order of NEFA with respect to increasing magnitude of ratios of fractional turnover rates. From this, we predict a certain ordered list of fatty acids according to decreasing fractional turnover rate regardless of pool size: LN > PA > LA > OL > EPA > DHA > arachidonic acid (ARA) > stearic acid (ST). This means that relative to each other, LN will have the highest and ST the lowest fractional turnover rate. When the same calculation is applied to previously reported kinetic constants obtained from purified acylCoA substrates (11), the relative order becomes: LN > ARA > LA > EPA > PA > DHA > OL > ST (calculated values not shown). For both types of substrates, NEFA or acylCoA, LN exhibits the highest and ST the lowest fractional turnover rate relative to all the other fatty acids. It appears, however, that ARA exhibits the most dramatic increase in relative fractional turnover rate when presented as a CoA ester rather than as NEFA. This would suggest that mitochondrial acylCoA synthetase, perhaps ACS5, plays a role in shunting ARA (as NEFA) away from β-oxidation.

An important point to be made is that the absolute rates of utilization of any given substrate will always be diminished by the presence of another substrate acting as a competitive inhibitor, and vice versa. This does not contradict the prediction that the fractional turnover rate of LN will always be higher than that of any other fatty acid. The physical meaning of this is that the pool size of LN will diminish faster than any other pool of fatty acid due to acylcarnitine synthesis. This is because no matter the size of the LN pool, a constant percentage of that pool is being transformed to acylcarnitine, a percentage that is higher than the corresponding values for any other fatty acid.

In conclusion, we succeeded in developing predictive equations for the selectivity of the acylcarnitine synthesis pathway and confirmed their validity by comparing the mathematically derived predictions with actual data from experiments. To our knowledge, this is the first time multisubstrate kinetics has been used to model and analyze a lipid metabolic pathway in the complex milieu of a subcellular fraction. The derived equations allow comparisons of fractional turnover rates independent of substrate concentrations. This eliminates the imprecision of using maximal velocities alone (*V*), or saturability tendencies alone (*K*), to compare reaction rates of various substrates. The results, both experimental and theoretical, indicate that acylcarnitine synthesis exhibits selectivity that could be a factor in the control of the channeling of different types of fatty acids to either synthetic pathways or β-oxidation.

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Long-Chain Fatty Acid Uptake by Skeletal Muscle Is Impaired in Homozygous, but Not Heterozygous, Heart-Type-FABP Null Mice

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ABSTRACT: Previous studies with cardiac myocytes from homozygous heart-type fatty acid (FA)-binding protein (H-FABP)$^{-/-}$ mice have indicated that this intracellular receptor protein for long-chain FA is involved in the cellular uptake of these substrates. Based on the knowledge that muscle FA uptake is a process highly sensitive to regulation by hormonal and mechanical stimuli, we studied whether H-FABP would play a role in this regulation. A suitable model system to answer this question is provided by H-FABP$^{+/-}$ mice, because in hindlimb muscles the content of H-FABP was measured to be 34% compared to wild-type mice. In these H-FABP$^{+/-}$ skeletal muscles, just as in H-FABP$^{-/-}$ muscles, contents of FA transporters, i.e., 43-kDa FABPpm and 88-kDa FAT/CD36, were similar compared to wild-type muscles, excluding possible compensatory mech-anisms at the sarcolemmal level. Palmitate uptake rates were measured in giant vesicles prepared from hindlimb muscles of H-FABP$^{-/-}$, H-FABP$^{+/-}$, and H-FABP$^{+/+}$ mice. For comparison, giant vesicles were isolated from liver, the tissue of which expresses a distinct type of FABP (i.e., L-FABP). Whereas in H-FABP$^{-/-}$ skeletal muscle FA uptake was reduced by 42–45%, FA uptake by H-FABP$^{+/-}$ skeletal muscle was not different from that in wild-type mice. In contrast, in liver from H-FABP$^{-/-}$ and from H-FABP$^{+/-}$ mice, FA uptake was not altered compared to wild-type animals, indicating that changes in FA uptake are restricted to H-FABP expressing tissues. It is concluded that H-FABP plays an important, yet merely permissive, role in FA uptake into muscle tissues.

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Cytoplasmic fatty acid (FA)-binding proteins (FABP) now have been firmly established to play an important role in cellular utilization of long-chain FA. Heart-type FABP (H-FABP) is one of the nine currently known FABP types and displays a characteristic tissue distribution pattern (1,2). It is most abundant in heart and, to a lesser extent, in skeletal muscle and brain. In heart and muscle it is virtually the only FABP type that occurs, whereas in brain it is coexpressed with brain-type FABP. There is ample evidence that H-FABP, and also other FABP types, facilitate the intracellular transport of FA (1,3). Most of this evidence, however, is indirect, including (i) a correlation with the expression of other FA-metabolizing enzymes, and (ii) a correlation with cellular function (3). More direct evidence has been obtained by studying the transfer of FA between mitochondria and liposomes that are spatially separated from each other. Both liver-FABP (L-FABP) and H-FABP are able to enhance FA movement markedly between the two membrane compartments (4).

Besides its function as cytoplasmic FA carrier, FABP has been proposed to be involved in cellular uptake of FA (1,3). However, it is questionable whether this function is separable from enhancement of intracellular transport of FA. Namely, this enhanced transport will result in a lower intracellular concentration of FA, and hence a steeper transmembrane gradient. Because the transmembrane gradient of FA generally is regarded as the driving force for FA uptake (5), enhanced intracellular transport of FA, as mediated by FABP, is coupled to enhanced FA uptake. Apart from (or together with) this indirect effect on FA uptake, H-FABP may directly interact with either plasma membrane phospholipids or proteins. Evidence for a physical contact between FABP and phospholipid membranes can be inferred from movement of fluorescent FA derivatives from FABP to model acceptor vesicles in a fluorescence resonance energy transfer assay (3). In these studies, it appeared that most FABP types, including H-FABP but not L-FABP, deliver FA to acceptor membranes through a collisional process. In this way, H-FABP could facilitate the uptake process by extracting FA from the cytoplasmic side of the plasma membrane. Evidence for an interaction between H-FABP and plasma membrane proteins comes from immunoprecipitation experiments with bovine mammary gland. In these experiments H-FABP was found to bind to a cytoplasmic segment of the 88-kDa FA transporter (FAT)/CD36 (6), allowing the speculation that there is a direct transfer of FA from a sarcolemmal FA transporter to cytoplasmic FABP.

Notwithstanding this debate, evidence for a role of FABP in FA uptake mainly comes from a variety of studies with cell cultures transfected with different FABP types (1–3). In most studies these transfected cells display an enhanced FA uptake rate, albeit a rather moderate increase in FA uptake. With respect to H-FABP, one study reports no effect of its overex-

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Abbreviations: ECL, enhanced chemiluminescence; FA, fatty acid; FABP, fatty acid-binding protein; FABPpm, plasma membrane FABP; FAT, FA translocase; H-FABP, heart-type FABP; L-FABP, liver-type FABP.

pression in M6-myoblasts on cellular FA uptake (7), whereas in an earlier study there was an increase in FA uptake in the same cell line (8). Also in human breast cancer cells, there was a moderate increase in FA uptake upon transfection with H-FABP (9). Transfection studies with other FABP types, notably adipocyte-FABP (10) and L-FABP (11), are confirmatory to the H-FABP transfection studies in that there is only a modest (less than twofold) increase in FA uptake.

New insights into the role of FABP in cellular FA utilization have been obtained by the generation of H-FABP knockout mice. Mice lacking the H-FABP gene exhibit acute exercise intolerance as well as cardiac hypertrophy later in life (12). More related to FA utilization, these mice display a diminished accumulation of systemically administered radiolabeled FA derivatives in peripheral tissues (12). When specifically examining FA uptake into cardiac myocytes from these null mice, it appeared that the complete absence of H-FABP resulted in a marked (45%) inhibition compared to cardiac myocytes from wild-type mice from the same genetic background (13). This drop in FA uptake cannot be explained by a parallel downregulation of other lipid metabolic enzymes, since in FABP null cardiac myocytes the oxidative capacity is completely preserved. Therefore, this mouse model provides the most convincing evidence that FABP is an important player in cellular FA uptake.

Evidence is accumulating that FA uptake is a protein-mediated process in muscle tissues (14,15). For instance, the rate of FA uptake into different types of muscle is positively correlated with the sarcolemmal amounts of 43 kDa plasma membrane FABP (FABPpm) and 88 kDa FAT/CD36. Notably, both proteins are more abundant in red vs. white skeletal muscle and most abundant in heart, and are involved in bulk uptake of FA into muscle tissues (15). In addition, H-FABP increases in content from white to red skeletal muscle to heart. Hence, it is difficult to attribute the changes in FA uptake to changes in abundance of sarcolemmal FA transporters or to that of cytoplasmic FABP. Although we do know that H-FABP is an important player in FA uptake, we do not know to what extent FA uptake is sensitive to changes in the muscle content of FABP. In other words, we do not know the flux control coefficient of H-FABP in muscle FA uptake.

To study whether there is a possible role for H-FABP in regulation of cellular uptake of FA, one would like to have a muscle model with variable amounts of H-FABP but with a constant amount of sarcolemmal FA transporters. In the present study, we investigated the possibility that H-FABP$^{+/-}$ mice can offer insight into the role of altered H-FABP content in muscle FA uptake. For determination of FA uptake into muscle, we used the model of giant vesicles because the uptake process can be determined separate from metabolism. Another advantage is that cytoplasmic FABP are retained within the lumen of these giant vesicles. Moreover, the concentration of FABP in giant vesicles reflects that of the tissue they are derived from (15,16). This is likely a result of the random sequestration of cytoplasm during the budding process induced by KCl treatment of tissue, which gives rise to vesicle formation (16). To investigate whether there are changes in FA uptake in tissues that do not express H-FABP in these H-FABP transgenic mice, we also studied FA uptake into giant vesicles derived from liver. Furthermore, the existence of compensatory changes in protein levels of the sarcolemmal FA transporters, FABPpm and FAT/CD36, was investigated.

MATERIALS AND METHODS

Animals. Mice with a targeted deletion of the entire H-FABP locus were generated as previously described (12). Homozygous and heterozygous gene-disrupted mice had been backcrossed four times onto the C57Bl/6 background, and their wild-type littermates were used as controls. Mice used in experiments were 6–12 mon of age and were weight-, age-, and sex-matched. These mice were bred at the Centralized Animal Facility of Maastricht University, where they had unrestricted access to food (containing 27.5% protein, 42.5% carbohydrate, and 7.5% fat) and water. This study was approved by the local Ethical Committee on Animal Experimentation.

Materials. A standard commercial rodent diet (SRM-A) was obtained from Hope Farms BV. Radiolabeled products were from Amersham Pharmacia Biotech (Roosendaal, The Netherlands). BSA (fraction V) and collagenase type VII were purchased from Sigma Chemical (St. Louis, MO). Western blot reagents were from Bio-Rad Laboratories (Hercules, CA), and the enhanced chemiluminescence (ECL) kit was from Amersham Pharmacia Biotech (Buckingham, United Kingdom). A rabbit polyclonal antiserum directed against human CD36 was kindly provided by Dr. Narendra N. Tandon (Otsuka America Pharmaceutical, Rockville, MD). A rabbit polyclonal antiserum against rat hepatic membrane FABP was a gift from Dr. Jorge Calles-Escandon (Glaxo SmithKline, Miami, FL). Generation of a polyclonal antiserum against recombinant rat L-FABP has been described previously (17).

Determination of palmitate uptake by giant vesicles from skeletal muscle and liver. Giant vesicles were isolated from 0.5–1 g of muscle tissue from both upper and lower hindlimbs and from 1 g of liver of H-FABP$^{+/+}$, H-FABP$^{+/-}$, and H-FABP$^{-/-}$ mice exactly as described for the rat (18). For both tissues, the yield of vesicular protein was generally between 80 and 150 μg. Briefly, vesicle budding was initiated by incubation of incised tissue in iso-osmotic KCl in the presence of collagenase type VII. Vesicles were harvested with low-speed Percoll/Nycodenz gradient centrifugation. For both tissues, average vesicle diameters were between 10 and 15 μm. Palmitate uptake rates by these vesicles were determined by adding 40 μL of 14.5 μM unlabeled palmitate, 0.3 μCi [^{3}H]palmitate, and 0.06 μCi [^{14}C]mannitol in a KCl-MOPS solution containing 0.1% BSA to a 40 μL vesicle suspension containing 1–2 μg protein/μL. The reaction was carried out at room temperature for 15 s. Palmitate uptake was terminated by addition of 1.4 mL ice-cold KCl-MOPS, 2.5 mM $HgCl_2$ and 0.1% BSA. The sample was then quickly centrifuged at $10{,}000 \times g$ in a microfuge for 1 min. The supernatant was discarded, and radioactivity was determined in the material that

sedimented out at the bottom of the microfuge tube. Inclusion of the adherent fluid marker mannitol, which is unable to permeate biological membranes, allows a correction to be made for palmitate that has not been taken up, and is still present in the adherent fluid.

Sample preparation for determination of total tissue content of H-FABP. Total tissue homogenates were prepared from skeletal muscle (hindlimb) and from liver as previously described (16). Protein concentration was determined by the bicinchonic acid assay (Sigma).

Assay of FABPpm, FAT/CD36, and L-FABP by Western blotting. Samples of total tissue homogenates (10 μg) and of giant vesicles (5 μg) were separated on 10% polyacrylamide gels (150 V for 1 h). Proteins were then transferred to Trans-Blot^{R} pure nitrocellulose membranes (Bio-Rad Laboratories) (100 V for 90 min). Western blotting was carried out as previously described (18). Detection of FABPpm and of FAT/CD36 was performed as described (18). For detection of FAT/CD36 and of L-FABP, the respective polyclonal antisera were used in a 1:3000 dilution and a 1:1000 dilution. Immune complexes were detected with ECL by exposing the membranes to film (Hyperfilm-ECL) at room temperature according to the instructions of the manufacturer. Protein band densities were obtained by scanning the films with a MultiImager (FluorS; Bio-Rad) connected to a personal computer followed by densitometry using the computer program Quantity One (Bio-Rad Laboratories).

Assay of H-FABP by sandwich ELISA. The content of H-FABP in homogenates and in giant vesicle preparations was determined by an ELISA of the antigen-capture type (sandwich ELISA) as previously described (19), and using purified mouse H-FABP as standard.

Data presentation and statistics. Data are presented as means ± SD for the indicated number of preparations. The statistical difference between groups of observations was tested with a paired Student's *t*-test. *P* values equal to or less than 0.05 were considered significant.

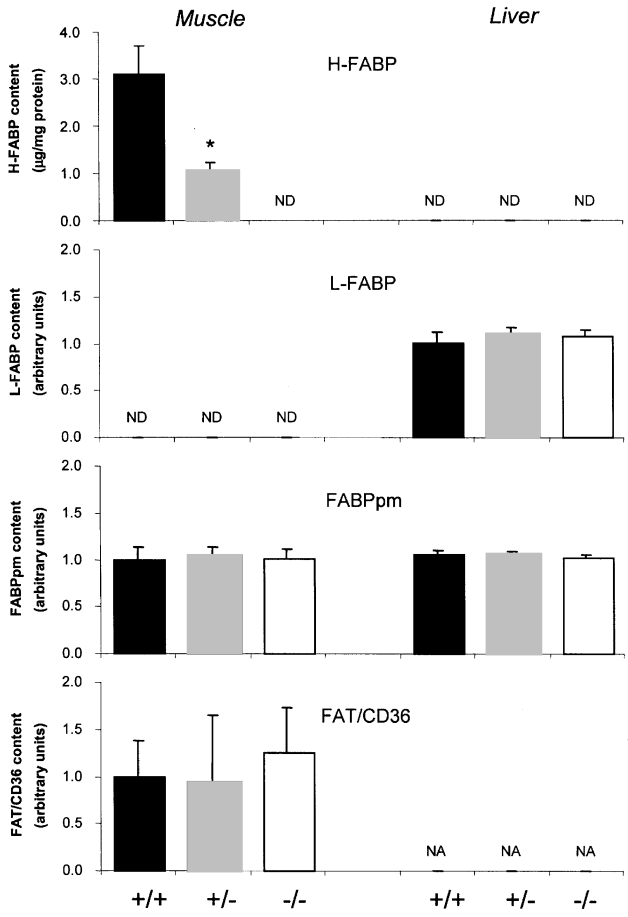

FIG. 1. Content of cytoplasmic fatty acid (FA)-binding protein (FABP) and sarcolemmal FA transporters in skeletal muscle and liver from heart π(H)-FABP$^{+/+}$, H-FABP$^{+/-}$ and H-FABP$^{-/-}$ mice. Homogenates from skeletal muscle and liver were analyzed by a sandwich ELISA on the content of H-FABP and by gel electrophoresis and Western blotting on the contents of liver (L)-FABP, FABPpm and FAT/CD36. Data are means ± SD for 4–8 experiments carried out with preparations from different animals. H-FABP and L-FABP were both detected at 15 kDa, FABPpm at 43 kDa, and FAT/CD36 at 88 kDa. ND, not detectable or detected at a level <0.01 mg/mg protein; NA, not analyzed. *Significantly different from "+/+" ($P < 0.05$).

RESULTS

Content of FABP and FA transporters in skeletal muscle and liver from H-FABP$^{+/+}$, HFABP$^{+/-}$, and HFABP$^{-/-}$ mice. The data on the tissue contents of FABP and FA transporters, presented in Figure 1, are arbitrarily from females. However, no gender difference was observed (data not shown). These protein contents were determined in total tissue homogenates only, and not in giant vesicles, because the bulk amount of vesicles obtained from the tissues of this small-size animal was needed for the palmitate uptake assay. It should be noted, however, that based on the mechanism of vesicle formation, which involves a budding process and random sequestration of cytoplasm, the relative abundance of a given protein should be similar between total tissue homogenates and giant vesicles. In this respect, we have previously shown that the relative abundance of cytoplasmic FABPs and FA transporters in different muscle types (white muscle < red muscle < heart) is retained in giant vesicles (15,16). Furthermore, the degree of alteration of the muscle content of cytoplasmic FABP and FA transporters due to pathological conditions, such as obesity (18), type 1 diabetes (20) or muscle denervation (Koonen, D.P.Y., unpublished observations), is of similar magnitude when measured in giant vesicles compared to total tissue homogenates. Therefore, it is very likely that a change in FABP measured in total tissue homogenates will also be reflected in the corresponding giant vesicles.

(i) H-FABP. In H-FABP$^{-/-}$ skeletal muscle, our sandwich ELISA was unable to detect this protein (Fig. 1). In H-FABP$^{+/-}$ skeletal muscle homogenates, the level of H-FABP was 34% of that of wild-type muscle (Fig. 1). Thus, notwithstanding the various possible mechanisms of transcriptional, translational, and posttranslational control that hypothetically could give rise to an unaltered protein level of

H-FABP, the 50% decrease in genomic H-FABP is reflected in a reduction of a similar magnitude at the protein level. In liver of all three mouse models, H-FABP was detected at levels below 0.01 μg/mg protein.

(ii) L-FABP. In skeletal muscle of all mouse models, L-FABP was undetectable with Western blotting. In liver, its content was not different between the transgenics and the wild-type mice.

(iii) FABPpm. This 43-kDa sarcolemmal FA transporter is known to be expressed in most mammalian tissues and cell lines (21), and, accordingly, was detected in both skeletal muscle and in liver (Fig. 1). Its content in muscle was identical in wild-type vs. transgenics. In liver, no differences were observed among the various mouse models.

(iv) FAT/CD36. In contrast to FABPpm, this 88-kDa sarcolemmal FA transporter is present in skeletal muscle and virtually absent in liver (21). This is in agreement with the lack of a functional role in FA uptake in the latter tissue based on the inability of sulfo-*N*-succinimidyloleate, a specific FAT/CD36 inhibitor, to block FA uptake (15). But just like FABPpm, its content in skeletal muscle was not different among the various mouse models.

FA uptake by skeletal muscle and liver giant vesicles from H-FABP$^{+/+}$, HFABP$^{+/-}$, and HFABP$^{-/-}$ mice. Palmitate uptake was studied at a physiologically relevant 1:1 molar ratio with BSA, resulting in a nonprotein-bound concentration of 5.1 nM, according to calculations of Richieri *et al.* (22). This concentration is below the Michaelis–Menten constant (K_m) for palmitate as determined in rat skeletal muscle, which was established to be 9.7 nM (15). At this concentration, palmitate uptake was linear with time for at least 1 min (data not shown). A linear increase also occurred with increasing quantities of vesicular protein (data not shown). In the present set of experiments, palmitate uptake was determined over a 15-s period with the use of 40–80 μg of total vesicular protein.

(i) Skeletal muscle. Under the conditions used, rates of palmitate uptake by giant vesicles from skeletal muscle from mouse were very similar to that from rat (for comparison, see Refs. 14–16). Palmitate uptake into muscle from heterozygous mice of both sexes was not significantly different from that of wild-type mice. However, in male homozygous knockout mice, FA uptake was reduced by 42% and in females by 45%.

(ii) Liver. Just as with skeletal muscle, rates of palmitate uptake into mouse (Fig. 2) and rat giant vesicles (16) were very similar. FA uptake into liver giant vesicles was 1.9- and 2.3-fold higher than in skeletal muscle giant vesicles from male and female wild-type mice, respectively. In both males and females, FA uptake into liver was not significantly different among wild-type, heterozygous, and homozygous H-FABP knockout mice.

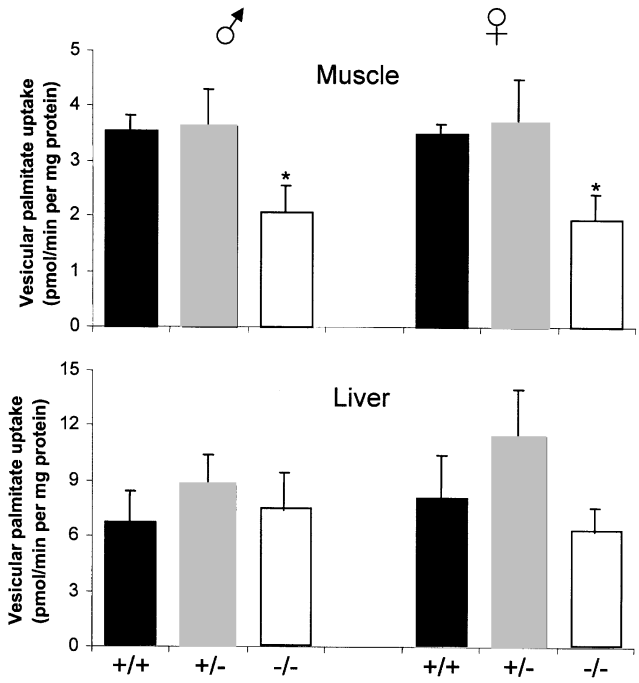

FIG. 2. FA uptake into skeletal muscle and liver from heart-type (H)-FABP$^{+/+}$, H-FABP$^{+/-}$, and H-FABP$^{-/-}$ mice. Giant vesicles were prepared from skeletal muscle and liver from both male and female transgenics for the determination of palmitate uptake (15 s). Data are means ± SD for 7 or 8 experiments carried out with giant vesicle preparations from different animals. *Significantly different from "+/+" ($P < 0.05$). For abbreviations see Figure 1.

DISCUSSION

A previous study with cardiac myocytes from null mice homozygous for the deletion of H-FABP resulted in a 45% inhibition of cellular FA uptake, as well as a 45% reduction of FA oxidation, indicating that this protein is an important player in the uptake process (13). In line with these previous results are the present measurements of FA uptake into giant vesicles from H-FABP$^{-/-}$ muscle, demonstrating a reduction in FA uptake of similar magnitude, i.e., 42–45%, independent of gender. Also in line with this previous study is the finding that there is no adaptation in muscle at the level of sarcolemmal FA transporters. That is, protein levels of FABPpm and of FAT/CD36 were unchanged. Since metabolism is absent in giant vesicles, which permits the proper study of uptake kinetics (14,15), this model system adds powerful evidence that H-FABP is indeed involved in the cellular FA uptake process. However, in the complete absence of H-FABP there is still a substantial (*ca.* 50%) rate of residual FA uptake. There are several possible explanations for why, in the absence of H-FABP, giant vesicles are still able to sequester FA from the medium, albeit at a reduced rate. First, FA taken up by sarcolemmal FA transporters remain within the sarcolemmal membranes of the giant vesicles. Although we cannot exclude this possibility, we regard it as unlikely. Namely, the solution applied to stop FA uptake by virtue of the disrupting action of HgCl on protein-mediated transport also contains BSA, which is able to extract FA residing within membranes efficiently (14). Second, other proteins present within the lumen of giant vesicles may be able to bind FA specifically or nonspecifically and therefore may serve as an alternative sink. In our opinion, this latter explanation is more likely because it

has been found with cytoplasmic preparations from heart tissue of H-FABP$^{-/-}$ mice that the capacity to bind oleic acid is not reduced to zero, but only by 55% (13). However, this alternative sink is most likely not made up of other FABP types, since radiochromatographic analysis of rat heart cytoplasm did not show FA binding by 14–15 kDa proteins (13).

The key question of this study is to what extent the FA flux into muscle can be modulated by changes in the intracellular content of H-FABP. H-FABP$^{+/-}$ mice proved to be a very suitable model to gain insight into this matter, because in skeletal muscle of these mice H-FABP levels are markedly lower than in wild-type mice. Furthermore, we observed that the content of sarcolemmal FA transporters, i.e., FABPpm and FAT/CD36, has not changed in FABP$^{+/-}$ muscles, which excludes the possibility that an upregulation of their levels could compensate for the diminished involvement of H-FABP in muscle FA uptake. Unlike their homozygous counterparts, heterozygous mice did not display a reduction in muscle FA uptake. Together, these data indicate that FABP plays a permissive role in cellular FA uptake and is not involved in regulation of this process. Hence, H-FABP must be present in muscle tissues in relative excess, meaning that the concentration has to be markedly reduced before FABP becomes rate-limiting for FA uptake. In this respect, FABP is known to be one of the most abundant proteins present within the cytoplasm of muscle cells, contributing to 1–2% of total cytoplasmic protein (1). However, under normal physiological conditions, only 2% of total cellular H-FABP has been calculated to be liganded by FA (1,23), indicating the presence of a large reserve intracellular binding capacity for FA. The benefit of maintaining very high levels of FABP could be that such levels allow a rapid adaptation to changes in substrate flux as occurring, for instance, during the transition from a resting to an exercising muscle (1). Furthermore, high levels of FABP may be part of a defense mechanism against detrimentally high intracellular FA concentrations as seen, for example, under ischemic conditions (1).

Using H-FABP$^{-/-}$ and H-FABP$^{+/-}$ mice, we have also investigated FA uptake into liver, a tissue that does not express H-FABP. Hepatocytes, making up >90% of the liver mass, express L-FABP, the content of which did not change in these H-FABP transgenics. In addition, no changes in plasma membrane FA transporters were found in liver. Furthermore, FA uptake into liver giant vesicles was unchanged in both transgenic models. Hence, a reduction of FA uptake in H-FABP$^{-/-}$ mice is restricted to tissues that normally express this FABP type, suggesting that there are no compensatory mechanisms at the level of FA transport across the plasma membrane in tissues, such as liver, that do not express H-FABP.

In conclusion, studies on H-FABP transgenics have shown that H-FABP plays an important, but nonetheless merely permissive, role in FA uptake into skeletal muscle tissues. Although not studied, this conclusion most likely will also apply to the role of H-FABP in FA uptake by heart muscle. Taken together, these data indicate that limited fluctuations in muscle H-FABP content are unlikely to have a major effect on muscle FA uptake and utilization.

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Trans-10,*cis*-12 CLA Increases Liver and Decreases Adipose Tissue Lipids in Mice: Possible Roles of Specific Lipid Metabolism Genes

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ABSTRACT: Although consumption of CLA mixtures has been associated with several health effects, less is known about the actions of specific CLA isomers. There is evidence that the *t*10,*c*12-CLA isomer is associated with alterations in body and organ weights in animals fed CLA, but the mechanisms leading to these changes are unclear. The purpose of this study was to determine the effects of two commonly occurring isomers of CLA on body composition and the transcription of genes associated with lipid metabolism. Eight-week-old female mice (n = 11 or 12/group) were fed either a control diet or diets supplemented with 0.5% *c*9,*t*11-CLA or *t*10,*c*12-CLA isomers or 0.2% of the peroxisome proliferator-activated receptor α (PPARα) agonist fenofibrate for 8 wk. Body and retroperitoneal adipose tissue weights were significantly lower (6–10 and 50%, respectively), and liver weights were significantly greater (100%) in the *t*10,*c*12-CLA and the fenofibrate groups compared with those in the control group; body and tissue weights in the *c*9,*t*11-CLA group did not differ from those in the control group. Livers from animals in the *t*10,*c*12-CLA group contained five times more lipids than in the control group, whereas the lipid content of the fenofibrate group did not differ from that in the control group. Although fenofibrate increased the mRNA for PPARα, *t*10,*c*12-CLA decreased it. These results suggest that PPARα did not mediate the effects of *t*10,*c*12-CLA on body composition. The CLA isomers and fenofibrate altered mRNA levels for several proteins involved in lipid metabolism, but the most striking difference was the reduction of mRNA for leptin and adiponectin in the *t*10,*c*12-CLA group. These initial results suggest that changes associated with energy homeostasis and insulin action may mediate the effects of *t*10,*c*12-CLA on lipid metabolism.

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CLA is a collective term for a group of isomers of linoleic acid that have conjugated double bonds. Depending on the position and geometry of the double bonds, several isomers of CLA have been reported (1). Most of the published studies have used a mixture of CLA isomers, which comprised two major forms, *cis* 9,*trans* 11-CLA (*c*9,*t*11-CLA) and *trans* 10,*cis* 12-CLA (*t*10,*c*12-CLA), and a number of minor isomers. The major dietary sources of *c*9,*t*11-CLA are dairy products and ruminant meat, whereas those of *t*10,*c*12-CLA are partially hydrogenated vegetable oils from margarines and shortenings (2).

Feeding a mixture of CLA isomers to animal models has been reported to alter chemically induced carcinogenesis, atherogenesis, diabetes, body composition, and immune cell functions (3). Since a mixture of CLA isomers has been used in most of the studies, it is not known which of the CLA isomers is responsible for its effects listed above. Studies conducted with purified isomers show that *c*9,*t*11-CLA and *t*10,*c*12-CLA did not differ in their effects on immune cell functions in mice (4), but other studies have reported that the isomer responsible for reducing body and adipose tissue weights in mice (5) and hamsters (6) and for altering mammary lipid metabolism in dairy cows (7) is the *t*10,*c*12-CLA isomer. Supplementing diets of mice with a mixture of CLA isomers also causes an increase in liver and spleen masses and a several-fold increase in the amount of hepatic lipids (8,9). Which isomer causes the increase in liver lipids is not known. The liver lipids of mice fed purified isomers of CLA were not examined (5), but in hamsters fed similarly liver lipids did not change (6). Furthermore, the mechanisms by which individual CLA isomers may alter liver and adipose tissue fat contents are poorly understood.

The purpose of this study was to examine the effects of two purified isomers of CLA (*c*9,*t*11-CLA, and *t*10,*c*12-CLA) on body weight, weight of liver and its lipid content, adipose tissue weight, and possible means by which the CLA isomers may alter body composition in mice. One approach was to compare CLA effects to those of fenofibrate, a known peroxisome proliferator-activated receptor α (PPARα) agonist; the other approach was to examine the mRNA for a number of proteins that can be involved in lipid metabolism. The mRNA levels examined included acyl CoA oxidase (ACO), a rate-limiting enzyme for β oxidation; lipoprotein lipase (LPL), an enzyme that removes FFA from TG-rich lipoproteins; apolipoprotein C-3 (apoC-3), an inhibitor of LPL; microsomal cytochrome P450A1 (CYP450A1), a catalyst of ω hydroxylation of FA; mitochondrial uncoupling protein-2

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Abbreviations: ACO, acyl-CoA oxidase; Acrp30, complement-related protein 30, (Adiponectin); apoA-I, apolipoprotein A-I; apoC-3, apolipoprotein C-3; CYP4A1, microsomal cytochrome P450 4A1; PPAR, peroxisome proliferator-activated receptor; UCP, mitochondrial uncoupling proteins.

(UCP2), a protein that uncouples ATP synthesis from oxygen consumption and results in the production of heat; apolipoprotein A-1 (apoA-1), the primary protein constituent of HDL; PPARα, a transcription factor that controls synthesis and catabolism of TG-rich lipoproteins; and two adipose tissue hormones (leptin and adiponectin) that play an important role in regulation of lipid metabolism. Our results show that the two isomers differed in their effects on liver and retroperitoneal adipose tissue lipids, and in their effects on the adipocyte-associated hormones, leptin and adiponectin.

MATERIALS AND METHODS

CLA isomers and diets. Highly enriched *c*9,*t*11-CLA, and *t*10,*c*12-CLA isomers in the form of FFA were a kind gift from Natural ASA (Hovdebygda, Norway). The analytical data for these isomers were provided by the supplier and confirmed in our laboratory. The preparation enriched in *c*9,*t*11-CLA contained 84.6% *c*9,*t*11-CLA; 7.7% *t*10,*c*12-CLA; 3.8% 18:1 *c*9; 2.0% *t*9,*t*11-CLA + *t*10,*t*12-CLA; and 1.9% other FA. The preparation enriched in *t*10,*c*12-CLA contained 88.1% *t*10,*c*12-CLA; 6.6% *c*9,*t*11-CLA; 2.5% *t*9,*t*11-CLA + *t*10,*t*12-CLA; 1.1% 18:1 *c*9; and 1.7% other FA. Fenofibrate was purchased from Sigma Chemical Co. (St. Louis, MO).

The concentration of CLA used in this study was 0.5 weight % (wt%) of the diet, which is comparable to the concentrations used in previous studies in rodent models, which ranged from 0.1 to 1.5 wt% of a mixture of CLA isomers. AIN-93G, the high-carbohydrate mouse diet, was used as the basal diet. The nutrient and FA composition of this diet has been previously reported (4). Briefly, the control diet contained (g/kg) cornstarch (417.5), casein (200), dextrinized cornstarch (132), sucrose (100), corn oil with tocopherol (50) (α-tocopherol 100 mg/kg corn oil), cellulose (50), mineral mixture (AIN-93G) (35), vitamin mixture (AIN-93) (10), L-cysteine (3), and choline bitartrate (2.5). For the two CLA-containing diets, CLA isomer-enriched oils were added by replacing 5 g/kg of corn oil with an equivalent amount of the CLA source. For the fenofibrate diet 2.0 g/kg of this compound replaced an equivalent amount of cellulose. Fenofibrate and the CLA isomers were first mixed with the corn oil and then mixed with the remainder of the dietary components. Diets were constantly flushed with nitrogen gas while being gently mixed in a blender. Diets were packaged in 30-g aliquots, flushed with nitrogen gas and stored at –20°C. Fresh dietary packets were served each day. Extraction of dietary lipids and FA analysis were performed according to previously published methods (10).

Animals, feeding, and tissue collection. Forty-six 8-wk-old, pathogen free C57BL/6N female mice were purchased from Charles River (Raleigh, NC). Female mice were chosen because of their docility for housing in groups. They were maintained in a sterile air curtain isolator at the animal facility of the University of California Medical School with controlled temperature (25°C) and a light and dark cycle (12 h each). They were fed the laboratory chow diet for the first 7 d and experimental diets for the last 56 d. Animals were divided into four groups at the start of experimental diets (study day 1), 11/group for the control and fenofibrate diets, and 12/group for the two CLA diets. They were housed 5 or 6/cage and were offered fresh diets every day (5 g/animal/d). This is based on our previous experience regarding the amount of food consumed by mice in the age range used in this study (11). All of the food was consumed within 4–6 h after it was offered, in all groups. This regime avoided the need for paired feeding and oxidation of the CLA if left for too long at room temperature. Animals were weighed on study days 10, 25, 35, 42, 49, and 56. All conditions and handling of the animals were approved by the Animal Care and Use Committee at the University of California, Davis.

At the end of the study, mice were anesthetized by interperitoneal injection of xylazine (0.7 mg/mL), ketamine (9.4 mg/mL), and acepromazine (0.2 mg/mL). Blood was collected into EDTA-containing syringes by heart puncture. The mice were then killed by cervical dislocation; spleens, livers, hearts, soleus muscles, and retroperitoneal fat pads were removed, weighed, frozen in liquid nitrogen, and stored at –70°C. Animals were without food for 16 h prior to sacrifice.

Blood lipid analysis. Total cholesterol, HDL, LDL, and TG were analyzed by commercially available diagnostic kits (catalog nos. 1554506, 1930672, 1985604, and 450032, respectively; Roche Diagnostics, Indianapolis, IN) and performed in duplicate on a Roche/Hitachi 902 analyzer. Between-day percent CV for all these kits ranged from 1 to 3%.

cRNA probe generation and ribonuclease protection assay. To generate cRNA probes for most of the target genes, cDNA fragments were amplified from commercially available mouse heart or liver cDNA (Ambion, Austin, TX) using sense/antisense primers designed to generate T7 promoter sites at the 5′ end of the fragment. Three fragments (ACO, apoA-I, and apoC-3) were subcloned into a vector containing a T7 or T3 promoter site and linearized for probe production. All primers used to generate the cDNA target templates were designed using Lasergene PRIMERSELECT software (DNASTAR Inc., Madison, WI) based on previously sequenced fragments of target genes. The primers used are listed from 5′ to 3′, where the nucleotide numbers indicate the primer location in the corresponding sequences of *Mus musculus* origin obtained from the GenBank/EMBL database (Table 1).

The identity of each fragment was confirmed by ribonuclease digestion at a predicted restriction site. All of the PCR templates that were not cloned were transcribed from the integrated T7 promoter site with the exception of the 18S loading control template fragment (Ambion) and apoA-I fragments, which were transcribed from the T3 promoter sites. All ^{32}P-labeled cRNA probes were synthesized using the Maxiscript *in vitro* transcription system according to the manufacturer's instructions (Ambion) and purified by PAGE.

To determine the steady-state transcript levels of the target genes, RNase protection assays were performed using a commercially available RNase protection assay kit (RPA III; Ambion). The cRNA probes were hybridized to target total RNA

TABLE 1
Primers[a] Used to Generate cDNA Target Templates

mRNA examined (accession #)	5′ primer[b]	3′ primer[b]
Adiponectin (NM_009605)	GTACCGGTACCTCCTGCCC AGTCATGCCGAAGAT	TAATACGACTCACTATAGGAA GCTTGCCCCACACTGAACGCTGAG
Apolipoprotein A-I (NM_009692)	GATGAAAACAAGCTTGCT GGCCGTGGCTCTGGTC	TGTAAGAAAGGTACCGCGG GGGTGGGGAGTGGAAGC
Apolipoprotein C-3 (L04150)	GATGTATAGGGATCCTTG CTGCTGGGCTCTGTG	TATCTGGAGAAGCTTGG TCCTCAGGGTTAGAATCC
Cytochrome P450 (NM_007822)	GAAATATACTCGGGCCAC CCACCCTGAGCACCAA	TAATACGACTCACTATAGGAAAGCTT GCAGAAAGATGAGATGACAGGA
Leptin (NM_008493)	TACACGGTACCTATCCGC CAAGCAGAGGGTCACT	TAATACGACTCACTATAGGAAGCT TATCTGCAGCACATTTTGGGAAGG
Lipoprotein lipase (NM_008509)	GGGGGGTACCGGATTGTT GCCGCTGTTTTGTTTTAC	TAATACGACTCACTATAGGAAGC TTGCATGTGGGTTGGTGTTCAGA
Peroxisomal acyl-CoA oxidase (AB034914)	CACAGTGACTGAATTCAACGCT GTGGCTTTGGTGGATG	CTTCCCGAGGTCTAGAGTCGG CCTGGGCTACTACTGC
PPAR-α (X57638)	CTGGCCAACTTGGTACCGAA GAGGGCTGAGCGTAGGTAAT	TAATACGACTCACTATAGGAAGCTTA GGGTGGCAGGAAGGGAACAGAC
Uncoupling protein-2 (AF111999)	CTTACAGGCTAAGCTTCGGG CTGGTGGTGGTCGGAGATA	TAATACGACTCACTATAGGGGTTATAAGGAA TTCGGGGTGCAGGAAATGGGAAGAGAA

[a]Primers were designed using Lasergene PRIMERSELECT software (DNASTAR Inc., Madison, WI) based on previously sequenced fragments of target genes.
[b]Primers are listed from 5′ to 3′, where the nucleotide numbers indicate the primer location in the corresponding sequences of *Mus musculus* originally obtained from the GenBank/EMBL database.

samples and digested using the conditions suggested by the manufacturer. Protected fragments were electrophoresed on a 6% denaturing polyacrylamide gel, and the signals were analyzed on a Storm phosphoimager (Amersham Pharmacia; Buckinghamshire, England).

RNA preparation and RNA blot analysis. Total RNA was isolated from liver, retroperitoneal adipose, soleus muscle, and heart tissues using the Totally RNA kit (Ambion) according to the manufacturer's recommended procedures. Because the amount of retroperitoneal adipose tissue from the *t*10,*c*12-CLA and fenofibrate groups was limiting, we chose to pool tissue samples for molecular analysis. For RNA extraction from each tissue, a 30-mg tissue sample from each animal in the group was pooled (11/group in the control and fenofibrate groups, and 12/group in the *c*9,*t*11-CLA and *t*10,*c*12-CLA groups). Five to 15 µg of total RNA was electrophoresed on a one percent, 1.1 M formaldehyde agarose gel and then blotted onto a nylon membrane. Membranes were subsequently hybridized (ULTRAHyb; Ambion) overnight with ^{32}P-labeled cRNA probes prepared with the cRNA described above and the Strip-EZ RNA labeling kit (Ambion). After each hybridization, the RNA blots were stripped. Each blot was reprobed with a transcript-specific probe three to seven times. After all clone-specific hybridizations were completed, the blots were restripped and probed with a T7 primed ^{32}P-labeled 18S ribosomal RNA probe (Ambion).

After quantification with a phosphoimager, hybridization signals were corrected for background hybridization and total RNA loading. To correct for nonspecific background hybridization, the signal from a sample of the blot to which the probe did not specifically hybridize was subtracted from the signal generated by a specifically bound region of equal area. This adjustment prevented an underestimation of gene expression differences between samples when the transcript-specific signal was not high. Hybridization signals were analyzed and quantified by phosphoimagery, and target mRNA levels were calculated relative to the 18S ribosomal RNA levels. Each of the transcripts was analyzed by two or three RNA blots and by one to three ribonuclease protection assays; data shown are from representative gels.

Statistical analysis. The data on an individual mouse basis were subjected to one-way ANOVA between and within diets, using the SAS software (12). The one-tailed version of Dunnett's test was used to make comparisons of the test diet means with the control diet. Levene's test was used to examine the homogeneity of variance assumption.

RESULTS

CLA isomers and fenofibrate induce different changes in body composition. At the end of the study, mean ± SEM for body weights of the control, *c*9,*t*11-CLA, *t*10,*c*12-CLA, and fenofibrate groups were 25.4 ± 0.3, 26.7 ± 0.5, 23.2 ± 0.3, and 21.6 ± 0.2 g, respectively (Fig. 1). Body weight of the fenofibrate group was 10% less ($P < 0.005$) and that of the *t*10,*c*12-CLA groups was 6% less ($P < 0.02$) than that of the control group.

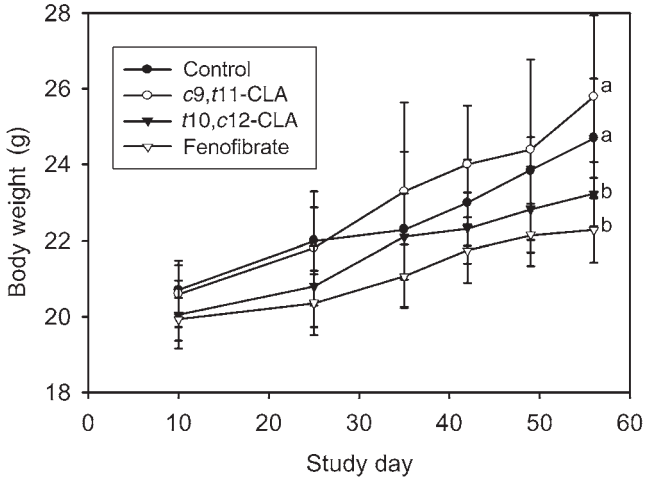

FIG. 1. Effect of CLA isomers and fenofibrate on body weights. Data are the mean ± SEM for 11 or 12 animals in each group. Means with different letters are significantly different ($P < 0.02$) using ANOVA.

Mean ± SEM for the liver weights in the control, *c*9,*t*11-CLA, *t*10,*c*12-CLA, and fenofibrate groups were 1.28 ± 0.03, 1.47 ± 0.06, 2.54 ± 0.07, and 2.52 ± 0.08 g, respectively (Fig. 2A). Mean liver weights in the fenofibrate and *t*10,*c*12-CLA groups were almost twice that of the mean liver weight in the control group ($P < 0.001$). Mean retroperitoneal adipose tissue weights in the control, *c*9,*t*11-CLA, *t*10,*c*12-CLA, and fenofibrate groups were 0.49 ± 0.04, 0.48 ± 0.05, 0.20 ± 0.01, and 0.26 ± 0.02 g, respectively (Fig. 2B). Thus, the retroperitoneal adipose tissue weights in the fenofibrate and *t*10,*c*12-CLA groups were about half of the corresponding value in the control group ($P = 0.001$). Body weight ($P = 0.18$), liver weight ($P = 0.11$), and adipose tissue weight ($P = 0.89$) for the *c*9,*t*11-CLA group did not differ from the corresponding values in the control group. Weights of hearts, soleus muscles, and spleens did not differ significantly among the four groups (data not shown).

Although the liver weights were increased in both the fenofibrate and *t*10,*c*12-CLA groups, this increase seemed to involve different mechanisms. In the *t*10,*c*12-CLA group, the liver lipids were fivefold greater than in the control group (mean ± SEM, 775 ± 119 vs. 147 ± 18 mg); liver lipids in the fenofibrate (234 ± 13 mg) and in the *c*9,*t*11-CLA groups (175 ± 13 mg) did not differ significantly from those in the control group (Fig. 3A). Lipids constituted 12% of the liver wet weights in the control and *c*9,*t*11-CLA groups; 30% in the

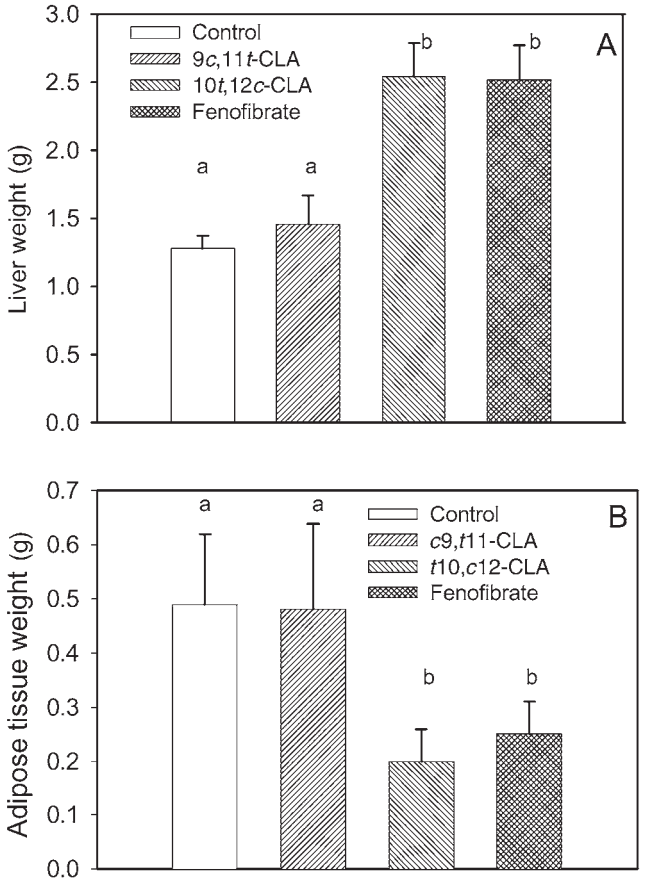

FIG. 2. Modulation of liver (A) and retroperitoneal adipose tissue (B) weights by dietary CLA isomers and fenofibrate. Data are the mean ± SEM for 11 or 12 animals in each group. Bars with different letters are significantly different ($P < 0.001$) using ANOVA.

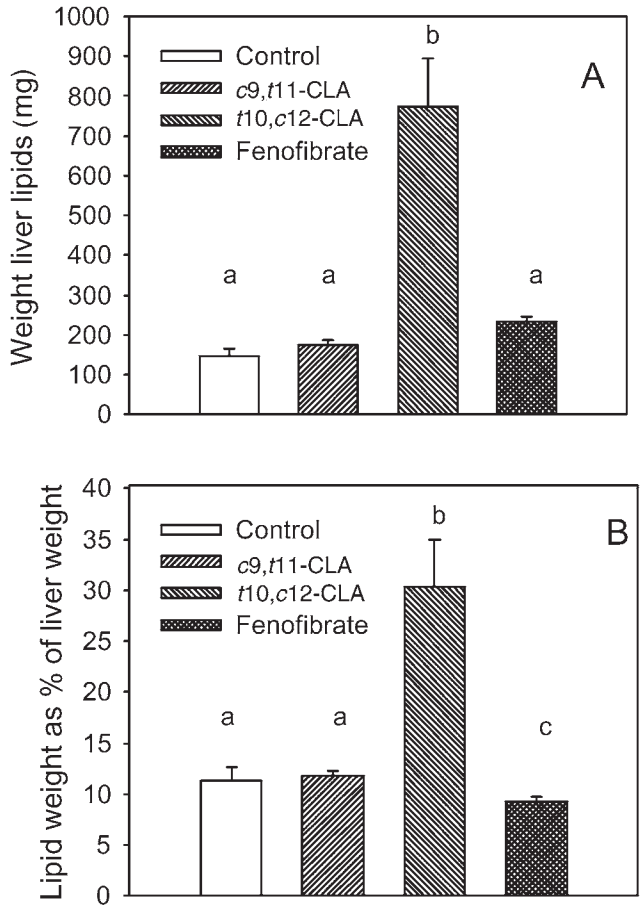

FIG. 3. Effect of CLA isomers and fenofibrate on liver lipids, as mg/liver (A) and as percentage of liver weight (B). Data are the mean ± SEM for 11 or 12 animals in each group. Bars with different letters are significantly different at $P < 0.0001$ (a vs. b) and $P < 0.0003$ (a vs. c) using ANOVA.

TABLE 2
Effect of feeding CLA Isomers and Fenofibrate on Serum TG; Total, LDL, and HDL Cholesterol (mg/dL)[a]

Diet	TG	Cholesterol	LDL	HDL
Control	19.6 ± 2.5	68.0 ± 7.4	7.8 ± 0.9	55.0 ± 6.0
*c*9,*t*11-CLA	20.3 ± 1.7	66.8 ± 4.8	8.3 ± 0.6	52.9 ± 4.2
*t*10,*c*12-CLA	17.8 ± 1.8	82.8 ± 5.0	9.9 ± 0.8	66.1 ± 3.7
Fenofibrate	15.8 ± 3.1	71.3 ± 4.2	10.1 ± 0.7	58.3 ± 3.1

[a]Mean value ± SEM, n = 10–12/group. Values within columns were not significantly different at $P \leq 0.05$ using ANOVA.

*t*10,*c*12-CLA group, and only 9% in the fenofibrate group (Fig. 3B, $P < 0.0003$ for both groups compared to the control group). Concentrations of serum TG, cholesterol, LDL, and HDL did not differ between the control and the three experimental groups (Table 2).

CLA isomers and fenofibrate result in differential steady-state transcript levels of mRNA for proteins associated with lipid metabolism and energy homeostasis. After 8 wk, the level of the apoC-3 transcript in liver was consistently reduced to an average of 47% of control in the animals fed fenofibrate. No differences in apoC-3 mRNA were detected for both the CLA isomers. No differences in the liver mRNA levels for the apoA-1 were observed between the four groups. Levels of the ACO transcript from liver and heart of animals fed the fenofibrate diet were increased (Figs. 4,5). The average increases of these transcripts were ninefold (SD = 1.4) and sixfold (SD = 0.4) over the control, respectively. ACO was also increased in the liver of animals fed *c*9,*t*11-CLA and *t*10,*c*12-CLA. In these tissues the transcript was increased 1.3-fold (SD = 0.2) and 1.2-fold (SD = 0.1) over the control value (Fig. 4). CYP4A1 mRNA was elevated in liver and adipose tissue when animals were fed fenofibrate (Figs. 4,6). The average increases in these tissues were eight (SD = 9.1) and 2.6 (SD = 0.9) times the control value, respectively. LPL was expressed fivefold more in the livers of mice fed the fenofibrate diet than that in the livers of mice fed control diet (Fig. 4). The CLA isomers had no effect on LPL mRNA levels. Interestingly, the PPAR-α transcript was increased over the control value in the *c*9,*t*11-CLA and fenofibrate conditions but suppressed in the *t*10,*c*12-CLA condition. The average differences were 1.6-fold (SD = 0.6), 2-fold (SD = 0.7), and 0.7-fold (SD = 0.2), respectively. The transcript level of UCP-2 in heart and soleus muscle (Figs. 5,7) and liver and adipose (data not shown) was not affected by any treatment.

Transcript level for leptin in the retroperitoneal adipose tissue of the mice fed *t*10,*c*12-CLA was reduced to 18% (SD = 0.06) of that in the control group (Fig. 6). Furthermore, adiponectin transcript levels were reduced in retroperitoneal adipose tissue, soleus muscle, and hearts of animals fed the *t*10,*c12*-CLA isomer when compared to the corresponding expression in the control group (Figs. 5–7). These reductions were 11 (SD = 0.07), 27 (SD = 0.07), and 38% (SD = 0.17) of the control values, respectively. In the control group, adiponectin mRNA levels in the heart and soleus muscle were 15 and 12% of the levels found in retroperitoneal adipose tissue (data not shown). *c*9,*t*11-CLA and fenofibrate did not alter the expression of adiponectin in these three tissues. In liver, the adiponectin transcript level was too low to be accurately assessed (data not shown).

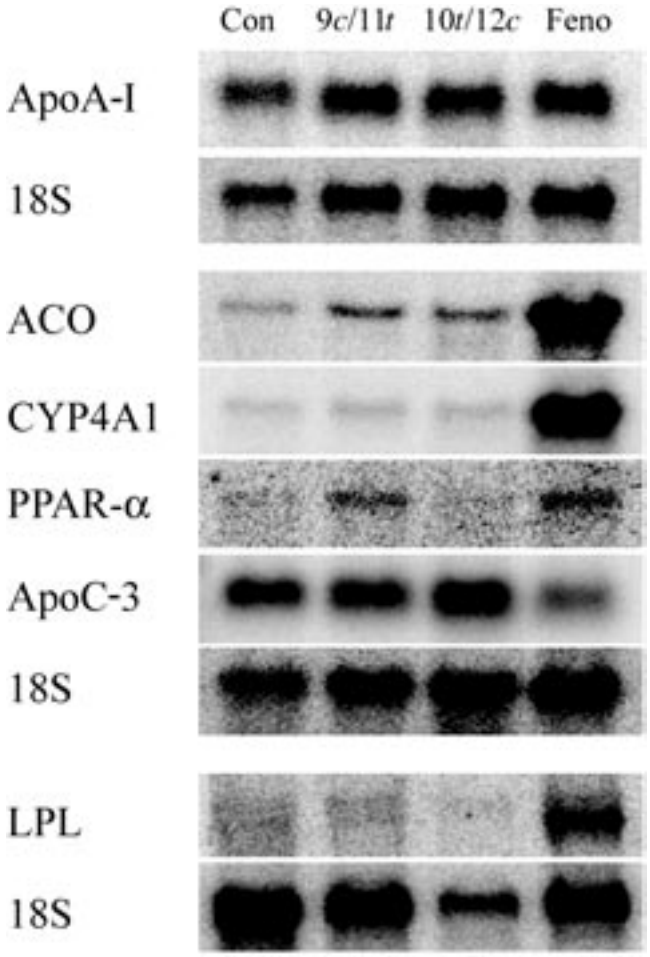

FIG. 4. Representative RNA blot analyses of lipid-metabolizing genes in liver from mice fed control (Con), 0.5% *c*9,*t*11-CLA (9*c*/11*t*), 0.5% *t*10,*c*12-CLA (10*t*/12*c*), or 0.2% fenofibrate (Feno) diets for 8 wk. The 18S loading control for individual blots is shown below the associated experimental panels above it. ApoA-1, apolipoprotein A-1; ACO, acyl-CoA oxidase; CYP4A1, cytochrome P450A1; PPAR-α, peroxisome proliferator-activated receptor α; ApoC-3, apolipoprotein C-3; LPL, lipoprotein lipase.

DISCUSSION

We compared the effects of feeding two purified isomers of CLA (*c*9,*t*11-CLA, and *t*10,*c*12-CLA) on body weights, liver and retroperitoneal adipose tissue lipids, and steady-state transcription of lipid-metabolizing enzymes that may mediate effects of CLA. Comparisons were made to the corresponding values found in mice fed a control diet and also in animals fed a known lipid-lowering agent, fenofibrate. Our results show that the two CLA isomers differed in their effects on body and organ weight as well as liver lipids. The *t*10,*c*12-CLA reduced the body and retroperitoneal adipose tissue weights significantly, compared with those found in the

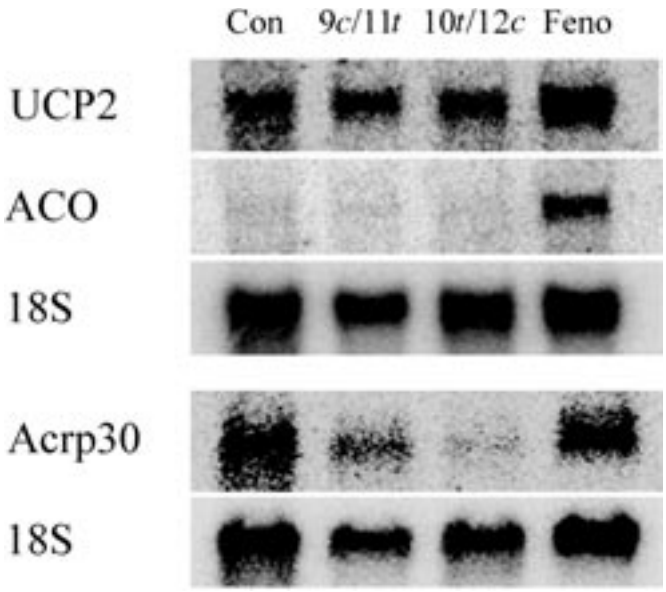

FIG. 5. Representative RNA blot analysis in heart from mice fed control, CLA, or fenofibrate diets. UCP2, mitochondrial uncoupling proteins; Acrp 30, complement-related protein 30 (= adiponectin); for other abbreviations see Figure 4. The 18S loading control for individual blots is shown below the associated experimental plots.

control group, but it caused a twofold increase in liver weights, which was largely due to an increase in liver lipids. The *c*9,*t*11-CLA did not alter any of these response variables. Our results regarding the effects of *t*10,*c*12-CLA on liver lipids are consistent with those previously reported with a mixture of CLA isomers (8,9). The increase in liver lipids in these studies was most likely due to *t*10,*c*12-CLA, because in

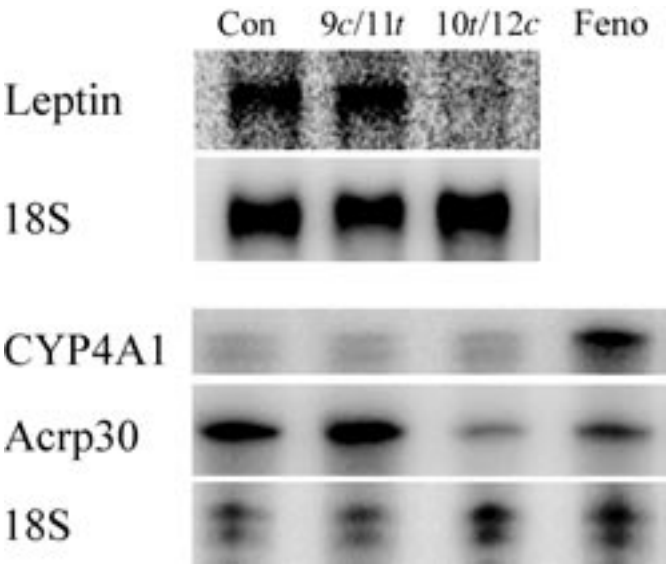

FIG. 6. Representative RNA blots for leptin and ribonuclease protection assay (RPA) for CYP4A1 and adiponectin (Acrp30) in adipose tissues from mice fed control, CLA, or fenofibrate diets for 8 wk. The 18S loading control for each experiment is shown below the associated experimental panel above it. The doublet observed in the 18S loading control for the RPA experiment is due to small differences in probe length due to radiolysis of this small probe. For abbreviations see Figures 4 and 5.

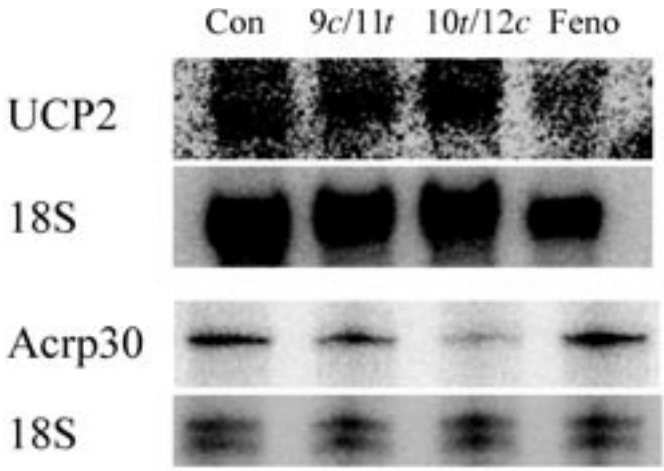

FIG. 7. Representative RNA blots of UCP2 and RPA (Acrp30) for muscle tissues from mice fed control, CLA-, or fenofibrate-containing diets. The 18S loading control for each experiment is shown below the experimental panel. The doublet observed in the 18S loading control for the RPA experiment is due to small differences in probe length due to radiolysis of this small probe. For abbreviations see Figures 4–6.

our current study *c*9,*t*11-CLA did not cause an increase in liver lipids. Our results regarding the effects of *t*10,*c*12-CLA on mice liver lipids differ from those reported with hamster liver lipids, where it did not alter the concentration of liver lipids (6). We are not certain of the reasons for this discrepancy, but hamster has been reported to more resistant to CLA than other species (13,14).

The changes in body, liver, and adipose tissue weights caused by *t*10,*c*12-CLA were similar to those caused by fenofibrate, a known PPARα agonist; however, different mechanisms may have mediated the effects of these two agents. Reduction in adipose tissue lipids in the *t*10,*c*12-CLA group was at the cost of storing lipids in the liver (five times more than in the control group), while the concentration of liver lipids in the fenofibrate group did not differ from those in the control group. Furthermore, *t*10,*c*12-CLA decreased the transcription of PPARα, whereas fenofibrate increased it. These results suggest that PPARα did not mediate the effects of *t*10,*c*12-CLA on body composition. This interpretation is consistent with the results from a study with the PPARα knock-out mice, where the effects of a mixture of CLA isomers on body and organ weights were similar to those found in the wild type (15).

Decreased transcription of leptin and of adiponectin in adipose tissue in the *t*10,*c*12-CLA group may have mediated its effects on liver and adipose tissue lipids. Leptin administration in animals has been reported to favor lipolysis and inhibit lipogenesis (16); the converse would be anticipated with a reduction in leptin levels. Adiponectin reduces hepatic glucose production (17); therefore, a reduction in adiponectin could increase hepatic glucose production that may be used for FA synthesis. Thus, reductions in leptin and adiponectin transcripts may account for the effects of *t*10,*c*12-CLA on liver and adipose lipids. These findings need to be corroborated by

investigating the changes in the proteins for these hormones. A reduction in the transport of lipids from liver to storage depots also could contribute to increased liver lipids. A reduction in adiposity is usually accompanied by a reduction in plasma leptin and increase in plasma adiponectin levels (18). However, in our study the mRNA for these molecules were reduced in mice fed *t*10,*c*12-CLA. These results suggest that adiponectin levels may be regulated by mechanisms not directly coupled to adiposity. This hypothesis can be supported by the work of Matsuzawa and colleagues (19) who have shown that although hypoadiponectemia is correlated with development of atherosclerosis, this effect is independent of well-known cardiovascular disease risk factors, such as body mass index.

Neither of the CLA isomers altered the mRNA for CYP4A1, LPL, or apoC-3. This is in contrast to a report indicating that *t*10,*c*12-CLA feeding decreased expression and activity of LPL in adipose and mammary lipids in dairy cows (20). Our results indicate that these genes may not be involved in mediating the effects of *t*10,*c*12-CLA on lipid metabolism. However, each of these PPARα-mediated transcripts was altered after fenofibrate treatment. These mRNA alterations are similar to what others have found with fibrate feeding (21,22). Both CLA isomers increased the transcripts for ACO, as previously reported by others feeding a mixture of CLA isomers (15). Since the ACO transcription was increased by both CLA isomers, it could not be responsible for the effects of *t*10,*c*12-CLA on liver and adipose tissue lipids.

The effects of *c*9,*t*11-CLA, and *t*10,*c*12-CLA on body composition differ from their effects on immune cell functions as previously reported (4). Both isomers increased secretion of inflammatory cytokines tumor necrosis factor α and interleukin 6 (IL-6) and decreased secretion of the T helper 2 cytokine IL-4; many other indices of immune status were not affected by either isomer. Comparison of the effects of CLA isomers on immune cell functions and body composition suggests that separate mechanisms may mediate the effects of CLA on immune cell functions and body composition.

In conclusion, results of our study show that the two isomers of CLA differed in their effects on body composition: *c*9,*t*11-CLA did not alter any of the response variables tested, whereas *t*10,*c*12-CLA reduced body and retroperitoneal adipose tissue weights, and increased liver weight and lipids. These effects of *t*10,*c*12-CLA were most likely the result of decreased transcription of leptin and adiponectin. Reduction of adipose tissue fat by *t*10,*c*12-CLA in mice and hamsters may be viewed as a beneficial effect; however, such benefits to humans are questionable. Supplementing diets of human volunteers with this isomer (3.4 g/d for 12 wk) did not change body weight or fat mass; however, it increased insulin resistance, fasting glucose, serum VLDL and C reactive protein, and lipid peroxidation, and decreased serum HDL and leptin (23–26). These metabolic changes indicate increased risk for diabetes and cardiovascular disease. It is therefore important to establish the risk/benefit ratio of this isomer before human use can be recommended.

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Dietary CLA Affects Lipid Metabolism in Broiler Chicks

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ABSTRACT: A total of 120 three-wk-old broiler chicks were randomly assigned to three diets containing 0, 2, or 3% CLA and fed for 5 wk. Fat content and FA composition of liver, plasma, and feces were analyzed. Key enzymes involved in FA synthesis and catabolism in liver, TG, cholesterol, and FFA content of plasma were also determined. Dietary CLA increased TG, total cholesterol, and HDL cholesterol levels in plasma. The increased plasma TG level could be caused by increased FA synthesis in the liver after CLA feeding, because the activity of FA synthase in the liver increased after dietary CLA treatment. Dietary CLA changed the FA composition of feces but had no effect on fat content. Compared to the amounts of linoleic and linolenic acids present in the control diet, the amounts excreted into the feces of CLA-treated birds were significantly higher. Liver weights of broilers significantly increased after CLA feeding, but there was no difference in liver fat content among the different CLA treatments. CLA treatment did not influence total FFA content in plasma; however, there was a significant difference in the composition of FFA. Dietary CLA reduced the content of linoleic and arachidonic acids in both plasma and liver.

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Dietary CLA is reported to reduce fat accumulation in certain animal models (1–3). Dietary CLA reduced retroperitoneal fat pad weight by 13, 25, and 32% in rats fed 0.25, 0.5, and 1.0% pure CLA, respectively ($P < 0.05$) (1). Similar effects were observed in parametrial fat pad (1). Feeding CLA at a low level produced a rapid, marked decrease in fat accumulation but increased protein content without any major effects on food intake (2). Rats fed 0.5% CLA in a diet had significantly reduced body fat but increased whole-body protein, water, and ash (3). Dietary CLA is also reported to improve feed efficiency in rats (4,5). The exact mechanism for the reduced fat accumulation by dietary CLA is not yet clear, but it can be related to the inhibition of lipid absorption and lipogenesis and the promotion of lipid oxidation.

In birds, the liver is the principal site of lipid synthesis. Unlike mammals, FA, rather than glucose, are the main energy source for birds, and the liver of birds has a very high capacity for lipogenesis. Numerous reports on the effect of dietary CLA on FA metabolism in mammals have been published, but few are available on birds (6,7). Further, most of the published reports have concentrated on milk synthesis and adipose tissue, and liver, an important organ in lipid metabolism, seems to have been ignored. Therefore, the objective of this study was to determine the effect of dietary CLA on FA status and key enzyme activities in the liver of broiler chicks.

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MATERIALS AND METHODS

Chicken feeding and sample preparation. A total of 120 three-wk-old broiler chicks were kept in 12 pens. Four pens were randomly assigned to one of three dietary treatments containing 0, 2, or 3% CLA (Tables 1 and 2). The CLA source, which contained 62% CLA, was obtained from a commercial company (Conlinco, Inc., Detroit Lakes, MN). Soybean oil and the CLA source were substituted on a weight/weight basis in different diets. After 5 wk dietary treatment, birds were slaughtered according to USDA guidelines, and feces were collected the night before slaughter. For feces collection, birds from each treatment group were placed into four containers, with five birds in each container. Birds were kept in containers for 2 h and all feces were collected and pooled. Fecal samples were then dried in a fume hood at 22°C for 48 h. Blood and whole liver were collected during slaughter. Whole liver was weighed, and then

TABLE 1
Percentage Composition of Diets Fed to Broiler Chicks

Ingredients	Diet (1 to 3 wk)	Diet (4 to 5 wk)
Corn	51.15	50.34
Soy meal	38.38	28.57
Wheat middlings	22.85	10.26
Meat and bone meal	3.00	3.00
Limestone	1.05	0.96
Dicalcium phosphate	0.85	0.85
Mineral premix[a]	0.30	0.30
Vitamin premix[b]	0.30	0.30
DL-Methionine	0.25	0.15
Sodium chloride (iodized)	0.09	0.25
BMD (bacitracin methylene disalicylate)	0.025	0.025
Soybean oil	4.61	5.0 to 0[c]
CLA source	0	0 to 5.0[c]
Calculated metabolizable energy (kcal/kg)	3,100	3,100

[a]Mineral premix provides (per kg of diet): Mn, 80 mg; Zn, 90 mg; Fe, 60 mg; Cu, 12 mg; Se, 0.147 mg; sodium chloride, 2.247 g.
[b]Vitamin premix supplies (per kg of diet): retinyl acetate, 8,065 IU; cholecalciferol, 1,580 IU; 25-hydroxy-cholecalciferol, 31.5 μg, DL-α-tocopheryl acetate, 15 IU; vitamin B_{12}, 16 μg; menadione, 4 mg; riboflavin, 7.8 mg; pantothenic acid, 12.8 mg; niacin, 75 mg; choline chloride, 509 mg; folic acid, 1.62 mg; biotin, 0.27 mg.
[c]In the 0% CLA group, soybean oil, 5.00%; CLA source, 0%. In the 2.0% CLA group, soybean oil, 2.67%; CLA source, 3.33%. In the 3.0% CLA group, soybean oil, 0%; CLA source, 5%.

TABLE 2
Crude Fat Content and FA Composition of Diets (4 to 5 wk)[a]

	0% CLA diet	2% CLA diet	3% CLA diet
Crude fat content	8.72 ± 0.40	8.68 ± 0.47	8.45 ± 0.38
FA composition			
Palmitoleic	0.32 ± 0.03^{b}	0.34 ± 0.02^{a}	0.37 ± 0.03^{a}
Palmitic	13.71 ± 0.25^{a}	9.43 ± 0.12^{b}	8.12 ± 0.11^{c}
Stearic	4.62 ± 0.05^{a}	2.76 ± 0.03^{b}	2.39 ± 0.07^{c}
Linoleic	46.45 ± 0.25^{a}	21.47 ± 0.07^{b}	15.14 ± 0.07^{c}
Oleic	30.96 ± 0.23^{b}	33.02 ± 0.27^{a}	33.37 ± 0.21^{a}
Linolenic	3.77 ± 0.17^{a}	1.67 ± 0.08^{b}	1.34 ± 0.09^{c}
CLA (*cis*-9,*trans*-11)	0 ± 0.00^{c}	9.20 ± 0.32^{b}	11.23 ± 0.39^{a}
CLA (*trans*-10,*cis*-12)	0 ± 0.00^{c}	11.69 ± 0.43^{b}	14.02 ± 0.40^{a}
CLA (*trans*-9, *trans*-11)	0 ± 0.00^{c}	4.88 ± 0.28^{b}	5.87 ± 0.34^{a}
Other CLA isomers	0 ± 0.00^{c}	5.04 ± 0.20^{b}	7.14 ± 0.38^{a}

[a]Means within a row with no common roman superscript (a–c) differ significantly ($P < 0.05$). Livers (1 g/bird) from five birds from the same pen were randomly selected and pooled, and four mixtures were prepared for analysis ($n = 4$).

a part of the liver was quickly frozen in liquid nitrogen and used for chemical analyses. Ten milliliters of blood was collected in test tubes containing 200 μL of 5 mM EDTA, and plasma was separated by centrifuging at 1500 × *g* for 15 min.

Analysis of total cholesterol, HDL cholesterol, and TG in plasma. Fifteen plasma samples for each treatment (three or four samples per pen) were randomly selected for analysis. Sigma kit methods (catalog nos. 352-20 and 336-20; Sigma-Aldrich, St. Louis, MO) were used to analyze plasma cholesterol, HDL cholesterol, and TG levels. Reagent (1.0 mL) was pipetted into a tube, and 10 μL of plasma sample was added. The tube was incubated for 10 min (for cholesterol) or 18 min (for TG) at 25°C. HDL cholesterol was measured after serum LDL and VLDL lipoproteins were selectively precipitated and removed by centrifugation (Sigma kit, catalog no. 352-7). Absorbance was read and recorded using a spectrophotometer at 500-nm wavelength.

Lipid extraction. Livers (1 g/bird, cut into small cubes) and plasma (0.5 mL/bird), respectively, from five birds from the same pen were randomly selected and pooled; thus, four mixtures each of liver and plasma were prepared. Two grams of liver from the pooled liver pieces (total, 5 g) or 2 mL of plasma mixtures were weighed into test tubes. The same method was also used for fecal samples. Ten volumes of chloroform/methanol solution (2:1, vol/vol) was prepared following the method of Folch *et al.* (8). This solution (Solution 1) was added to the samples, which were then homogenized with a Brinkman polytron (Type PT 10/35; Brinkman Instruments, Inc., Westbury, NY) for 10 s at high speed. Twenty-five micrograms of 10% BHA dissolved in 98% ethanol was added to each sample prior to homogenization. The homogenate was filtered through Whatman #1 filter paper into a 100-mL graduated cylinder and 0.25 vol (on the basis of Solution 1) of 0.88% NaCl solution was added. After the cylinder was capped with a glass stopper, the filtrate was mixed well. The inside of the cylinder was washed twice with 2 mL of $CHCl_3/CH_3OH/H_2O$ (3:47:48, by vol; Solution 2), and the contents were stored until the aqueous and organic layers were clearly separated. The upper layer was siphoned off, and the lower layer was moved to glass scintillation vials and dried at 50°C under nitrogen.

Separation of FFA from plasma. The dried plasma lipids were redissolved with chloroform to make a final concentration of 0.2 g lipid mL chloroform, and 50 mg of behenic acid (Sigma-Aldrich) was added as internal standard. The lipid/chloroform solution (150 μL) was loaded onto an activated (120°C for 2 h) silica gel plate (20 × 20 cm; Sigma-Aldrich). The plate was developed first in Solvent 3, composed of chloroform/methanol/water (65:25:4, by vol), until the solvent line reached the middle of the plate. The plate was air-dried and then redeveloped in Solvent 4, composed of hexane/diethyl ether (4:1, vol/vol), until the solvent reached 5 cm below the top of the plate. After air-drying for 10 min at room temperature (22°C), the plates were sprayed with 0.1% 2′,7′-dichlorofluorescein in ethanol. Lipid classes were identified under UV light, and the lane corresponding to FFA was scraped into a separate test tube. FFA were extracted three times using 5 mL of 1:1 (vol/vol) cholesterol/methanol. The solvent was dried under a nitrogen flow, and FFA were used for FA composition analysis.

Analysis of FA composition. One milliliter of methylating reagent (3 N anhydrous methanolic HCl; Sigma-Aldrich) was added into the test tube containing total lipids or FFA, capped tightly, and incubated in a water bath at 60°C for 40 min. After cooling to room temperature, 2 mL of hexane and 5 mL of water were added, mixed thoroughly, and left at room temperature overnight for phase separation. The top hexane layer containing methylated FA was used for GC analysis. Analysis of FA composition was performed with a gas chromatograph (HP 6890; Hewlett-Packard Co., Wilmington, DE) equipped with an autosample injector and an FID. A capillary column (HP-5, 0.25 mm i.d., 30 m, 0.25 μm film thickness; Hewlett-Packard Co.) was used. A splitless inlet was used to inject samples (1 μL) into the capillary column. Ramped oven temperature conditions (180°C for 2.5 min, increased to 230°C at 2.5°C/min, then held at 230°C for 7.5 min) were used for the analysis of FA composition of lipids from liver and feces. For the composition of FFA in plasma, the initial oven temperature was lowered to

140°C, held for 2 min, increased to 230°C at 5°C/min, and then held at 230°C for 10 min.

The temperatures of both inlet and detector were 280°C. Helium was used as a carrier gas, and a constant column flow of 1.1 mL/min was used. Detector (FID) air, H_2, and makeup gas (He) flows were 350, 35, and 43 mL/min, respectively. FA were identified using a mass selective detector (Model 5973; Agilent Technologies, Wilmington, DE). The ionization potential of the mass selective detector was 70 eV, and the scan range was 45 to 450 *m/z*. Identification of FA was achieved by comparing mass spectral data with those of the Wiley library and confirmed by comparing retention times with standards purchased from Matreya (Pleasant Gap, PA) and Nu-Chek-Prep (Elysian, MN). The FA compositions of lipids in liver and feces were reported as percentages, and the FFA in plasma were reported as actual amounts calculated by using behenic acid as an internal standard.

Enzyme activity analysis. Ten livers per treatment (two or three livers from each of the four pens) were randomly selected for enzyme activity analysis. All the chemicals used in the analyses of enzyme activities were purchased from Sigma-Aldrich.

(i) Preparation of mitochondria and cytosolic fractions. One gram of liver was homogenized in 5 vol of Solution 5 [0.25 M mannitol, 5 mM Hepes (pH 7.4), and 1 mM EGTA] using a polytron. One milliliter of homogenate was then transferred to Eppendorf vials and centrifuged at 2,000 × *g* for 10 min. The supernatant was collected and centrifuged again at 10,000 × *g* for 10 min to sediment the mitochondria. The resulting supernatant was used as a cytosolic solution for FA synthase and acetyl-CoA carboxylase analyses. The sediment (200 μL, mainly mitochondria) was added to 500 μL of Solution 5, mixed, and used for the analysis of carnitine palmitoyltransferase activity. The protein contents of cytosolic and mitochondrial solutions were analyzed with a protein analysis kit (Sigma-Aldrich).

(ii) Carnitine palmitoyltransferase I and II. Carnitine palmitoyltransferase I and II activities were analyzed by measuring the release of CoA catalyzed by carnitine palmitoyltransferases (9). Briefly, two sets of Eppendorf vials were prepared. Vial A contained a reaction mixture (200 μL) composed of 100 mM Hepes buffer (pH 7.8), 1.25 mM EGTA, 1 mM DTNB, 0.15 mM palmitoyl-CoA, 1.25 mM carnitine, and an aliquot of mitochondrial mixture (0.6 mg protein). Vial B contained substances identical to Vial A but without 1.25 mM carnitine. After 3 min of incubation at room temperature, the reaction was stopped by placing the vials in a boiling water bath. Samples were cooled to room temperature and centrifuged at 8,000 × *g* for 3 min; absorbance of the supernatant was then measured at 412 nm. To calculate the standard curve between absorbance and CoA formation, a known amount of CoA was added instead of the mitochondrial mixture and the same procedure was followed as above. The enzyme activity was calculated by the difference in absorbance between Vials A and B. Enzyme activity was expressed as CoA formation min^{-1} mg $protein^{-1}$.

(iii) Acetyl-CoA carboxylase. A reaction mixture (200 μL) composed of 20 mM sodium citrate, 20 mM magnesium chloride, 1.0 mM DTT, 0.5 mg/mL FA-free BSA, 50 mM Hepes buffer (pH 7.4), 200 μM acetyl-CoA, 5 mM ATP, 30 mM [^{14}C]sodium bicarbonate, and an aliquot of cytosolic fraction (0.6 mg protein) was incubated for 8 min at 37°C; the reaction was then stopped by adding 40 μL of HCl (6 N). The samples were evaporated to dryness at room temperature and transferred to scintillation vials for reading. Enzyme activity was expressed as [1-^{14}C]bicarbonate incorporation into FA min^{-1} mg $protein^{-1}$ (10,11).

(iv) FA synthase. A reaction mixture (200 μL) composed of 100 mM Hepes (pH 7.4), 3.0 mM EGTA, 1.0 mM dithioerythritol, 0.062 mM (4 Ci/mol) [1-^{14}C]acetyl-CoA, 1.25 mM NADP, 12.5 mM glucose-6-phosphate, 0.7 U glucose-6-phosphate dehydrogenase, 0.30 mM malonyl-CoA, and an aliquot of cytosolic fraction (0.6 mg protein) was incubated at 37°C for 8 min. The reaction mixture was then extracted directly with 1 mL of Solution 1 and mixed vigorously. After phase separation, the bottom (chloroform) layer was used for scintillation counts. Enzyme activity was expressed as [1-^{14}C]acetyl-CoA incorporation into FA min^{-1} mg $protein^{-1}$ (10,11).

Statistical methods. Effects of dietary CLA on FA composition, weight, plasma TG, plasma cholesterol, and enzyme activities were analyzed using SAS software (12). The Student–Newman–Keuls multiple range test was used to compare differences among mean values ($P < 0.05$). Mean values and SEM were reported.

RESULTS AND DISCUSSION

The ingredients in the diets of the broiler chicks are shown in Table 1, and the fat content and FA composition of the diets are shown in Table 2. There was no difference in fat content among the diets. The content of linoleic acid in diets decreased as the amount of CLA increased. The level of linolenic acid in the CLA diets was lower, but that of oleic acid was higher than in the control. The major CLA isomers present in the diets were *cis*-9,*trans*-11 and *trans*-10,*cis*-12.

The TG and cholesterol levels of plasma are shown in Table 3. TG levels in plasma increased significantly with CLA diets,

TABLE 3
Plasma TG, Total Cholesterol, and HDL Cholesterol Content of Broilers[a]

	0% CLA diet	2% CLA diet	3% CLA diet
TG	42.1 ± 6.82[b]	49.8 ± 9.85[a]	50.2 ± 11.44[a]
Total cholesterol	126.3 ± 12.57[c]	152.9 ± 16.02[b]	170.4 ± 23.70[a]
HDL cholesterol	38.2 ± 4.18[b]	46.8 ± 5.30[a]	48.3 ± 2.99[a]
Calculated LDL + VLDL cholesterol	88.2	106.8	122.1

[a]Means within a row with no common roman superscript (a–c) differ significantly ($P < 0.05$). Plasma samples were randomly selected ($n = 15$) from 15 birds per treatment.

TABLE 4
Activities of Selected Enzymes Related to FA Metabolism in Livers[a]

	0% CLA diet	2% CLA diet	3% CLA diet
FA synthase	0.38 ± 0.03[b]	0.46 ± 0.04[a]	0.46 ± 0.06[a]
Acetyl-CoA carboxylase	2.97 ± 1.28	3.46 ± 1.01	3.84 ± 1.76
Carnitine palmitoyl-CoA transferase	11.41 ± 0.81	11.99 ± 1.12	12.24 ± 0.96

[a]Means within a row with no common roman superscript (a,b) differ significantly ($P < 0.05$). Livers were randomly selected for analysis from 10 birds per treatment ($n = 10$).

in agreement with previous reports: In rats, plasma TG concentrations were elevated significantly ($P \leq 0.01$) after CLA feeding (13,14). Feeding diets containing up to 1% CLA increased VLDL TG (80 and 61%) in hamsters (15). Dietary CLA also increased plasma cholesterol levels in broilers, an unexpected result (Table 3). The total cholesterol level in plasma increased from 126.3 mg/dL in the control diet to 152.9 and 170.4 mg/dL, respectively, in the 2 and 3% CLA diets, an increase mainly due to the increase in VLDL and LDL cholesterol. This result was different from previous reports: Nicolosi *et al.* (16) reported that hamsters fed up to 1.1% CLA-containing diets for 11 wk had significantly reduced levels of plasma total cholesterol, non-HDL cholesterol, and TG with no effect on HDL cholesterol. Diets containing CLA mixtures of 3 and 5% exhibited marked reductions of LDL and HDL cholesterol compared with rats receiving no CLA (17). However, in pigs there was an increase in total plasma cholesterol after CLA feeding, and the ratio of LDL cholesterol to HDL cholesterol was significantly increased in CLA diets (18). Our study indicated that up to 1% dietary CLA did not influence plasma TG and cholesterol levels in broiler chicks (data not shown). Animal species, dose of CLA, and duration of treatment could be responsible for the different responses in plasma cholesterol levels after CLA feeding.

The reason for the increased plasma TG and cholesterol levels in CLA-treated birds was not clear, but it could be related to the changes in enzyme activities associated with lipid metabolism in the liver. In birds, liver is the main site of lipid synthesis. Table 4 shows the activities of FA synthesis, acetyl-CoA synthase, and carnitine palmitoyl-CoA transferase in the liver. A significant increase in FA synthase activity and an increase (although not significant) in acetyl-CoA carboxylase activity in the liver were observed with CLA feeding. Acetyl-CoA carboxylase and FA synthase are the main enzymes controlling FA synthesis. The increase in FA synthase activity could account in part for the increased TG levels in plasma. The effect of dietary CLA on enzymes of adipose tissues and mammary glands has been reported previously, but changes in FA synthase and acetyl-CoA carboxylase activities in the liver after CLA feeding have not yet been reported. In the mammary glands of sows, FA synthase and acetyl-CoA carboxylase activities decreased after feeding CLA diets (19). Dietary CLA was also involved in reducing the *de novo* FA synthesis and desaturation process in adipose tissues and mammary glands in sows (20). In the adipose tissue of AKR/J mice, however, dietary CLA increased fat oxidation but had no effect on *de novo* fat biosynthesis (21). In adipose cell culture, the mRNA expression of FA synthase was not reduced by dietary CLA (22). These results indicate that dietary CLA reduces lipogenesis in adipose tissues and mammary glands but not in liver. This could be the reason CLA is ineffective in reducing fat accumulation in birds (13), in which lipogenesis is concentrated in the liver. Adipose tissues are important for FA synthesis in mice, rats, and pigs, and inhibiting lipogenesis in adipose tissue by CLA could significantly reduce fat accumulation in these animal species. No change was shown in acetyl-CoA carboxylase activity in the liver of rabbits fed 0.5% CLA, but its activities in adipose tissue were inhibited (23). The authors suggested that the activities of lipogenic enzymes in the adipose tissues and liver of rabbits are regulated differently (23). The overall FA synthase and acetyl-CoA carboxylase activities measured in this study were quite low (Table 4), a result that could be associated with the high fat content of the diets due to 5% oil addition (Table 1).

There was no difference in the activity of carnitine palmitoyl-CoA transferase with CLA feeding (Table 4). Jones *et al.* (24) fed male Wistar rats a semipurified diet containing 0, 1.5, or 5.0 energy percentage CLA for 4 wk and found that dietary CLA did not change the activities of hepatic palmitoyl-CoA oxidase and carnitine acetyl transferase. In hamsters, palmitoyl-CoA oxidase and carnitine acetyl transferase activities were not increased by CLA (15). In rats, the activity of carnitine palmitoyltransferase I was not changed by dietary CLA either in liver or muscle, but its activity did increase more than 30% compared to the control value in epididymal adipose tissue, showing that dietary CLA might increase FA oxidation in adipose tissues (25).

The lipid content and FA composition of liver are shown in Table 5. Liver weight increased as the level of dietary CLA increased, in agreement with the result of DeLany and West (21) in mice. No difference in liver fat content was observed (Table 5). However, a recent study showed that dietary CLA reduced the fat content in chicken liver (7). The proportions of saturated FA, palmitic acid, and stearic acid increased as dietary CLA level increased, and the content of monounsaturated FA decreased. This was in agreement with our previous report (26).

The FFA content in plasma from birds fed CLA diets is shown in Table 6. Dietary CLA had no effect on the content of FFA in plasma, whereas the content of individual FA changed. Dietary CLA decreased the levels of palmitoleic, linoleic, and arachidonic acids, whereas CLA isomers increased. Other FFA, including stearic, palmitic, myristic, lauric, and capric acids, did not change (Table 6). In pigs, a 1% level of CLA for 6 wk reduced plasma concentrations of nonesterified FA by 38% but

TABLE 5
Weight, Crude Fat Content, and FA Composition of Liver[a]

	0% CLA diet	2.0% CLA diet	3.0% CLA diet
Liver weight	62.1 ± 9.94[b]	64.2 ± 9.55[b]	70.9 ± 9.12[a]
Crude fat content	3.8 ± 0.53	3.6 ± 0.98	4.2 ± 1.20
FA composition			
Myristic	0.23 ± 0.06	0.19 ± 0.01	0.19 ± 0.04
Palmitoleic	0.65 ± 0.09[a]	0.32 ± 0.07[b]	0.55 ± 0.18[a]
Palmitic	18.02 ± 0.63[c]	21.46 ± 0.54[b]	24.34 ± 1.32[a]
Margaric	0.23 ± 0.03[a]	0.20 ± 0.01[b]	0.18 ± 0.01[b]
Linoleic	25.47 ± 1.79[a]	18.97 ± 1.18[b]	15.02 ± 2.82[c]
Oleic	21.29 ± 2.11[a]	18.57 ± 1.17[b]	15.84 ± 1.14[c]
Stearic	12.56 ± 0.85[c]	19.39 ± 0.93[b]	25.05 ± 1.01[a]
Linolenic	1.40 ± 0.12[a]	1.22 ± 0.08[b]	0.81 ± 0.06[c]
CLA (*cis*-9,*trans*-11)	0 ± 0.00[c]	1.28 ± 0.13[b]	2.01 ± 0.35[a]
CLA (*trans*-10,*cis*-12)	0 ± 0.00[c]	1.86 ± 0.30[b]	2.58 ± 0.66[a]
CLA (*trans*-9,*trans*-11)	0 ± 0.00[b]	0.59 ± 0.15[a]	0.71 ± 0.15[a]
Other CLA isomers	0 ± 0.00[c]	0.81 ± 0.10[b]	1.12 ± 0.22[a]
Arachidonic	11.07 ± 2.74[a]	8.78 ± 1.19[b]	6.01 ± 0.89[c]
Eicosapentaenoic	0.52 ± 0.05[a]	0.45 ± 0.31[a]	0.25 ± 0.03[b]
Docosahexaenoic	2.80 ± 0.55[a]	2.01 ± 0.31[b]	0.95 ± 0.18[c]
Unconfirmed	4.21 ± 1.12	4.59 ± 1.22	3.55 ± 0.61

[a]Means within a row with no common roman superscript (a–c) differ significantly ($P < 0.05$); $n = 40$ for liver weight, and $n = 4$ for the analysis of fat content and FA composition.

the change was not statistically significant (18). The FFA content in serum has been shown to be associated with human diseases (27).

As Table 7 shows, there is no difference in the extractable lipid content in feces from birds fed different CLA diets, indicating that CLA had no influence on total lipid excretion. No differences in energy digestibility and metabolizable energy between the control and CLA diets were reported in pigs (28). A significant difference in the FA composition of feces was found (Table 7). The content of CLA isomers in feces was much higher in CLA-treated birds. Although the content of linoleic acids in all treatments was similar, the linolenic acid level was significantly higher in CLA-treated groups. When comparing the contents of linoleic and linolenic acids in the diet, their levels were more than two times lower in CLA diets than in the control diet (Table 2), but the contents in feces of birds treated with CLA were similar for linoleic acid and even higher for linolenic acid. This indicates that the excretion of linoleic and linolenic acids in birds fed CLA diets could be much higher than in the control diet. This might be related to an increased saturated FA content in the plasma and liver (Table 5).

This study showed that high-level dietary CLA increased plasma TG and cholesterol levels. The increase in TG level could be due in part to increased FA synthase activity in the liver. Dietary CLA decreased the contents of linoleic and arachidonic

TABLE 6
Composition of FFA in Plasma (μg/mL plasma)[a]

	0% CLA diet	2% CLA diet	3% CLA diet
FFA content	501.7 ± 68.3	592.1 ± 80.3	552.3 ± 67.2
Individual FA content (μg/mL)			
Capric	3.1 ± 0.93	2.9 ± 0.77	3.0 ± 0.81
Lauric	4.1 ± 1.88	4.8 ± 1.25	7.2 ± 2.44
Myristic	2.7 ± 0.40	3.6 ± 0.43	3.8 ± 0.59
Palmitic	133.8 ± 22.1	163.9 ± 19.9	163.0 ± 24.4
Palmitoleic	10.8 ± 1.0[a]	6.6 ± 0.8[b]	6.0 ± 0.7[b]
Stearic	78.3 ± 10.3	105.4 ± 15.8	99.6 ± 13.8
Oleic	156.6 ± 18.3	164.0 ± 22.8	141.6 ± 19.6
Linoleic	91.3 ± 13.1[a]	90.1 ± 8.8[a]	64.3 ± 6.4[b]
CLA (*cis*-9,*trans*-11)	0 ± 0.00[c]	13.2 ± 3.4[b]	18.6 ± 3.7[a]
CLA (*trans*-10,*cis*-12)	0 ± 0.00[c]	13.8 ± 2.9[b]	19.0 ± 3.7[a]
CLA (*trans*-9,*trans*-11)	0 ± 0.00[c]	4.7 ± 1.8[b]	9.6 ± 1.7[a]
Other CLA isomers	0 ± 0.00[c]	5.8 ± 2.3[b]	10.0 ± 2.8[a]
Arachidonic	20.4 ± 2.7[a]	13.3 ± 2.2[b]	6.7 ± 1.7[c]

[a]Means within a row with no common roman superscript (a–c) differ significantly ($P < 0.05$). Plasma (0.5 mL/bird) from five birds from the same pen were randomly selected and pooled, and four mixtures were prepared for analysis ($n = 4$). FFA in plasma were reported as actual amounts calculated by using behenic acid as an internal standard.

TABLE 7
Crude Fat Content and FA Composition of Feces from Broilers[a]

	0% CLA diet	2.0% CLA diet	3.0% CLA
Crude fat content	5.34 ± 0.78	5.55 ± 0.73	5.28 ± 0.84
FA composition			
Myristic	0.17 ± 0.07	0.12 ± 0.05	0.16 ± 0.03
Palmitoleic	0.21 ± 0.03	0.27 ± 0.04	0.26 ± 0.02
Palmitic	14.08 ± 0.45[a]	12.23 ± 0.15[b]	11.44 ± 0.16[c]
Margaric	0.20 ± 0.08	0.13 ± 0.06	0.10 ± 0.06
Stearic	4.21 ± 0.65[a]	3.06 ± 0.18[b]	3.21 ± 0.20[b]
Oleic	21.37 ± 5.95	22.86 ± 6.39	26.92 ± 0.31
Linoleic	52.30 ± 5.33[a]	45.45 ± 4.97[b]	41.13 ± 1.27[b]
Linolenic	0.90 ± 0.03[b]	1.16 ± 0.17[a]	1.33 ± 0.15[a]
CLA (*cis*-9,*trans*-11)	0 ± 0.00[c]	2.86 ± 0.48[b]	4.85 ± 0.20[a]
CLA (*trans*-10,*cis*-12)	0 ± 0.00[c]	2.88 ± 0.52	5.00 ± 0.25[a]
CLA (*trans*-9,*trans*-11)	0 ± 0.00[c]	1.48 ± 0.28[b]	2.77 ± 0.63[a]
Other CLA isomers	0 ± 0.00[c]	1.23 ± 0.08[b]	2.89 ± 0.41[a]

[a]Means within a row with no common roman superscript (a–c) differ significantly ($P < 0.05$). Feces collected from five birds in the same container were pooled and dried; four containers were used ($n = 4$).

acids in the liver and the level of FFA in plasma. There was no difference in the crude lipid content of feces among chickens treated with different levels of dietary CLA. Even though the contents of linoleic and linolenic acids in the CLA diets were much lower than in the control diet, their contents in feces were very similar to or even higher than in the control.

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Impact of Dietary FA and Energy Restriction on Plasma Leptin and ob Gene Expression in Mice

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ABSTRACT: The aim of the present study was to elucidate whether the qualitative composition of dietary fat influences plasma leptin and adipose tissue ob gene expression differentially. Two high-fat diets and a diet rich in carbohydrate were each administered both *ad libitum* and with a 25% energy restriction. The high-fat diets contained 58 energy percent as either monounsaturated FA (MUFA) or saturated FA (SAFA), whereas the carbohydrate-rich diet (CH) contained 7 energy percent as fat. We aimed at obtaining the same final weight for the animals in the *ad libitum* group as in the energy-restricted groups. This goal was reached at the same time (days 22–24) for all groups except for the *ad libitum* animals fed on saturated fat (day 36). The plasma leptin concentrations on *ad libitum* CH and MUFA diets did not differ significantly (24.3 ± 2.1 and 34.7 ± 6.7 ng/mL, respectively) whereas the saturated fat diet caused a lower concentration (13.9 ± 1.9 ng/mL; $P < 0.05$). Interestingly, no differences in plasma leptin levels between groups were seen in the energy-restricted groups (mean 8.0 ± 1.0 ng/mL). The type of diet did not alter the ob gene expression in intraabdominal white adipose tissue; however, a lower expression level was found in the energy-restricted groups. The percentage of body fat in the three *ad libitum* fed groups did not differ (23 ± 1%). Thus, short-term administration of a diet rich in SAFA suppresses circulating leptin levels without altering the adipose tissue ob gene expression. This indicates that saturated fat may alter protein handling by adipose tissue or the whole body clearance of leptin.

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The discovery of the obese (ob) gene in 1994 (1) has enlarged our knowledge on the disordered molecular biology of obesity. A high correlation between circulating leptin levels and body weight consistently has been reported (2,3). Obesity is, however, not ascribed to one or a few genetic mutations *per se* but rather to an excess energy intake and fat overeating. As a result of excessive calorie intake for example, leptin resistance has been proposed to provoke diet-induced obesity in animals and humans (3). Most studies have shown that high-fat diets can both increase the ob gene expression in adipose tissue and augment circulating leptin levels (4–6). However, controversy exists since high-fat diets also have been associated with a suppression of plasma leptin levels (7). Even energy restriction can decrease the circulating leptin levels independently of the total fat mass. How the quality of ingested fat influences the level of plasma leptin and ob gene expression is not clear. The present work was undertaken to study the association in normal mice between the circulating levels of leptin and the adipose tissue expression of the ob gene secondary to short-term changes elicited by diets rich in saturated FA (SAFA) or monounsaturated FA (MUFA) compared to a carbohydrate-rich (CH) diet. The potential impact of the qualitative composition of dietary fat was tested after *ad libitum* feeding and after a 25% energy restriction.

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Abbreviations: CH, carbohydrate-rich (control) diet; MUFA, diet rich in monounsaturated FA; SAFA, diet rich in saturated FA.

MATERIALS AND METHODS

Animals, diet, and study protocol. Eight-week-old female NMRI mice (Taconic M&B, Ry, Denmark) were allocated to the study groups after 1 wk of acclimatization in the local animal care facilities. Before the experiment the animal diet for all groups was standard chow (Altromin GmbH, Lage, Germany). Hereafter, the dietary treatments were started (see below). Animal weight and food consumption were measured. Animals were housed individually. Food consumption by the animals fed *ad libitum* was determined by weighing out 12 g of food and 24 h later weighing the remainder in the tray. The light/dark cycle was 12/12 h. Tap water was given *ad libitum*. The study was approved by the Danish Veterinary Authorities, The Ministry of Justice.

Six study groups were examined; half were given the diets *ad libitum* and the other half were fed a 25% energy-restricted diet. The following six groups were examined: groups A (CH *ad libitum*), B (MUFA *ad libitum*), C (SAFA *ad libitum*), D (CH energy-restricted), E (MUFA energy-restricted), and F (SAFA energy-restricted). Twelve animals were allocated to each of the study groups. The diets rich in either carbohydrate, SAFA or MUFA were made by Altromin (C1010, D1057, and C1057, respectively) and stored at 4°C until use. The composition of the three diets is shown in Table 1. The high-fat diets contained approximately 60 energy percent as fat (Table 1), as this percentage previously has been shown to influence intermediary metabolism (e.g., Ref. 8). The diets were composed upon our request and a detailed description was given by the manufacturer.

We wanted to study animals with comparable body weights in order to reduce the influence of differences in body weights between groups. A pilot study revealed differences in weight

gain rates with the different diets. Animals fed the carbohydrate-rich diet were chosen to determine the ending weight of the two remaining groups, and the experiment was extended for one group to achieve similar ending weights. The aim for the animals on restricted energy intake was to maintain stable weight during the 4-wk study period. These animals were allowed 75% of the diet given to the *ad libitum* fed animals (9) based on the cumulative intake during the preceding week. Blood sampling was performed at 8:00–9:00 P.M. on the day of the experiment after a 16-h fasting period (water *ad libitum*). The animals were anesthetized with pentobarbital (50 mg/kg) intraperitoneally, and blood was subsequently drawn from the retroorbital plexus in chilled tubes with 10 μL of a solution containing heparin and aprotinin (7.7 mg/mL and 2300 IE/mL, respectively). The blood was used for determination of plasma leptin, plasma glucose, and plasma lactate. Intraabdominal white adipose tissue was removed and immediately frozen in liquid nitrogen for later determination of ob gene expression. The remainder of the carcass was frozen at −18°C for later determination of body fat. Total body fat was determined using the method described by Bligh and Dyer (10). In brief, after freeze-drying and homogenization, extractions using methanol, chloroform, and water were performed thrice for each animal. The amount of fat in the chloroform phase was determined, and the percentage of total body fat calculated.

Analyses. Plasma leptin was analyzed by use of a rat leptin kit (Linco Res. Inc., St. Charles, MO). Plasma glucose and lactate were determined using an YSI Stat 2000 Glucose Analyzer (YSI Inc., Yellow Springs, OH).

RNA was isolated using the Trizol reagent (Gibco BRL, Grand Island, NY). The reverse transcription and the amplification were performed with 25 ng total mRNA using Amplitaq Gold DNA polymerase and hexamer primers as described by the manufacturer (GeneAmp PCR kit; PerkinElmer Cetus, Norwalk, CT). The leptin primers, used in the PCR, spanned a cDNA product of 352 base pairs. The sense leptin-primer corresponds to nucleotide number 85 to 107 and the antisense strand of nucleotide 417 to 437 (1) β-Actin mRNA was amplified as a housekeeper marker, and a semiquantitative multiplex PCR method "primer-dropping" (11) was used to monitor mRNA expression. Semiquantitative multiplex PCR estimates the relative amount of mRNA to a known housekeeping gene (β-actin) working as internal control of the sample variability. Initial experiments were done for each set of primers to decide the cycle numbers for exponential amplification of cDNA (data not shown). The target primers were run 11 PCR cycles before the β-actin primers were dropped (primer-dropping); both sets were brought into the middle of the exponential range after an additional 22 cycles. The PCR products were loaded on a 2% agarose gel stained with ethidium bromide and analyzed using the Bio Rad Gel Doc 1000 system. The CV was 10.2%.

Statistics. ANOVA and the Newman–Keuls test for multiple differences were used for statistical comparisons (using the SPSS for Windows v. 8.0, Chicago, IL). A P value <5% was considered significant. Data are presented as mean ± SEM.

TABLE I
Energy Composition of the Three Diets[a] (given as energy %)

Diet	CH	MUFA	SAFA
Fat	7.3	57.8	57.8
10:0 - Capric acid	0.0	0.0	3.6
12:0 - Lauric acid	0.0	0.0	25.7
14:0 - Myristic acid	0.0	0.0	11.0
16:0 - Palmitic acid	0.4	6.8	6.3
18:0 - Stearic acid	0.2	1.7	4.3
18:1 - Oleic acid	1.5	42.6	3.2
18:2 - Linoleic acid	5.0	4.6	3.1
18:3 - Linolenic acid	0.0	1.1	1.1
Carbohydrates	79.5	27.5	27.5
Monosaccharides	5.2	4.2	4.2
Disaccharides	71.9	9.6	9.6
Polysaccharides	2.4	13.8	13.8
Protein	13.2	14.7	14.9

[a]CH, carbohydrate-rich diet; MUFA, diet rich in monounsaturated FA; SAFA, diet rich in saturated FA.

RESULTS

Animal weights and food consumption. The animals fed *ad libitum* gained approximately 5 g, with no statistical differences between groups, by the end of the study period (Table 2). However, the relative weight gains per day were different between groups. The time for the animals fed the SAFA diet *ad libitum* (E) to gain a weight similar to groups A and B was 36 d (vs. 22 for groups A and B). The animals on the energy-restricted diets were weight stable during the study period with no differences between groups. The daily food intake was significantly different among groups ($P < 0.01$) at all time points in the following order: CH > MUFA > SAFA. The amount of food ingested at the start, at half of the study period, and at the end of the study is provided in Table 2.

Body composition. After the study period, the relative percentages of total body fat in the *ad libitum* fed groups A, B, and C were similar (Table 2).

Plasma leptin levels. The plasma leptin level in a control group of mice at the beginning of the study was 16 ± 5 ng/mL. The leptin levels of animals fed *ad libitum* on the CH (A) or the MUFA diet (B) did not differ between the beginning and the end of the study period. The plasma leptin level in the MUFA group, however, tended to increase during the study period (Fig. 1). Interestingly, the leptin levels in the carbohydrate and MUFA groups were both higher than in the SAFA diet group (C) ($P < 0.05$). The leptin levels in the energy-restricted animals were lower compared to those of the animals fed *ad libitum*. Thus, energy restriction reduced leptin levels significantly in the CH and the MUFA groups ($P < 0.001$), whereas the tendency for energy restriction to lower leptin did not reach statistical significance in the SAFA-treated group. No differences in leptin levels appeared between the three energy-restricted groups.

Ob gene expression in adipose tissue. The ob gene expression in intraabdominal adipose tissue was examined using reverse transcriptase-PCR (Table 2). The diet composition (CH, MUFA, and SAFA) had no influence on ob-mRNA levels.

TABLE 2
Animal Weights at Start and End of Experiment, Food Intake, Fasting Plasma Glucose, Total Body Fat, and ob Gene Expression[a]

Group diet	A CH	B MUFA	C SAFA	D CH	E MUFA	F SAFA
Days of treatment	22	22	36	23	22	24
Start weight (g)	29.5 ± 0.4	29.2 ± 0.4	28.9 ± 0.5	29.2 ± 0.2	28.5 ± 0.3	27.9 ± 0.6
End weight (g)	36.4 ± 0.8	35.7 ± 1.1	34.8 ± 0.7	29.8 ± 0.4	29.4 ± 0.4	28.7 ± 0.7
Food intake (g)						
Start	6.1 ± 0.2	5.1 ± 0.2	4.8 ± 0.2	4.0	3.5	3.0
Middle	5.0 ± 0.1	4.2 ± 0.2	4.3 ± 0.2	3.5	3.0	3.0
End	4.0 ± 0.1	3.4 ± 0.1	3.4 ± 0.2	3.0	3.0	3.0
All period	4.9 ± 0.1	4.4 ± 0.1	4.0 ± 0.1	3.5 ± 0.1	3.2 ± 0.1	3.0 ± 0.0
P-glucose (mM)	9.3 ± 0.3	9.9 ± 1.3	6.0 ± 0.3	7.8 ± 0.8	8.3 ± 0.7	3.6 ± 0.4
Total body fat (%)	23 ± 3	24 ± 2	21 ± 2	ND	ND	ND
Ob mRNA	2.8 ± 0.5	2.5 ± 0.4	2.5 ± 0.5	1.3 ± 0.3	1.4 ± 0.2	1.4 ± 0.2

[a]Animal weight at start and end of the dietary regimen. Food intake is reported as the mean of 3 d of intake at the start, middle, and end of the experiment (see Materials and Methods section). Fasting plasma glucose (P-glucose) at the end of the study is shown. Total body fat is determined as described in the Materials and Methods section. Ob gene expression (arbitrary units, see the Materials and Methods section) is determined in intraabdominal adipose tissue. Groups A, B, and C were fed *ad libitum*. Groups D, E, and F were on energy-restricted diets. Data are presented as mean ± SEM. ND, not determined. $n = 11$ or 12 in each group.

However, energy restriction in all diet groups was associated with lower expression levels compared to the groups fed *ad libitum*. The relative decreases induced by energy restriction for the CH, MUFA, and SAFA groups were approximately 53, 44, and 44%, respectively.

Plasma glucose levels. Both the *ad libitum* and energy-restricted SAFA (C and F) groups had fasting plasma glucose levels significantly lower than the CH- and MUFA-rich groups (Table 2) ($P < 0.01$). The levels in the energy-restricted animals were not significantly different from the *ad libitum* group except for the SAFA-rich group where the levels in the restricted group were lower ($P < 0.01$). No differences in plasma lactate levels between groups were found (data not shown).

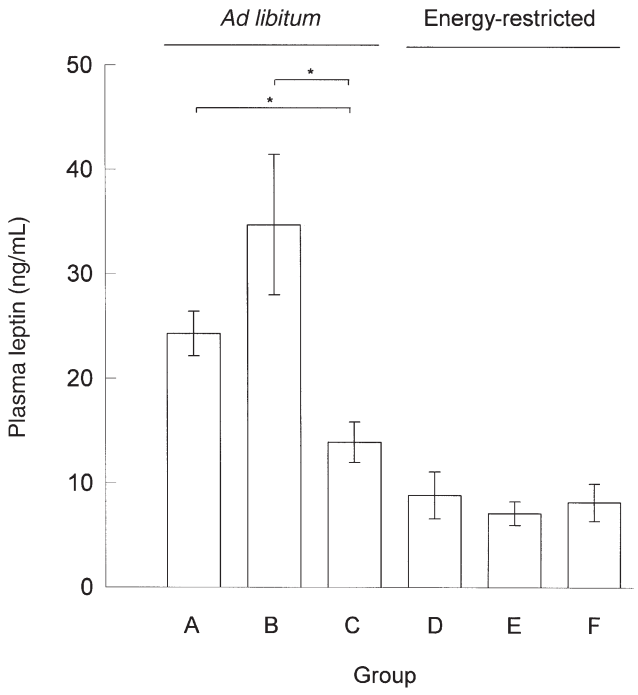

FIG. 1. Influence of dietary fat and energy restriction on plasma leptin levels in normal mice. Groups A and D were on a carbohydrate-rich diet, groups B and E on a diet rich in monounsaturated FA, and groups C and F on a diet rich in saturated FA. Mean ± SEM is shown; $n = 11$ or 12 in each group. *$P < 0.05$.

DISCUSSION

The present data indicate that the fat quality differentially can alter the circulating leptin levels in normal mice fed *ad libitum*. In comparison to a CH diet, a diet rich in MUFA tended to increase the circulating leptin levels whereas the SAFA-rich diet reduced it. These changes are not related to changes in the percentage of total body fat or body weight. A possible explanation for the differential impact of the dietary fat quality on the circulating leptin levels could be that the diets induced differential changes in the ob gene expression. Therefore, we tested whether changes in the ob gene mRNA levels in white adipose tissue were associated with the observed differences in the circulating leptin levels. Interestingly, similar ob gene expression levels were detected among the three groups of animals fed *ad libitum*, indicating that dietary manipulation does not induce changes in the ob gene expression level. This raises the possibility that the quality of fat may change the clearance of circulating leptin. Leptin is cleared mainly by the kidney and to a lesser degree within the splanchnic bed (12); however, until now a change in the regulation of leptin clearance has been detected only in renal failure. In this context, it should be noted that our animals were healthy. It cannot be ruled out that dietary manipulations could alter the rate of ob gene translation in adipose tissue. Leptin secretion usually appears to be constitutive (13). Our results corroborate those of Cha and Jones (14), who observed differential effects of diets independent of body weight and notably also independent of total body fat percentage. In the study by Cha and Jones it was found that 10 wk of

feeding rats with fish oil or safflower oil increased plasma leptin compared to a group receiving a diet rich in beef tallow (14). However, the beef tallow diet contained a considerable amount of 18:1 and differs from the SAFA diet used in our study. Another difference between the two studies was that our SAFA group had a slower weight gain, for which we compensated by extending the feeding period so that similar weight gain and final weights were obtained in all three groups in our study.

The finding that plasma leptin levels were different despite unchanged total body fat seems not to support a direct link between circulating leptin and fat mass. Our method for determination of total fat does not, however, elucidate whether a redistribution of fat stores from, e.g., subcutaneous to intraperitoneal stores might have taken place. This issue seems relevant since the leptin gene expression is regulated by the fat quality to a different extent in adipose tissue at different anatomical locations (15).

Whether the total energy intake plays a major role on the impact of the fat quality on leptin levels was investigated through studies on energy-restricted animals. The primary aim was to determine whether the potential differential effects of high-fat diets were also present under conditions of weight stability. Interestingly, energy restriction seemed to dilute the effect of the fat quality, i.e., the observed differences in the levels of circulating leptin disappeared. It was expected, however, that energy restriction would suppress ob gene expression (14) and circulating leptin levels (2). To our knowledge, the combination of dietary fat manipulations and energy restriction has not previously been investigated in animals with similar body weights. Ainslie *et al.* (7) found that in the short term a high-fat diet is associated with relatively low circulating leptin levels and higher body weights. The differential impact of the dietary fat observed in the *ad libitum*-fed groups on the circulating leptin levels disappeared in the energy-restricted animals. Also, no differences in ob gene expression between the dietary groups in the energy-restricted animals were found.

We have chosen a fasting period of 14–16 h before blood and tissue sampling. The energy-restricted animals were fed their meals at 9.00 A.M. and finished eating after 5–6 h. Therefore, the lengths of the fasting periods for both *ad libitum* and the energy-restricted animals were considered similar. In rats, ob gene expression previously has been demonstrated to decrease after fasting for 12 to 24 h (16). A 24-h fast has been shown to decrease ob gene expression in lean mice, in contrast to obese mice that had unchanged levels (17). So the fasting period before obtaining the blood and tissue samples may have reduced plasma leptin levels as well as the ob gene expression. However, as the fasting period is similar in all three groups, the length of fasting period cannot explain our findings.

The lower circulating leptin levels in the SAFA-fed group cannot be explained by the slightly more advanced age of these animals since leptin levels normally increase with age. Therefore, the possibility exists that the circulating leptin levels reflect the rate of weight gain in these nonobese mice. It is unclear why the *ad libitum* SAFA-fed animals had a slower weight increase, but obviously it may be related to a lower daily food intake. It is worth keeping in mind that the volumetric intake of the three isocaloric diets was lower for the MUFA and the SAFA groups compared to the CH group. However, in contrast to the SAFA group, this does not result in a slower weight gain in the MUFA group.

Since all three animal groups on energy-restricted diets obtained similar final weights on the same day, it is very unlikely that the SAFA diet was absorbed to a lesser extent than the other two diets. As expected, blood glucose levels were higher in the *ad libitum* than in the energy-restricted state. It is worth noting that the fasting plasma glucose levels were significantly lower in both SAFA-treated groups of animals. An association between adipose tissue ob gene expression and plasma glucose was suggested earlier (18). We did not find any differences in the ob gene expression levels within the *ad libitum* and the energy-restricted groups. The reason why SAFA feeding was associated with lower blood glucose levels is not known.

Ad libitum-administered diets rich in either MUFA or SAFA fatty acids altered the plasma leptin levels in a differential manner in mice, whereas the adipose tissue ob gene expression remained unchanged, pointing to potential changes in adipose tissue protein handling or the clearance of leptin. This impact of dietary fat quality, however, seems to fade after energy restriction.

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Pitavastatin Ameliorates Severe Hepatic Steatosis in Aromatase-Deficient (Ar–/–) Mice

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ABSTRACT: Tamoxifen is a potent antagonist of estrogen, and hepatic steatosis is a frequent complication in adjuvant tamoxifen for breast cancer. Impaired hepatic FA β-oxidation in peroxisomes, microsomes, and mitochondria results in progression of massive hepatic steatosis in estrogen deficiency. This impairment, although latent, is potentially serious: About 3% of the general population in the United States is now suffering from nonalcoholic steatohepatitis associated with obesity and hyperlipidemia. Therefore, in the present study we tried to restore impaired hepatic FA β-oxidation by administering a novel statin, pitavastatin, to aromatase-deficient (Ar–/–) mice defective in intrinsic estrogen synthesis. Northern blot analysis of Ar–/– mice liver revealed a significant restoration of mRNA expression of essential enzymes involved in FA β-oxidation such as very long fatty acyl-CoA synthetase in peroxisome, peroxisomal fatty acyl-CoA oxidase, and medium-chain acyl-CoA dehydrogenase. Severe hepatic steatosis observed in Ar–/– mice substantially regressed. Consistent findings were obtained in the *in vitro* assays of FA β-oxidation activity. These findings demonstrate that pitavastatin is capable of restoring impaired FA β-oxidation *in vivo via* the peroxisome proliferator-activated receptor-α-mediated signaling pathway and is potent enough to ameliorate severe hepatic steatosis in mice deficient in intrinsic estrogen.

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Uptake, utilization, and secretion rates of FA are tightly controlled to meet energy demands and to maintain the cellular lipid content in hepatocytes. The major lipid catabolic pathways in the liver are peroxisomal and mitochondrial FA β-oxidation, which are regulated at the level of gene expression during development and in response to diverse physiological stimuli (1–3). The deterioration of gene expression of FA-metabolizing enzymes in hepatocytes may result in massive hepatic steatosis.

FA metabolism is impaired in the aromatase-deficient (Ar–/–) mouse. It has a reduced gene expression of FA-metabolizing enzymes in hepatocytes and spontaneously develops massive hepatic steatosis, a condition observed in one-third of breast cancer patients treated with the estrogen antagonist tamoxifen (4,5). Thus, it is an elegant model of estrogen deficiency mimicking that of breast cancer patients treated with tamoxifen.

Adjuvant tamoxifen became a standard treatment for women with early breast cancer in the 1990s, and although some adverse effects have been reported (6), this treatment undoubtedly outweighs the risks of the adverse effects. A major problem, however, is that tamoxifen induces nonalcoholic steatohepatitis (NASH) and liver cirrhosis (7–10). Rapid progression of hepatic steatosis was also noted in 36% of nonobese, nondiabetic breast cancer patients treated with tamoxifen (11), where a body mass index (BMI: kg/m^2) greater than 23 was reported as a significant risk factor for progressive hepatic steatosis (5).

Today, hepatic steatosis is no longer regarded as a benign lesion, since chronic accumulation of hepatic TG sometimes leads to NASH, liver fibrosis, and cirrhosis (12). In two-thirds of the general population in the United States, the BMI exceeds 23 and about 3% of the general population is now suffering from NASH associated with obesity (13). Twenty percent of NASH patients are expected to develop liver cirrhosis within 10 yr (14), yet breast cancer patients are advised to take tamoxifen for 5 yr (15). It is difficult to ignore such a risk for 5 yr when a breast cancer patient is obese, particularly since tamoxifen is known to impair FA β-oxidation in at least in one-third of nonobese nondiabetic Japanese women (4,11,16). In breast cancer patients treated with tamoxifen, physicians need medicines that can either decrease fat deposition in the liver or prevent the development of hepatic steatosis. Fibrates, one type of peroxisome proliferators, are the first-line medicines because they were shown to ameliorate massive hepatic steatosis in tamoxifen-treated patients by activating FA β-oxidation in the liver through peroxisome proliferator-activated receptor-α (PPAR-α)-mediated signaling (4,5,16).

Recently, however, hypercholesterolemia has become a frequent concern both in the general population and in breast cancer patients. Fibrates sometimes fail to reduce plasma cholesterol levels sufficiently in these patients. Coadministration of a statin with a fibrate is one option, but it is not recommended since such coadministration is believed to increase the incidence rate of rhabdomyolysis, a rare but serious com-

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Abbreviations: AOX, peroxisomal acyl-CoA oxidase; Ar+/+ mouse, wild-type mouse; apo, apolipoprotein; Ar–/– mouse, aromatase-deficient mouse; BMI, body mass index; CYP2E1, microsomal cytochrome P450 2E1; CYP4A1, microsomal cytochrome P450 4A1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; NASH, nonalcoholic steatohepatitis; PPAR-α, peroxisome proliferator-activated receptor-α; VLACS, very long fatty acyl-CoA synthetase.

plication (17). For this reason, a statin with intrinsic agonistic effects on PPAR-α such as pitavastatin, would be preferable for those patients (18–20).

In the present study, we investigated the hypolipidemic effects of pitavastatin and its fat-eliminating capacity on spontaneously developed fatty liver in Ar–/– mice.

MATERIALS AND METHODS

Mice. The aromatase gene (*cyp 19* gene) was disrupted by homologous recombination (21). In brief, an 87-bp fragment located within exon 9 of the *cyp 19* gene in E14-1 cells (embryonic stem cells) was replaced with a neomycin resistance gene derived from *pMC1*-neo. Selected embryonic stem cells were micro-injected into the C57BL/6J blastocytes to generate chimeric mice. Chimeric male mice were then mated with C57BL/6J female mice to generate mice heterozygous for the mutation. Heterozygous mice were mated to obtain aromatase null (Ar–/–) mice because of the infertility of homozygous males and females. Ar–/– male mice aged 16 wk and their wild-type male siblings (Ar+/+, C57BL/6J) were used in the present study. A conventional maintenance diet (CE-2) containing 25.4 w/w% of protein and 4.4 w/w% of fat (Clea Japan, Suita, Japan) was used in this study, which approximates the composition of the human diet. The Ar+/+ group was fed a CE-2 diet for 8 wk. Ar–/– mice were divided into two groups and also fed for 8 wk: The Ar–/– group was fed a CE-2 diet, and the Ar–/– + pitavastatin group was fed a CE-2 diet supplemented with 0.014% pitavastatin, a potent novel statin with an intrinsic agonistic effect on PPAR-α (kindly provided by Kowa, Nagoya, Japan). Genotypes of mice were determined by a PCR using genomic DNA isolated from tail tips. Animal care and experiments were carried out in accordance with institutional animal care regulations.

Light microscopic observations. Liver tissues were routinely fixed in 10% phosphate-buffered formalin (pH 7.4), embedded in paraffin, and sectioned for hematoxylin and eosin staining. The degree of hepatic steatosis in whole specimens was classified into four grades according to the distribution pattern of the fat vacuoles as follows: 0 = no or few fat droplets in the lobules, I = a few fat droplets in the lobules, II = fat droplets restricted to zone 3, III = fat droplets in zones 3 and 2, IV = numerous fat droplets in zones 3 and 2.

Analysis of mRNA expression for enzymes involved in FA β-oxidation. mRNA analysis was performed by Northern blotting. Total liver RNA was obtained from fresh liver using the acid guanidinium thiocyanate/phenol/chloroform extraction method. RNA was separated on 1% agarose gel and transferred to a nylon membrane. The membranes were incubated with ^{32}P-labeled cDNA probes and analyzed on a Fuji system analyzer (Fuji Photo Film, Tokyo, Japan). The cDNA used for Northern blotting included catalase, very long fatty acyl-CoA synthetase (VLACS), peroxisomal acyl-CoA oxidase (AOX), medium-chain acyl-CoA dehydrogenase (MCAD), cytochrome P450 2E1 (CYP2E1), cytochrome P450 4A1 (CYP4A1), apolipoprotein A4, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (4). Changes in mRNA levels were estimated by densitometric scanning of autoradiograms and analyzed by NIH Image 1.52 to show a relative ratio to the findings in Ar+/+. The number of animals used for mRNA analysis was five in each group.

FA β-oxidation activity. FA β-oxidation activity was measured as described previously (22). In brief, fresh liver was homogenized in 4 vol of 0.25 M sucrose containing 1 mM EDTA in a Potter–Elvehjem homogenizer using a tight-fitting Teflon pestle. Homogenate (1–10 mg) was incubated with the assay medium in 0.2 mL of 150 mM KCl, 10 mM HEPES (pH 7.2), 0.1 mM EDTA, 1 mM potassium phosphate buffer (pH 7.2), 5 mM malonate, 10 mM $MgCl_2$, 1 mM carnitine, 0.15% BSA, 5 mM ATP, and 50 μM of each FA (10^5 cpm for radioactive substrates; 55 mCi/mmol; American Radiolabeled Chemicals, St. Louis, MO): [1-^{14}C]tetracosanoic acid (24:0), [1-^{14}C]palmitic acid (16:0), or [1-^{14}C]lauric acid (12:0). The reaction was run for 30 min at 25°C and stopped by the addition of 0.2 mL of 0.6 N perchloric acid. The mixture was centrifuged at 2,000 × *g* for 10 min, and the unreacted FA in the supernatant was removed using 2 mL of *n*-hexane with three extractions. Radioactive degradation products in the water phase were counted. FA β-oxidation activity was expressed as nmol/min/liver. In some experiments using [1-^{14}C]palmitic acid, KCN, a potent inhibitor of the mitochondrial respiratory chain, was added to the assay medium to inhibit potent mitochondrial activity.

FA analysis. The liver was homogenized and TG were extracted using chloroform/methanol (2:1, vol/vol). TG were then further extracted with water/chloroform (1:1, vol/vol) and quantified.

Statistical analysis. Data were analyzed using Student's *t*-test or Wilcoxon's signed rank test.

RESULTS

Light microscopy observation. Hepatic steatosis was limited to zone 3 (centrilobular zone in the lobules) in Ar–/– mice at 10 wk of age. However, the mice progressively developed massive hepatic steatosis, although there was a slight variation in the severity (Fig. 1A). Steatosis of liver cells in zone 1 (periportal) was absent or slight. Liver cells in zones 3 and 2 (centrilobular and intermediate zones in the lobules) showed marked microvesicular steatosis in Ar–/– mice. Some large vacuoles were also seen. This zonal difference of steatosis within the liver lobules was clear. Grade IV hepatic steatosis regressed to grades II or III in Ar–/– mice when treated with pitavastatin ($P < 0.03$; Table 1, Fig. 1B). Their wild-type siblings (Ar+/+) did not develop hepatic steatosis (Fig. 1C).

mRNA analysis. To clarify whether pitavastatin could restore deteriorated hepatic lipid metabolism, mRNA expression of hepatic peroxisomal (catalase, VLACS, AOX), mitochondrial (MCAD), and microsomal (CYP2E1 and CYP4A1) enzymes was analyzed by Northern blot analyses in the Ar+/+, Ar–/–, and Ar–/– + pitavastatin groups (Fig. 2).

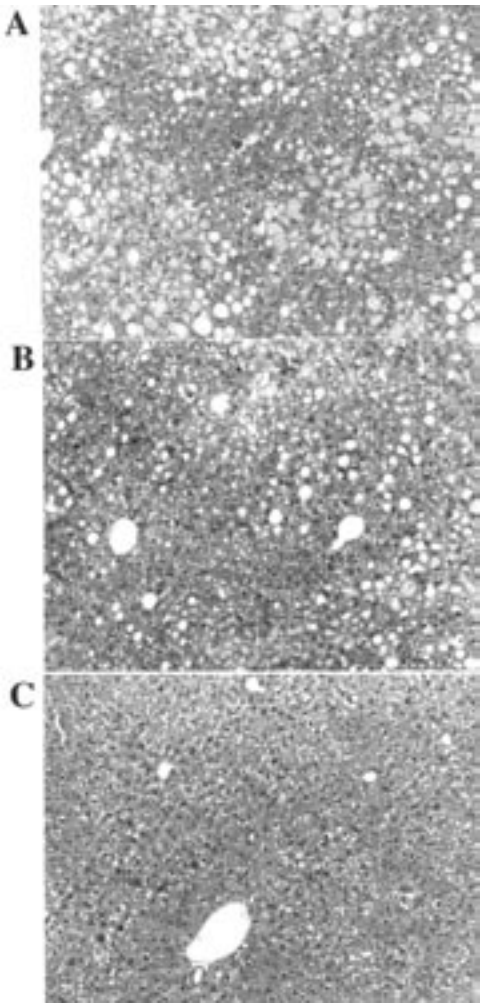

FIG. 1. Steatosis of the liver in aromatase (Ar)–/– mice and its attenuation by pitavastatin. Ar–/– mice spontaneously developed massive steatosis of the liver. Light micrographs show livers of Ar–/– mice at 24 wk of age. (A) Numerous fat droplets in zones 3 and 2 (centrilobular and intermediate zones in the lobules) of Ar–/– mice liver at 24 wk of age; (B) a few fat droplets in the lobules of Ar–/– mice liver at 24 wk of age treated with pitavastatin; (C) no fat droplets in the lobules of Ar+/+ mice liver at 24 wk of age.

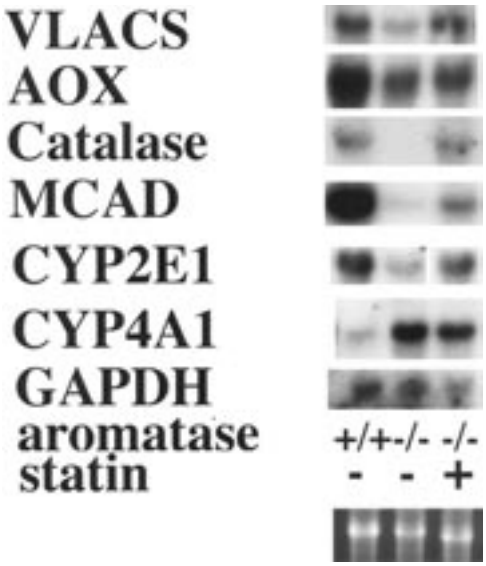

FIG. 2. Northern blot analysis of Ar–/– liver. mRNA expression of hepatic peroxisomal, mitochondrial, and microsomal enzymes was analyzed. The expression of most enzymes for FA β-oxidation was diminished in Ar–/– mice and pitavastatin was partially recovered. In contrast, microsomal cytochrome P450 4A1 (CYP4A1) mRNA expression was enhanced in Ar–/– mice and pitavastatin attenuated the enhanced expression. VLACS, very long chain fatty acyl-CoA synthetase; AOX, peroxisomal acyl-CoA oxidase; MCAD, medium-chain acyl-CoA dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CYP2E1, cytochrome P450 2E1; for other abbreviation see Figure 1.

mRNA expression of mitochondrial MCAD and of three peroxisome-associated enzymes (VLACS, AOX, and catalase) was significantly lower in Ar–/– mice than in Ar+/+ mice (0.23 ± 0.12, 0.33 ± 0.13, 0.28 ± 0.11, and 0.18 ± 0.09 fold-change, respectively, compared to Ar+/+), whereas microsomal CYP4A1 mRNA expression was significantly higher in the Ar–/– mice (7.64 ± 1.07 fold-change compared to Ar+/+; $P = 0.043$ in the Wilcoxon signed rank test). Administration of pitavastatin significantly restored mRNA expression of MCAD and of VLACS, AOX, and catalase (3.15 ± 0.44, 2.39 ± 0.23, 1.41 ± 0.15, and 3.59 ± 0.42 fold-change, respectively, compared to Ar–/–; $P = 0.043$ in the Wilcoxon signed rank test), whereas microsomal CYP4A1 mRNA expression (0.38 ± 0.18) was attenuated by pitavastatin treatment ($P = 0.043$ in the Wilcoxon signed rank test), although CYP4A1 is well known to be induced by PPAR-α stimulation (23).

FA β-oxidation activity. The basal levels of FA β-oxidation activity in Ar–/– mice were lower compared with those in C57BL/6J using [1-^{14}C]24:0 (1.82 ± 0.21 vs. 6.55 ± 0.39 nmol/min/g protein, $P < 0.001$), [1-^{14}C]16:0 (35.5 ± 5.8 vs. 123.7 ± 5.5 nmol/min/g protein, $P < 0.001$), or [1-^{14}C]12:0 (7.7 ± 0.5 vs. 25.6 ± 2.1 nmol/min/g protein, $P < 0.01$) as substrates (Fig. 3). FA β-oxidation activities were significantly enhanced by administration of pitavastatin (2.77 ± 0.23, 48.6 ± 7.0, and 11.6 ± 2.6 nmol/min/g protein; $P < 0.01$, 0.05, and 0.05, respectively), whereas they were significantly lower

TABLE 1
Grade of Hepatic Steatosis in three Groups of Mice

Grade	Description[a]	Ar+/+ group (n = 10)	Ar–/– group (n = 10)	Ar–/–+ statin group (n = 10)
0	No or few fat droplets in the lobules	8	0	0
I	A few fat droplets in the lobules	1	0	0
II	Fat droplets restricted to zone 3	1	0	3
III	Fat droplets in zones 3 and 2	0	3	5
IV	Numberous fat droplets in zones 3 and 2	0	7	2

[a]Zone 3, centrilobular area; zone 2, intermediate area.

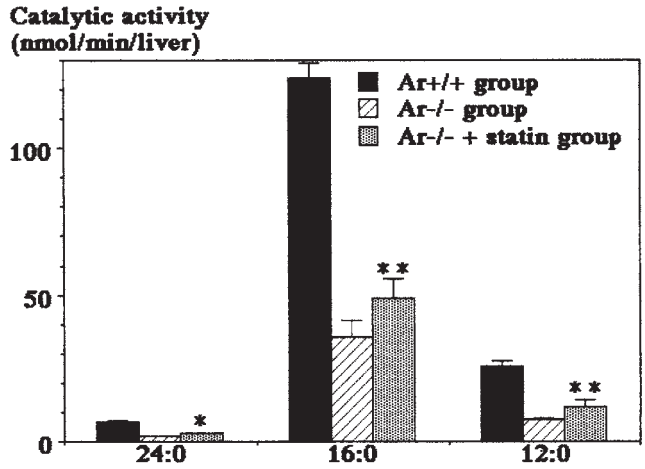

FIG. 3. Effects of pitavastatin on lipid β-oxidation activity in the liver. Peroxisomal β-oxidation capacity was assessed using tetracosanoic acid (24:0) as substrate and mitochondrial β-oxidation capacity using palmitic acid (16:0) and lauric acid (12:0). Bars represent the mean ± SD from at least five samples in each group, and asterisks denote a statistically significant difference ($P < 0.001$) compared with the values obtained with the wild-type group (Ar+/+) and the Ar–/– mice group treated with pitavastatin (Ar–/– + statin).

than those in wild-type mice (Ar+/+) ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively). The specificity of the β-oxidation assay was confirmed by KCN, which inhibited the assay at a rate of 91–94%.

Plasma and hepatic TG content. Levels of plasma TG (128.1 ± 36.2 mg/dL) and total cholesterol (107.5 ± 19.6 mg/dL) of Ar–/– mice were higher than those of Ar+/+ mice (80.6 ± 20.3 mg/dL and 71.7 ± 22.1 mg/dL, respectively, $P < 0.05$). Pitavastatin administration resulted in a significant reduction in levels of plasma TG (44.0 ± 8.2 mg/dL, $P < 0.01$) and total cholesterol (47.5 ± 8.7 mg/dL, $P < 0.001$) of Ar–/– mice. The hepatic content of TG of Ar–/– mice was clearly higher than that of Ar+/+ mice (126.1 ± 7.9 vs. 29.4 ± 3.3 mg/g liver, $P < 0.001$). In Ar–/– mice the hepatic content of TG was significantly diminished by administration of pitavastatin (126.1 ± 7.9 vs. 101.3 ± 17.2 mg/g liver, $P < 0.001$) (Fig. 4).

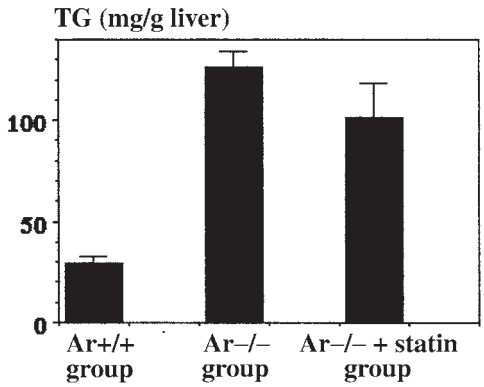

FIG. 4. TG content in the liver. Hepatic TG content in Ar–/– mice was clearly higher than that in Ar–/– mice treated with pitavastatin (Ar–/– + statin) or their wild-type siblings (Ar+/+).

DISCUSSION

The clinical benefits of cholesterol reduction by HMG-CoA reductase inhibitors (statins) have been established in large-scale primary and secondary prevention studies for coronary heart disease (24,25). However, successful reduction of LDL-cholesterol has revealed the contribution of other atherogenic lipoproteins, such as TG-rich lipoproteins, in the development of coronary events. Therefore, statins possessing TG-lowering properties have been newly developed.

Pitavastatin is a potent novel synthetic inhibitor of HMG-CoA reductase, that has shown lowering effects on plasma total cholesterol and TG (19,20). Two possible mechanisms appear to cooperate in lowering plasma TG levels during pitavastatin treatment (18). One mechanism is an inhibition of the assembly and secretion of VLDL, a common mechanism observed in statins. The other is an enhanced cycling of hepatic LDL receptors. These pathways contribute to the elimination of plasma TG but result in hepatic accumulation of TG unless they are oxidized efficiently.

FA are physiological ligands for PPAR-α, and PPAR-α has been implicated in the control of cellular lipid utilization (4). TG are accumulated in Ar–/– mouse liver, but our molecular and enzymological analyses revealed that mRNA expression and activities of enzymes involved in peroxisomal and mitochondrial FA β-oxidation are markedly reduced in Ar–/– mouse liver. Therefore, this is the most suitable model for investigating whether pitavastatin is able to activate the peroxisomal and mitochondrial FA β-oxidation and CYP450 pathways sufficiently. Otherwise, an inhibition of the assembly and secretion of VLDL induced by pitavastatin may easily exaggerate hepatic steatosis further in the liver of Ar–/– mice.

VLACS and AOX are involved in the first two steps of peroxisomal FA β-oxidation, by which very long chain FA are exclusively metabolized, and MCAD is a rate-limiting enzyme catalyzing the mitochondrial oxidation of medium-chain fatty acyl thioesters produced by peroxisomal β-oxidation of long-chain FA (26,27). Northern blot analysis and enzyme activities revealed that pitavastatin efficiently activated PPAR-α-dependent FA metabolizing pathways in Ar–/– mice. Indeed, not only plasma levels of total cholesterol and TG but also fat deposition in hepatocytes were reduced by pitavastatin treatment. These observations suggest that pitavastatin has an agonistic effect on PPAR-α and that pitavastatin can ameliorate severe hepatic steatosis in Ar–/– mice through the PPAR-α-mediated activation of the peroxisomal and mitochondrial FA β-oxidation pathways (26–28), including most known PPAR-α target genes encoding enzymes involved in hepatocellular FA β-oxidation. This is the first evidence *in vivo* that pitavastatin activates the peroxisomal and mitochondrial FA β-oxidation pathways.

Our knowledge is still limited about the regulatory mechanisms involved in maintaining cellular lipid content under normal physiological conditions or in the context of disease states. As such, present laboratory findings are important, as they support the idea of administering pitavastatin to tamox-

ifen-treated breast cancer patients with fibrate-resistant hypercholesterolemia to lower both plasma total cholesterol and TG. It is worth noting as well that physicians need to minimize the effects of obesity and diabetes mellitus that deteriorate FA homeostasis in the liver when adjuvant tamoxifen is being administered to breast cancer patients.

ACKNOWLEDGMENTS

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Liver Desaturase Activities and FA Composition in Monkeys. Effect of a Low-Protein Diet

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ABSTRACT: The aim of the present study was to measure Δ9-, Δ6-, and Δ5-desaturase activities in liver microsomes, as well as phospholipid FA composition of liver and erythrocytes in monkeys fed a control or low-protein diet during the postweaning period. Ten *Saimiri sciureus boliviensis* (Cebidae) of both sexes were employed; at 12 mon of age they were separated into two groups fed *ad libitum* on a control or a low-protein diet for 24 mon. *Saimiri sciureus* had active Δ9, Δ6, and Δ5 liver desaturase enzymes, and these activities were influenced by the diet. A low-protein diet produced a significant reduction in Δ5-desaturation capacity, an increase in Δ9-desaturase activity, and no change in Δ6-desaturase activity ($P < 0.05$). These changes, evoked by protein deprivation, were reflected in the liver phospholipid FA composition. Increases in the proportion of saturated FA and in monounsaturated oleic acid (18:1n-9) and a decrease in the proportion of PUFA of the n-6 and n-3 series were produced in the animals fed a low-protein diet ($P < 0.0001$). Differences between the two dietary groups were less pronounced in the FA composition of erythrocyte phospholipids.

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FA in general, and PUFA in particular, are an integral part of the structural and dynamic nature of cellular membranes on which normal physiology is crucially dependent. Moreover, PUFA play important functions as precursors of eicosanoids and as second messengers in the process of signal transduction (1,2). In animals, PUFA are synthesized from the EFA linoleic and α-linolenic acids, which are derived mainly from vegetables (1). The endogenous synthesis of PUFA starts with desaturation at the 6-position, catalyzed by the Δ6-desaturase enzyme, followed by an alternating sequence of malonyl CoA-dependent elongation and position-specific desaturation steps, in which Δ5-desaturase plays an important role (3–5). From the non-EFA palmitic and stearic acids, monounsaturated FA can be produced through the activity of the Δ9-desaturase enzyme, which introduces a *cis*-double bond at the Δ9-position.

Δ9-, Δ6-, and Δ5-desaturase activities are highly dependent on nutritional and hormonal factors (6). Previous studies carried out in our laboratory demonstrated that liver FA desaturase activities in pregnant rats were markedly affected by protein deficiency (7). The adverse effect of protein deprivation upon Δ6-desaturation activity was also demonstrated in lactating rats (8). Protein-energy malnutrition during gestation and lactation is detrimental not only to EFA metabolism but also to growth and brain development (9). In infants, the relationship between EFA metabolism and protein-energy malnutrition and the importance of dietary PUFA on visual function have been demonstrated (10,11).

Previous reports on the striking differences in desaturase activities among different animal species (12) and the lack of evidence about direct measures of liver desaturase activities in the monkey led us to study liver desaturase enzyme activities in normal monkeys as well as how they were affected after feeding on a low-protein diet.

EXPERIMENTAL PROCEDURES

Chemicals. [1-^{14}C]Palmitic (59 mCi/mmol, 99% radiochemically pure), [1-^{14}C]linoleic (55.6 mCi/mmol, 99% radiochemically pure), and [1-^{14}C]eicosatrienoic (47.0 mCi/mmol, 99% radiochemically pure) acids were purchased from New England Nuclear Corp. (Boston, MA). The pure unlabeled FFA and cofactors used for enzymatic reactions were obtained from Sigma Chemical Co. (St. Louis, MO). All chemicals and solvents were analytical grade. Solvents for HPLC were provided by Carlo Erba (Milan, Italy).

Animals and diets. Ten animals of both sexes of *Saimiri sciureus* (Cebidae) were used. They were born in captivity at the Centro Argentino de Primates (CAPRIM, Argentina). After weaning (7 mon), the animals were raised at the Centro de Investigaciones en Genética Básica y Aplicada (CIGEBA) and were fed for 5 mon on a standard diet for their adaptation to the new environment. When the animals were 1 yr old, they were divided according to diet into two experimental groups that were fed *ad libitum* from weaning to 36 mon of age. One of them received a control diet containing 20% protein (six animals) and the other a low-protein diet containing 9% protein (four animals) (Table 1). The control and low-protein diets had the same fat content (21.6% of total calories). These diets were sufficient in EFA: They supplied linoleic acid (18:2n-6) at 11.5 mg/g and α-linolenic acid (18:3n-3) at 1.5 mg/g in the control diet, whereas in the protein-deficient diet these levels were 11.1 and 1.1 mg/g, respectively. These values represented approximately 4% and 0.4–0.5% of total calories for linoleic and α-linolenic acids, respectively. The percent distribution of FA in both diets also can be seen in Table 1. The animals were

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TABLE 1
Diet Composition

Component	Control diet[a]		Low-protein diet[b]	
	Amount (g)	Protein content (%)	Amount (g)	Protein content (%)
Soybean meal	28.0	13.0	9.9	4.6
Wheat meal[c]	14.7	1.8	8.0	1.0
Glucose	0.0	0.0	6.7	0.0
Skim milk	10.6	3.7	4.9	1.7
Wheat bran	5.6	1.0	5.6	1.0
Sucrose	3.5	0.0	3.5	0.0
Rice meal	3.3	0.3	6.6	0.6
Cornstarch	3.0	0.0	21.4	0.0
Margarine	4.2	0.0	6.7	0.0
Egg	7.0	0.2	3.2	0.1
Vitamin mixture	1.5	0.0	1.5	0.0
Salt mixture	1.5	0.0	1.5	0.0
Water	17.1	0.0	20.5	0.0
Total	100.0	20.0	100.0	9.0

[a]FA composition of total diet: 16:0, 14.8%; 18:0, 6.7%; 18:1n-9, 26.8%; 18:1n-7, 3.8%; 18:2n-6, 41.5%; 18:3n-3, 5.6%.
[b]FA composition of total diet: 16:0, 13.9%; 18:0, 8.4%; 18:1n-9, 29.9%; 18:1n-7, 2.8%; 18:2n-6, 39.5%; 18:3n-3, 5.1%.
[c]Whole wheat flour.

weighed and sacrificed by decapitation under ether anesthesia; livers were removed, weighed, and maintained in an ice-bath until analysis.

Maintenance and treatment of the animals were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals (13).

Assay for in vitro *desaturation. (i) Microsome isolation.* Livers were homogenized in 3 mL of homogenizing solution (14) for each gram of liver, and the homogenate was centrifuged at 10,000 × *g* for 20 min. The pellet was discarded, and the supernatant fraction was centrifuged again at 110,000 × *g* for 60 min in an ultracentrifuge to obtain the microsomal fraction. The entire isolation procedure was carried out at 0–4°C (14). The microsomal protein was estimated by the method of Lowry *et al.* (15).

(ii) Desaturase assays. Δ5-, Δ6-, and Δ9-desaturase activity was determined in liver microsomes by measuring the conversion of [1-^{14}C]16:0 (palmitic acid), [1-^{14}C]18:2n-6 (linoleic acid), or [1-^{14}C]20:3n-6 (eicosatrienoic acid) to [1-^{14}C]16:1 (palmitoleic acid), [1-^{14}C]18:3n-6 (γ-linolenic acid), and [1-^{14}C]20:4n-6 (arachidonic acid), respectively, according to Garda *et al.* (16). The assays were performed using 0.10 μCi of labeled acid with the corresponding unlabeled pure FA up to an amount of 16.6 nmol per tube. The FA were separated using RP-HPLC in an apparatus equipped with an L-6200 solvent delivery system, and the column eluate was monitored at 205 nm using an L-4200 UV/VIS Detector (Merck-Hitachi) as described previously (16). A 250 × 4.6 mm Econosil C18 column (Alltech Associates, Inc., Deerfield, IL) coupled to a 10 × 4 mm guard column and packed similarly was used. The flow rate was 1 mL/min. HPLC peaks were identified on the basis of their retention times relative to appropriate FA standards. Radioactivity was detected with a Radiomatic Model Flo-One/Beta radioactivity flow detector (Packard Instruments, Downers Grove, IL) using the liquid scintillation cocktail Ultima Flo-M (Packard Instruments). Desaturase activities are expressed as picomoles of [^{14}C] product of desaturation formed per minute per milligram of microsomal protein (pmol·min^{-1}·mg prot^{-1}).

Liver and erythrocyte FA analysis. Erythrocytes were separated from plasma by centrifugation. Lipids from erythrocytes and an aliquot of liver homogenate were extracted by the method of Folch *et al.* (17). The phospholipid fraction was isolated from the lipid extract by silicic acid column chromatography (Bio-Rad Laboratories, Richmond, CA) according to the method of Hanahan *et al.* (18). FAME were prepared with boron trifluoride/methanol (19) and analyzed in a chromatograph (model GC-9A; Shimadzu Corp., Kyoto, Japan) equipped with an Omegawax 250 capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA). Authentic standards of FA were purchased from Sigma Chemical Co.

Statistical analysis. Statistical analyses were performed by one-way ANOVA. When differences were detected ($P < 0.05$), means were tested with Tukey's test (GB-STAT Professional Statistics and Graphics 4.0; Dynamic Microsystems Inc., Silver Spring, MD).

RESULTS

Figure 1 shows the growth curves for the control and low-protein-fed monkeys. From the beginning of the experiment and up to 6 mon of dietary treatment, the mean body weights were almost equivalent. However, the growth of the monkeys fed

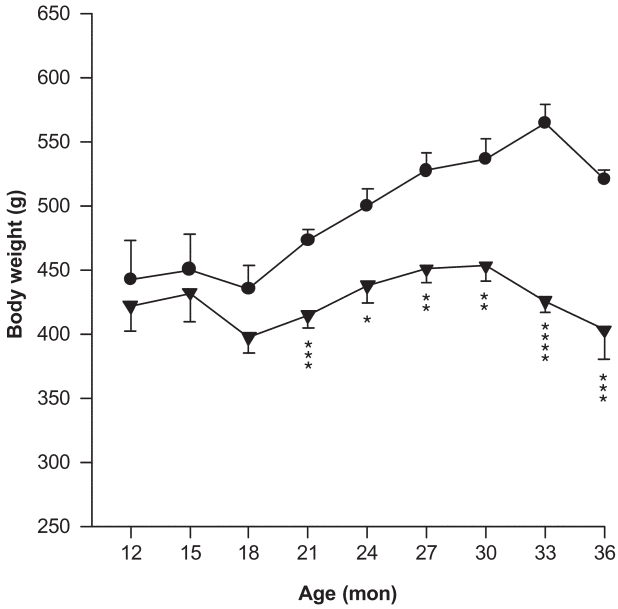

FIG. 1. Body weights of monkeys fed control (●; $n = 6$ animals) and low-protein (▼; $n = 4$) diets recorded during the entire experimental period. Values are means ± SEM. Significant differences relative to control group are indicated by asterisks: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

on a low-protein diet were retarded, and the body weights were significantly lower than the control ones. Differences between the two groups increased with age. Liver weights from the control group also were significantly higher than those of the low-protein-fed group (12.2 ± 0.6 vs. 9.7 ± 0.7 g, $P < 0.05$).

From the results shown in Table 2, it is clear that *S. sciureus* have active Δ9-, Δ6-, and Δ5-desaturase enzymes. This fact was demonstrated by the conversion of the respective radioactive precursors into their desaturated products by the three enzymes in the liver microsomal fraction during the postweaning period. Moreover, these desaturase activities were influenced by nutritional factors. The highest value was obtained for the Δ5-desaturase, followed by those for Δ9- and Δ6-. The low-protein diet had different effects on the desaturase activities; a significant reduction in Δ5-desaturation capacity was observed in the animals fed a 9% protein diet compared with the control ones. On the other hand, an increase in Δ9-desaturase activity was obtained when animals were fed the low-protein diet. However, in our experimental conditions, Δ6-desaturase activity was not modified according to the diet protein level.

The changes observed in the desaturation activities evoked by dietary protein level in monkeys were reflected in the liver phospholipid FA composition (Table 3). A significant increase in the proportion of saturated FA of 12 and 14 carbons as well as in the monounsaturated oleic acid (18:1n-9) was found in low-protein-diet animals compared to the control. A marked decrease in the proportion of PUFA of the n-6 series, such as 20:2n-6, 20:4n-6, and 22:4n-6, and in the major component of the n-3 series, 22:6n-3, in monkeys fed on a low-protein diet relative to the control was also shown. These results evidenced a significant decrease in the total PUFA, especially those of the n-3 series in the low-protein-fed group when compared to the control one. On the contrary, the sum of total n-9 FA was higher in the phospholipid fraction of animals fed a low-protein diet than that from controls. Table 3 also shows the effect of the deficient diet on the unsaturation index calculated in the total phospholipid fraction, which was significantly diminished compared to the controls.

In the present study we also analyzed the FA composition of erythrocyte phospholipids from monkeys (Table 4). Differences between the two dietary groups were smaller than those found in the liver phospholipid fraction. A significant increase in the proportion of monounsaturated 18:1n-9 acid as well as in the 20:5n-3 level was detected in the erythrocyte phospholipid fraction compared with the control. On the contrary, a significantly lower proportion of 22:6n-3 was observed in monkeys fed a low-protein diet compared with the controls.

TABLE 2
Desaturase Activities of Liver Microsomes from Monkeys Fed on Control and Low-Protein Diet (pmol·mg prot^{-1}min^{-1})[a]

Desaturase enzymes	Substrate/product	Control diet ($n = 6$)	Low-protein diet ($n = 4$)
Δ5	20:3n-6/20:4n-6	88.0 ± 4.0	71.7 ± 3.5*
Δ6	18:2n-6/18:3n-6	29.6 ± 1.8	27.2 ± 2.5
Δ9	16:0/16:1	40.6 ± 3.6	72.0 ± 3.5**

[a]Values are mean ± SEM of *n* determinations. Probability value relative to control monkey data. *$P < 0.05$; **$P < 0.0001$.

TABLE 3
FA Composition[a] (mol%) of Liver Phospholipid from Monkeys Fed on Control and Low-Protein Diets

FA	Control diet ($n = 6$)	Low-protein diet ($n = 4$)
12:0	0.28 ± 0.03	1.78 ± 0.05***
14:0	0.25 ± 0.07	1.22 ± 0.16***
16:0	19.12 ± 0.87	19.16 ± 0.33
16:1n-7	0.60 ± 0.08	0.78 ± 0.24
16:2n-4	0.65 ± 0.14	0.43 ± 0.01
18:0	21.94 ± 0.81	21.24 ± 0.92
18:1n-9	4.72 ± 0.36	7.25 ± 0.50**
18:1n-7	2.48 ± 0.25	2.90 ± 0.81
18:2n-6	20.85 ± 1.15	21.68 ± 0.56
18:3n-6	0.22 ± 0.02	0.17 ± 0.02
18:3n-3	0.28 ± 0.02	0.23 ± 0.03
20:0	0.15 ± 0.01	0.13 ± 0.01
20:1n-9	0.15 ± 0.02	0.22 ± 0.04
20:2n-6	0.82 ± 0.02	0.52 ± 0.02***
20:3n-6	1.41 ± 0.09	1.56 ± 0.14
20:4n-6	14.57 ± 0.37	13.42 ± 0.24*
20:3n-3	0.19 ± 0.04	0.18 ± 0.02
20:5n-3	0.19 ± 0.02	0.06 ± 0.03
22:1n-9	0.16 ± 0.01	0.70 ± 0.03***
22:4n-6	1.74 ± 0.10	0.74 ± 0.06***
22:4n-3	1.51 ± 0.10	0.84 ± 0.12**
22:5n-6	1.00 ± 0.08	1.06 ± 0.13
22:5n-3	0.49 ± 0.08	0.37 ± 0.10
22:6n-3	6.20 ± 0.32	4.33 ± 0.22**
Σ Saturated	42.01 ± 1.65	43.87 ± 0.92
Σ Monounsaturated	8.10 ± 0.58	11.85 ± 1.14
Σ Polyunsaturated	50.10 ± 0.56	45.59 ± 0.53***
Σ n-9	5.03 ± 0.38	8.17 ± 0.49***
Σ n-6	40.60 ± 1.15	39.15 ± 0.42
Σ n-3	8.85 ± 0.42	6.01 ± 0.32***
Unsaturation Index	177.13 ± 4.43	157.34 ± 1.27**

[a]Values are mean ± SEM of *n* determinations. Some minor components have been omitted. Probability values relative to data from control monkeys: *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$.

DISCUSSION

The number of FA double bonds is an important factor in determining membrane structure, packing, fluidity, and function. Δ9-Desaturase is widely distributed in different kinds of animals. However, there is considerable variation between animal species in their ability to synthesize the C_{20} and C_{22} PUFA. High rates of desaturation are observed in small species, particularly the rat (20,21).

It was reported that some animals, notably cats and lions, which are carnivores, have a very limited ability to synthesize C_{20} and C_{22} PUFA and consequently have a strict requirement for a dietary source of preformed C_{20} and C_{22} PUFA (22–25). However, recent reports showed that cats would be able to convert 18:2n-6 and 18:3n-3 to longer-chain PUFA after feeding on an EFA-deficient diet for 6 mon (26).

Although indirect evidence has been reported in favor of the presence of Δ6- and Δ5-desaturases in monkeys, an indirect

TABLE 4
FA Composition (mol%) of Erythrocyte Phospholipid from Monkeys Fed on Control and Low-Protein Diets[a]

FA	Control diet (n = 6)	Low-protein diet (n = 4)
12:0	0.09 ± 0.03	0.13 ± 0.11
14:0	0.19 ± 0.03	0.28 ± 0.02
16:0	22.13 ± 0.34	21.82 ± 0.93
16:1n-7	0.23 ± 0.06	0.51 ± 0.03
16:2n-4	0.52 ± 0.01	0.42 ± 0.02
18:0	18.00 ± 0.29	17.75 ± 0.37
18:1n-9	10.26 ± 0.31	11.90 ± 0.11*
18:1n-7	5.59 ± 0.53	4.75 ± 0.61
18:2n-6	21.56 ± 0.41	21.16 ± 0.73
18:3n-6	0.08 ± 0.04	0.17 ± 0.05
18:3n-3	0.05 ± 0.02	0.06 ± 0.02
20:0	0.44 ± 0.02	0.52 ± 0.01
20:1n-9	0.48 ± 0.03	0.35 ± 0.05
20:2n-6	0.84 ± 0.04	0.56 ± 0.04**
20:3n-6	0.70 ± 0.07	0.87 ± 0.06
20:4n-6	9.34 ± 0.31	9.50 ± 0.22
20:5n-3	0.32 ± 0.05	0.59 ± 0.03*
22:1n-9	0.41 ± 0.08	0.20 ± 0.06
22:4n-6	2.16 ± 0.18	2.78 ± 0.17
22:4n-3	1.33 ± 0.08	1.30 ± 0.03
22:5n-6	0.75 ± 0.05	1.11 ± 0.11
22:5n-3	0.21 ± 0.03	0.35 ± 0.02
22:6n-3	3.88 ± 0.11	2.31 ± 0.08**
Σ Saturated	41.28 ± 0.45	41.25 ± 1.34
Σ Monounsaturated	16.97 ± 0.83	17.71 ± 0.73
Σ Polyunsaturated	41.67 ± 0.75	41.11 ± 0.75
Σ n-9	11.15 ± 0.33	12.45 ± 0.20
Σ n-6	35.36 ± 0.63	36.15 ± 0.81
Σ n-3	5.80 ± 0.19	4.54 ± 0.08**
Unsaturation Index	147.67 ± 2.22	145.48 ± 2.44

[a]Values are mean ± SEM of n determinations. Some minor components have been omitted. Probability values relative to data from control monkeys: *$P < 0.01$; **$P < 0.001$.

measurement of these enzymatic activities has been published. Su *et al.* (27) have reported that fetal organs from pregnant baboons who consumed a long-chain PUFA-free diet and received [U-^{13}C]linoleic acid in their third trimester of gestation could accumulate 18:2 acid within a day of a maternal dose and convert much of it to 20:4 within weeks (27). Another approach to the study of desaturase activities in monkeys was made by Kanazawa *et al.* (28), who studied the synthesis of chain elongation–desaturation products from linolenate by brain microsomes of Japanese monkeys *Macaca fuscata fuscata,* demonstrating that it was markedly influenced by age. However, in none of these works was the contribution of each desaturase in the final conversion of linoleic or α-linolenic acid to arachidonate or docosahexaenoate, respectively, clearly determined, and the rate of conversion was very low.

On the other hand, previous research carried out in our laboratory demonstrated that liver FA desaturating activities were profoundly affected by protein deficiency in pregnant rats; this fact would alter the normal supply of PUFA to the fetus (9). An alteration in the FA composition was also observed in malnourished nursing infants compared to normal ones (10,29). However, in these cases the direct measurement of desaturase activities could not be carried out. In postweaning monkeys fed on a low-protein diet, we observed changes in liver and erythrocyte FA composition similar to the ones in malnourished infants (10,29), as well as an increment in saturated and monounsaturated acid with a concomitant decrease in PUFA from the n-6 and n-3 series (Tables 3 and 4). Although we could not observe any alteration in Δ6-desaturase activity, we were able to demonstrate that the changes in FA composition in monkeys fed a low-protein diet correlated with a significant decrease in Δ5- and an increase in Δ9-desaturase. All these results would imply an important contribution to the study of the desaturase activities in primates since they could be directly measured in an animal model closely related to the human.

This is the first direct measurement of desaturase activity in primates, and these enzymatic activities were found to depend on the diet. Moreover this kind of monkey responded to the low-protein diet in the same way as already observed in other mammals in spite of the evolutionary difference between rats and humans.

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Regulation by Carbohydrate and Clofibric Acid of Palmitoyl-CoA Chain Elongation in the Liver of Rats

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ABSTRACT: Regulation of palmitoyl-CoA chain elongation (PCE) and its contribution to oleic acid formation were investigated in rat liver in comparison with stearoyl-CoA desaturase (SCD). Hepatic PCE activity was induced by the administration of 20% wt/vol glucose or fructose in the drinking water of normal rats. In streptozotocin-induced diabetic rats, the activities of both PCE and SCD were suppressed, and fructose, but not glucose, feeding caused an increase in the activities of both enzymes. Treatment of normal rats with clofibric acid in combination with carbohydrate further increased PCE, but not SCD, activity. FA analysis of hepatic lipids revealed that the proportion of oleic acid (18:1n-9) increased upon administration of carbohydrate or clofibric acid. The treatment of rats with clofibric acid in combination with carbohydrate greatly increased the proportion of 18:1n-9. A significant correlation was observed between PCE activity and the hepatic proportion of 18:1n-9 (r^2 = 0.874, $P < 0.01$), whereas the relationship between SCD activity and the proportion of 18:1n-9 was not significant (r^2 = 0.552, $P > 0.05$). Taken together, these results suggest that carbohydrate induces PCE as well as SCD activity to increase the hepatic 18:1 content in rat liver, and the increased PCE activity seems to be responsible for the further increase in 18:1n-9 when carbohydrate is administered in combination with clofibric acid.

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Biosynthesis of FA plays an important role in both storing energy and providing the components of biomembranes in mammalian liver. The major parts of FA are the C_{16}–C_{18} saturated and monounsaturated FA that are initially synthesized *de novo* by FA synthase using acetyl-CoA and malonyl-CoA. However, the end product of FA synthase is usually palmitic acid (16:0), and further desaturation and chain elongation are required to synthesize major C_{16}–C_{18} FA such as stearic acid (18:0), palmitoleic acid (16:1n-7), oleic acid (18:n-9), and vaccenic acid (18:1n-7) (1,2). Stearoyl-CoA desaturase (SCD) was identified in 1986 as an enzyme responsible for the desaturation of both 16:0 and 18:0 to form 16:1n-7 and 18:1n-9, respectively (3). It is known that SCD activity is greatly enhanced in response to feeding a fat-free diet, refeeding after fasting, and insulin, whereas it is suppressed by feeding PUFA or by starvation (4–6). Recent progress has revealed the molecular mechanisms responsible for the regulation of SCD (7–9). By contrast, information on FA chain elongation has been limited, although several lines of indirect evidence have suggested that the endoplasmic reticulum contains at least three different enzymes catalyzing the elongation of FA (1,10–13). Among them, palmitoyl-CoA chain elongation (PCE), which catalyzes stearic acid (18:0) formation from palmitic acid (16:0), was regulated by refeeding after fasting, insulin, and PUFA (10,11,14–16). In addition, various xenobiotics such as clofibric acid and diethylhexylphthalate induced the activities of both SCD and PCE (10,11,17,18), although the mechanism of the induction has not yet been clarified. These observations suggest that PCE is regulated by a mechanism similar to SCD. At the same time, it raises the question of how these enzymes individually contribute to 18:1n-9 formation. To answer this question, in the present work we studied whether administration of carbohydrate increases PCE activity in rat liver in combination with clofibric acid and the relative contribution of PCE and SCD to 18:1n-9 formation. The physiological significance of PCE in 18:1n-9 formation in the liver is discussed.

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Abbreviations: FA are designated by the number of carbon atoms and double bonds: 16:0, palmitic acid; 16:1n-7, palmitoleic acid; 18:0, stearic acid; 18:1n-9, oleic acid; 18:1n-7, vaccenic acid. 1-acyl-GPC, 1-acylglycerophosphocholine; clofibric acid, 2-(*p*-chlorophenoxy)-2-methylpropionic acid; PCE, palmitoyl-CoA chain elongation; PPARα, peroxisome proliferator-activated receptor α; PPRE, peroxisome proliferator responsive element; SCD, stearoyl-CoA desaturase.

MATERIALS AND METHODS

Materials. Stearoyl-CoA, palmitoyl-CoA, malonyl-CoA, 2-(*p*-chlorophenoxy)-2-methylpropionic acid (clofibric acid), and BSA were purchased from Sigma (St. Louis, MO). Triheptadecanoin and methylheptadecanoate were from Nu-Chek-Prep Inc. (Elysian, MN); 1-acylglycerophosphocholine (1-acyl-GPC, from egg PC) was from Avanti Polar Lipids, Inc. (Alabaster, AL); NADH, NAD, and CoA were from Oriental Yeast Co. (Tokyo, Japan); [2-^{14}C]malonyl-CoA was from Moravek Biochemicals Inc. (Brea, CA); [1-^{14}C]16:0 was from American Radiolabeled Chemicals Inc. (St. Louis, MO); and horseradish peroxidase was from Boehringer Mannheim (Mannheim, Germany). All other chemicals used were of analytical grade.

Animals. All procedures were approved by Josai University Animal Care and Use Committee and complied with the *Guide for the Care and Use of Laboratory Animals.* Male Wistar rats, 5 wk of age, were purchased from SLC (Hamamatsu, Japan). After acclimatization for 1 wk, some rats were given drinking water containing 20% wt/vol glucose or fructose for 2–7 d. In a separate experiment, rats were given drinking water

containing 20% wt/vol glucose or fructose for 4 d. During the administration of glucose or fructose, half of each group was injected subcutaneously with clofibric acid at a dose of 100 mg/kg body weight twice a day for 4 d. Male Wistar rats 4 wk of age were injected intravenously with streptozotocin dissolved in citrate buffer (pH 4.5) at a dose of 60 mg/kg body weight. Three weeks after streptozotocin injection, these rats were fed a fat-free diet (modified AIN93M in which carbohydrate and oil were substituted with glucose or fructose) for 4 d. Blood samples were collected under light ether anesthesia and rats were then killed by decapitation. Livers were quickly excised, perfused with ice-cold 0.9% wt/vol NaCl, and rinsed in 0.25 M sucrose/1 mM EDTA/10 mM Tris-HCl (pH 7.4). Livers were then homogenized with 4 vol of the same solution in a Potter glass-Teflon homogenizer. An aliquot of the homogenates was frozen in liquid nitrogen and stored at −80°C for lipid analysis and assay of peroxisomal β-oxidation. The other part of the homogenates was centrifuged at $18,000 \times g$ for 20 min; the supernatant was recentrifuged under the same conditions. The resulting supernatant was centrifuged at $105,000 \times g$ for 60 min. The pellet was resuspended in 0.25 M sucrose/0.1 mM EDTA/10 mM Tris-HCl (pH 7.4) and recentrifuged under the same conditions. The resulting pellet (microsomes) was resuspended in a small volume of 0.25 M sucrose/0.1 mM EDTA/10 mM Tris-HCl (pH 7.4) and used as an enzyme source. All the operations mentioned above were carried out at 0–4°C. Protein concentrations were determined by the method of Lowry *et al*. (19) using BSA as a standard.

Enzyme assays. Acyl-CoA oxidase was assayed by measuring palmitoyl-CoA-dependent H_2O_2 production according to the method of Small *et al.* (20), using homogenates as an enzyme source. Briefly, 1 mL of the reaction mixture contained 50 nmol leuco-dichlorofluorescein, 0.2 mg horseradish peroxidase, 4 μmol aminotriazole, 50 nmol palmitoyl-CoA, 0.02% Triton X-100, 20 mM potassium phosphate buffer (pH 7.4), and 20 μg protein of the liver homogenates. After preincubation in the absence of palmitoyl-CoA at 30°C, the reaction was started by adding 50 nmol palmitoyl-CoA, and the change in absorbance at 502 nm was monitored. The activity was calculated assuming an absorption coefficient for leucodichlorofluorescein of 1.42×10^5 $M^{-1}cm^{-1}$.

SCD activity was assayed spectrophotometrically by the method of Oshino *et al.* (21) as the stearoyl-CoA stimulated reoxidation of NADH-reduced cytochrome b_5. The rate of cytochrome b_5 oxidation was measured by recording the changes in absorbance between 424 and 409 nm at 30°C. The initial incubation mixture contained 1.2 mg microsomal protein and 100 mM Tris-HCl buffer (pH 7.4). Cytochrome b_5 was reduced by adding 2 nmol NADH, and the reoxidation was recorded. When the reoxidation was completed, 20 nmol stearoyl-CoA was added, and cytochrome b_5 was reduced again by 2 nmol of NADH. The first-order rate constant for the reoxidation of NADH-reduced cytochrome b_5 was calculated as described by Oshino and Sato (22). The rate constant for the reoxidation of cytochrome b_5 was measured in the presence (k) and in the absence (k^-) of stearoyl-CoA; the rate constant for SCD was given by $k^+ = k - k^-$ (11,23). Microsomal activities of NADH-cytochrome *c* reductase and NADH-ferricyanide reductase and the content of cytochrome b_5 were determined as described previously (24).

FA chain elongation was assayed as described previously (14). The incubation mixture contained 15 nmol palmitoyl-CoA, 100 nmol [2-^{14}C]malonyl-CoA, 0.5 μmol NADH, 0.5 μmol NADPH, 0.5 μmol KCN, and 250–500 μg microsomal protein in 0.5 mL of 100 mM Tris-HCl buffer (pH 7.4). The mixture was incubated at 37°C for 4 min under nitrogen. The incubation mixture without palmitoyl-CoA was run simultaneously. After stopping the enzymatic reaction by the addition of 1 mL of 10% KOH/90% methanol, the mixture was heated at 80°C for 30 min under nitrogen and then acidified by adding 2 mL of 6 M HCl. FA were extracted with 3 mL of *n*-hexane four times. The combined *n*-hexane extract was washed with 4 mL of acidic water, transferred to a counting vial, and taken to dryness. The remaining FA were dissolved in a toluene scintillator, and radioactivity was measured by a liquid scintillation counter.

1-Acylglycerophosphocholine (1-acyl-GPC) acyltransferase was assayed essentially according to Lands and Heart (25). The reaction mixture contained 20–30 nmol oleoyl-CoA, 150 nmol 1-acyl-GPC, 1 μmol DTNB, and 50–75 μg microsomal protein in a final volume of 1 mL of 100 mM Tris-HCl buffer (pH 7.4). After preincubation in the absence of oleoyl-CoA at 30°C, the incubation was initiated by adding oleoyl-CoA, and the increase in absorbance at 412 nm was monitored. The value determined in the absence of 1-acyl-GPC was subtracted as a background to provide a net acyl transfer rate.

Acyl-CoA synthetase was assayed using [^{14}C]palmitic acid as a substrate according to Tanaka *et al*. (26).

Assays for blood glucose and insulin. Serum levels of glucose and insulin were determined using the Glucose-Test Wako (Wako Pure Chemicals Inc, Osaka, Japan) and the Rat Insulin ELISA kit (Mercodia, Uppsala, Sweden), respectively.

Lipid analyses. Total lipids were extracted from liver homogenates by the method of Bligh and Dyer (27). For quantification of FA, a known amount of triheptadecanoin was added to the homogenates as an internal standard. After the solvent was evaporated, total lipids were added to 1 mL 10% KOH/90% methanol and then heated at 80°C for 60 min for saponification. Nonsaponified lipids were removed by extracting three times with 3 mL of hexane. After the addition of 1 mL 6 M HCl, FFA were extracted three times with 3 mL of hexane. The extract was taken to dryness; to the residue was added methanolic BF_3 and the mixture was heated at 100°C for 10 min. The FAME formed were extracted with hexane and subjected to GLC analysis [Shimadzu GC-14A (Kyoto, Japan), equipped with an FID and a SUPELCOWAX 10 column (0.32 mm i.d. × 30 m)].

Statistics. ANOVA was used to test for significant differences between control, glucose-administered, and fructose-administered rats. Where differences were significant, the statistical significance between any two means was determined using Sheffé's multiple range test. The statistical significance between clofibric acid-treated and untreated rats was analyzed

by Student's *t*-test or Welch's test after the *F*-test for two means.

RESULTS

Induction of PCE and SCD by glucose and fructose. Administration of glucose caused a significant increase in PCE activity in rat liver. At least 4 d were required to reach the maximum activity (Fig. 1). Fructose induced PCE, as was observed with glucose. There was no significant difference between glucose and fructose in their potency to induce PCE. Next, we examined whether clofibric acid, a drug known to induce PCE (11), further increased PCE activity in rats administered glucose or fructose. Clofibric acid treatment caused a significant increase in PCE activity that was 1.9, 1.8, and 1.4 times that of the untreated control in normal, glucose-, and fructose-administered rats, respectively (Fig. 2A). The administration of glucose and fructose increased hepatic terminal desaturase activity 4.2 and 4.8 times, respectively, in the SCD system (Fig. 2B). Nevertheless, clofibric acid did not cause an additional increase in terminal desaturase activity in glucose- or fructose-administered rats (Fig. 2B). In other components of the SCD system, NADH-ferricyanide reductase activity and the content of cytochrome b_5 were not altered by carbohydrate administration or by clofibric acid treatment, whereas NADH-cytochrome *c* reductase activity was reduced by clofibric acid in both normal and carbohydrate-administered rats (data not shown).

Clofibric acid is known as a peroxisome proliferator and causes the induction of both acyl-CoA oxidase and acyl-CoA synthetase *via* peroxisome proliferator-activated receptor α (PPARα) activation (28). The drug also induces 1-acyl-GPC acyltransferase; the induction was positively correlated to peroxisomal β-oxidation or acyl-CoA oxidase activity (29). Glucose or fructose administration did not significantly affect the activities of these enzymes (Table 1). In addition, clofibric acid-induced activities of these three enzymes were not different between control, glucose-administered, and fructose-administered rats (Table 1).

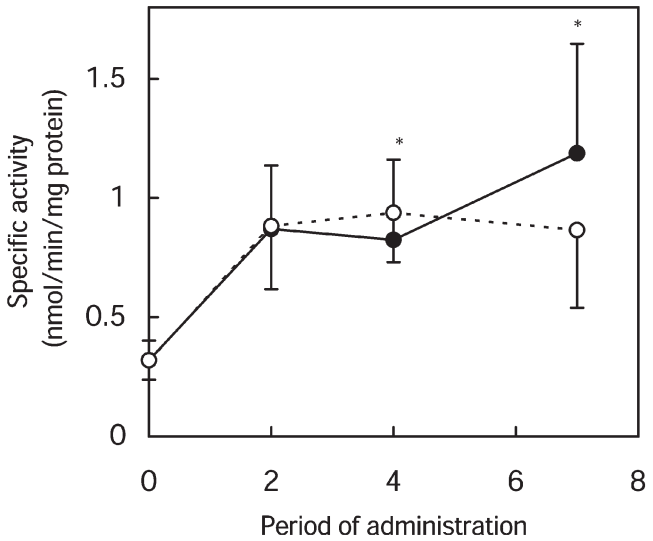

FIG. 1. Time course of the induction of hepatic palmitoyl-CoA chain elongation (PCE) by the administration of glucose and fructose. Liver microsomes were prepared from the rats that were given a 20% wt/vol solution of glucose (●) or fructose (○) in drinking water. Values are means ± SD for three to eight rats. *Significantly different from control ($P < 0.05$).

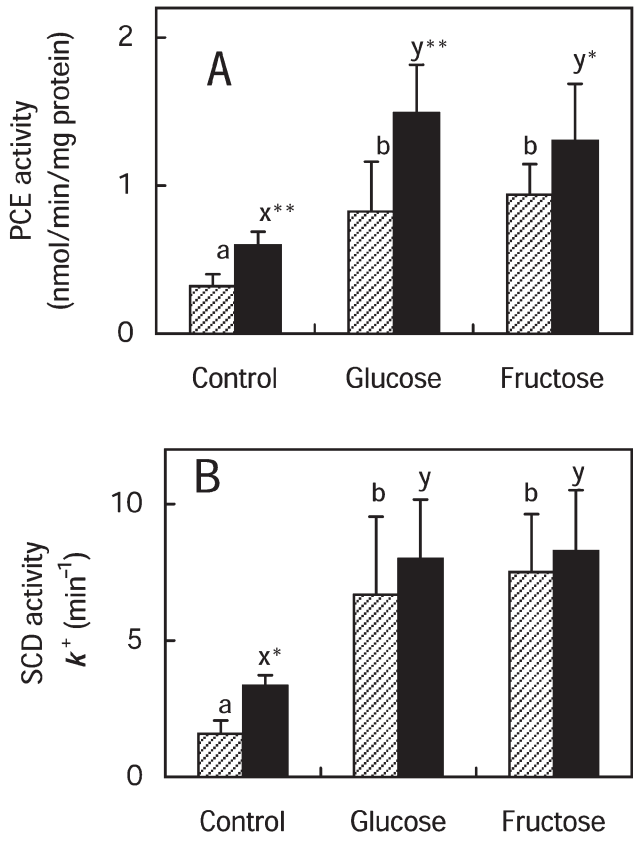

FIG. 2. Effects of glucose and fructose in combination with clofibric acid on the activities of PCE and stearoyl-CoA desaturase (SCD) in rat liver. Rats were given a 20% wt/vol solution of glucose or fructose to drink for 4 d with (solid bar) or without (hatched bar) a subcutaneous injection of clofibric acid twice a day at a dose of 100 mg/kg body weight. Values are means ± SD for four to eight rats. (A) PCE; (B) SCD. [a,b]Values for control, glucose-, and fructose-administered rats without a common superscript are statistically significant ($P < 0.05$). [x,y]Values for control, glucose-, and fructose-administered rats with clofibric acid treatment without a common superscript are statistically significant ($P < 0.05$). *,**Significantly different from the value of clofibric acid-untreated rats ($P < 0.05$ and $P < 0.001$, respectively).

In streptozotocin-induced diabetic rats, the activities of both PCE and SCD were reduced by 70% (Fig. 3). In diabetic rats, fructose feeding increased the activities of both PCE and SCD, whereas glucose feeding did not change these activities (Fig. 3).

Effects of clofibric acid on serum levels of insulin and glucose. Serum levels of glucose were not significantly different among control, glucose-, and fructose-administered normal rats (153.2 ± 12.0, 173.7 ± 14.0, and 144.9 ± 3.5 mg/dL, respectively). Clofibric acid treatment did not alter the serum glucose level in control and glucose-administered rats (146.4

TABLE 1
Effects of Glucose, Fructose, and Clofibric Acid on the Activites of Acyl-CoA Synthetase, Acyl-CoA Oxidase, and 1-Acyl-GPC Acyltransferase in the Liver of Rats[a]

	Acyl-CoA synthetase (nmol/min/mg protein)	Acyl-CoA oxidase (nmol/min/mg protein)	1-Acyl-GPC acyltransferase (nmol/min/mg protein)
Control	73.9 ± 6.2[a,b]	4.58 ± 0.28[a]	96.3 ± 9.4
Control + clofibric acid	143.8 ± 7.5*	31.78 ± 3.98*	184.2 ± 12.4*
Glucose	87.5 ± 9.5[b]	4.53 ± 0.66[a]	100.0 ± 8.4
Glucose + clofibric acid	147.5 ± 11.3*	31.10 ± 2.10*	188.6 ± 11.5*
Fructose	65.5 ± 7.4[a,*]	5.63 ± 0.43[b]	86.0 ± 7.0
Fructose + clofibric acid	128.8 ± 11.5*	29.10 ± 0.47*	197.8 ± 16.9*

[a]Rats were given 20% wt/vol glucose or fructose solution to drink for 4 d. Half were injected subcutanously with clofibric acid (100 mg/kg body weight) twice a day. Values are means ± SD for three to five rats. *Significantly different from clofibric acid-untreated rats ($P < 0.001$). [a,b]Values for control, glucose-, and fructose-administered rats without a common superscript are statistically significant. Where no superscript appears, differences between the three groups are not statistically significant. 1-acyl-GPC, 1-acylglycerolphosphocholine.

± 5.5 and 169.5 ± 6.8 mg/dL, respectively) and slightly increased it in fructose-administered rats (152.3 ± 4.2 mg/dL). In diabetic groups, serum levels of glucose in control, glucose-, and fructose-fed rats were 497.2 ± 22.9, 657.5 ± 97.5, and 750.6 ± 145.9 mg/dL, respectively. There was no significant difference in serum insulin levels between the six experimental groups (average value of all rats was 0.785 ± 0.456 ng/mL). In all diabetic rats, the serum insulin level was below the detection limit (0.07 ng/mL).

Effects of glucose and fructose administration and clofibric acid treatment on the composition of hepatic FA. Figure 4 shows the effects of carbohydrate administration in combination with clofibric acid treatment on the proportion of 18:1n-9 in liver lipids. The amounts of total FA did not differ among the six experimental groups. The proportion of 18:1n-9 in the hepatic lipids of clofibric acid-treated rats was 1.7 times higher than that in untreated rats. Glucose and fructose administration caused increases of 1.9- and 1.8-times, respectively, in 18:1n-9 (Fig. 4). Treatment of glucose- and fructose-administered rats with clofibric acid further increased 18:1n-9 by 2.1 and 1.6 times, respectively (Fig. 4).

To estimate the relative contribution of SCD and PCE to 18:1n-9 formation, linear regression analyses were performed on the proportion of 18:1n-9 vs. the activity of PCE or SCD in the liver (Fig. 5). There was a highly significant correlation between the proportion 18:1n-9 and PCE activity (r^2 =

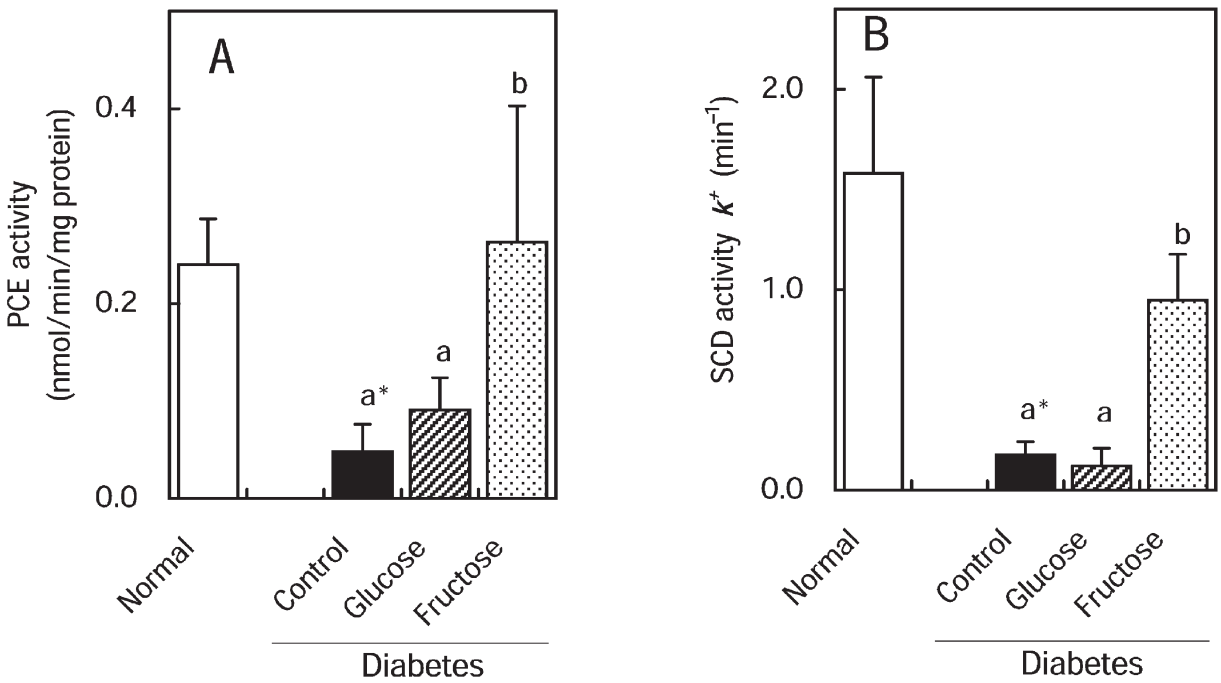

FIG. 3. Effects of carbohydrate on the activities of PCE and SCD in the liver of streptozotocin-induced diabetic rats. Rats were injected intravenously with streptozotocin (60 mg/kg body weight). Three weeks after the injection, these rats were fed a fat-free diet containing glucose or fructose as a carbohydrate for 4 d and simultaneously received a subcutaneous injection of clofibric acid (100 mg/kg body weight) twice a day. Microsomes were prepared from the rats and the activities of PCE (A) and SCD (B) were determined. *Significantly different from normal rats ($P < 0.05$). [a,b]Values for control, glucose-, and fructose-administered diabetic rats without a common superscript are statistically different ($P < 0.05$). For abbreviations see Figures 1 and 2.

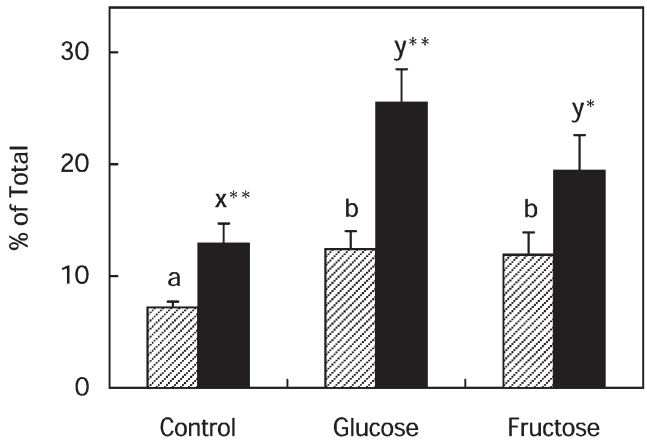

FIG. 4. Effects of glucose and fructose in combination with clofibric acid on the hepatic proportion of 18:1n-9. Rats were given a 20% wt/vol solution of glucose or fructose for drinking for 4 d with (solid bar) or without (hatched bar) a subcutaneous injection of clofibric acid twice a day at a dose of 100 mg/kg body weight. FA composition of total lipids was analyzed in the liver. Values are means ± SD for three to four rats. [a,b]Values for control, glucose-, and fructose-administered rats without a common superscript are statistically significant ($P < 0.05$). [x,y]Values for control, glucose-, and fructose-administered rats with clofibric acid treatment without a common superscript are statistically significant ($P < 0.05$). *,**Significantly different from the value of clofibric acid-untreated rats ($P < 0.05$ and $P < 0.01$, respectively).

0.8743, $P < 0.01$), whereas the correlation between the proportion of 18:1n-9 and SCD activity was not statistically significant ($r^2 = 0.5521$, $P > 0.05$).

DISCUSSION

Induction of PCE by glucose and fructose. In the present study, we demonstrated that both glucose and fructose induced PCE as well as SCD in the liver of rats (5). The effects of the carbohydrates were not mediated by insulin, a known inducer of both PCE and SCD, because the serum insulin level was not altered by the administration of carbohydrates in normal and diabetic rats. As compared to normal rats, only fructose, but not glucose, increased the activities of PCE and SCD in diabetic rats (Fig. 3). Although the mechanism by which fructose induces PCE and SCD in diabetic animals has not yet been clarified, it is plausible that intermediates of the glycolytic pathway play a pivotal role, because fructokinase was not affected whereas glucokinase was greatly depressed in the diabetic state (30).

Comparison of the induction of SCD and PCE. Early studies showed that PCE activity increased concomitantly with an increase in SCD activity by administration of clofibric acid (11,24,31), diethylhexylphthalate (10), refeeding of a fat-free diet after fasting, and treatment of diabetic rats with insulin (10,11); in contrast, a diabetic state and starvation reduced the activities of both SCD and PCE (5,10,15). These findings suggest that both PCE and SCD are regulated by similar mechanisms. Recent studies have revealed that SCD is transcriptionally regulated by insulin, carbohydrate, and PUFA

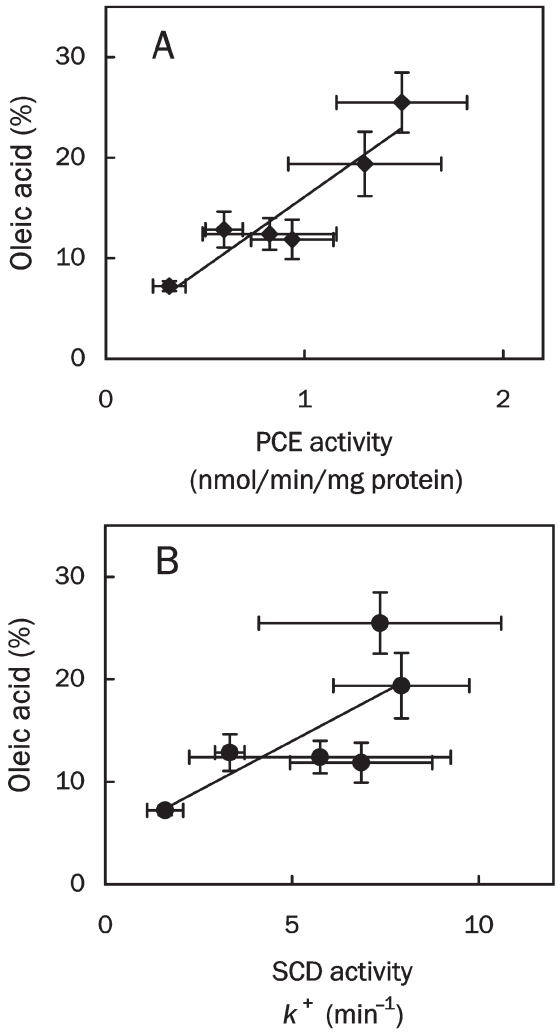

FIG. 5. Relationship between the activity of PCE or SCD and proportion of oleic acid in the liver. (A) The relationship between the activity of PCE (data from Fig. 2A) and the proportion of oleic acid (data from Fig. 4) was determined as $Y = 13.955X + 2.152$ ($r^2 = 0.8743$, $P < 0.01$). (B) The relationship between the activity of SCD (data from Fig. 2B) and the proportion of oleic acid was calculated as $Y = 1.934X + 4.294$ ($r^2 = 0.5521$, $P > 0.05$). For abbreviations see Figures 1 and 2.

via sterol regulatory element-binding protein (7–9,32). In contrast to SCD, little attention has been paid to the regulation of PCE except in early studies on changes in activity with various physiological conditions (10,11,15). More recent studies have shown the possibility that mRNA expression of FA chain elongase is regulated by sterol regulatory element binding protein-1 (33,34). Therefore, the possibility exists that the induction of PCE by carbohydrates is mediated by sterol regulatory element-binding protein. Induction of PCE and SCD by carbohydrate was independent of PPARα, because carbohydrate did not induce acyl-CoA synthetase, acyl-CoA oxidase, or 1-acyl-GPC acyltransferase and did not modify their induction with clofibric acid (Table 1).

Both PCE and SCD are known to be induced by peroxisome proliferators, such as clofibric acid and diethylhexylphthalate (10,11,24,31). Peroxisome proliferators induce

TABLE 2
Comparison of the Effects of Clofibric Acid on FA Contents of Hepatic Lipids in Glucose- or Fructose-Administered Rats[a]

	Control		Glucose		Fructose	
Clofibric acid	−	+	−	+	−	+
FA	(μmol/g liver)					
16:0	17.7 ± 1.3	18.2 ± 2.2^{x}	21.9 ± 3.4	23.9 ± 1.5^{y}	21.0 ± 2.8	20.2 ± 1.8^{x,y}
16:1n-7	1.2 ± 0.2^{a}	1.1 ± 0.2^{x}	4.6 ± 1.3^{b}	3.5 ± 0.8^{y}	4.9 ± 1.1^{b}	2.5 ± 1.0^{x,y,*}
18:0	12.2 ± 1.2	13.6 ± 0.5^{x}	12.9 ± 1.1	11.4 ± 1.5^{x,y}	10.9 ± 0.9	9.6 ± 1.0^{y}
18:1n-9	5.0 ± 0.5^{a}	8.7 ± 1.0^{x,***}	9.6 ± 2.1^{b}	20.5 ± 3.1^{y,**}	8.8 ± 2.4^{a,b}	14.2 ± 3.3^{z,*}
18:1n-7	2.9 ± 0.2^{a}	1.7 ± 0.1^{x,***}	3.7 ± 0.7^{a,b}	3.3 ± 0.5^{y}	4.2 ± 0.6^{b}	2.6 ± 0.6^{x,y,*}
18:2n-6	13.1 ± 1.5^{a}	10.3 ± 1.3^{x,***}	8.9 ± 0.6^{b}	6.6 ± 0.6^{y,**}	9.3 ± 0.7^{b}	8.3 ± 1.6^{x,y}
18:3n-3	0.3 ± 0.1^{a}	0.1 ± 0.1*	0.1 ± 0.0^{b}	0.1 ± 0.0*	0.3 ± 0.1^{a}	0.1 ± 0.0**
20:3n-9	0.1 ± 0.1	0.1 ± 0.0^{x}	0.2 ± 0.1	1.3 ± 0.1^{y,***}	0.5 ± 0.4	0.5 ± 0.2^{z,*}
20:3n-6	0.7 ± 0.1^{a}	1.2 ± 0.1^{x,***}	1.0 ± 0.2^{b}	1.6 ± 0.1^{x,y,**}	0.9 ± 0.1^{a,b}	1.8 ± 0.3^{y,**}
20:4n-6	10.8 ± 1.1^{a}	10.7 ± 3.0^{x}	8.4 ± 0.8^{b}	8.7 ± 0.2	7.2 ± 0.3^{b}	9.5 ± 1.1**
20:5n-3	0.8 ± 0.2	0.2 ± 0.1^{x,***}	1.1 ± 0.2	0.8 ± +0.1^{y,*}	1.0 ± 0.1	0.4 ± 0.1^{z,***}
22:5n-3	1.4 ± 0.1^{a}	0.4 ± 0.1^{x,***}	1.0 ± 0.1^{b}	0.6 ± 0.1^{y,***}	1.0 ± 0.1^{b}	0.5 ± 0.0^{x,y,***}
22:6n-3	3.8 ± 0.5	1.8 ± 0.6**	3.6 ± 0.2	2.5 ± 0.1***	3.2 ± 0.3	2.4 ± 0.1**
Total	69.8 ± 5.4	68.2 ± 8.0	76.9 ± 7.9	84.6 ± 7.9	73.4 ± 7.0	72.6 ± 6.2

[a]Rats were given 20% wt/vol glucose or fructose solution to drink for 4 d. Half were injected subcutanously with clofibric acid (100 mg/kg body wt) twice a day. Values are means ± SD for three to four rats. [a,b]Values for control, glucose-, and fructose-administered rats without a common superscript are statistically significant. [x,y]Values for control, glucose-, and fructose-administered rats with clofibric acid treatment without a common superscript are statistically significant ($P < 0.05$). If no superscript appears, the differences between the three groups are not statistically significant. *,**,***Significantly different from clofibric acid-untreated rats ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively).

various enzymes and proteins that are responsible for FA transport and oxidation by transcriptional activation *via* PPARα binding to a peroxisome proliferator responsive element (PPRE) in their promoter region (27,35). Treatment of mice with clofibrate (ethyl ester of clofibric acid) increased the mRNA level of SCD only twofold (36), which was far less than the increase in mRNA levels of FA oxidizing enzymes (28). It is therefore unclear whether clofibric acid induces PCE and SCD by direct activation of PPARα, although Miller and Ntambi (37) found a PPRE-like sequence in the promoter region of mouse SCD1. We observed that clofibric acid did not increase SCD activity further in carbohydrate-administered rats (Fig. 2). It is plausible that clofibric acid indirectly induces SCD by altering FA metabolism. Even though the induction of SCD by clofibric acid is dependent on PPARα, the physiological significance seems to be low compared to its regulation by carbohydrate. By contrast, PCE was additively induced by clofibric acid and carbohydrate. The question of whether PPARα directly promotes PCE expression remains to be elucidated.

Role of PCE in 18:1n-9 formation. The similarity in the regulation of PCE and SCD raised the question of which enzyme is more important in the regulation of oleic acid formation. Our present study demonstrated a difference in induction between SCD and PCE upon clofibric acid treatment in combination with carbohydrate. Namely, clofibric acid treatment additionally increased PCE activity in carbohydrate-treated rats, whereas no additional increase in SCD activity was observed with clofibric acid treatment (Figs. 2 and 3). These results imply that SCD and PCE contribute differently to oleic acid formation in response to various stimuli. In fact, the hepatic content of oleic acid was significantly increased whereas only PCE activity was increased by the treatment of rats administered carbohydrate with clofibric acid (Table 3). The highly significant correlation found between the proportion of oleic acid and PCE activity ($r^2 = 0.8743$, $P < 0.01$), but not SCD activity ($r^2 = 0.5521$, $P > 0.05$), clearly indicates the physiological importance of PCE in oleic acid formation. To our knowledge, this is the first report demonstrating the importance of PCE in comparison with SCD in oleic acid formation. The present study indicates that carbohydrate can induce not only SCD but also PCE and that PCE plays a crucial role in the formation of 18:1n-9.

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Differences in Δ^9 Desaturase Activity Between Jersey- and Limousin-Sired Cattle

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ABSTRACT: An experiment examined Δ^9 desaturase activity and FA composition in subcutaneous adipose tissue in two differing breeds of cattle. Jersey-sired cattle had significantly higher rates of desaturase activity than Limousin-sired cattle (1.55 vs. 0.75 nmol/mg protein/min). This difference was also demonstrated by a lower concentration of individual (e.g., 18:0) and total saturated FA (38.3 vs. 45.1 wt%), and a higher concentration of individual (e.g., 16:1) and total monounsaturated FA (58.2 vs. 52.7 wt%) in the Jersey animals. Other indices of desaturation calculated from the FA composition showed this same difference. The slip point of adipose tissue of Jersey cattle (36.8°C) was significantly lower than that of Limousin cattle (39.2°C), but Jersey adipose tissue had a greater content of β-carotene. The positive relationship between adipose tissue β-carotene and desaturation opposes the negative relationship between dietary β-carotene and desaturation determined elsewhere. These results, however, lead to the hypothesis that some cattle have a reduced capacity to metabolize β-carotene to various forms of vitamin A, a compound that can reduce Δ^9 desaturase enzyme activity. In addition, the higher level of intramuscular fat in Jersey cattle (6.97 vs. 3.82%) is possibly related to a lack of inhibition of the adipocyte differentiation genes by vitamin A.

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The quality of meat in terms of texture and taste is improved when there is an increase in the ratio of monounsaturated FA (MUFA) to saturated FA (SFA) in its fat component (1,2). This ratio is displayed in some cattle, particularly animals raised in Japan (3,4). SFA are converted to MUFA by the enzyme Δ^9 desaturase (EC 1.14.99.5), the activity of which has been measured in rat liver (5,6) and in bovine liver and adipose tissue (7). The levels in cattle adipose tissue are of the same order as those of rat liver, but no activity is present in bovine liver. No difference in enzyme activity was found between Angus and American Wagyu breeds of cattle (8).

It appears, however, that some dietary components reduce the activity of the Δ^9 desaturase enzyme. Two known examples of this are, first, a cyclopropenoic acid found in some varieties of cottonseed that is sometimes included in cattle feed (1) and, second, β-carotene or its metabolite, retinoic acid, when fed to rats (6) or sheep (9). Both these latter findings demonstrate a negative relationship between dietary β-carotene and desaturation. However, in some cattle that have yellow fat (caused by β-carotene accumulation) a positive relationship exists between fat color and MUFA concentration (10). The present study examines the difference in the activity of Δ^9 desaturase between Jersey- and Limousin-sired cattle and the effect of this breed difference on the FA profile of subcutaneous fat.

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Abbreviations: C16 desaturation index, 100·(*cis*-16:1Δ9)/(16:0 + *cis*-16:1Δ9); C18 desaturation index, 100·(*cis*-18:1Δ9)/(18:0 + *cis*-18:1Δ9); MUFA, monounsaturated FA; SFA, saturated FA.

MATERIALS AND METHODS

Animals and management. The animals used were progeny of a large crossbreeding program that involved crossing sires of various breed types with Hereford dams (11). Those sampled for the purposes of this study were 10 randomly selected Jersey × Hereford steers and 10 randomly selected Limousin × Hereford steers. The animals were weaned at about 8 mon of age. They were then raised on a mixed pasture of strawberry clover and phalaris for approximately 12 mon. At slaughter, the mean live weight and carcass weight of the animals sampled were not significantly different ($P > 0.05$) from those of all the animals of that breed type (10 of 13 Jerseys, 10 of 22 Limousins). The mean live weight of the Jersey-sired group (487 kg) was not significantly ($P > 0.05$) different from the Limousin-sired group (520 kg).

Sample collection. Subcutaneous adipose tissue (~100 g) samples were removed from carcasses in a chiller within 60 min of slaughter, placed in liquid nitrogen, and stored at −80°C until analyzed. Muscle samples (~200 g) were removed from the 12th/13th rib interface from the carcass in the chiller on the day following slaughter and were frozen and stored at −20°C until analyzed.

FA composition and m.p. Samples of adipose tissue (~5 g) were heated at 100°C for 30 min before subsamples (20 mg) were placed in 1.5 mL acidified methanol (1% H_2SO_4) for the preparation of methyl esters by heating at 60°C overnight in sealed culture tubes (12). After cooling, 3 mL H_2O and 5 mL petroleum ether (40–60°C b.p.) were added and the tubes sealed and vortexed. The upper phase containing the FAME was removed and the sample extracted a further time with petroleum ether. The pooled extracts were evaporated to dryness under a stream of N_2 at 40°C. The residue was dissolved in 300 μL of iso-octane. Separation of individual FAME was

carried out by GLC using a Hewlett-Packard gas chromatograph (5890A II) fitted with a capillary column (BPX70; SGE, Melbourne, Australia). Hydrogen was used as the carrier gas. The system allowed the identification of *cis/trans* isomers. Methyl esters were identified by comparison with retention times of authentic standards. MUFA reported here were identified relative to the carboxyl end (Δ convention). Total saturated and total monounsaturated species were expressed as the sum of the individual FA (∑SFA and ∑MUFA). *cis* isomers were identified separately and total *cis* MUFA (∑*cis* MUFA) are reported as such. Desaturation was calculated as 100 × *cis*-18:1 Δ9/(18:0 + *cis* 18:1 Δ9) and reported as a C18-desaturation index (%). A similar calculation was performed for C16-desaturation index. This calculation is the same as used in calculating Δ^9 desaturase activity (see below). At the time subsamples of fat were taken for FA analysis, a further two subsamples were taken in 1-mm capillary tubes for m.p. as determined from the "slip point" (13,14).

Δ^9 desaturase activity. The method described by Yang *et al*. (1) was used for this measurement. Frozen adipose tissue samples (~50 g) were diced and homogenized for 1 min in 2 vol of 0.02 M phosphate buffer (pH 7.4) containing 0.15 M KCl, 1 mM EDTA, and 1 mM DTT. The homogenate was filtered through washed kitchen toweling into a flask. It was then centrifuged at 1,500 × *g* for 10 min and filtered through glass wool. The microsomal fraction was isolated by centrifugation of the resultant supernatant at 17,300 × *g* for 30 min and the supernatant filtered through glass wool. The fat-free supernatant was centrifuged 104,000 × *g* for 60 min and the microsomal pellet suspended in 1 mL of cold buffer. Protein concentration was determined using the method of Bradford (15).

The desaturase enzyme assay was carried out in duplicate. The reaction mixture (final volume 1.0 mL) contained 5 mM $MgCl_2$, 50 mM K_2HPO_4, 5 mM ATP, 0.5 mM NADH, and 0.05 mM [1-^{14}C]palmitoyl-CoA (specific activity = 57.1 mCi/mmol). The reaction was initiated by the addition of 1 mg of microsomal protein and was incubated at 37°C for 5 min before being stopped by the addition of 2 mL 10% KOH in methanol. Blanks were obtained by adding 2 mL of the 10% KOH to the reaction mixture prior to the addition of the microsomal protein. The tubes were next heated at 70°C for 30 min, and the solution was then acidified with 6 mL 3 M HCl. FFA were extracted three times with 6 mL petroleum ether (40–60°C b.p.), and the combined extracts were washed with 6 mL acidic water (pH 3.0). The pooled upper layer was evaporated to dryness, and the FA were methylated in 1 mL acidified dried methanol (5% H_2SO_4) at 60°C overnight. Subsequently, 1 mL of purified water was added, and the methyl esters were extracted three times with petroleum ether and then pooled and evaporated under nitrogen before being redissolved in 100 μL of petroleum ether. The saturated and unsaturated species of the methyl esters were separated by thin-layer argentation chromatography (3% $AgNO_3$) on freshly activated commercial silica gel G plates. They were developed with a petroleum ether/diethyl ether mixture (97:3, vol/vol), and the species were identified under UV light after spraying with 0.02% dichlorofluorescein. The different species were then cut from plates individually and placed in 4 mL of scintillant (10% aqueous solution), and the radioactivity was determined in a scintillation counter (LS3801; Beckman Instruments, Irvine CA). Δ^9 Desaturase activity was calculated as the percentage of counts for the monounsaturated species over the sum of the saturated and monounsaturated species and expressed as nmol palmitoleic acid formed per mg protein per min.

Intramuscular fat content. Muscle samples were trimmed of all visible fat and a subsample (~100 g) was homogenized in a food processor. Fat content was determined from an accurately weighed subsample (~2 g) of this material using chloroform/methanol (2:1, vol/vol) as the solvent (12).

β-Carotene and color of fat. β-Carotene content was calculated from fat color using equations derived from a large number of animals involved in the cross-breeding experiment (16). Fat color was assessed at slaughter by AUS-MEAT® (17) assessors on a 0 (white) to 9 (yellow) scale.

Source of chemicals and radioisotopes. All biochemicals were purchased from Sigma Chemical Co. (Sigma-Aldrich, Sydney, Australia). The radioisotope [1-^{14}C]palmitoyl-CoA was purchased from NEN (Boston, MA).

Statistical analysis. The results were expressed as mean ± SE and all data were analyzed as single-factor ANOVA (Microsoft Excel, Version 5).

RESULTS

Jersey-sired cattle at pasture had a significantly lower ($P < 0.05$) concentration of SFA (∑SFA), a significantly higher ($P < 0.05$) concentration of *cis* MUFA (∑*cis* MUFA), and a significantly higher ($P < 0.05$) ratio of 16:1(*c*9)/18:0 than the Limousin-sired cattle (Table 1). These ∑SFA differences occurred chiefly because of the highly significant difference between the breeds of 18:0 ($P < 0.01$) and, to some degree, 16:0 ($P < 0.07$) and 14:0. All MUFA values were higher for Jersey- than Limousin-sired cattle, although only when summed (∑*cis* MUFA) did the difference reach significance. The PUFA [18:2n-6 (linoleic) and 18:3n-3 (linolenic)] were low in concentration and not significantly different between the breeds. Although low in concentration, an isomer of linoleic, CLA, was significantly higher ($P < 0.05$) in Jersey-sired animals. The β-carotene content of the Limousin animals was significantly lower ($P < 0.001$) than that of the Jersey animals. Conversely, the mean slip point of fat from Jersey cattle was 2.4°C lower ($P < 0.05$) than that from Limousin cattle. Intramuscular fat content was significantly greater ($P < 0.001$) in Jersey-sired cattle, reaching nearly 7.0% compared with 3.8% in Limousin-sired cattle.

Desaturase enzyme activity was significantly higher in Jersey-sired cattle than in Limousin-sired cattle ($P < 0.001$) (Table 2) and significantly correlated with β-carotene content ($P < 0.01$). When indices of desaturation were calculated from FA composition values, the Δ^9C16 and Δ^9C18 values were significantly greater ($P < 0.001$) in Jersey-sired cattle.

TABLE 1
Mean (± SE) Values of the FA Components (wt%) of Subcutaneous Fat from Limousin- and Jersey-Sired Cattle

FA	Limousin	Jersey	Statistics[a]
14:0	3.90 ± 0.05	3.30 ± 0.27	NS
14:1 (*c*9)	1.90 ± 0.18	2.31 ± 0.19	NS
15:0	0.59 ± 0.05	0.44 ± 0.03	NS
16:0	25.54 ± 1.24	22.70 ± 0.93	$P < 0.07$
16:1 (*c*7)	0.16 ± 0.05	0.43 ± 0.22	NS
16:1 (*t*7)	0.26 ± 0.06	0.37 ± 0.05	NS
16:1 (*c*9)	5.91 ± 0.43	7.39 ± 0.39	$P < 0.05$
17:0	1.04 ± 0.04	0.79 ± 0.07	NS
18:0	12.34 ± 0.84	9.51 ± 0.85	$P < 0.01$
18:1 (*t*11)	1.70 ± 0.47	2.06 ± 0.40	NS
18:1 (*c*9)	42.99 ± 1.80	46.20 ± 1.24	NS
18:1 (*c*11)	1.89 ± 0.14	2.63 ± 0.20	$P < 0.05$
19:0	0.24 ± 0.11	0.24 ± 0.10	NS
18:2 (*c*9,*c*12)	0.92 ± 0.11	1.08 ± 0.11	NS
18:3 (*c*9,*c*12,*c*15)	0.28 ± 0.05	0.38 ± 0.04	NS
18:2 (*c*9,*t*11) (CLA)	0.337 ± 0.068	0.538 ± 0.075	$P < 0.05$
16:1 (*c*9)/18:0	0.52 ± 0.08	0.82 ± 0.09	$P < 0.05$
∑SFA[b]	45.10 ± 1.95	38.30 ± 1.37	$P < 0.05$
∑*cis* MUFA[c]	52.70 ± 1.74	58.16 ± 1.25	$P < 0.05$
β-Carotene (μg/g)	2.3 ± 0.00	2.7 ± 0.07	$P < 0.001$
Slip point (°C)	39.2 ± 0.51	36.8 ± 1.10	$P < 0.05$
IMF (%)[d]	3.82 ± 0.06	6.97 ± 0.10	$P < 0.001$

[a]NS, not significant.
[b]∑SFA, total saturated FA.
[c]∑*cis* MUFA, total *cis* monounsaturated FA.
[d]IMF (%), intramuscular fat (g/100 g w/w).

There was a significant ($P < 0.05$) relationship between measured enzyme activity and the C16 index of desaturation (Fig. 1).

DISCUSSION

The degree of desaturation of subcutaneous fat in Jersey-sired cattle was shown to be different from that of Limousin-sired cattle. The difference was exhibited in a number of measurements. First, a significant difference was determined in the activity of the Δ^9 desaturase enzyme extracted from subcutaneous fat immediately after slaughter. The activity in Jersey cattle was found to be significantly ($P < 0.001$) higher than that in Limousin cattle. It was also apparent in a difference in the concentration of the SFA and MUFA present in the same fat. For example, the mean concentration of stearic acid (18:0) was 12.3%, significantly higher ($P < 0.01$) than that found in Jersey cattle (9.5%). ∑SFA and ∑*cis* MUFA were significantly ($P < 0.05$) higher and lower, respectively, in Limousin cattle than in Jersey cattle. A ratio sometimes used to illustrate desaturation [16:1 (*c*9)/18:0] (4,18) was significantly greater in Jersey cattle, as were two other indices of desaturation, calculated from C16 and C18 FA concentrations. Both indices were found to be significantly correlated with measured enzyme activity (e.g., Fig. 1). Although lower in concentration, a further example of this difference in desaturase activity was found in the concentration of CLA, a FA produced by the action of Δ^9 desaturase

TABLE 2
Mean (± SE) Values of Measured Desaturase Enzyme Activity and Calculated Indices of Desaturation in Adipose Tissue from Limousin- and Jersey-Sired Cattle

Sire breed	Desaturase activity[a]	Desaturation index (Δ^9C16)[b]	Desaturation index (Δ^9C18)[c]
Limousin	0.75 ± 0.08	25.02 ± 1.37	77.64 ± 1.39
Jersey	1.55 ± 0.10	33.43 ± 1.67	82.78 ± 0.94
Statistics	$P < 0.001$	$P < 0.001$	$P < 0.01$

[a]Palmitoleic acid (16:1) formed from palmitic acid (16:0) (nmol/mg protein/min).
[b]Desaturation (Δ^9C16) = index of desaturation of 16:0 FA = 100 × (*cis*-16:1Δ9)/(16:0 + *cis*-16:1Δ9).
[c]Desaturation (Δ^9C18) = index of desaturation of 18:0 FA = 100 × (18:1)/(*cis*-18:1Δ9 + 18:0).

in tissues from *trans* vaccenic acid (18:1, *t*11), a FA that is formed in the rumen (18).

The value for total MUFA (including *trans* FA) reported here for Limousin-sired cattle (~54%) was very similar to that reported for Angus-cross cattle at pasture (1) but not as high as the nearly 60% found in two different studies of Japanese lot-fed cattle (3,4). Similar values were found in meat fat of imported Korean lot-fed cattle (Siebert, B.D., unpublished data). The value for Jersey-sired cattle in the present experiment (60.6%), however, was similar to the high Asian cattle values. To a large extent, MUFA are derived from SFA, and ∑SFA in the present study was similar to that recorded in studies of cattle in Japan (3,4). Apart from these indicators of desaturation derived from FA concentrations, measured desaturase enzyme activity of Jersey cattle at pasture in this study was similar to that reported for Angus-cross cattle at pasture (1). Both of these values are considerably higher than those recorded with American cattle (7,8), although there were some differences in the assay method (*viz.* different substrates), and direct comparisons may not be valid.

The concentration of the MUFA 16:1 and 18:1 increased with age and degree of maturity (19). Despite this general increase, however, there appear to be other reasons for the differences in desaturation between mature cattle in different countries. Japanese cattle have much higher levels of desaturation

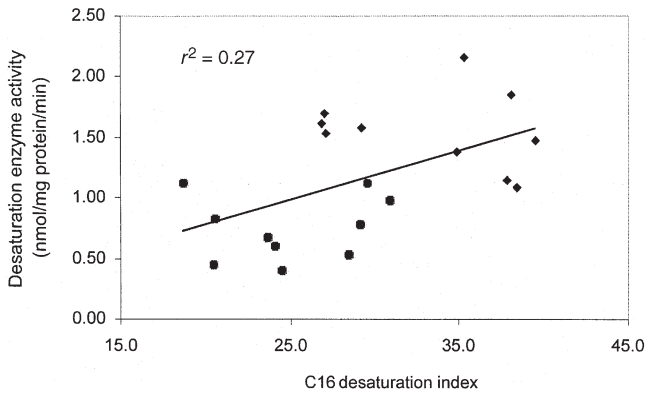

FIG. 1. Relationship between measured desaturase enzyme activity and the C16 index of desaturation calculated from the FA composition of adipose tissue from Limousin-sired (●) and Jersey-sired (◆) cattle.

(e.g., low ∑SFA or high ∑MUFA) than Australian cattle (4) or American cattle (20), but a group of Australian cattle transported to Japan and fattened in a feedlot had values similar to local Japanese cattle (4).

Australian cattle grown in feedlots sometimes receive feed containing cottonseed. Some varieties of cottonseed contain a cyclopropenoic acid that can inhibit the desaturase enzyme (21) and could possibly explain the relatively low values (1). Alternatively, Australian cattle grown at pasture consume β-carotene, a compound that has been shown also to reduce Δ^9 desaturase enzyme activity (6). This reduction has been noted in sheep when animals fed a high β-carotene diet had lower levels of MUFA than animals fed a low β-carotene diet (9). The sheep receiving a high β-carotene diet had significantly greater levels of retinyl ester in liver (fivefold) than those receiving low β-carotene (Siebert, B.D., unpublished data). Japanese cattle known to have high levels of monounsaturates (3) are raised on low β-carotene feeds. These results or practices demonstrate a negative relationship between dietary β-carotene and desaturase activity. β-Carotene is also present in green hay and silage. Its intake by cattle will be subject to pasture type, locality, and season and may well explain the relatively low value of MUFA in Australian and New Zealand pasture-fed cattle.

Some cattle, particularly those of Channel Island origin (e.g., Jersey), exhibit higher concentrations of desaturated FA than others and have colored fat resulting from β-carotene accumulation (22). Contrary to the negative relationship between dietary β-carotene and desaturation referred to above, significant positive relationships have been found between fat β-carotene (or fat color) and MUFA concentrations in Australian and New Zealand cattle (10,23). Similar results were found in the experiment reported here; Jersey cattle had higher color scores and higher desaturation than Limousin cattle. The reason for this opposing relationship is most probably because in some cattle there is less activity of the dioxygenase, an enzyme present in intestinal mucosa that catalyzes the splitting of β-carotene into two molecules of retinal. This leads to the accumulation of this carotenoid in adipose tissue and causes yellow fat (24). In animals where β-carotene is metabolized completely or to a large extent, retinal and, subsequently, retinoic acid are formed from the splitting of β-carotene molecules. Retinoic acid has been shown to inhibit the enzyme Δ^9 desaturase (6). In sheep, the β-carotene dioxygenase is more active than in cattle (24), and desaturation is lower in sheep, which leads to a higher concentration of SFA and higher fat m.p. (9). Apart from the fact that Japanese cattle consume feeds very low in β-carotene, they may also have a reduced ability to metabolize β-carotene, a trait inherited possibly from crossing Japanese inbred lines with British dairy cattle. This ability not to reduce β-carotene, however, does not appear to be as great as that found in Jersey-cattle because fat color in Wagyu-sired cattle is less than that in Jersey sired cattle (11).

Vitamin A (retinoic acid) also has been found to inhibit the differentiation of adipocytes, thus limiting lipid deposition. It has been proven that this inhibition occurs at a DNA level (25). It is noteworthy that apart from greater desaturation of adipose tissue, Jersey breed cattle in the present study also had a higher intramuscular fat concentration (6.97%) than Limousin breed cattle (3.82%).

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Development of Intestinal Alkaline Sphingomyelinase in Rat Fetus and Newborn Rat

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ABSTRACT: Sphingomyelin metabolism is a novel signal transduction pathway related to cell differentiation, proliferation, and apoptosis. Alkaline sphingomyelinase (alk-SMase) is specifically present in the intestinal tract of many species. The enzyme is important in digestion of dietary sphingomyelin. Milk is the only exogenous source of sphingomyelin for an infant, and digestion of milk sphingomyelin may be important for development of intestinal mucosa. It is unknown whether alk-SMase is present before birth and whether it changes after birth and during the suckling period. We studied activities, expression, and distribution of alk-SMase in rat fetus and newborn. The changes of acid and neutral SMase as well as alkaline phosphatase were analyzed for comparison. Little activity of alk-SMase was identified up to gestation day 20, but increased 10 times during the following 2 d. After birth, the activity continued to increase during the following 4 wk. Western blot using IgY antibody against rat alk-SMase failed to identify the enzyme at gestation day 20 but clearly showed the protein at day 22. The distribution pattern of the enzyme along the intestinal tract in fetus was largely the same as in adult animals, but became more pronounced after birth. Short-term weaning had no effect on alk-SMase activity. The activities of acid and neutral SMase were high at gestation day 20 and decreased significantly before birth. The changes of alk-SMase also differed from those of alkaline phosphatase, another brush border enzyme. Thus, we conclude that alk-SMase is rapidly expressed during the last days of gestation and that the newborn rat acquires the ability to digest milk sphingomyelin early in life.

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Sphingomyelin (SM) is a type of sphingolipid and is hydrolyzed by sphingomyelinase (SMase) to ceramide and phosphocholine. The interest in SM metabolism has increased during recent years because the hydrolytic products may have important signaling effects in cell proliferation, differentiation, and apoptosis (1,2). To date, three different groups of enzymes—named acid, neutral, and alkaline SMase (alk-SMase)—have been identified. Both acid and neutral SMases are present in many tissues, and some of them have been purified and their genes cloned (3–12). Alk-SMase, which hydrolyzes SM at an optimal pH of 9.0, was first found in human and pig intestinal contents by Nilsson (13). The activity of the enzyme was low in the duodenum, increasing in the small intestine and reaching the peak level in the distal part of the jejunum (14). The enzyme from rat intestine was recently purified and polyclonal antibody against the enzyme raised (15). Both biochemical and immunological studies showed that the enzyme may be expressed only in the intestinal mucosa in rats (15).

SM is a dietary component that is present in milk, meat, and seafood (16,17). When rats are fed dietary SM, the digestion and absorption of SM are found to occur mainly in the middle part of small intestine where alk-SMase is abundant, indicating that the enzyme may play an important physiological role in digestion of SM (18). Milk is rich in SM, where it occurs at about 0.2%. For a newborn mammal, milk is the only source of SM. Thus, it would be of interest to find out whether alk-SMase activity appears in the intestinal tract before birth and how it changes after birth.

In this work we studied the activity and expression of alk-SMase in the gastrointestinal tract of rat fetus and newly born rats and examined short-term effects of weaning on enzyme activity. For comparison, the activities of acid SMase, neutral SMase, and intestinal alkaline phosphatase before and after birth also were determined.

MATERIALS AND METHODS

Materials. Under the permission of the Ethical Committee of Lund, Sweden, pregnant Sprague-Dawley rats with known day of conception were obtained from B&K Universal AB (Sollentuna, Sweden) and housed in a temperature-controlled room under a 12 h light and dark regime. Rats were fed with standard food (Beekay Feeds, B&K Universal AB) and had free access to water. SM was purified from milk according to Nyberg *et al.* (19) and the purity analyzed by HPLC. The purified SM was labeled with [N-^{14}C-CH$_3$]choline by the methods of Stoffel (20). Specific activity of labeled SM was 56 μCi/mg, and radiochemical purity was more than 98%. The purification of rat intestinal alk-SMase and the development of IgY antibody against the enzyme were performed in the laboratory and have been described in detail elsewhere (15). Anti-IgY conjugated with alkaline phosphatase was purchased from Sigma Chemical Co. (Stockholm, Sweden). Taurocholate (TC), taurodeoxycholate (TDC), glycocholate (GC), glycochenodeoxycholate (GCDC), phenylmethylsulfonyl fluoride

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Abbreviations: alk-SMase, alkaline sphingomyelinase; GC, glycocholate; GCDC, glycochenodeoxycholate; PMSF, phenylmethylsulfonyl fluoride; SM, sphingomyelin; SMase, sphingomyelinase; TC, taurocholate; TDC, taurodeoxycholate.

(PMSF), and benzamidine were purchased from Sigma Chemical Co. (St. Louis, MO).

Study design. Pregnant animals with known date of conception were killed at gestation day 10, 20, 21, 22, or birthday, which in this study always was on day 23. Newborn rats were investigated 0.5 to 2 h after birth, on their second day (24 h after birth), and after 1, 3, and 4 wk. Some rats were weaned at the age of 3 wk and investigated 1 wk later. These animals were compared with 4-wk-old unweaned animals.

Preparation of samples. Pregnant rats were anesthetized with diethyl ether and killed by cervical dislocation on gestation day 10, 20, 21, and 22. A caesarean section was performed and the fetus brought out. Newborn rats, 2-d, 1-, 3-, and 4-wk-old rats were anesthetized with diethyl ether. Laparotomy was performed and the gastrointestinal tract identified and brought out under a dissecting microscope. In the 10-d fetus this was impossible owing to its size. The fetal gastrointestinal tract was divided in 4 or 5 segments while that of older animals was divided into 7 or 8 segments. In all animals, the ventriculum (stomach) was identified. In fetus and newborn rats it was impossible to distinguish between small and large intestine, and the cecum was not developed. The division between small and large intestine was thus an approximation in rats younger than 1 wk. The intestinal segments were homogenized in 0.5–2 mL buffer containing 0.25 mol/L sucrose, 5 mmol/L $MgCl_2$, 0.15 M mol/L KCl, and 50 mmol/L KH_2PO_4 (pH 7.4), 10 mmol/L TC, 1 mmol/L benzamidine, and 1 mmol/L PMSF followed by sonication and centrifugation. Acid, neutral, and alk-SMase activities and protein contents in the supernatant were analyzed.

Assay of SMase activity. The activity of SMase was determined as described previously (21) with some modifications. For alk-SMase determination, 5-µL samples were added to 75 µL Tris buffer, pH 9.0 containing 50 mmol/L Tris, 0.15 mol/L NaCl, 2 mmol/L EDTA, and 3 mmol/L bile salt mixture with a molar ratio of TC/TDC/GC/GCDC of 3:2:1.8:1. The addition of EDTA to the buffer served to inhibit neutral SMase, which is magnesium-dependent and has a pH optimum of 7.5 (22). The reaction was started by adding 20 µL of ^{14}C-SM (80 pmol, 8,000 dpm) suspension containing 0.15 M NaCl and 3 mM bile salt mixture. The samples were incubated at 37°C for 30 min. The reaction was stopped by addition of 0.4 mL chloroform/methanol (2:1). After phase partition and centrifugation, an aliquot of the upper phase was taken and the radioactivity was counted by liquid scintillation. The activities of acid and neutral SMase were determined in a similar way as for alk-SMase assay with modifications of the buffers. The buffer for acid SMase assay was 50 mM Tris-maleate buffer containing 0.15 M NaCl, and 0.12% Triton X100, pH 5.0, and that for neutral SMase assay was 50 mM Tris-HCl buffer, containing 0.15 M NaCl, 0.12% Triton X100, and 4 mM Mg^{2+}, pH 7.4. Triton X100 at this concentration completely blocks alk-SMase activity (15).

Western blot. Western blot using purified IgY was performed according to Murata *et al*. (23). Homogenates of intestinal mucosa from the fetus at gestation days 20 and 22, and from rats at the age of 4 wk (75 µg protein in each sample) were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane electrophoretically. The membrane was washed with 20 mM Tris buffer pH 7.5, containing 0.15 M NaCl, 7.5% dried milk, and 0.05% Tween 20 and then probed with purified IgY antibody at 1:500 dilution in Tris buffer containing 0.15 M NaCl, 2% dried milk, and 0.25% Triton X-100 for 2 h. After rinsing, the membrane was incubated with anti-IgY conjugated with alkaline phosphatase at 1:100,000 dilution for 2 h. The bands were then visualized using a kit from Bio-Rad Co. (Hercules, CA).

Other biochemical assays. Alkaline phosphatase was determined as described (24), using *p*-nitrophenylphosphate as substrate and pure *p*-nitrophenyl as a standard. The activities were expressed as unit/mg protein. One activity unit is the production of 1 mol *p*-nitrophenyl per min. The protein was determined by a kit from Bio-Rad Co. using BSA as a standard.

RESULTS

Changes of alk-SMase activity. Alk-SMase activity was determined in the entire gastrointestinal tract of fetuses and newborn rats. The highest alk-SMase activities in the intestinal segment of the rats are presented in Figure 1. There was a 10-fold increase in activity from the 20th until the 22nd day of gestation. In the newborn there was a slight decrease in activity, but from the second day there was a continuous increase during the observation time. In 10-d fetuses the activity was very low, 0.14 ± 0.01 nmol/mg. Whether there were similar changes for acid and neutral SMases before and after birth was also examined, and the results are shown in Table 1. Both acid and neutral SMase activities were high at the 20th day of gestation and sharply decreased at the 22nd day. The activities at the age of 4 wk were higher than those at the birth but failed to reach the levels at gestation day 20.

Expression of alkaline SMase in fetus. As shown in Figure 2, Western blot using purified anti-alk-SMase antibody

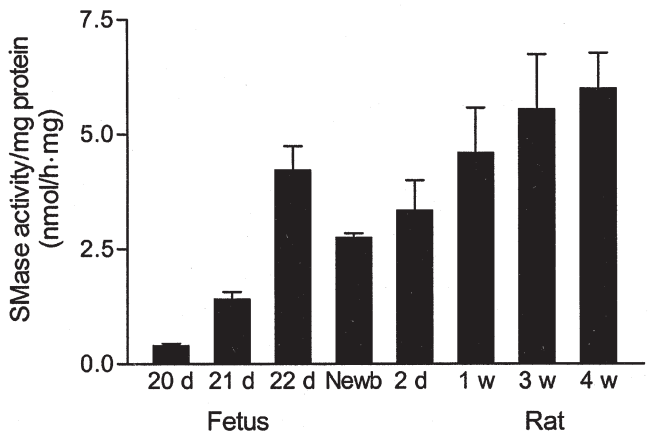

FIG. 1. Peak alkaline sphingomyelinase (alk-SMase) activity in the intestinal tract in 20–22-d fetus and newborn to 4-wk-old rats. The intestinal segments were homogenized, sonicated, and centrifuged. The alk-SMase activities were determined. Results are mean ± SEM from four fetuses in each group, six newborn rats, and five 4-wk-old rats.

TABLE 1
Changes in the Activities of Acid SMase and Neutral SMase in Rat Intestinal Tract Before and After Birth[a]

Age	Acid SMase (pmol/h/mg)	Neutral SMase (pmol/h/mg)
20 d gestation	5550 ± 210	196 ± 48
22 d gestation	210 ± 160	42 ± 14
Newborn	340 ± 80	74 ± 6
4 wk after birth	620 ± 320	134 ± 38

[a]The small intestine was removed from rats in each group and cut into four or five segments depending on the ages. The mucosa was scraped and homogenized. The activities of acid and neutral SMases were determined. The table shows the peak activity in the small intestine. Results are obtained from three rats in each group. SMase, sphingomyelinase.

hardly identified the alk-SMase band in the sample from the fetus at gestation day 20, but clearly showed the band in the samples of gestation day 22 and of 4 wk after birth. This is a representative figure, and similar results were obtained from the samples of other rats at the same ages. Sometimes, a few faint bands below the major SMase band were demonstrated. These bands were also identified in one of our previous studies (15).

Distribution of alk-SMase in the gastrointestinal tract. Determination of alk-SMase activity in different parts of the intestinal tract in fetuses and newly born rats is presented in Figure 3. The highest levels always were found in the middle of the small intestine. Activities were low in the duodenum and colon compared to the small intestine. In four rats, it was possible to determine the activities in both cecum and colon. The alk-SMase activity in cecum and colon was 0.20 ± 0.10 and 0.10 ± 0.04 nmol/h/mg protein, respectively (statistically not different).

We also compared alk-SMase activity in unweaned rats with those in rats weaned for 1 wk at the age of 4 wk. There was no difference in either levels or distribution pattern of the enzyme activity after a short-term weaning (data not shown).

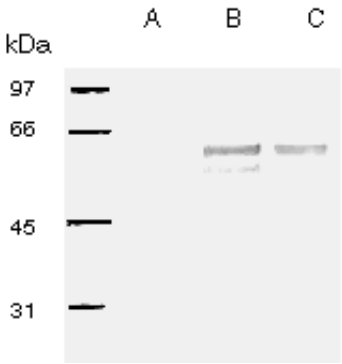

FIG. 2. Western blot of intestinal alk-SMase in rat fetus and rats. The homogenates of intestinal mucosa (75 μg protein in each) from rat fetus of gestation days 20 and 22 and from rat 4 wk after birth were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blotted with anti-alk-SMase antibody (IgY) followed by incubation with anti-IgY antibody conjugated with alkaline phosphatase. (A) 20-d rat fetus, (B) 22-d rat fetus, and (C) 4-wk-old rat. The standard molecular sizes are indicated on the left. For abbreviation see Figure 1.

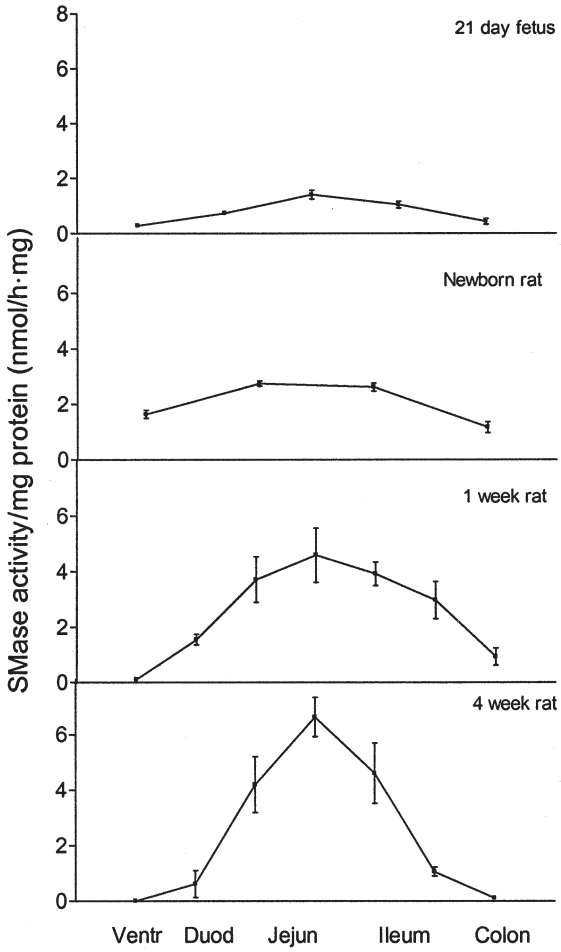

FIG. 3. Distribution pattern of alk-SMase activity in 21-d rat fetus, newborn, 1-wk, and 4-wk-old rats. Alk-SMase activity and protein concentration were determined in homogenized, sonicated, and centrifuged intestinal segments. Results are mean ± SEM. The number of animals are: four for 21-d fetus, six for newborn rats, and five for 1- and 4-wk-old rats. For abbreviations see Figure 1.

Changes of alkaline phosphatase activity. Alkaline phosphatase activity was determined in the entire gastrointestinal tract of fetuses and newborn rats. The intestinal segment with the highest activity is presented in Figure 4. Little activity was identified at day 22, and considerable activity was found in the newborn rats. The activity increased to a high level 2 d and 1 wk after birth, followed by a dramatic decrease in the third and fourth week. This pattern was the same in every animal but clearly different from that of alk-SMase shown in Figure 1.

DISCUSSION

In this work we studied the development of alk-SMase in rat fetus and neonatal rats. We found that at gestation day 20 the activity in the intestine was very low. It increased dramatically at day 22, one day before the birth, whereas the acid and neutral SMase activities were inversely changed. In agreement with the alk-SMase activity determination, Western blot

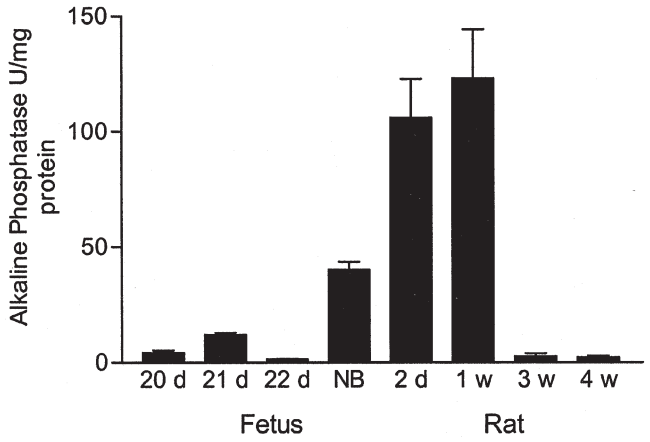

FIG. 4. Peak alkaline phosphatase activities in 20–22-d fetus and newborn to 4-wk-old rats. Activity was determined in homogenized, sonicated, and centrifuged intestinal segments. Results are mean ± SEM. n = 4 for 10–22-d fetus, 6 for newborn rats, and 5 for 4-wk-old rats.

showed the enzyme protein in the intestinal mucosa at day 22, but not at day 20. After birth, the alk-SMase activity steadily increased and seemed to approach a plateau at 4 wk. Short-term weaning did not show a significant effect on the levels of alk-SMase.

SM has been found in milk, meat, and fish (16,17), and for an infant, milk is the only source of dietary SM. Recent studies indicate that milk may contain components that have an impact on cell differentiation, proliferation, and apoptosis (25), thus affecting the development of the intestinal tract in infants (26). Based on the current knowledge of the signaling effects of sphingolipid and the changes of sphingolipids with postnatal maturation of the intestine (27), digestion of SM in the milk may play an important role in regulation of the development of the intestinal tract. Although acid, neutral, and alk-SMases are present in the gut, studies indicate that the alk-SMase may be the most important one in digestion of dietary SM for the following reasons. First, the specific activity of alk-SMase is much higher than those of acid and neutral SMase. Although the optimal pH of alk-SMase is 9.0, which is above the pH values under physiological conditions, the enzyme at pH 7.4 has about 60% of the maximal activity (15), which is well above either the acid or neutral SMase in the mucosa. Second, the alk-SMase is resistant to trypsin digestion, whereas acid and neutral SMases are not (14). Third, digestion of dietary SM was found to occur at the middle part of the small intestine, where alk-SMase is higher and acid SMase is very low (14). Finally, as shown in this paper, the expression of alk-SMase dramatically increased just 1 d before birth, whereas the activities of acid and neutral SMase decreased by 80 to 95%. The increase of expression of alk-SMase before birth could have important physiological significance for digestion of milk SM. Notably, the development of alk-SMase in the fetus resembles that of lactase in rat (28). Lactase protein is also expressed before birth, with a continuous increase with age. The rapid expression of alk-SMase together with lactase just 1 d before birth indicates that nature has provided the newborn with enzymes that are important for digestion and absorption of nutritional components in the milk. In addition, lactase is identical to intestinal glycosyl ceramidase that has been purified from intestinal mucosa (29,30). Thus, the enzymes necessary to hydrolyze both SM and glycosphingolipids in milk are expressed at birth in the rat.

In addition to the nutritional aspect, it is noteworthy that the rapid expression of alk-SMase before birth and the increase in the activity after birth may contribute to the differentiation and development of the small intestine. It has already been shown that the differentiation of rat intestine occurs late in the 18- to 22-d gestation period and continues after birth, and that the small intestine of rats reaches maturity about 3 wk after birth (31,32), which correlates well with the express of alk-SMase and the changes in enzyme activity.

The distribution of intestinal alk-SMase has been studied in several species. The level is highest in the distal part of jejunum, although the enzyme also is found in the proximal part of the small intestine and in the ileum (14,33). In this study we found that this distribution pattern was already established in the fetus as early as gestation day 21. This pattern was strengthened with time, with a distinct peak in the middle of the small intestine in 4-wk-old rats. No activity was found in the stomach, and very low activities were detected in colon and cecum. Interestingly, lactase previously was found to have a similar distribution pattern, with a peak in proximal jejunum and lower levels in the duodenum and ileum (34). Weaning showed no effect on either activity or distribution of alk-SMase. However, the weaning time in this study was only 1 wk. The question whether SM in milk or other food constituents affects alk-SMase awaits further study.

To assess the specificity of the results of alk-SMase, the changes in alkaline phosphatase, another brush border enzyme in the intestine, were determined as a comparison. High alkaline phosphatase activity levels were seen in the beginning of the suckling period, with a dramatic decrease at the time of weaning. These findings confirmed previous studies (35,36) but showed a different pattern from alk-SMase, indicating the changes in alk-SMase activity in gestation were specific.

This study demonstrated the opposite changes of acid and neutral SMase activities in the gut to that of alk-SMase before birth. To the authors' knowledge, this is the first time that the activities of intestinal acid and neutral SMases in the fetus were examined. The high activities of both acid and neutral SMases at gestation day 20 followed by a rapid reduction before birth emphasize that these enzymes may have other functions than alk-SMase. Whether they are important in early development and differentiation of the fetus gut during the gestation period should be of interest for investigation.

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Marine Lipid-Based Liposomes Increase *in vivo* FA Bioavailability

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ABSTRACT: Liposomes made from an extract of natural marine lipids and containing a high n-3 PUFA lipid ratio were envisaged as oral route vectors for FA supplements in order to increase PUFA bioavailability. The absorption of FA in thoracic lymph duct-cannulated rats, after intragastric feeding of dietary fats in the form of liposomes or fish oil, was compared. Lipid and FA analyses were also performed on feces. Five mole percent α-tocopherol was added to fish oil and incorporated into the liposome membrane. The influence of α-tocopherol on FA lymph recovery was also investigated. *In vivo,* FA absorption in rats was favored by liposomes (98 ± 1%) compared to fish oil (73 ± 6%). In the same way, the DHA proportion in lymph was higher after liposome ingestion (78%) than after fish oil ingestion (47%). However, phospholipid (PL) concentration in lymph was not affected by the kind of dietary fat ingested, suggesting a PL regulation due to *de novo* TAG synthesis. The influence of the intramolecular distribution of n-3 PUFA in dietary lipids (TAG and PL) on the intramolecular FA distribution in TAG of chylomicrons was also investigated. The results obtained showed that the distribution of n-3 PUFA esterified on the *sn*-2 position of chylomicron TAG depended on the lipid source administered. All these results correlated, at least partly, with *in vitro* liposome behavior under conditions that mimic those of the gastrointestinal tract. As a whole, this study pointed out that marine PL may constitute an attractive material for the development of liposomes as oral PUFA supplements.

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Evidence of the involvement of n-3 FA in cardiovascular (1–3) and inflammatory disease prevention (4–6) is growing. Owing to the unique range of FA in marine oils, particularly the long-chain FA, a number of industrial and food applications have been developed (7–9). Thus, fish oil in the TAG form and, to a lesser extent, free acids and ethyl esters are widely marketed at drug and health food stores. However, fish oils, in natural form or chemically modified, are not absorbed to a similar degree *in vivo* (10–14). In addition, individual lipid metabolism and intestinal lipid bioavailability are consequences of chemical parameters that affect absorption, including enzymatic hydrolysis and/or micellar solubilization processes. Although free PUFA are essential to life, they show poor bioavailability when delivered by the oral route using traditional forms of administration, at least partly because of their oxidative lability in the gastrointestinal tract. The contribution by pancreatic lipase of the low *in vitro* hydrolysis rate of EPA (20:5n-3) and DHA (22:6n-3) at the *sn*-1,3 positions of TAG to less effective PUFA absorption is more controversial (10–12,15,16). Intestinal lipid absorption also requires the solubilization of the hydrolysis products in mixed bile salt–lipid micelles. This process is based on micellar solubility, which depends on the chemical form of the lipid (FFA or *sn*-2 MAG) (17), the degree of unsaturation of the FA (17), and the presence of other lipids (18). Moreover, lipolytic enzymes are known to hydrolyze lipids at the oil–water interface (19). Thus, the physical characteristics of the fat globules (size and ultrastructure) also may be of particular importance for PUFA absorption (20).

In this context, different approaches were proposed to increase PUFA bioavailability: modification of the intramolecular FA distribution of TAG (21), fish oil-containing lipid emulsions (22,23), microencapsulated fish oil (9), and PUFA-enriched oils such as phospholipids (PL) (24,25). For several years, we have been investigating the potential for using liposomes, based on a natural lipid mixture extracted from a marine organism, for PUFA supplementation. The esterification of PUFA in the *sn*-2 position of PL associated to the supramolecular organization of the lipids in bilayer structures ensured oxidative stability of the n-3 FA even in conditions as drastic as those present in the gastrointestinal tract (acid pH, 37°C) (26,27). Moreover, although *in vitro* acidification simultaneously induced changes in morphology of lipid bilayers and aggregation processes, liposome structures resist pH variations relevant to the digestive processes (26,27).

In the present study, the *in vivo* absorption of fish oil and of a natural lipid mixture, principally constituted by PL, was compared in thoracic lymph duct-cannulated rats following intragastric feeding. These dietary lipids differed both in their chemical nature and in the stereochemical configuration of their acylglycerols, but their FA compositions were similar. Moreover, because, in water, PL spontaneously form bilayer structures, preformed liposomes were administered to rats, whereas fish oil was given in a nonemulsified form. α-Tocopherol, the principal active form of vitamin E, was either added to fish oil or incorporated into the liposome membrane. The influence of this natural antioxidant on FA lymph recovery was investigated.

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Abbreviations: MUFA, monounsaturated FA; PL, phospholipid; SFA, saturated FA.

MATERIALS AND METHODS

Materials. The crude marine lipid mixture used for liposome preparation was supplied by Phosphotech (Nantes, France). The average lipid content was: 69 wt% of PL, 27 wt% of cholesterol, and 4 wt% of TAG (26–28). Fish oil was obtained from Pronova Biocare (EPAX 0525 TG; Sandefjord, Norway). In marine lipids as well as in fish oil, α-tocopherol was initially present at 0.01 mol%. When added to marine lipids or fish oil, α-tocopherol (Fisher Scientific, Elancourt, France) was used without further purification. [^{14}C]Triolein (specific radioactivity 3 GBq mmol^{-1}) was obtained from NEN Life Science Products (Boston, MA). Internal lipid standards were purchased from Sigma France (Saint Quentin Fallavier, France). The solvents were of analytical grade or distilled when needed.

Liposome preparation. Liposomes were prepared by filtration of the marine lipid suspension as described before (26,29). Briefly, marine lipids were dissolved in chloroform/methanol (2:1, vol/vol). Organic solvents were removed under a nitrogen stream followed by a lyophilization step overnight. Additional lipids, [^{14}C]triolein, or, when used, α-tocopherol (5 mol%) were added to the initial marine lipid extract in order to be incorporated into the liposome membranes. HEPES buffer (10 mM HEPES, 145 mM NaCl, pH 7.4) was used for the hydration step of the lyophilized lipid film. The liposome suspension was obtained after a single extrusion of the lipid suspension (100 mg mL^{-1}) through polycarbonate membranes of 5-μm pore diameters (Millipore Corp., Bedford, MA). Phase-contrast and polarized light microscopy observations (IM35 microscope; Zeiss, Jena, Germany) indicated that a mixture of spherical vesicles with a variable number of lamellae was obtained. The liposomes prepared with or without α-tocopherol were characterized by a mean diameter of about 4 μm as measured with a photon correlator spectrophotometer (Malvern Mastersize Ver. 214).

Animals and surgical procedures. Official French regulations for the care and use of laboratory animals were followed. Male Wistar rats weighing 250–300 g were obtained from Elevage Janvier (Saint-Berthein, France). They were housed for 1 wk before the study in a controlled environment, with constant temperature and humidity. They were fed a fat-free diet (UAR, Epinay, France) and allowed free access to water for 24 h before surgery. Rats were anesthetized with pentobarbital (1 μL g^{-1} body wt) and subjected to thoracic cannulation with a polyethylene catheter (i.d. 0.86 mm, o.d. 1.27 mm; Biotrol, Paris, France) as described in Reference 30. Following surgery, the rats were placed in individual restraining cages, in a warm environment, with tap water freely available. A few hours after the surgical operation, 0.4 g of lipids (radioactive liposome suspension, 187 kBq g^{-1} of marine lipids; or fish oil, 93 kBq g^{-1} of oil) was administered through the gastrostomy tube followed by 1 mL of water in the case of fish oil. The lymph was collected for 24 h without fractionation into weighed tubes maintained in an ice bath. During the collection period, lymph flow averaged 0.6 mL h^{-1}. These 24-h samples were immediately treated for lipid extraction and analysis. At least five cannulated rats were used for each lipid ingestion study condition.

In another set of experiments, 0.4 g of radioactive lipids was administered through the gastrostomy tube to fasted rats, and the feces were collected for 24 h. The fecal material was stored at −20°C under nitrogen until lipid analysis.

Analytical methods. Lipids from intestinal lymph samples were extracted with 20 vol of chloroform/methanol (2:1, vol/vol) according to the method of Folch *et al.* (31). Feces were weighed, hydrated overnight at 4°C, and ground in a blender. Fecal lipids were extracted according to the Folch *et al.* technique with slight modifications, i.e., an additional organic extraction with chloroform was performed on the first aqueous phase obtained.

Radioactivity of lymph samples and fecal lipid extracts was determined using a liquid scintillation spectrometer (LS1801 scintillation spectrometer, Beckman) and Ready Gel (Beckmann) as scintillation mixture.

TLC was performed with Merck silica gel (60H) spread on 20 × 20 cm glass plates, 0.35 mm thick, activated at 110°C for 1 h. The Folch *et al.* extract was applied to a TLC plate for either PL and TAG separation using diethyl ether/acetone (60:20, vol/vol) as a developing solvent or nonpolar lipid separation using hexane/ether/acetic acid (90:10:1, by vol) (32). Total lipid extract or lipid fractions (PL, TAG) were transmethylated in the presence of a boron trifluoride/methanol complex (33) with tri-[*cis*-9-tetradecenoyl]glycerol as internal standard for total lipids and the TAG analysis and with L-α-PC-diarachidoyl as internal standard for the PL analysis. FAME of total lipids, TAG, and PL were subjected to GC on a BPX 70 capillary column (60-m long, 0.25-μm film, 0.25-mm i.d.; SGE, Melbourne, Australia; hydrogen as carrier gas, split ratio of 1:80). The GC system consisted of a gas chromatograph (Hewlett-Packard, HP 4890) provided with an FID maintained at 250°C. The injector was at 250°C. The column temperature was programmed from 150 to 200°C (1.5°C/min), held for 15 min, from 200 to 230°C (20°C/min), held for 20 min, and from 230 to 235°C (20°C/min), and then held for 10 min. Data were collected and integrated by a Chromjet SP 4400 integration system (Spectra-Physics). FA from Sigma and natural extracts of known composition were used as standards for column calibration. Variation in determination of area under the curves for GC peaks between injections was less than 2%.

For analysis of positional FA distribution, PL were hydrolyzed by *Naja mossambica mossambica* phospholipase A_2. A mixture containing 10 mg of PL dissolved in 1 mL of diethyl ether and 0.15 mL of 3-mM $CaCl_2$ containing 1500 IU of phospholipase A_2 was incubated for 50 min at 30°C. The reaction was stopped by evaporation of diethyl ether under nitrogen. FFA and lyso-PL were separated by TLC with chloroform/methanol/water (65:25:4, by vol) as a developing solvent (32), then transmethylated and analyzed by GC under the same conditions as described above.

The intramolecular FA distribution in TAG of dietary fish oil and chylomicrons was determined by Grignard degradation

using allylmagnesium bromide (34,35). This procedure eliminated the possible discrimination of n-3 PUFA due to slow hydrolysis previously reported for pancreatic lipase (15). The resulting *sn*-2 MAG and 1,3-DAG were separated by TLC using chloroform/acetone (96:4, vol/vol) as developing solvent (36). Respective fractions were transmethylated, and FAME were analyzed by GC as previously described.

The PL content in lymph samples was determined by the method of Ames (37). Cholesterol was quantified either by using an enzymatic kit (CHOD-PAP; Boehringer Mannheim, Mannheim, Germany) or by GC. In this latter case, after saponification of total lipids, in the presence of stigmasterol as internal standard, cholesterol was derivatized into trimethylsilyl ether (38) and then analyzed by GC on an OV-17 capillary column [30 m long, 0.3 μm film, 0.32 mm i.d., a.m.l. Chromato (Peyrelavade, France); hydrogen as carrier gas, split ratio of 1:11]. The column, injector, and detector temperatures were 280, 320, and 320°C, respectively. The TAG content in lymph samples was determined by enzymatic assay using standard kits (GPO-PAP; Boehringer Mannheim).

Relative lymphatic absorption. The lymphatic recovery of each FA was calculated as reported in References 39 and 40 using the amount of FA isolated from dietary and lymphatic lipids compared to that of the radioactivity of oleic acid, used as internal standard, in the same samples. When liposomes were administered, complete intestinal triolein absorption was considered. In the case of fish oil administration, intestinal triolein absorption determined on fecal lipid radioactivity was found to be 92 ± 4% ($n = 5$).

Intestinal lipid absorption. The relative PL absorption following liposome ingestion by rats was determined on the basis of phosphorus analysis of fecal lipids as follows:

$$\text{PL relative absorption} = (Q_{\text{PLin}} - Q_{\text{PLex}}) \times 100/Q_{\text{PLin}} \quad [1]$$

where Q_{PLin} is the quantity of dietary PL ingested by the rat and Q_{PLex} is the quantity of PL excreted in fecal lipids. It is worth noting that the amount of PL measured in fecal lipids corresponded to nonabsorbed PL species from dietary marine lipids plus endogenous PL from bile secretion and cell desquamation. The Q_{PLex} took into account this latter amount determined for each rat.

A similar calculation was applied to determine the relative absorption of each FA on the basis of FA quantification of dietary fats (marine lipids and fish oil) and fecal lipids.

Statistical analysis. Differences between two groups were tested for significance using Student's *t*-test. Data were expressed as mean values ± SEM.

RESULTS

Intestinal absorption of fish oil. Analysis of fecal lipids by TLC revealed the absence of TAG, suggesting that all ingested TAG were, at least partly, hydrolyzed by lipases. In contrast, the presence of FFA and MAG indicated that only part of the hydrolysis products were absorbed at the intestinal level. Comparison of FA compositions of feces and lymph lipids indicated a similar absorption level of the different monounsaturated FA (MUFA), whereas PUFA absorption was overestimated with fecal lipids. For example, relative DHA absorption was 47 ± 6% and 85 ± 6%, after lymph and feces lipids analysis, respectively. These results presumably accounted for the *in vivo* PUFA oxidation processes. The absorption of fish oil was calculated using the absorption of saturated FA (SFA) and MUFA from the fecal lipid analysis associated with the absorption of PUFA from lymph lipid analysis. It was found to be equal to 73 ± 6% ($n = 5$).

Intestinal absorption of liposomes. After liposome administration, fecal PL quantification indicated the presence of only 2% of the initial marine PL ingested. These results, added to the fact that no FFA were detected in the fecal lipid by TLC analysis, showed that the intestinal absorption of liposomes was 98 ± 1% ($n = 4$). Similar results (97 ± 1%, $n = 5$) were found for intestinal absorption of α-tocopherol associated-liposomes.

Lipid species recovery in lymph. Lymphatic lipid composition is shown Table 1 for lipid-fasted rats and rats following intragastric administration of liposomes and fish oil, with or without 5 mol% α-tocopherol. The results of absorption of α-tocopherol are presented and discussed elsewhere (29).

TABLE 1
Lipid Fraction Concentrations in Lymph of Rats After Intragastric Administration of Different Dietary Lipid Sources

	Lymph composition (g L^{-1})[a]		
Lipid source[b]	Phospholipids	TAG	Cholesterol
Fat-free diet ($n = 2$)	4.2 ± 0.8[a]	6.2 ± 0.9[a]	0.6 ± 0.1[a]
Liposomes ($n = 5$)	4.5 ± 1.9[a]	12.8 ± 5.3[b]	1.7 ± 0.7[b]
α-Toco-associated liposomes ($n = 6$)	5.3 ± 1.1[a]	16.7 ± 8.1[b]	2.5 ± 0.8[b]
Fish oil ($n = 6$)	5.3 ± 0.4[a]	20.2 ± 6.1[b]	0.8 ± 0.2[a]
Fish oil + α-toco ($n = 6$)	4.7 ± 0.8[a]	15.0 ± 5.0[b]	0.7 ± 0.3[a]

[a]Lymph was collected over 24 h. The lipid fractions were extracted and analyzed.
[b]Liposomes were prepared by a filtration technique using 5-μm pore diameter filters. Lipid ingestion by rats corresponded to 0.4 g in the form of liposome suspension or fish oil. Marine lipids and fish oil initially contained 0.01 mol% of α-tocopherol (α-toco). When added to fish oil or incorporated into liposome membranes, α-toco was at 5 mol%. Values are means ± SEM for the number of rats per group. Values with different superscript letters (a, b) in the same column are significantly different at $P < 0.05$.

There were no noticeable differences in the lymphatic PL composition of lipid-fasted rats and the treatment groups. The TAG concentrations measured in lymph were significantly higher when lipids were given to rats compared to fat-free diet conditions, whereas they were not significantly different within the different rat groups. This showed that the TAG increase in lymph was independent of the nature of the lipid source. As for chylomicron cholesterol, only administration of liposomes that initially contained 27% cholesterol led to an increased amount of this sterol in the lymph. The addition of α-tocopherol to fish oil or liposome membranes did not influence the lymph profile of lipid species.

Nonendogenous lymphatic FA. The composition of the two lipid sources used and that of lipid-fasted rat lymph differed from five FA referred to as nonendogenous FA. Relative lymphatic absorption of the latter was calculated. Data are shown in Table 2. Following administration of liposomes to rats, the lymphatic recovery of MUFA varied from 39 to 48%. There were no detectable differences in lymphatic MUFA absorption between liposome and fish oil treatment groups. In both cases, α-tocopherol significantly increased the proportion of MUFA in the lymph. Absorption levels of nonendogenous PUFA were twice as high after liposome ingestion compared to fish oil ingestion, independent of the presence of α-tocopherol.

In lymph, total FA were distributed at 85% in TAG and 15% in PL whatever dietary lipid source was considered and whether α-tocopherol was present. The distribution of nonendogenous FA in TAG and PL of chylomicrons as a function of the lipid source is presented in Table 3. Nonendogenous PUFA were similarly distributed in TAG and PL as far as the dietary lipid source was concerned. In contrast, in PL, MUFA proportions in the liposome rat group were significantly lower than in the fish oil group. Whatever the dietary lipid source, the ratio of α-tocopherol did not modify the distribution of MUFA or PUFA in TAG or PL.

FA composition and distribution of FA in dietary lipids and resultant chylomicrons. FA composition of fish oil and the intramolecular FA distribution in TAG are shown in Table 4. In fish oil, the major sources of n-3 PUFA were DHA and EPA. DHA was concentrated in the *sn*-2 position of the TAG

TABLE 2
Relative Composition of the Nonendogenous FA of Lymph in Rats After Intragastric Administration of Different Dietary Lipid Sources

	Relative lymphatic absorption (%)[a]				
Lipid source[b]	20:1n-9	22:1n-9	24:1n-9	22:5n-3	22:6n-3
Liposomes ($n = 5$)	48 ± 7	43 ± 20	39 ± 15	95 ± 5	78 ± 4
α-Toco-associated liposomes ($n = 6$)	84 ± 8*	89 ± 17**	72 ± 14**	91 ± 4	91 ± 3**
Fish oil ($n = 6$)	50 ± 6	57 ± 8	36 ± 7	42 ± 7*	47 ± 6**
Fish oil + α-toco ($n = 6$)	88 ± 10*	100 ± 14*	77 ± 14**	59 ± 7*	65 ± 6*

[a]The lymphatic recovery of FA was calculated using the amount of the considered FA isolated from dietary and lymphatic lipids, respectively, compared to that of the radioactivity of oleic acid, considered as internal standard, in the same samples. Intestinal triolein absorption was taken to be equal to 100 and 92%, respectively, in the case of liposome administration and fish oil administration.
[b]Liposomes were prepared by a filtration technique using 5-μm pore diameter filters. Lipid ingestion by rats corresponded to 0.4 g in the form of liposome suspension or fish oil. Marine lipids and fish oil initially contained 0.01 mol% of α-toco. When added to fish oil or incorporated into liposome membranes, α-toco was at 5 mol%. Values are means ± SEM for the number rats per group. *Significantly different from intragastric liposome administration to rats ($P \leq 0.01$). **Significantly different from intragastric liposome administration to rats ($P \leq 0.05$). For abbreviations see Table 1.

TABLE 3
Relative Incorporation of the Nonendogenous FA into Phospholipids and TAG in Lymph of Rats After Intragastric Administration of Different Dietary Lipid Sources

	Relative lymphatic composition (%)				
Lipid source[a]	20:1n-9	22:1n-9	24:1n-9	22:5n-6	22:6n-3
TAG					
Liposomes	90 ± 6[a]	92 ± 4[a]	91 ± 2[a]	91 ± 3[a]	92 ± 8[a]
α-Toco-associated liposomes	88 ± 7[a]	92 ± 3[a]	93 ± 3[a]	93 ± 4[a]	92 ± 3[a]
Fish oil	82 ± 5[a]	83 ± 5[a]	85 ± 2[b]	93 ± 2[a]	94 ± 5[a]
Fish oil + α-toco	85 ± 4[a]	86 ± 3[a]	84 ± 3[b]	93 ± 3[a]	94 ± 4[a]
Phospholipids					
Liposomes	10 ± 2[a]	8 ± 2[a]	9 ± 2[a]	9 ± 2[a]	8 ± 2[a]
α-Toco-associated liposomes	12 ± 1[a]	8 ± 1[a]	7 ± 4[a]	7 ± 4[a]	8 ± 2[a]
Fish oil	18 ± 1[b]	17 ± 2[b]	15 ± 2[b]	7 ± 2[a]	6 ± 1[a]
Fish oil + α-toco	15 ± 2[b]	14 ± 4[b]	16 ± 2[b]	7 ± 2[a]	6 ± 1[a]

[a]Liposomes were prepared by a filtration technique using 5-μm pore diameter filters. Lipid ingestion by rats corresponded to 0.4 g in the form of liposome suspension or fish oil. Marine lipids and fish oil initially contained 0.01 mol% of α-tocopherol. When added to fish oil or incorporated into liposome membranes, α-tocopherol was at 5 mol%. Values are means ± SEM for five or six rats per group. Values with different superscript letters (a, b) are significantly different at $P < 0.05$. For abbreviation see Table 1.

(71%), whereas EPA was more evenly distributed between the *sn*-2 (44%) and *sn*-1,3 positions (66%) of the TAG molecules. Oleic acid (18:1) and palmitic acid (16:0) were esterified mainly in the *sn*-1,3 positions. Concerning liposomes, half of the total FA amount consisted of PUFA, among which EPA and DHA represented more than 90% (Table 5). The intramolecular structure of marine PL was significantly different from that of fish oil TAG. Indeed, EPA and DHA were specifically acylated in the *sn*-2 position (75 and 67% for EPA and DHA, respectively).

The FA composition of TAG and PL of chylomicrons and the intramolecular FA distribution of TAG following administration of fish oil and marine lipid-based liposomes were examined (Tables 4 and 5). For the rat group dosed with fish oil, the FA composition of chylomicron TAG resembled the composition of the dietary lipid source as far as SFA and MUFA were concerned (Table 4). The ratio of PUFA in the chylomicron TAG and the oil administered was similar. However, compared with the dietary oil, linoleic acid (18:2n-6) and arachidonic acid (20:4n-6) proportions in chylomicrons increased, whereas EPA and DHA were reduced. The structural analysis of the chylomicron TAG showed that EPA and DHA were equally distributed between the internal and the two external positions of TAG molecules. An enrichment of n-6 PUFA and SFA at the *sn*-2 position was observed. For instance, 14% of total 18:2n-6 was esterified in the internal position of the fish oil TAG; this proportion increased to as much as 58% when the chylomicron TAG composition was considered.

For the liposome rat group, the FA composition of chylomicron TAG did not reflect the composition of the dietary lipid source, both in percentages and in intramolecular distribution (Table 5). Compared with dietary liposomes, total MUFA significantly increased in chylomicron TAG at the expense of total PUFA, especially EPA and DHA. The structural analysis of the chylomicron TAG showed that EPA and DHA were preferentially incorporated into the external positions of TAG molecules, i.e., 70% into *sn*-1,3 positions vs. 30% into the *sn*-2 position, whereas n-6 PUFA essentially from the endogenous pool were present mainly at the internal position.

The FA composition of the chylomicron PL also reflected the contribution of FA of endogenous origin, as the contents of stearic acid (18:0, 16.1 ± 0.9 and 15.8 ± 1.8 wt%, following fish oil and liposome administration, respectively), linoleic

TABLE 4
Composition (wt%) of the Main FA of Fish Oil and of TAG and Phospholipids (PL) of the Resultant Rat Chylomicrons

	Fish oil		Chylomicrons[a]		
FA	TAG[b]	*sn*-2 MAG[c]	TAG	*sn*-2 MAG[c]	PL
14:0	4.0	5.4	2.6 ± 0.3	1.7	0.7 ± 0.1
15:0	1.2	1.0	0.9 ± 0.1	0.4	0.7 ± 0.1
16:0	20.4	7.3	21.6 ± 1.4	15.6	25.0 ± 0.8
17:0	1.3	0.0	0.7 ± 0.1	0.0	0.7 ± 0.2
18:0	5.7	0.0	8.5 ± 0.4	6.4	16.1 ± 0.9
20:0	1.1	1.6	0.7 ± 0.0	1.0	0.0 ± 0.0
ΣSFA	33.7		35.0		43.2
16:1n-7	5.1	2.6	4.9 ± 0.5	2.5	2.1 ± 0.3
17:1	0.9	0.4	0.5 ± 0.1	0.1	0.2 ± 0.0
18:1n-9	12.2	2.2	11.2 ± 0.6	0.9	5.8 ± 0.2
18:1n-7	2.6	1.4	3.9 ± 0.3	0.2	3.5 ± 0.5
20:1n-9	0.9	0.3	0.5 ± 0.1	0.1	0.3 ± 0.0
20:1n-7	0.1	0.0	1.2 ± 0.6	0.0	1.0 ± 0.2
22:1n-9	0.3	0.1	0.1 ± 0.0	0.0	0.1 ± 0.1
24:1n-9	0.6	0.1	0.2 ± 0.1	0.0	0.2 ± 0.0
ΣMUFA	22.7		22.5		13.2
18:2n-6	1.4	0.6	11.9 ± 1.3	20.6	15.9 ± 0.6
18:3n-3	0.5	0.3	0.6 ± 0.1	0.5	0.2 ± 0.0
20:3n-6	0.2	0.3	0.4 ± 0.0	1.0	0.7 ± 0.1
20:4n-6	2.1	3.1	8.8 ± 0.6	19.7	16.7 ± 2.1
20:5n-3	6.6	8.7	3.9 ± 0.3	5.9	2.9 ± 0.3
22:5n-6	2.2	4.6	0.9 ± 0.1	0.4	0.4 ± 0.1
22:5n-3	1.2	1.5	0.9 ± 0.1	0.0	0.3 ± 0.1
22:6n-3	26.4	56.3	12.7 ± 0.8	20.8	4.8 ± 0.4
ΣPUFA	40.6		40.1		41.9

[a]Lipid ingestion by rats corresponded to 0.4 g in the form of fish oil. Chylomicrons were collected from six animals, and the lipid fractions were extracted. Following separation on TLC, TAG and PL were analyzed for FA composition by GC.
[b]Data represent the average of three different determinations.
[c]The FA composition of *sn*-2 MAG of fish oil and the chylomicron TAG were determined by Grignard degradation followed by isolation and analysis of the MAG. In the case of chylomicron TAG, *sn*-2 MAG analysis was performed on pooled samples of lymph. MUFA, monounsaturated FA; SFA, saturated FA.

TABLE 5
Composition (wt%) of the Main FA of Marine Lipid-Based Liposomes and of TAG and PL of the Resultant Rat Chylomicrons

	Liposomes[a]		Chylomicrons[b]		
FA	Total lipids[c]	FFA[d]	TAG	*sn*-2 MAG	PL
14:0	1.5	0.3	1.9 ± 0.4	1.0	0.6 ± 0.1
15:0	0.4	0.1	0.6 ± 0.1	0.3	0.5 ± 0.1
16:0	31.4	5.5	26.1 ± 3.3	29.3	25.9 ± 1.0
17:0	0.6	0.1	0.6 ± 0.1	0.3	0.6 ± 0.1
18:0	3.6	0.7	8.0 ± 0.6	3.6	15.8 ± 1.8
ΣSFA	37.5		37.2		43.4
16:1n-7	0.5	0.2	2.3 ± 0. 7	1.4	1.4 ± 0.4
18:1n-9	3.5	1.3	7.0 ± 1.0	3.8	5.2 ± 1.0
18:1n-7	1.1	0.2	3.4 ± 0.3	1.1	2.9 ± 0.3
20:1n-9	3.6	0.5	1.8 ± 0.4	0.0	0.9 ± 0.2
20:1n-7	0.1	0.0	1.2 ± 0.5	0.0	0.9 ± 0.1
22:1n-9	0.8	0.1	0.1 ± 0.1	0.0	0.1 ± 0.0
24:1n-9	0.6	0.0	0.3 ± 0.1	0.0	0.3 ± 0.1
ΣMUFA	10.2		16.1		11.7
18:2n-6	0.3	0.2	9.3 ± 3.6	16.4	12.5 ± 2.7
20:3n-6	0.0	0.0	0.3 ± 0.1	0.4	0.7 ± 0.3
20:4n-6	1.8	3.3	7.7 ± 1.3	16.6	14.8 ± 2.3
20:5n-3	14.5	26.0	9.8 ± 1.5	7.3	7.4 ± 1.9
22:5n-6	0.3	0.8	0.2 ± 0.1	0.0	0.2 ± 0.0
22:5n-3	0.4	0.7	0.6 ± 0.1	0.0	0.2 ± 0.0
22:6n-3	32.9	59.8	16.6 ± 3.9	16.8	6.5 ± 1.6
ΣPUFA	50.2		44.5		42.3

[a]Liposomes were prepared by a filtration technique using 5-μm pore diameter filters.
[b]Lipid ingestion by rats corresponded to 0.4 g in the form of liposome suspension. Chylomicrons were collected from five animals, and the lipid fractions were extracted. Following separation on TLC, TAG and PL were analyzed for FA composition by GC.
[c]The data represent the average of three different determinations.
[d]The FA distribution of PL was determined after hydrolysis by *Naja mossambica mossambica* phospholipase A_2. FFA and lyso-PL were separated by TLC, transmethylated, and analyzed by GC. The FA composition of the *sn*-2 MAG of the chylomicron TAG was determined by Grignard degradation followed by isolation and analysis of the MAG. The *sn*-2 MAG analysis was performed on pooled samples of lymph. For abbreviations see Tables 1 and 4.

acid (15.9 ± 0.6 and 12.5 ± 2.7 wt%), and arachidonic acid (16.7 ± 2.1 and 14.8 ± 2.3 wt%) were high, whereas these FA were present in dietary lipids only in low amounts. The FA composition of PL from the two populations of chylomicrons differed mainly in EPA and DHA contents. Chylomicron PL from the liposome rat group were enriched in these two PUFA compared with the fish oil rat group.

DISCUSSION

Increasing the oral delivery of FA, especially of n-3 PUFA, is a potential strategy for providing additional lipids for developing tissues. This strategy was tested by comparing the effects of liposomes (FA esterified in PL, organized in bilayer structures) with those of fish oil (similar FA esterified in TAG, given without any preliminary emulsification process). The influence of the presence of α-tocopherol in the initial formula was also pointed out. To our knowledge, this is the first time that liposome absorption following oral administration has been quantified. The present results show that the level of liposome absorption was significantly higher than that of fish oil. The total FA recovery in the case of fish oil-fed rats (73 ± 6%) was in agreement with other studies that have demonstrated that the ratio of FA absorbed after fish oil feeding was low (41). An increase in lipid bioavailability in the case of liposome administration was actually demonstrated by a better assimilation of some nonendogenous FA, especially DHA (Table 2). The highest rate of liposome absorption may also account for a better digestibility of SFA. It is well known that, when released into the intestinal tract, these FA are partially excreted in the feces through the formation of calcium soap (42). In the fish oil used in this study, SFA were preferentially esterified in the *sn*-1,3 positions of TAG (Table 4) and thus were present essentially as FFA in the intestinal tract. In contrast, in the case of liposomes, most SFA were esterified in the *sn*-1 position of PL (Table 5), and thus they should be easily inserted into mixed micelles in the form of lyso-PL.

The fact that PL/TAG ratios in lymphatic chylomicrons were similar, whatever the lipid source used (Table 2), confirmed once more that lymphatic lipid composition is regulated. However, in the present case, it should be noted that two metabolic pathways were involved in this regulation, i.e., the 2-MAG pathway for TAG synthesis in the case of fish oil and the *de novo* TAG synthesis through α-glycerophosphate pathway in the case of liposome ingestion.

In the liposome-treated rat group, the results obtained for lymphatic absorption of nonendogenous FA pointed out the influence of the nature of the FA, i.e., chain length and degree of unsaturation, on lipid bioavailability. Indeed, PUFA absorption was higher than that of the three MUFA quantified (Table 2). Surprisingly, MUFA bioavailability increased when α-tocopherol was added to liposome membranes. This may account for specific interactions between vitamin E and MUFA chains that could promote lipid intestinal absorption. This influence of the presence of α-tocopherol on MUFA digestibility was also demonstrated for fish oil administration (Table 2). MUFA showed similar lymphatic absorption as far as PL and TAG sources were concerned. Thus, for these FA, bioavailability was not influenced by the chemical and/or the physical form under which they were ingested. In contrast, the lymphatic enrichment in PUFA after liposome ingestion, compared with fish oil, reflected the role of the chemical lipid structure in bioavailability. Similar results concerning the influence of the chemical lipid form were found for healthy preterm infants fed with formula enriched in PUFA derived from PL (24). DHA was better absorbed from the PL enriched-formula than from the other infant formulas and even from breast milk. It is worth noting that, although DHA bioavailability depended on the lipid source and the presence of α-tocopherol, it was incorporated into PL and TAG in a similar ratio, whatever the lipid source administrated to rats (Table 3).

Several studies have reported the effect of intramolecular distribution of n-3 PUFA in dietary lipids on their intramolecular distribution in chylomicron TAG (18,43). In the present study, the influences of both the nature of the dietary lipids (TAG and PL) and the intra-molecular distribution of n-3 PUFA in these lipids on the FA distribution in the resultant chylomicron TAG were compared. The data obtained after fish oil ingestion were consistent with other studies demonstrating that the FA composition of chylomicrons reflects that of the dietary oil (43,44). As already known, the FA found at the *sn*-2 position of dietary TAG are mainly retained during the absorption process (43) owing to the positional specificity of pancreatic lipase. After incorporation of these hydrolysis products into the mucosal pool, most of the 2-MAG are reacylated to TAG that are incorporated into chylomicrons secreted into lymph. Nevertheless, the percentage of n-3 PUFA esterified in the *sn*-2 position of chylomicron TAG was reduced compared to that in the fish oil administered. This may be attributed to a total degradation of some MAG, to a special utilization of MAG containing n-3 PUFA (43), or to endogenous FA dilution. Indeed, chylomicron TAG contained a high percentage of endogenous FA that were supplied by bile (45,46). In agreement with other studies (18,43), endogenous FA mainly occupied the *sn*-2 position of chylomicron TAG. These TAG were actually synthesized by the PA pathway. In the case of liposome administration, the proportion of endogenous FA esterified in the *sn*-2 position of chylomicron TAG was similar to that observed in the fish oil treatment group. The increase in n-3 PUFA in chylomicron TAG following liposome ingestion compared to fish oil (Tables 4 and 5) was consistent with increasing DHA bioavailability (Table 2). Part of the EPA and DHA was incorporated into the *sn*-2 position of TAG, suggesting that the supply of endogenous FA bound to the *sn*-2 position of TAG was insufficient during the active absorption of PUFA. The fact that significantly more n-3 PUFA were esterified in the *sn*-1,3 positions after liposome administration compared with fish oil administration may be of peculiar importance for the potential use of liposomes for nutritional purposes. Indeed, several studies have demonstrated that lipid structure affects the clearance of TAG and the removal of chylomicron remnants (47,48). Moreover, metabolic differences between FA esterified in internal and external positions of TAG may be a cause of different physiological functions. It has been shown that DHA and EPA have stronger TAG-lowering activity in plasma and the liver when distributed at the *sn*-1,3 positions of TAG compared with the *sn*-2-position (36,44).

The FA compositions of PL chylomicrons of the liposome and fish oil rat groups were very similar except for EPA and DHA levels, which were higher after liposome ingestion (Tables 4 and 5). Consistent with other reports dealing with TAG intake (43), the present study showed that, whatever the lipid source considered, PL seemed to transport n-3 PUFA of dietary origin to a lesser degree, especially when the amount of PL (26–35 wt%) relative to TAG in chylomicrons was taken into consideration. As the phosphatidic pathway mainly contributes to PL synthesis, the enrichment of n-3 PUFA in PL after liposome intake also argued in favor of a better bioavailability of these FA compared with fish oil. In contrast with EPA, DHA from fish oil or from liposomes was not incorporated equally into lymphatic TAG and PL. This was already observed in the case of fish oil and attributed to the TAG structure in fish oil, the positional specificity of pancreatic lipase, and the pathways for lipid synthesis in the enterocyte (22). In the case of liposome ingestion, EPA and DHA were nearly totally present as FFA in the mucosal pool. The difference in DHA distribution in lymphatic PL and TAG may arise from a different specificity of acylases toward these two PUFA.

On the whole, the present study demonstrated that FA absorption and n-3 PUFA bioavailability were higher after liposome administration than after fish oil administration. Lipid bioavailability involves different digestion processes according to the lipid source considered. Several hypotheses may account for the best lipid bioavailability using liposomes instead of fish oil. The lipolytic enzymes, i.e., pancreatic phospholipase A_2 and lipases, involved in hydrolysis of PL and TAG, respectively, differ both in nature and activity. Each enzyme presents a peculiar specificity with respect to its substrate (FA positional distribution, degree of unsaturation, chain length). Fish oils are resistant, at least *in vitro*, to hydrolysis by pancreatic lipase (13,15), whereas phospholipase A_2 activity is not influenced by the presence of PUFA. This may result in a faster hydrolysis of PL than TAG. Moreover, in the case of liposomes, the lipid–water interface should be preserved until the intestine is reached, as suggested by *in vitro*

studies (26,27,29). In contrast, in the case of fish oil, the interface has to be created by mechanical mixing by the stomach and intestines and by bile compounds. The solubilization process of the hydrolysis products may also account for the differences in lipid bioavailability when the two lipid sources are considered. The presence of additional lyso-PL resulting from liposome PL degradation may influence lipid micelle solubilization. Although products of hydrolysis of fish oil TAG by pancreatic lipase, and of liposomes by phospholipase A_2, are both surface active, they may affect lipid emulsification differently. Indeed, FFA and esterified FA in lyso-PL or in 2-MAG present different micelle solubilities (17). Moreover, in the case of liposomes, the presence of high amount of lyso-PL may facilitate the transport of the hydrolysis products to the unstirred water layer of enterocytes. Finally, the better bioavailability of n-3 PUFA bound to PL compared with TAG may account for the oxidative stability of PUFA esterified in PL (49,50) and associated in liposome membranes (51) compared to fish oil.

This study was undertaken to increase the efficiency of FA absorption without using TAG structure chemical modifications but rather a natural mixture of marine lipids. It demonstrated that liposomes based on natural PL rich in n-3 PUFA favor n-3 PUFA bioavailability and modify FA distribution in chylomicron TAG compared to standard fish oil. In addition to the potential use of such liposomes for PUFA supplementation, the quasi-quantitative recovery of PL may also be interesting for choline and ethanolamine delivery.

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Analysis of FA Contents in Individual Lipid Fractions from Human Placental Tissue

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ABSTRACT: Data on FA contents in the human placenta are limited. Different methods have been used for the FA analysis, and only percentage results have been presented. We developed and evaluated a method for the determination of FA concentrations in placental tissue. Lipids were extracted from placental tissue with a chloroform/methanol mixture; and phospholipids (PL), nonesterified FA (NEFA), TG, and cholesterol esters (CE) were isolated by TLC. Individual lipid fractions were derivatized with methanolic hydrochloric acid, and the FAME were quantified by GC with FID. The CV of intra-assay (n = 8) of absolute concentrations were evaluated for FA showing a tissue content >0.01 mg/g. CV ranges were 4.6–11.0% for PL, 6.4–9.3% for NEFA, 6.1–8.9% for TG, and 11.4–16.3% for CE. The relative FA composition across a term placenta indicated no differences between samples of central and peripheral locations of maternal and fetal site (CV 0.5–9.9%), whereas the absolute FA concentrations were only reproducible in the PL fraction (CV 7.0–12.8%). The method shows a reasonably high precision that is well suited for physiological and nutritional studies.

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The supply of EFA and long-chain PUFA (LCPUFA) across the placenta is of major importance for fetal growth and development (1). The placental transfer of FA is a complex process involving several cytosolic and membrane-associated fatty acid-binding proteins (FABP) and transfer proteins (2). In experiments using placenta perfusion techniques, different transfer rates for individual FA have been described (3,4). Observational studies indicate a preferential transfer of LCPUFA to the fetal circulation relative to EFA (5). These data are in agreement with *in vitro* studies, which have described a higher affinity and binding capacity of individual FABP for LCPUFA compared with EFA (6,7). Thus, many studies have investigated the active transfer process and the molecular mechanisms in the placenta, but only one study has reported the placental FA composition and transfer (8), and very few studies have described the effect of the dietary FA intake on the FA composition of the placenta (9,10). The placental FA content might reflect the nutrient supply to the fetus, and its analysis together with other biological assays may further elucidate the placental FA transfer. Thus, a reliable and accurate method for FA analyses of placental tissue is required.

The analysis of FA in biological samples involves mainly three steps: extraction of lipids into an organic solvent (11,12), acid- or base-catalyzed derivatization to obtain volatile FAME (13,14), and quantification of individual FAME by GC with FID detection (15,16). However, there is no validated method for the analysis of the FA content in placental tissue (9,10,17).

The present paper describes a method that allows quantitative determination of FA compositions of phospholipids (PL), nonesterified FA (NEFA), TG, and cholesterol esters (CE) of placental tissue with a high reproducibility. Factors that may influence the results obtained were investigated.

MATERIALS AND METHODS

Sampling. Placental tissue was collected from four healthy pregnant women undergoing planned caesarean section. Tissue was obtained within 10 min after delivery, dissected, and washed several times in cold sodium chloride solution (0.9%, 4°C) to eliminate blood residues. Once the tissue was cleaned, it was immediately frozen in liquid nitrogen and stored at –80°C.

For intra- and interassay, eight samples per placenta, and for time-dependent analysis, one sample per time-point per placenta were obtained from the center of the parenchyma to ensure sampling homogeneity. Attached decidua and connective tissue were removed. Furthermore, 12 samples from different randomly chosen locations from each of two additional placentas were collected to evaluate possible differences in the FA composition inside the placenta.

Sample preparation. FA of placental tissue were extracted using a modification of the method of Folch *et al.* (12). All reagents used were obtained from Merck (Darmstadt, Germany). Approximately 0.3 g of tissue was weighed, and internal standard (25 μg FA each of pentadecanoic acid, tripentadecanoin, PC dipentadecanoyl, and cholesteryl heptadecanoate, dissolved in 100 μL chloroform/methanol; Sigma, Deisenhofen, Germany) was added. Samples were homogenized for 1 min in 3 mL chloroform/methanol mixture (2:1, vol/vol) with BHT using a metal-blade homogenizer (DIAX 100; Heidolph, Schwabach, Germany). Different concentrations (0.5, 2, 3.5, 5, 6 g/L) of BHT were used to avoid oxidation of PUFA during sample preparation. Homogenizer and tube were rinsed with 9 mL chloroform/methanol, and the

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Abbreviations: CE, cholesterol esters; FABP, fatty acid-binding protein; LCPUFA, long-chain PUFA; NEFA, nonesterified FA; PL, phospholipids; PLA_2, phospholipase A_2.

washing solution was combined with the extraction solution. Subsequently, the mixture was heated to 35°C for 20 min, filtered through a glass fiber filter (GMF 1; Sartorius, Bärenstein, Germany), and 4 mL potassium hydroxide solution (0.1 M) was added. The samples were shaken carefully and centrifuged at 900 × *g* at 10°C for 30 min. Thereafter, the aqueous phase was discarded, and the organic phase containing the lipids was filtered over sodium sulfate and taken to dryness under reduced pressure. The remaining lipids were dissolved in 400 µL of chloroform/methanol (1:1, vol/vol) and deposited on a TLC plate (Merck, Darmstadt, Germany). PL, free cholesterol, NEFA, TG, and CE were separated using heptane, diisopropylether (J.T.Baker, Deventer, Holland), and acetic acid (60:40:3, by vol) as mobile phase (18). After visualization of the components with 2′,7′-dichlorofluorescein, the bands with the lipid fractions were scraped from the TLC plate and transferred into 4-mL glass tubes equipped with Teflon-lined screw caps (14). FAME from the fractions were obtained by reaction with 3 M methanolic hydrochloric acid (Supelco, Bellefonte, PA) at 85°C for 45 min in closed glass tubes. After neutralization with sodium carbonate/sodium hydrogen carbonate/sodium sulfate buffer, 1 mL of hexane was added. After centrifugation at 400 × *g* for 3 min, the hexane layer was transferred into another vial, the extraction was repeated, and the combined extracts were taken to dryness under a gentle stream of nitrogen. FAME were analyzed by capillary GLC as described previously and identified by comparison with authentic standards (Nu-Chek-Prep, Elysian, MN) (5).

Statistics. Results are expressed as absolute (mg/g wet weight) and as percentage (% w/w) values of all detected FA with a chain length of 14–24 carbon atoms. All data are presented as mean ± SD. Time-dependent experiments are evaluated using paired *t*-tests with SPSS for Windows, Release 10.0 (SPSS Inc., Chicago, IL) and expressed as absolute values in mg/g. $P < 0.05$ is considered statistically significant.

RESULTS

BHT concentration. To prevent oxidation of PUFA during sample preparation, BHT at concentrations of 0.5, 2, 3.5, 5, and 6 g/L was added to the chloroform/methanol used for the extraction. The variation in the results, expressed as the CV ($n = 8$), was improved for DHA with increasing concentrations of BHT in the solvent. The CV decreased from 13% (0.5 g/L BHT) to 5.5% (5 g/L BHT). The CV of stearic acid and arachidonic acid concentrations were <5% in all cases, which indicated a small variation and independence from the BHT content. If 6 g BHT per liter was added to the extraction solution, the antioxidant displaced the TG on the TLC plate, leading to a distribution of TG over a larger area. A clear identification of the band under UV light was no longer possible. Therefore, all further samples were processed with 5 g/L BHT in the extraction solution.

Method development. The absolute FA contents (mg/g) of the different lipid fractions studied in placental tissue are presented in Table 1. PL constituted the major part of lipids in placental tissue with 87.5 ± 4.2% (mean ± SD), followed by NEFA (5.7 ± 0.4%), TG (3.8 ± 0.2%), and CE (3.1 ± 0.4%). The detection limit for the analyses was 0.001 mg/g, but the limit of quantification was approximately 0.01 mg/g based on CV values less than 10%. Many of the NEFA, TG, and CE FA showed concentrations <0.01 mg/g, and with some exceptions quantification of these fractions was not reproducible. For FA concentrations >0.01 mg/g, the method indicated a good reproducibility for the PL FA of 4.6–11.0% (CV), for NEFA (6.4–9.3%; DHA 13.9%), for TG (6.1–8.9%), and for CE (11.4–16.3%).

TABLE 1
Intra-assay ($n = 8$) Reproducibility of FA Concentrations of Placental Phospholipids (PL), Nonesterified FA (NEFA), TG, and Cholesterol Esters (CE) (mg/g)

FA	PL		NEFA		TG		CE	
	Mean	CV	Mean	CV	Mean	CV	Mean	CV
Saturated FA								
14:0	0.031	6.9	0.003	10.6	0.004	5.6	0.004	19.2
16:0	1.728	5.3	0.102	7.5	0.075	6.5	0.038	15.5
18:0	0.771	5.3	0.064	6.4	0.031	7.4	0.008	29.7
20:0	0.023	9.1	0.002	16.3	ND		ND	
22:0	0.089	4.9	0.003	10.2	ND		ND	
24:0	0.121	5.8	0.004	13.8	ND		ND	
Monounsaturated FA[a]								
16:1n-7	0.028	4.9	0.004	6.4	0.004	7.0	0.007	8.8
18:1n-7	0.100	5.0	0.007	7.2	0.005	8.4	0.004	21.7
18:1n-9	0.523	4.8	0.051	6.5	0.045	8.5	0.042	16.3
20:1n-9	0.015	6.3	0.001	8.1	ND		ND	
24:1n-9	0.089	7.1	0.003	12.1	ND		ND	
n-9 PUFA								
20:3n-9	0.013	11.0	0.001	9.4	ND		ND	
n-6 PUFA								
18:2n-6	0.500	4.6	0.033	6.4	0.026	6.7	0.078	11.4
18:3n-6	0.005	16.9	0.001	9.8	0.001	7.3	0.002	8.9
20:3n-6	0.309	5.9	0.019	9.3	0.017	6.1	0.006	25.6
20:4n-6	1.591	5.9	0.088	8.0	0.042	8.9	0.023	12.4
22:5n-6	0.062	5.4	0.004	9.5	0.003	10.5	0.001	14.9
n-3 PUFA								
18:3n-3	0.003	14.4	0.001	9.3	0.001	7.8	0.001	16.6
20:5n-3	0.007	6.3	0.002	7.6	0.001	8.4	0.001	16.2
22:5n-3	0.053	5.8	0.003	9.7	0.003	7.3	ND	
22:6n-3	0.301	7.4	0.017	13.9	0.012	7.0	0.005	21.9

[a]Erucic acid (22:1n-9) content is not reported because of its coelution with an unidentified peak. ND, concentrations <0.001 mg/g.

Table 2 shows the relative FA composition of the lipid fractions of placental tissue. In PL, NEFA, and TG, palmitic acid and arachidonic acid are quantitatively the most important FA. In contrast, CE showed a different FA pattern, with a high abundance of linoleic and oleic acids. The CV ranges for the relative FA composition were 0.4–10.5% for PL; 1.2–9.7% for NEFA; 1.8–9.0% for TG; and 2.0–7.5% for CE, for FA found in concentrations >0.01 mg/g.

To determine interassay variation, eight tissue samples from one placenta were analyzed within a 2-mon period (Table 3). The reproducibility of FA of the PL and CE fractions was similar compared with the results of intra-assay. However, the FA from NEFA and TG fractions showed higher CV, which might be due to lipolytic alterations during storage.

TABLE 2
Intra-assay (n = 8) Reproducibility of the FA Composition of Placental PL, NEFA, TG, and CE (% w/w)

FA	PL		NEFA		TG		CE	
	Mean	CV	Mean	CV	Mean	CV	Mean	CV
Saturated FA								
14:0	0.48	7.0	0.77	8.0	1.47	8.2	1.71	8.8
16:0	27.10	0.4	24.67	2.3	26.97	1.8	17.02	2.0
18:0	12.10	2.4	15.45	3.1	11.28	3.0	3.58	17.6
20:0	0.35	10.5	0.40	14.9	ND		ND	
22:0	1.39	2.7	0.81	7.0	ND		ND	
24:0	1.90	4.1	0.91	9.3	ND		ND	
Monounsaturated FA[a]								
16:1n-7	0.43	4.8	0.86	3.4	1.34	3.6	3.34	7.5
18:1n-7	1.58	1.1	1.59	1.2	1.93	2.4	1.65	8.9
18:1n-9	8.21	2.3	12.30	2.2	16.26	3.0	18.87	2.7
20:1n-9	0.24	6.4	0.34	3.2	ND		ND	
24:1n-9	1.39	7.0	0.66	7.7	ND		ND	
n-9 PUFA								
20:3n-9	0.20	8.8	0.29	10.4	ND		ND	
n-6 PUFA								
18:2n-6	7.84	1.9	8.00	2.2	9.30	5.3	35.23	5.1
18:3n-6	0.07	14.2	0.10	7.4	0.20	7.5	0.69	6.4
20:3n-6	4.85	2.1	4.64	4.9	6.18	4.9	2.60	13.3
20:4n-6	24.96	2.7	21.32	2.3	15.22	4.5	10.56	4.5
22:5n-6	0.98	4.3	0.92	5.9	1.22	6.5	0.64	9.1
n-3 PUFA								
18:3n-3	0.04	13.2	0.22	11.7	0.30	7.7	0.54	3.7
20:5n-3	0.11	8.3	0.41	6.5	0.27	4.5	0.45	4.7
22:5n-3	0.83	3.3	0.77	4.8	1.02	4.2	ND	
22:6n-3	4.72	5.5	3.99	10.7	4.50	9.0	2.08	11.3

[a]Erucic acid (22:1n-9) content is not reported because of the coelution with an unidentified peak. ND, concentrations <0.001 mg/g. For abbreviations see Table 1.

TABLE 3
Inter-assay (n = 8) of the FA Content in Placental Tissue Expressed as CV[a] (in %)

	CV of the absolute FA concentration				CV of the relative FA composition			
FA	PL	NEFA	TG	CE	PL	NEFA	TG	CE
Saturated FA								
14:0	7.5	**10.6**	**14.3**	**16.4**	10.9	**20.4**	**20.4**	**12.8**
16:0	4.6	17.4	13.7	8.0	1.8	6.3	12.4	3.6
18:0	5.4	18.7	22.4	**12.9**	1.7	4.4	6.5	**14.0**
20:0	13.5	**10.7**	ND	ND	14.3	**14.4**	ND	ND
22:0	7.6	**14.1**	ND	ND	7.2	**16.3**	ND	ND
24:0	8.2	**26.1**	ND	ND	11.6	**20.4**	ND	ND
Monounsaturated FA								
16:1n-7	6.5	**36.3**	**18.0**	**16.9**	8.5	**20.6**	**31.9**	**14.3**
18:1n-7	6.2	**31.0**	**16.9**	**14.1**	5.3	**10.4**	**11.2**	**10.4**
18:1n-9	5.5	26.6	16.8	13.8	4.4	5.9	5.4	4.4
20:1n-9	5.2	**27.7**	ND	ND	6.0	**9.6**	ND	ND
24:1n-9	10.2	**33.1**	ND	ND	12.8	**21.3**	ND	ND
n-9 PUFA								
20:3n-9	14.3	ND	ND	ND	12.7	ND	ND	ND
n-6 PUFA								
18:2n-6	6.2	28.2	21.7	9.7	3.0	8.5	4.7	2.5
18:3n-6	**10.2**	ND	ND	ND	**11.1**	ND	ND	ND
20:3n-6	12.0	36.1	30.5	**14.0**	12.8	15.4	16.5	**12.3**
20:4n-6	9.5	31.2	30.5	13.8	5.3	12.1	12.3	7.4
22:5n-6	12.9	**30.2**	**36.9**	17.9	9.8	**15.9**	**24.3**	17.3
n-3 PUFA								
18:3n-3	**13.5**	ND	ND	ND	**13.2**	ND	ND	ND
20:5n-3	**11.5**	**34.1**	**35.0**	**31.3**	**7.0**	**19.6**	**24.1**	**23.1**
22:5n-3	10.4	**33.9**	**35.8**	**15.8**	10.1	**19.2**	**19.2**	**18.2**
22:6n-3	9.0	**32.9**	35.4	**12.9**	5.0	**13.5**	20.8	**6.7**

[a]Boldface data show the CV of concentrations <0.01 mg/g. For abbreviations see Table 1.

NEFA content at different times after placenta delivery. If a lipid degradation caused by enzyme activity, e.g., phospholipase A_2 (PLA_2) or TG hydrolase, takes place, the resultant FA should accumulate in the NEFA fraction. Results indicate a significant increase in the NEFA content after 14.5, 24.5, 34.5, and 44.5 min compared with the initial content (Fig. 1). Furthermore, accumulation of individual NEFA was shown. The most striking increase was observed for arachidonic acid, dihomo-γ-linolenic acid, and DHA, whereas percentages of all saturated FA decreased (data not shown).

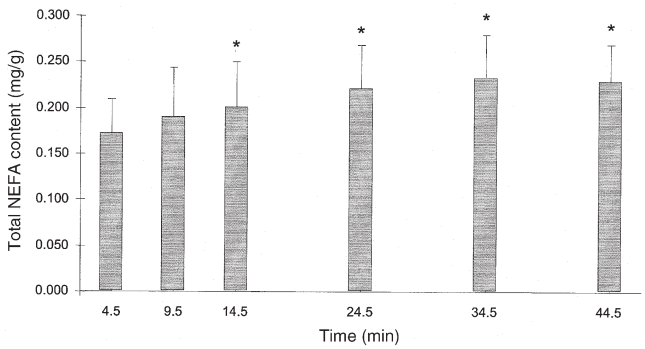

FIG. 1. Nonesterified FA (NEFA) content (mean ± SD) in central placenta parenchyma samples (n = 4) obtained over 45 min after placenta delivery. Significant differences from the first sample (4.5 min) were observed for all samples collected at 14.5 min or later. *$P < 0.05$.

FA content in randomly chosen locations of placental tissue. To clarify whether the lipid contents in various parts of the placenta are similar, 2 × 12 randomly chosen samples from two placentas were analyzed (Table 4). The relative composition of the FA with a concentration >0.01 mg/g in all studied lipid fractions showed a high reproducibility (CV 0.5–9.9%), and no systematic pattern was detected. This indicates that the proportional distribution of FA in all locations of the placenta was similar.

The absolute FA concentration in the PL fraction showed a low variation (7.0–9.3%) with the exception of DHA (12.8%). This indicates a homogeneous concentration of FA in PL in the whole placenta. However, NEFA, TG, and CE showed differences in the FA content, which resulted in a high variation (CV 6.1–42.1%).

DISCUSSION

The described method enabled the analysis of absolute concentrations of FA from placental tissue lipid fractions with a satisfactory precision.

Lakin *et al.* (9) determined the relative FA composition of total placental lipids in 10 English women, and their results were similar to ours. This similarity might reflect an almost identical daily intake of FA reported in the Lakin study and in Germany (19). Matorras *et al.* (10) described the relative FA

TABLE 4
CV (in %) of Absolute FA Concentrations and of Relative FA Compositions Within Different Placenta Locations of Two Placentas[a]

	CV of the absolute FA concentration				CV of the relative FA composition			
FA	PL	NEFA	TG	CE	PL	NEFA	TG	CE
Placenta 1 (n = 12)								
16:0	7.0	12.2	25.0	31.5	0.5	3.9	5.9	6.6
18:0	7.1	10.0	17.3	**31.0**	2.7	3.4	6.9	**14.1**
18:1n-9	7.1	10.3	25.1	37.7	1.7	4.3	5.3	1.8
18:2n-6	7.2	10.4	27.9	42.1	1.8	4.0	7.6	7.5
18:3n-6	**26.6**	ND	ND	ND	**23.5**	ND	ND	ND
20:4n-6	7.4	13.4	21.1	35.8	1.3	4.5	5.8	8.7
22:5n-6	8.3	**8.3**	**12.8**	**31.7**	4.9	**6.1**	**18.9**	**19.6**
18:3n-3	**15.4**	ND	ND	ND	**15.7**	ND	ND	ND
20:5n-3	**16.0**	ND	ND	ND	**11.2**	ND	ND	ND
22:5n-3	9.3	**14.5**	**20.9**	ND	5.0	**10.1**	**12.7**	ND
22:6n-3	12.8	**11.6**	**15.9**	**40.4**	9.1	**11.7**	**20.8**	**9.1**
Placenta 2[b] (n = 11)								
16:0	5.7	8.0	6.3	11.4	1.6	2.7	4.0	8.7
18:0	5.6	8.2	10.4	**23.6**	1.9	3.5	4.3	**17.2**
18:1n-9	5.9	11.3	6.1	13.4	3.4	4.9	5.8	9.3
18:2n-6	6.9	12.9	7.7	10.7	1.8	5.5	2.8	9.9
18:3n-6	**11.0**	ND	ND	ND	**11.1**	ND	ND	ND
20:4n-6	9.0	13.0	12.7	14.0	3.2	4.7	7.4	8.5
22:5n-6	6.3	**17.7**	**11.4**	**23.6**	5.8	**10.0**	**8.3**	**19.1**
18:3n-3	**15.1**	ND	ND	ND	**10.4**	ND	ND	ND
20:5n-3	**14.3**	ND	ND	ND	**10.1**	ND	ND	ND
22:5n-3	8.6	**16.0**	**20.1**	ND	5.1	**9.6**	**14.9**	ND
22:6n-3	9.0	**16.2**	**18.2**	**17.5**	5.6	**8.5**	**14.4**	**14.2**

[a]Boldface data show the CV of concentrations <0.01 mg/g. For abbreviations see Table 1.
[b]The total FA content of one sample differed by more than four SD from the mean and was therefore considered an outlier.

composition in placental PL of 78 Spanish women. Differences from these results may be explained by a higher consumption of marine fish and olive oil in Spain compared with Germany (19,20), resulting in higher contents of oleic acid (13.88 vs. 12.10%), EPA (0.40 vs. 0.11%), and DHA (5.63 vs. 4.72%), and lower contents of arachidonic acid (22.56 vs. 24.96%) in the Spanish samples. Such diet-induced effects have been observed with respect to plasma lipid composition in supplementation studies with fish oil and olive oil (21).

Data on the precision of tissue FA analysis could not be found, but such data have been reported for the analysis of relative FA content in foods. CV ranging from 0.6 to 10.7% were reported only for those FA with concentrations above 0.1 mg per g fresh sample (22). With the method presented here, the quantification limit was lower, at only 0.01 mg per g sample. A CV of 10% is sufficient for the detection of clinically relevant differences of the FA content in biological samples in physiological or nutritional studies.

A few papers have reported absolute FA concentrations in plasma or isolated lipoproteins (Refs. 23 and 24, respectively). Only one publication has reported absolute FA concentrations in placental tissue (25). Absolute concentrations may be more informative than the percentage composition for studying time-dependent processes as well as placental transfer mechanisms. Our method has a good precision for the determination of absolute FA concentrations in PL, TG, and NEFA. CE showed a higher variation, possibly because their solubility in methanolic hydrogen chloride is more limited. A higher derivatization temperature and a longer incubation time might enhance the precision here (18).

The interassay shows the same precision as the intra-assay only in the PL fraction. Possibly, the high amount of PL in placental tissue compensated for small alterations during storage and sample preparation, whereas changes in the NEFA, TG, and CE fractions led to stronger relative variations of the results. CV of interassay for C_8–C_{26} FA in plasma were reported to range from 4.6 to 22.9% (26), compared with the CV in our study ranging from 4.6 to 36.1%.

With respect to the particular importance of NEFA in placental FA transfer and metabolism, it is very important to avoid alterations in this fraction as much as possible. PLA_2 and TG hydrolase catalyze the cleavage of FA from PL and TG, which accumulate in the NEFA fraction (27,28). During 45 min after delivery, the NEFA concentration increased significantly, but within the first 10 min no significant changes occurred (Fig. 1). We did not observe significant alterations in the quantitatively small TG fraction, which indicates a low activity of TG hydrolase. Therefore, FA appear to be contributed primarily by hydrolysis from the PL fraction, which is abundant in placental tissue. The proportional composition of total PL and of liberated FA differs because of the preference of PLA_2 for the *sn*-2 position (29) and the asymmetric distribution of acyl chains in PL. Saturated FA are generally esterified at the *sn*-1 position, whereas unsaturated FA are preferentially esterified at the *sn*-2 position (30). This is in agreement with our results, where arachidonic acid in NEFA showed the highest proportional increase over time followed by dihomo-γ-linolenic acid and linoleic acid, while the proportions of all saturated FA decreased. We conclude that in order to minimize alterations in the NEFA fraction, placental tissue should be sampled as fast as possible and frozen immediately.

It has been reported that addition of EDTA to the washing solution inhibits PLA_2 activity in a concentration-dependent manner (31). It was assumed that the penetration of EDTA through an intact plasma membrane is limited; therefore, the effect of EDTA in the washing solution to inhibit enzyme activity should be of little benefit, and this was therefore not used.

The distribution of FA in different regions of the placenta is determined by transfer, placental metabolism, and release to the fetal circulation (2). Data on the FA distribution are not available, but differences across the placenta might be assumed. Thus, samples from different randomly chosen locations of the placenta were analyzed including central and peripheral locations of the maternal and fetal side and the parenchyma. The analysis of the relative FA composition of all lipid fractions indicated no significant differences between the investigated locations. The absolute concentrations of the PL-bound FA showed a homogeneous distribution, in contrast to the FA in NEFA, TG, and CE. Therefore, for investigations of

the relative FA composition in placental lipid fractions, the location of sampling does not need to be exactly defined, but determinations of absolute FA concentrations require explicit definition of the location, usually the center of the parenchyma.

In conclusion, the method presented here shows a reasonable precision for the analysis of FA in different lipid fractions of placental tissue. The good reproducibility of the absolute FA concentrations offers opportunities for further investigations of time-dependent processes and placental transfer functions and for the study of different nutritional and physiological factors.

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Nonpolar Lipid Composition of *Chenopodium album* Grown in Continuously Cultivated and Nondisturbed Soils

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ABSTRACT: *Chenopodium album* L. plants grown in continuously cultivated and in nondisturbed soils were compared in terms of the compositions of nonpolar extracts of the corresponding aerial parts. Both light petroleum ether extracts of *C. album* L. were analyzed by high-performance thin-layer LC, capillary GC, and capillary GC-EI-MS. Further percolation and medium-pressure LC, along with EI-MS analysis, permitted the separation and identification of the chemical constituents. Differences were observed between mean contents of the chemical constituents of *C. album* L., with respect to nonpolar extracts, obtained from continuously cultivated and from nondisturbed soils, in particular in linear and branched long-chain hydrocarbons, FA and their esters, and long-chain linear alcohols and aldehydes. The most remarkable features of the disturbed soils were a pronounced increase in the amounts of linear hydrocarbons and a decrease in the relative proportions of FA.

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Owing to the growing world population, crop management involves continuous and intensive exploitation of land based on the use of fertilizers and pesticides in order to achieve higher productivities. The overuse of agrochemicals results in severe alterations to the agroecosystem (1,2), particularly in the physical, chemical, and biological properties of the soil and in groundwater contamination (3–7).

The quality of a soil is defined in terms of its capacity to sustain productivity in natural or managed ecosystems, to maintain or enhance water and air quality, and to support human needs (8). The potential use of some soil properties (organic C; total N; P, K, B, Ca, and Zn concentrations; and cation exchange capacity) and of crop characteristics (total dry matter at physiological maturity, grain yield, kernel number, and prolificacy) as indicators for soil quality, has been demonstrated by multivariate techniques (9). The Rolling Pampa of Argentina (10) has been subjected to continuous cropping in the last two decades, with a nearly total loss of the traditional mixed farming with cattle grazing (11). Thus, soil quality has been reduced by nutrient exploitation, soil compaction, and negative changes in biotic conditions (9,12–16). Different levels of soil deterioration were attributed to farming. Organic C, total N, extractable P, pH, soil aggregate stability (SA_s) and infiltration rate showed the greatest changes compared to the same parameters in pristine conditions (12). Among them, SA_s and P concentration seem to be the most critical parameters in detecting soil deterioration in the Rolling Pampa (9).

Weeds, a group of plants with a great adaptability to environmental changes, have colonized most of the places disturbed by humans, particularly lands undergoing continuous agricultural exploitation. The occurrence of biologically active principles is known in several weed species (17–19); a number are secondary metabolites that are related to the ecological fitness of the producing organism. *Chenopodium album*, a species native to Europe and Asia and called "fat hen" in Europe and "lamb's-quarters" in the United States, causes significant economic loss in agriculture (20). This broadleaf weed grows in association with most common crops in Argentina and has developed cross- and multiple-resistance to synthetic agrochemicals (21). Although *C. album* competes strongly with crops, it also has growth-inhibiting allelopathic effects (22–24), feeding deterrence potential (20), and the ability to control viruses (25), fungi (26), and soil nematodes (27). The bioactive role of *Chenopodium* spp. is appreciated mainly in developing countries as reflected in the use of their raw extracts (18–20). The potential of *Chenopodium* spp. to control pests has been related to a variety of chemicals, such as hydrocarbons (23), saponins (28), flavonoids (29), terpenoids, and steroids (20). Among them, less polar compounds such as hydrocarbons, FA and their methyl esters (30–32), long-chain aldehydes, and alcohols (33) are present in the epicuticular wax layer. Wax deposition plays an important role in reducing water loss; the other major function is related to plant protection against herbivores and pathogens (33). The purpose of our experiment was to compare the nonpolar lipid compositions of *C. album* collected from continuously cultivated and noncultivated areas.

EXPERIMENTAL PROCEDURES

Organic solvents were obtained from Sintorgan (Buenos Aires, Argentina) and were glass-distilled before use; *n*-hexane was freed from olefins followed by distillation. All solvent mixtures are expressed in volumes (vol/vol). Authentic samples were purchased from Sigma-Aldrich.

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Abbreviations: HPTLC, high-performance TLC; MPLC, medium-pressure LC; SA_s, soil aggregate stability; UBA, Universidad de Buenos Aires.

Plant sample collection. *Chenopodium album* L. specimens were collected in their vegetative stage during 1998 summertime from a set of fields in the maize production area of Argentina (32–35°S and 58–62°W) on silty clay loam soil, located in the highlands within the Arroyo Dulce soil series (34). We chose four fields with a long cropping history (more than 15 yr since last pasture) and characterized by a low stability index (less than 20%), expressed as (100 × deteriorated soil mean-weighted diameter/pristine soil mean-weight diameter) of aggregates (16). Within each field we collected at random specimens of entire plants with similar height from two kind of sites: (i) continuously cultivated, and (ii) nondisturbed. Chemical and physical properties (Table 1) that define continuously cultivated soils and nondisturbed soils as deteriorated and nondeteriorated were previously determined (9).

Since all specimens were collected on the same day from fields in the same area, all environmental conditions were the same except for soil exploitation level. A voucher specimen of each sample was deposited (no. 24038) in the Herbarium of the Cátedra de Botánica, Facultad de Agronomía, UBA, Buenos Aires, Argentina.

Extraction. Aerial parts (stems and leaves) of *C. album* were separated from roots, and immediately submitted to drying conditions at 40°C, under ventilation, the same day that they were collected. The dry material was milled to a coarse powder prior to Soxhlet extraction with light petroleum ether. Four samples (100 g each) of dry plant material from each kind of soil were submitted to continuous extraction (300 mL). Each extract was evaporated *in vacuo* to dryness and successively percolated with *n*-hexane, methylene chloride, acetone, and methanol to give the corresponding subextracts. Further separation procedures included medium-pressure liquid chromatography (MPLC). All extracts, subextracts, and MPLC fractions were qualitatively analyzed by TLC and submitted to capillary GC and to GC–MS analysis.

Chromatographic conditions. TLC was performed on silica gel GF-254 (250 µm layers; Merck, Darmstadt, Germany) plates; high-performance TLC (HPTLC) was carried out on commercial plates (silica gel F-254, Merck). Mobile phases were as follows: (A) *n*-hexane/chloroform (95:5), (B) *n*-hexane/chloroform (6:4), and (C) light petroleum ether/ethyl ether/acetic acid (90:10:1). Spots were visualized under UV light at 254 nm and/or with sulfuric acid/acetic acid (1:1), with heating at 110°C for 5 min. Selective chromogenic reactions included bromothymol blue for lipids; anisaldehyde/sulfuric acid for steroids and terpenes; vanillin/sulfuric acid for higher alcohols and ketones; silver nitrate/ammonia for aldehydes; and silver nitrate/pyrogallol for acids.

TABLE 1
Chemical and Physical Indicators of Soil Quality[a]

Indicator (mean value)	Continuously cultivated soil (CC)	Nondisturbed soil (ND)
C_o (organic carbon), g kg^{-1}	20b	28a
N_t (total nitrogen), g kg^{-1}	2.1b	2.9a
P, mg kg^{-1}	17b	111a
B, mg kg^{-1}	0.3b	0.8a
Zn, mg kg^{-1}	1.9b	6.5a
SA_s (soil aggregate stability), mm	1.66c	0.31a
pH	6.2a	6.4a

[a]Within a row, means followed by a different letter show significant differences among soil types ($P < 0.05$).

MPLC was carried out under nitrogen pressure on silica gel H (Merck) columns, eluting with gradients of *n*-hexane/ethyl acetate (5:1 to 1:5); ethyl acetate; and gradients of ethyl acetate/methanol (49:1 to 1:5); main components were isolated, identified, and quantified.

GC was performed on a Hewlett-Packard 5890 gas chromatograph with a temperature program from 100 to 280°C at a rate of 15°C/min and then isothermally, by using a FID and a RSL-150 capillary column (50 m × 0.20 mm i.d. × 0.25 µm thickness; Alltech, Deerfield, IL) and nitrogen as carrier. Capillary GC–EI–MS analysis was performed at 70 eV on a Trio 2-VG quadrupole spectrometer, under programmed temperature from 100 to 280°C at a rate of 6°C/min. EI–MS data were processed using a Lab Base GC–MS data system; relative abundances (as percentages) were registered vs. retention times. Library matching was used to identify compounds tentatively. Comparing mass spectra with those of authentic commercial samples provided the final identification.

Statistical analysis. Statistical analysis was performed by means of an ANOVA, and mean values were compared by Tukey's test.

RESULTS

TLC analysis. Saturated hydrocarbons and waxes were detected as the main components in the *n*-hexane subextract, together with minor spots corresponding to terpenes and steroids (mobile phase A). Terpenoids, steroids, and aldehydes were detected as the main constituents of the dichloromethane subextract. The acetone subextract gave two major spots, characterized as aldehydes and alcohols; FA were also present. Alcohols and FA were the main components of the methanol subextract. MPLC confirmed these results and allowed the identification of minor components by GC–EI–MS and the isolation and quantification of the main ones. The contribution of different compounds to total dry matter was then calculated relative to those main compounds already quantified, by their relative abundances in the corresponding GC chromatogram.

Comparison of GC chromatograms (Fig. 1) of nonpolar extracts from aerial parts of *C. album* grown in continuously cultivated and in nondisturbed soils showed differences in the relative chemical composition of mainly hydrocarbons (Table 2). No major differences in the composition of the bulk lipid matrix were found, except for remarkably higher levels of long-chain hydrocarbons and related long-chain derivatives, along with lower relative levels of FA.

The EI–MS spectra [*m/z* (%)] of representative compounds are presented below.

(i) Tritetracontane (Peak 26, Table 2). 41 (14), 43 (49), 55 (25), 56 (17), 57 (100), 58 (6), 69 (20), 70 (12), 71 (68), 82

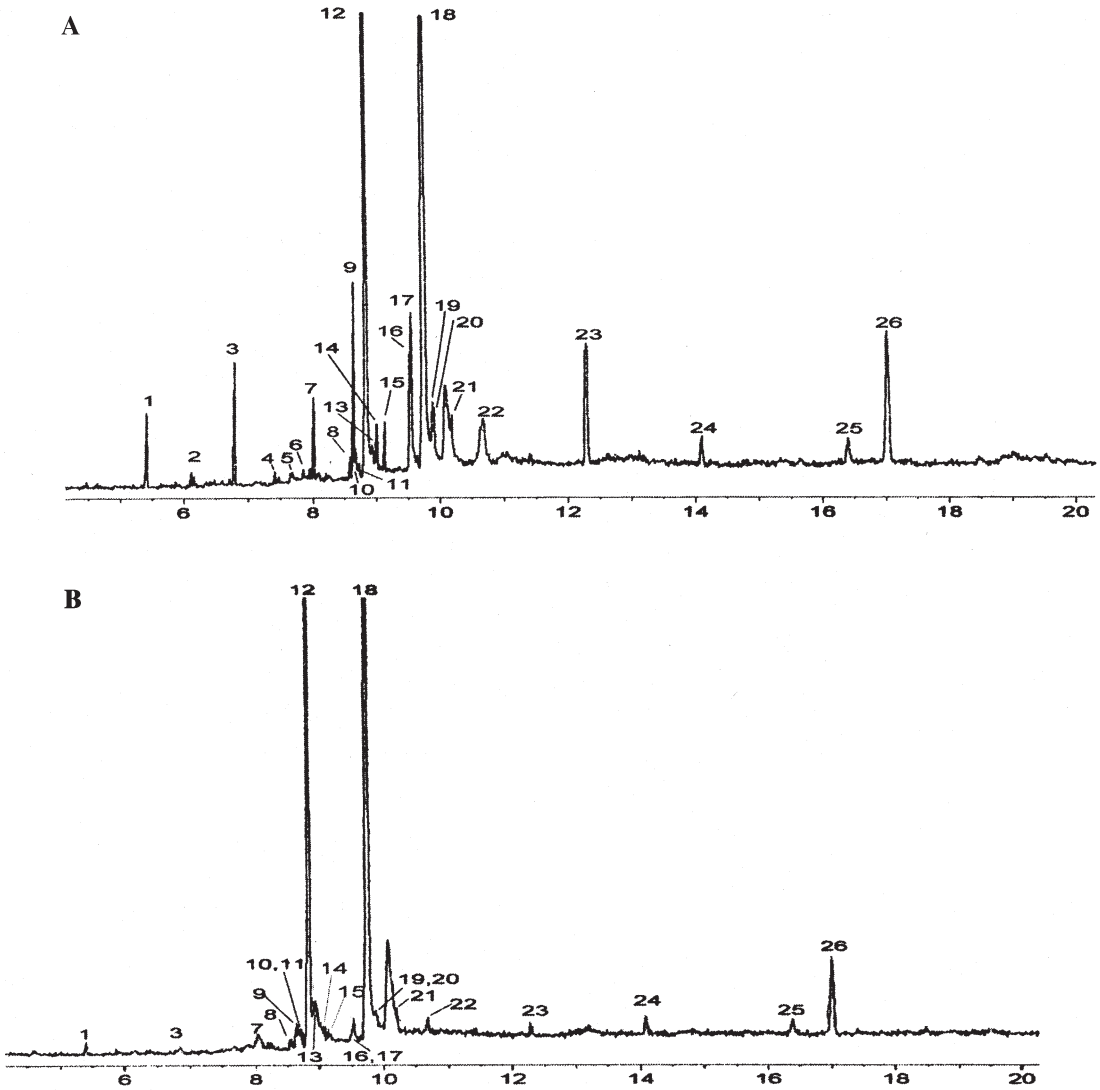

FIG. 1. Gas chromatograms of nonpolar extracts from aerial parts of *Chenopodium album* growing in continuously cultivated (A) and nondisturbed (B) soils.

(6), 83 (20), 84 (7), 85 (39), 97 (15), 98 (5), 99 (11), 111 (8), 113 (7), 127 (6), 141 (5).

(ii) Tetracontane (Peak 23, Table 2). 41 (10), 43 (49), 55 (19), 56 (11), 57 (100), 69 (13), 70 (9), 71 (77), 82 (5), 83 (16), 84 (6), 85 (48), 97 (15), 98 (5), 99 (19), 111 (9), 113 (8), 127 (13), 141 (10), 155 (6), 169 (5), 183 (5).

(iii) Pentatriacontane (Peak 22, Table 2). 41 (12), 42 (5), 43 (59), 55 (23), 56 (18), 57 (100), 69 (20), 70 (11), 71 (73), 83 (18), 84 (7), 85 (50), 97 (15), 98 (5), 99 (15), 111 (9), 112 (6), 113 (11), 125 (5), 126 (5), 127 (7), 141 (5), 492 (7).

(iv) n-*Nonacosane (Peak 21, Table 2).* 41 (19), 42 (5), 43 (79), 55 (23), 56 (14), 57 (100), 58 (5), 69 (18), 70 (12), 71 (74), 83 (17), 84 (8), 85 (53), 97 (12), 98 (5), 99 (20), 111 (7), 113 (15), 125 (5), 127 (11), 141 (10), 155 (6), 169 (5).

(v) n-*Heneicosane (Peak 15, Table 2).* 41 (26), 42 (5), 43 (61), 55 (20), 56 (11), 57 (100), 58 (6), 69 (11), 70 (10), 71 (72), 82 (10), 83 (10), 84 (6), 85 (57), 97 (7), 98 (6), 99 (20), 113 (10), 127 (7), 141 (5), M^+ 296 (1.8).

(vi) n-*Eicosane(Peak 14, Table 2).* 41 (43), 42 (10), 43 (90), 55 (30), 56 (17), 57 (100), 58 (5), 69 (15), 70 (10), 71 (68), 83 (10), 84 (9), 85 (50), 97 (7), 98 (5), 99 (9), 113 (8), 127 (6), 141 (5), M^+ 282 (7).

(vii) n-*Hexadecanal (Peak 24, Table 2).* 41 (89), 42 (22), 43 (100), 44 (40), 45 (15), 53 (5), 54 (14), 55 (63), 56 (27), 57 (85), 58 (5), 66 (8), 67 (34), 68 (40), 69 (37), 70 (19), 71 (30), 72 (6), 81 (24), 82 (53), 83 (31), 84 (10), 85 (11), 95 (20), 96 (28), 97 (18), 109 (8), 110 (8), 111 (6).

(viii) n-*Octadecanal (Peak 25, Table 2).* 41 (86), 42 (19), 43 (100), 44 (28), 45 (10), 53 (5), 54 (14), 55 (75), 56 (23), 57 (79), 58 (5), 66 (6), 67 (33), 68 (40), 69 (42), 70 (17), 71 (34), 72 (6), 81 (30), 82 (63), 83 (41), 84 (11), 85 (17), 95 (28), 96 (29), 97 (28), 98 (7), 99 (5), 109 (14), 110 (14), 111 (10), 123 (7), 124 (7), 137 (5), 138 (5).

DISCUSSION

The most remarkable feature in samples from continuously cultivated soils was a pronounced enhancement of long-chain

TABLE 2
Mean Contents (mg/g dry weight) of Nonpolar *Chenopodium album* Constituents from Plants Grown in Continuously Cultivated (CC) and Nondisturbed (ND) Soils

Peak no.[a]	Compound	CC (mean value)	ND (mean value)	*P*
1	*n*-Tetradecane	0.246 ± 0.077	0.034 ± 0.005	<0.001
2	*n*-Pentadecane	0.068 ± 0.0073	Trace[b]	
3	*n*-Hexadecane	0.286 ± 0.014	0.024 ± 0.004	<0.001
4	*n*-Heptadecane	0.052 ± 0.009	Trace	
5	2,6-Dimethylheptadecane	0.068 ± 0.012	Trace	
6	2-Methyloctadecane	0.040 ± 0.006	Trace	
7	*n*-Octadecane	0.213 ± 0.012	0.020 ± 0.003	<0.001
8	2,6,10,14-Tetramethylheptadecane	0.054 ± 0.007	0.013 ± 0.002	<0.001
9	Methyl *n*-hexadecanoate (methyl palmitate)	0.363 ± 0.038	0.053 ± 0.005	<0.001
10	Ethyl *n*-hexadecanoate (ethyl palmitate)	0.088 ± 0.011	0.037 ± 0.005	<0.001
11	Methyl *n*-octadecanoate (methyl stearate)	0.037 ± 0.003	0.019 ± 0.002	<0.001
12	*n*-Hexadecanoic acid (palmitic acid)	3.581 ± 0.264	4.385 ± 0.387	<0.001
13	*n*-Octadecanoic acid (stearic acid)	0.125 ± 0.011	0.205 ± 0.020	<0.001
14	*n*-Eicosane	0.213 ± 0.036	0.030 ± 0.005	<0.001
15	*n*-Heneicosane	0.207 ± 0.03	0.029 ± 0.004	<0.001
16	Methyl-(18:2) Δ9,12-*cis,cis*-octadienoate or methyl-9*Z*,12*Z* octadienoate or methyl linoleate	0.339 ± 0.022	0.016 ± 0.002	<0.001
17	Methyl (18:3) Δ9,12,15-*cis,cis,cis*-octadecatrienoate or methyl 9*Z*,12*Z*,15*Z*-octadecatrienoate or methyl linolenate	0.407 ± 0.025	0.076 ± 0.006	<0.001
18	(18:2) Δ9,12-*cis,cis*-octadienoic acid or 9*Z*,12*Z*-octadienoic acid or linoleic acid	4.076 ± 0.233	5.070 ± 0.301	<0.001
19	(18:2) Δ9,12-*cis,cis*-octadecadien-1-ol or 9*Z*,12*Z*-octadecadien-1-ol	0.205 ± 0.023	0.113 ± 0.011	<0.029
20	*n*-Octacosane	0.182 ± 0.078	0.055 ± 0.006	<0.001
21	*n*-Nonacosane	0.195 ± 0.032	0.099 ± 0.012	<0.001
22	*n*-Pentatriacontane	0.413 ± 0.080	0.047 ± 0.004	<0.001
23	*n*-Tetracontane	0.322 ± 0.025	0.040 ± 0.004	<0.001
24	*n*-Hexadecanal	0.146 ± 0.015	0.089 ± 0.005	<0.001
25	*n*-Octadecanal	0.157 ± 0.017	0.086 ± 0.004	<0.001
26	*n*-Tritetracontane	0.583 ± 0.033	0.351 ± 0.012	<0.001

[a]Numbers of the compounds refer to peaks in Figure 1.
[b]Traces (<0.01 mg/g dry weight).

linear hydrocarbons—such as hexadecane, eicosane, heneicosane, nonacosane, pentatriacontane, tetracontane, and tritetracontane—and methyl and ethyl esters derived from common FA, in comparison with samples from undisturbed soils. Smaller increments were found in long-chain aldehydes (*n*-hexadecanal and *n*-octadecanal). There was also an increment in the relative amount of unsaturated long-chain alcohols and branched long-chain hydrocarbons. Likewise, the relative proportion of FFA (palmitic, linoleic, and stearic acids) was diminished.

Long-chain hydrocarbons are ubiquitous in the waxy coating of leaves. Biosynthetically related to FA, they may play a role in plant disease resistance (35). The various biochemical steps of wax biosynthesis are known (36–38). The impact of variations in environmental parameters on the production of secondary metabolites by plants is well known (39–42). In response to biotic and abiotic stresses, plants produce more secondary metabolites. Higher levels of certain products of secondary metabolism, found under stress conditions, can negatively affect potential predators and may reflect natural selection, resulting in increased resistance to herbivores or to disease organisms and to future damage (40).

For example, water stress increases the levels of long-chain alkanes in cotton epicuticular waxes, *n*-tetratriacontane being the mayor wax constituent in leaves and *n*-triacontane in boll and bract waxes (43). Since many of these allelochemicals may possess multiple modes of action (20), their occurrence in higher levels under stress conditions can negatively affect potential predators. Changes in relative proportions of the different epicuticular wax components like FA and hydrocarbons may affect herbivory through their influence on oviposition (44). Differences in the proportions of *n*-alkanes, long-chain FA, esters, and aldehydes of *Brassica oleracea* epicuticular waxes found as a result of changes in growth conditions have also been related to the defense mechanisms of this organism (45).

The production of larger amounts of allelopathic compounds in a stressful environment can also be interpreted as an adaptive strategy to suppress the increase of competition for nutrients (42).

As a result of the overuse of agrochemicals, many herbivores, pathogens, and weeds have developed resistance to synthetic agrochemicals, making it necessary to find new methods of pest control based on natural products (20). The possible management of the chemical properties of weed species with potential biologic activities may help lessen and even revert agroecosystem damage (46).

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Occurrence of a Novel *cis,cis,cis*-Octadeca-3,9,12-trienoic (*Z,Z,Z*-octadeca-3,9,12-trienoic) Acid in *Chrysanthemum (Tanacetum) zawadskii* Herb. (Compositae) Seed Oil

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ABSTRACT: A new octadecatrienoic acid (6.9%), found as a component of *Chrysanthemum zawadskii* Herb. (Asteraceae) seed oil, was shown to be the hitherto unknown *cis,cis,cis*-octadeca-3,9,12-trienoic acid. The oil also contained 8.6% of crepenynic acid in addition to the other common FA. The structures of the new unusual FA and other FA were confirmed by chromatographic (TLC, GC), spectroscopic (IR, UV, and NMR), and MS methods by using different chemical derivatizations (preparation of methyl ester, pyrrolidide, picolinyl esters, and dimethyloxazoline derivatives).

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One of the characteristics of Asteraceae (Compositae) is the great biosynthetic potential to produce a number of unusual seed oils. Unusual FA, such as Δ3 *trans* (1–4), Δ5 *cis* (5), Δ6 *cis* (6,7), conjugated (8), acetylenic (9–11), epoxy (12), and hydroxy (13) acids, were previously found in different genera of this family, and these have been reviewed elsewhere (14–16).

Chrysanthemum (Tanacetum), a genus of the Asteraceae, has many useful species (17). *Chrysanthemum parthenium* (feverfew) is an ornamental medicinal herb, said to be antiseptic, and used as an antidote, aperient, carminative, sedative, and vermifuge for hysteria and parturition (18). Garland (*Chrysanthemum coronarium* L.) possesses antioxidant activities (19). The ligulate flowers of the edible chrysanthemum (*C. morifolium* Ramat.) were found to contain compounds that showed a strong inhibitory effect on tumor promotion in mouse skin (20). *Chrysanthemum cinerarifolium* (pyrethrum or Dalmatian insect-flower) is cultivated for the dried inflorescences, which are used in the manufacture of insecticides and parasiticides (21).

The genus *Chrysanthemum* has been little studied for its seed oils. *Chrysanthemum coronarium* (22,23) has been reported to contain epoxy acids (vernolic and coronaric) as seed oil components. *Chrysanthemum corymbosum* (24) was recently found to contain a novel *trans,trans*-conjugated acetylenic acid (*trans,trans*-octadeca-8,10-dien-12-ynoic) in addition to crepenynic acid (*cis*-octadeca-9-en-12-ynoic) as seed oil components.

In continuation of our exploration of the potential of less common oil seeds, the structure and composition of the seed oil from *Chrysanthemum (Tanacetum) zawadskii* growing in Mongolia (25) were investigated by means of chromatographic, spectroscopic, and spectrometric methods and two unusual FA were identified. This paper describes the structural identification of a new unusual FA in this hitherto unstudied species.

EXPERIMENTAL PROCEDURES

Seeds. Seeds (about 10 g) of *Tanacetum* (*Chrysanthemum*) *zawadskii* Herb. were collected from a series of the plant in the same area of Tereljiin davaa, Mongolia, during September 2000. The herbarium in which the plant is deposited is the Biological Institute, National University of Mongolia, Ulaanbaatar.

Extraction of seed oil. Air-dried seeds were isolated from other plant tissues and ground with an electric mill (Junke & Kunkel GmbH IKA-WERK, Staufen, Germany). Ground seeds (6.44 g) were mixed with anhydrous sodium sulfate and sea sand and extracted with *n*-hexane (80 mL) in a Soxhlet apparatus for 5 h. The extraction gave 0.83 g of seed oil with a yellow color.

Preparation of FAME. Mixed FAME (180 mg) were prepared by transesterification of the seed oil (200 mg) with 2.0 M sodium methoxide in methanol (0.4 mL). They were then purified by preparative TLC separation in an *n*-hexane/diethyl ether (80:20, vol/vol) system (6).

Preparation of picolinyl esters. The free acids (1 mg), prepared by hydrolysis with 0.1 M ethanolic potassium hydroxide, were converted to the picolinyl esters by the method of Balazy and Nies (26). The product was dissolved in isohexane containing BHT (50 ppm) for GC–MS analysis (27).

Preparation of dimethyloxazoline (DMOX) derivatives. DMOX derivatives were prepared in a manner similar to that previously reported (28,29). Ten milligrams of mixed FAME were hydrolyzed with 1 N ethanolic potassium hydroxide to

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Abbreviations: DMOX, 4,4-*N,N*-dimethyloxazoline; ECL, equivalent chain length; RRT, relative retention time.

FFA. The FFA (5 mg) were added to a screw-capped reaction tube, and a threefold excess (w/w) of 2-amino-2-methyl-1-propanol was added. The tube was purged with argon gas and heated at 170°C for 0.5 h in an oven. The reaction mixture was then transferred to a separatory funnel containing 40 mL petroleum ether and 50 mL water and was shaken. Saturated NaCl was added to break the emulsion, and the aqueous layer was removed. The petroleum ether was washed with water and then dried over anhydrous sodium sulfate and concentrated to an appropriate volume with argon gas.

Preparation of pyrrolidide derivatives. The FAME (1 mg) were dissolved in freshly distilled pyrrolidine (1 mL), acetic acid (0.1 mL) was added, and the mixture was heated at 100°C for 1 h. Excess pyrrolidine was evaporated in a stream of nitrogen at 50°C, and then the residue was taken up in 8 mL of *n*-hexane/diethyl ether (1:1, vol/vol) and was washed three times with water (4 mL portions). After drying over anhydrous sodium sulfate, the required product was obtained on evaporation of the solvent (30,31).

TLC. To obtain additional information on unusual components in oils, analytical TLC of oils and FAME was carried out on a 0.25-mm layer of silica gel G (Merck, Darmstadt, Germany) using a solvent system of *n*-hexane/diethyl ether (70:30 or 80:20, vol/vol). Spots were visualized by spraying with phosphomolybdic acid (5% in ethanol) and heating at 100°C.

Capillary GLC. Capillary GLC analysis of FAME was carried out using a GC-380 GL Sciences gas chromatograph (GL Sciences, Tokyo, Japan) equipped with an FID and a 50 m × 0.25 mm CP-Sil 88 capillary column (Chrompack, Middelburg, The Netherlands) under the following conditions. The temperature was programmed from 70°C (held for 4.0 min.) to 175°C (held 27.0 min) at 13°C/min, then to 215°C at 4.0°C/min, and held at this point for 31 min before cooling again to 70°C. FID and split/splitless injector temperatures were 250°C. Hydrogen was the carrier gas at a flow rate of 2.0 mL/min. Components were quantified by electronic integration. Identification of FAME was confirmed by chromatographic comparison of equivalent chain lengths (ECL) with those of standards or previously known seed oils.

GC–MS. The derivatives were submitted to GC–MS with a Hewlett-Packard 5890 Series II Plus gas chromatograph attached to an HP model 5989 MS engine. The latter was used in the EI mode at 70 eV with a source temperature of 250°C. The GC was fitted with on-column injection. For all derivatives, a capillary column of fused silica coated with Supelcowax 10™ (30 m × 0.25 mm, 0.25 micron film; Supelco UK, Poole, United Kingdom) was used. The column temperature was programmed from 80°C (held 3 min) to 180°C at 20°C/min, then to 280°C at 2°C/min, and held 15 min at 280°C. Helium was the carrier gas at a constant flow rate of 1 mL/min.

UV absorption spectroscopy. UV spectra were determined from solutions of the seed oil FAME in *n*-hexane using a JASCO Ubest-35 scanning spectrophotometer (Japan Spectroscopic Co., LTD, Tokyo, Japan) with a focusing attachment to check the presence of any unusual (conjugated) FA.

FTIR spectroscopy. FTIR spectra were determined from oil films on sodium bromide cells using a Nicolet Model 410 Impact FTIR spectrometer (Nicolet Instrument Corporation, Madison, WI) with a focusing attachment to check the presence of any unusual (*trans* unsaturated or oxygenated) FA.

^1H and ^{13}C NMR. The NMR spectra were recorded on a Varian Unity Inova 600 NMR spectrometer (600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR) after dissolving FAME in $CDCl_3$.

RESULTS AND DISCUSSION

n-Hexane extraction of *C. zawadskii* Herb. seeds yielded 12.9% oil. TLC of the seed oil and the mixed FAME did not show any oxygenated FA.

The FTIR spectrum of FAME from the *C. zawadskii* Herb. seed oil showed strong peaks at 2925, 2854 (C–H), 1753 (C=O), 1435 (C≡C), 1360 (C–H), 1170 (C–O), and 723 cm^{-1} (C=C; *cis*). It did not exhibit any characteristic absorption at 960 cm^{-1}, which is indicative of an isolated *trans* double bond group (1–4). This gave us evidence that double bonds in unsaturated FA of the oil have the all-*cis* configuration. It was also demonstrated that no hydroxy- and epoxy-FA were contained in the seed oil by FTIR analysis. The UV spectrum of the oil did not show any characteristic absorption spectrum for a conjugated FA (24).

^1H and ^{13}C NMR were measured for mixed FAME from the *C. zawadskii* Herb. seed oil. ^1H NMR gave complicated signals due to olefin (5.36–5.45 ppm), methylene (1.28–3.01 ppm), terminal methyl (0.87 ppm), and carboxylmethyl protons (3.61 ppm). Signals found at 2.86–3.01 ppm seemed to correspond especially to methylene group protons situated between a double bond and carboxyl ester group. ^{13}C NMR also gave complicated signals due to olefin (127–130 ppm), methylene (13.9–37.8 ppm), terminal methyl (14.0 ppm), and carboxylmethyl groups (174 ppm). It gave characteristic signals at 76.7–77.3 ppm, showing the presence of an acetylene group. Moreover, two signals due to a methylene group adjacent to an acetylene group were observed at 17.1 and 18.7 ppm.

The GLC separation of mixed FAME from *C. zawadskii* Herb. on a SUPELCOWAX 10 is illustrated in Figure 1. Table 1 shows the data for the FA composition of the seed oil and their chromatographic separation characteristics on a CP-Sil 88 capillary column. As can be seen from Table 1, major FA were linoleic (63.9%) and oleic (9.8%). However, GLC analysis of the mixed FAME from *C. zawadskii* Herb. seed oil revealed two unusual components along with common FA (Fig. 1). The first unknown compound (6.9%) eluted at a retention time of 27.6 min, had an ECL value of 20.30 on the CP-Sil 88 column, and appeared to be a C_{18}-triene. The second unusual component (8.6%) eluted at a retention time of 37.7 min and had an ECL of 21.82. Preliminary identification of methyl crepenynate as the second unusual FA in *C. zawadskii* Herb. seed oil was based on the appearance of a single peak, following co-injection into GLC with *Crepis sibirica*

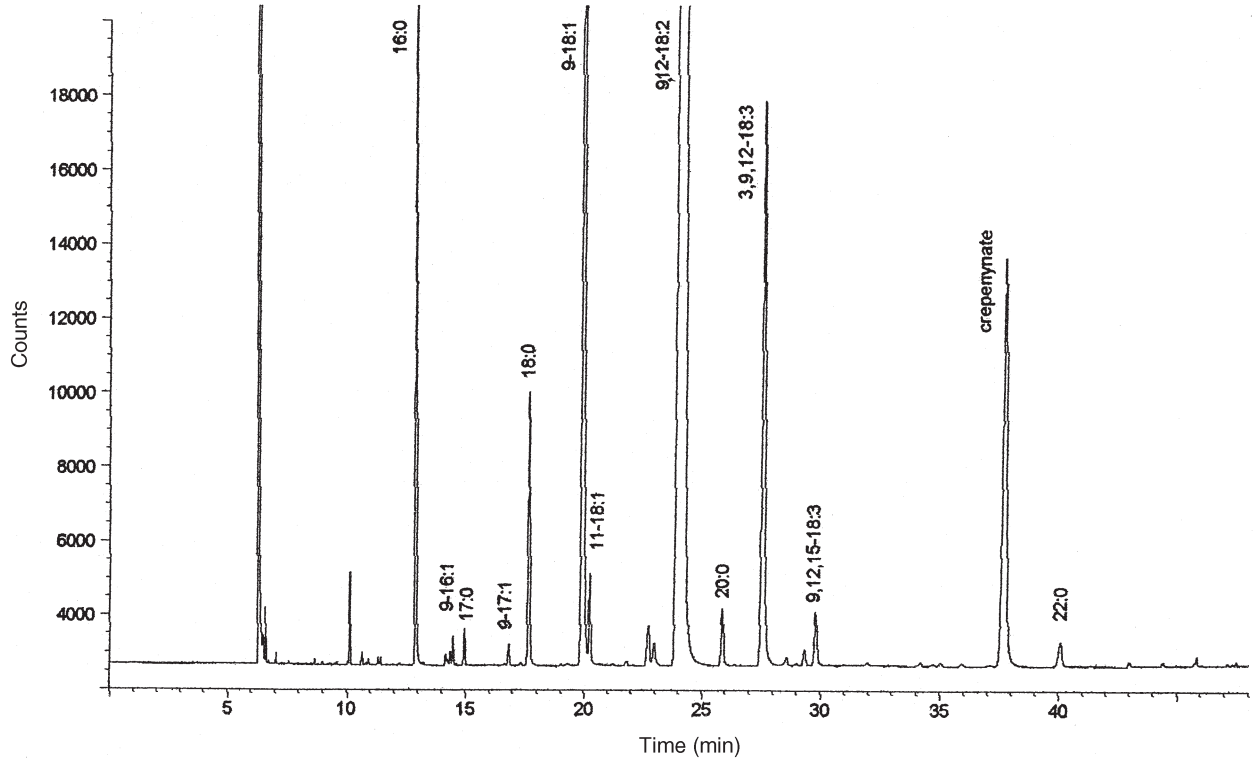

FIG. 1. The gas chromatogram of the mixed FAME from *Chrysanthemum zawadskii* Herb. seed oil separated on a SUPELCOWAX 10™ column.

seed oil FAME, known to contain crepenynic acid, and this was subsequently confirmed by GC–MS.

The structures of the two unusual FA were determined based on MS of various derivatives by methods described elsewhere (32,33). The MS of the first unusual methyl ester gave the molecular ion at *m/z* = 292, suggesting a C_{18}-trienoic methyl ester with other abundant fragments not relevant for the characterization of structure. The MS of the picolinyl ester of this acid is illustrated in Figure 2 (upper). In addition to the expected ions at *m/z* = 92 (the base peak), 108, 151, and 164, the molecular ion is at *m/z* = 369 followed by a series of ions 14 amu apart representing cleavage at successive methylene groups, i.e., at *m/z* = 354, 340, 326, 312, and 298. The gaps of 26 amu between *m/z* = 232 and 258 and between 272 and 298 locate double bonds in positions 9 and 12, respectively. The characteristic fingerprint ions at *m/z* = 190/191 and 204, formed by cleavage with allylic rearrangements (27), locate the remaining double bond in position 3. This suggested that the component was octadeca-3,9,12-trienoic acid.

TABLE 1
FA Composition and GLC Separation Characteristics of FAME in the Seed Oil of *Chrysanthemum (Tanacetum) zawadskii* (Asteraceae)[a]

FA	GLC area (%)	RRT	ECL on CP-Sil 88
14:0	0.1	0.8607	14.00
16:0	4.8	1.0000	16.00
16:1Δ7*c*	0.1	1.0555	16.50
16:1Δ9*c*	0.2	1.0656	16.59
18:0	2.0	1.2225	18.00
18:1Δ9*c*	9.8	1.3159	18.55
18:1Δ11*c*	0.7	1.4371	19.26
18:2Δ9c,12*c*	63.9	1.5082	19.68
20:0	0.5	1.5637	20.00
18:3Δ3*c*,9*c*,12*c*	6.9	1.6357	20.30
20:1Δ11*c*	0.2	1.6716	20.45
20:1Δ13*c*	0.2	1.7047	20.59
18:3Δ9*c*,12*c*,15*c*	0.6	1.7220	20.67
18:2Δ9*c*,12a	8.6	1.9969	21.82
22:0	0.3	2.0386	22.00
22:1Δ13*c*	0.1	2.1654	22.84
24:0	0.2	2.3395	24.00
Others	0.8		

[a]RRT, relative retention time; ECL, equivalent chain length; CP-Sil 88 (Chrompack, Middelburg, The Netherlands); *c, cis*; a, acetylene.

The mass spectra of the DMOX and pyrrolidide derivatives of this acid are illustrated in Figure 2 (middle, lower). Fortuitously, pyrrolidides and DMOX derivatives have exactly the same M.W. and the diagnostic ions are in the same place (usually clearer with DMOX derivatives). In this instance, the molecular peak is at *m/z* = 331. In the spectrum are the expected ions at *m/z* = 113 (the McLafferty ion) and at 126. In the high mass range, there is a series of ions 14 amu apart from *m/z* = 316 to 260 for cleavage at successive methylene groups. The gaps of 12 amu between *m/z* = 194 and 206, and between 234 and 246, locate the double bonds in positions 9 and 12, respectively (34). The double bond in position 3 is defined by the characteristic fingerprint ion at *m/z* = 152 for DMOX derivatives (35) and pyrrolidides (30). This ion is the base peak in the MS of DMOX derivatives and is the second-most prominent ion in that of the pyrrolidide.

The mass spectrum of the second unusual methyl ester

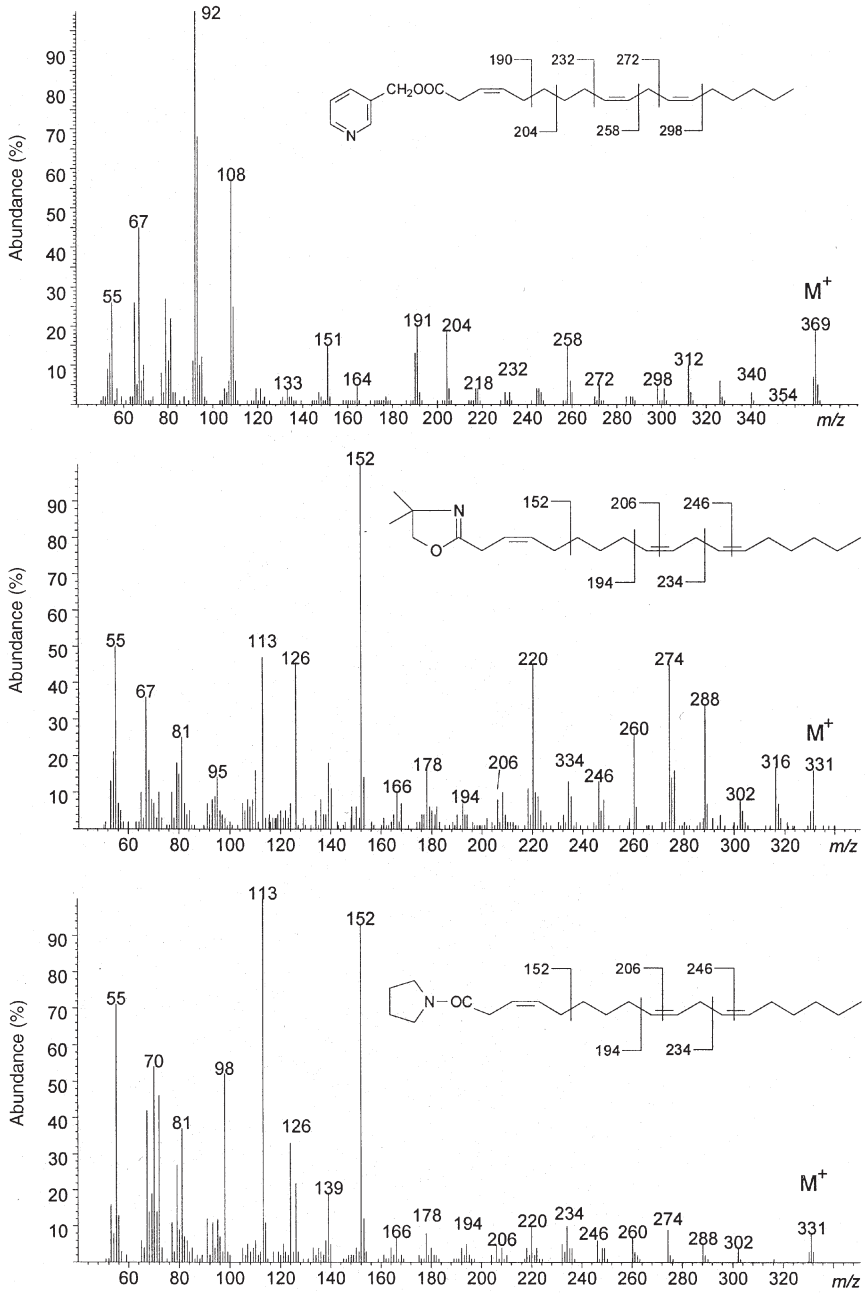

FIG. 2. Mass spectra of picolinyl ester (upper), 4,4-*N,N*-dimethyloxazoline derivative (middle), and pyrrolidide derivative (lower) of *cis,cis,cis*-octadeca-3,9,12-trienoic acid from *Chrysanthemum zawadskii* Herb. seed oil.

gave a molecular ion at *m/z* = 292 and several abundant fragments closely resembling those published for methyl crepenynate (but not suitable for characterization of structure). However, the mass spectra of picolinyl esters and DMOX derivatives of this FA were identical to those of the same derivatives of crepenynic acid published elsewhere (31).

In the mass spectrum of the DMOX derivatives of this FA, the expected ions at *m/z* = 113 (the McLafferty ion) and at 126 (the base peak) are of high abundance (Fig. 3, upper). The molecular ion is at *m/z* = 331, and it is followed by a series of ions 14 amu apart from *m/z* = 316 to 260 for cleavage at successive methylene groups. The presence of a gap of 10 amu between *m/z* = 246 and 236 in the spectrum located the triple bond in position 12, whereas a gap of 12 amu between *m/z* = 208 and 196 showed the double bond in position 9.

The picolinyl ester of a second unusual FA gave the molecular ion at *m/z* = 369 followed by a series of ions 14 amu apart representing cleavage at successive methylene groups from *m/z* = 354 to 298 as expected (35) (Fig. 3, lower). The triple bond in position 12 is indicated by a gap of 24 amu between *m/z* = 298 and 274. A further gap of 14 amu to *m/z* = 260, then one of 26 amu to *m/z* = 234, located the double bond in position 9. These data proved the second unusual FA to be crepenynic acid.

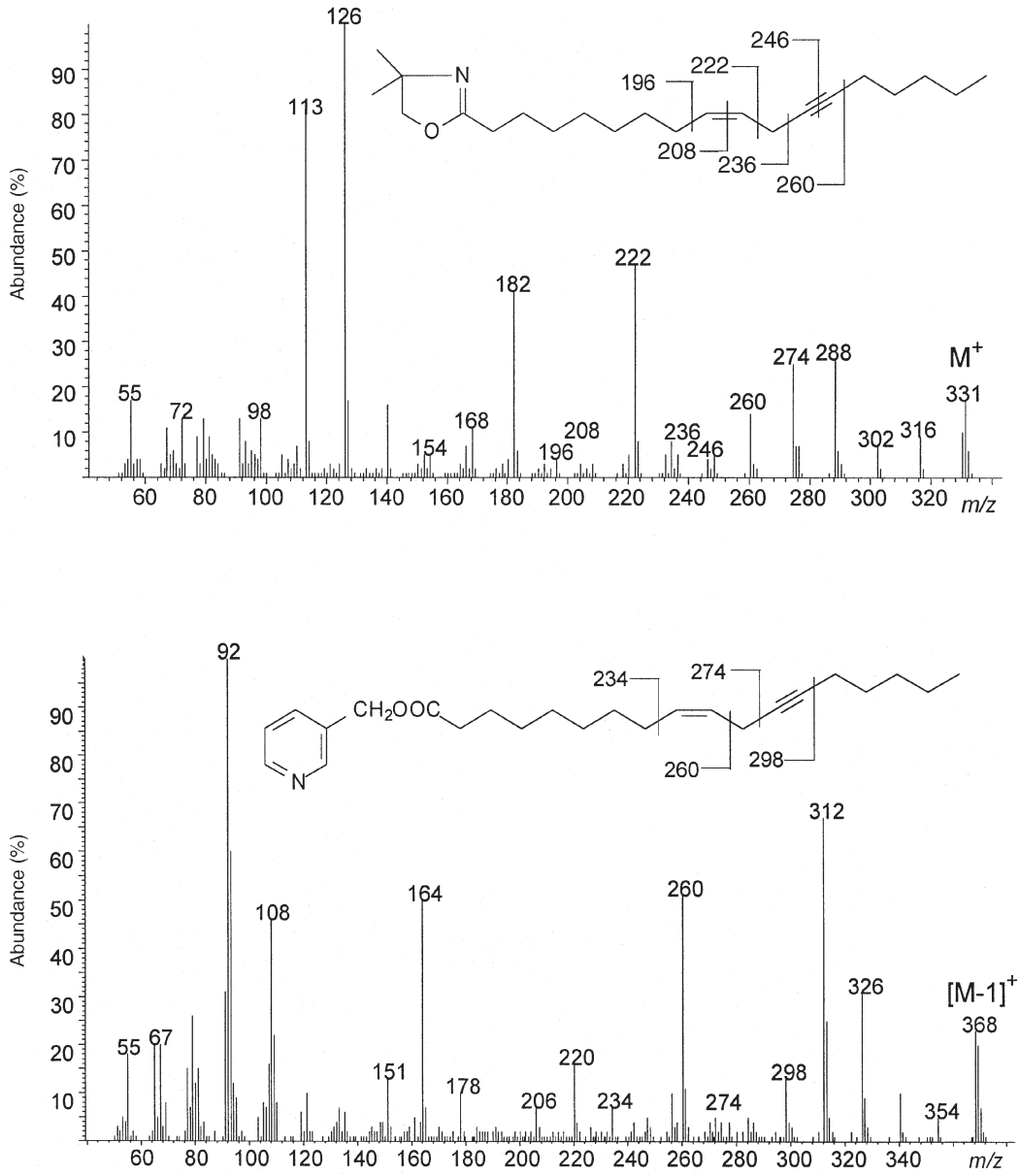

FIG. 3. Mass spectra of 4,4-*N,N*-dimethyloxazoline derivative (upper) and picolinyl ester (lower) of crepenynic acid from *Chrysanthemum zawadskii* Herb. seed oil.

Together with the spectrometric, spectroscopic, and chromatographic evidence, the mass spectroscopic study of chemical derivatives of the unknown FA confirmed that the unknown was *cis,cis,cis*-octadeca-3,9,12-trienoic acid, which has not been reported previously in nature. For some other genera of Asteraceae, 3-*trans* mono-, di-, or triunsaturated C_{16} or C_{18} FA have been reported, but *cis*-3 unsaturated isomers have never been reported as seed oil components (1–4).

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Synthesis and Isolation of *trans*-7,*cis*-9 Octadecadienoic Acid and Other CLA Isomers by Base Conjugation of Partially Hydrogenated γ-Linolenic Acid

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ABSTRACT: CLA is of considerable interest because of reported potentially beneficial effects in animal studies. CLA, while not yet unambiguously defined, is a mixture of octadecadienoic acids with conjugated double bonds. The major isomer in natural products is generally considered to be *cis*-9,*trans*-11-octadecadienoic acid (*c*9,*t*11), which represents >75% of the total CLA in most cases. Other isomers are drawing increased attention. The *t*7,*c*9 isomer, which is often the second-most prevalent CLA in natural products, has been reported to represent as much as 40% of total CLA in milk from cows fed a high-fat diet. The need for a reference material became apparent in a recent study directed specifically at measuring *t*7,*c*9-CLA in milk, plasma, and rumen. A suitable standard mixture was produced by stirring 0.5 g of γ-linolenic acid (all *cis*-6,9,12-$C_{18:3}$) with 100 mL of 10% hydrazine hydrate in methanol for 2.5 h at 45°C. The solution was diluted with H_2O and acidified with HCl. The resulting partially hydrogenated FA were extracted with ether/petroleum ether, dried with Na_2SO_4, and conjugated by adding of 6.6% KOH in ethlylene glycol and heating for 1.5 h at 150–160°C. Approximately 20 mg each of *cis*-6,*trans*-8; *trans*-7,*cis*-9; *cis*-9,*trans*-11; and *trans*-10,*cis*-12 were obtained along with other FA. Methyl esters (FAME) of these four *cis*/*trans* isomers were resolved by Ag^+ HPLC (UV 233) and partially resolved by GC/(MS or FID) (CP-Sil 88). Treatment of these FAME with I_2 yielded all possible *cis*/*trans* (geometric) isomers for the four positions 6,8; 7,9; 9,11; and 10,12.

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Isomers of octadecadienoic acid with conjugated double bonds are referred to individually or collectively as CLA. There are 14 positional isomers of octadecadienoic acid with conjugated double bonds (from 2,4-$C_{18:2}$ to 15,17-$C_{18:2}$). Each positional isomer has four geometric isomers (*cis,cis; cis,trans; trans,cis; trans,trans*), so there are 56 CLA isomers.

Twenty or so isomers among these possible isomers have been identified in natural products and dietary supplements (1). Only six of them are commercially available in relatively pure form. To date, no single technique has been able to identify and quantify the CLA isomers present in extracts of natural products, although NMR has been applied to mixtures of CLA isomers that did not contain co-extractives (2). If one is interested only in total CLA in a properly extracted and methylated sample portion (3), FAME that elute at the GC retention time of *c*9,*t*11/*t*7,*c*9 will give ~90% accuracy in the analysis of natural products, e.g., milk (4). Although the major isomer in natural products is thought to be *cis*-9,*trans*-11-octadecadienoic acid (*c*9,*t*11), which averages >75% of the total CLA in most cases (4), other isomers are drawing attention. The *t*10,*c*12 isomer has been reported to be responsible for the repartitioning (i.e., the redistribution of fat to muscle) effect of CLA (5). The *c*11,*t*13 has been reported to accumulate selectively in heart phospholipids of pigs (6). The *t*7,*c*9 isomer, which is usually the second-most prevalent CLA in natural products (4), has been reported to represent as much as 40% of total CLA in milk from cows fed a high-fat diet (7). Analysis of CLA isomers present at low levels requires procedure(s) such as silver ion (Ag^+ HPLC) (8–11) that complement GC analysis. The current work was initiated as a result of collaborative efforts to identify the source of *t*7,*c*9 in bovine milk, rumen, and plasma (12).

In this work, the well-established techniques of partial hydrogenation with hydrazine (13) and KOH/ethylene glycol conjugation (14) were performed using γ-linolenic acid (GLA) to produce specific *cis*/*trans* isomers, particularly the *t*7,*c*9 isomer. Partial reduction of FA double bonds with hydrazine did not move double bonds to new positions or change the *cis*/*trans* geometry of the remaining unsaturated bonds. The *cis*/*trans* isomers that were formed in the conjugation reaction followed established rules for base conjugation (15,16), *viz.*, double bonds that did not shift position during the conjugation maintained their *cis*/*trans* geometry, but *cis* double bonds that migrated to adjacent positions converted selectively (~99%) to the *trans* configuration. In the case of GLA, double bonds that remained at carbon positions 6, 9, and 12 maintained the *cis* configuration, and double bonds that shifted to carbon positions 7, 8, 10, and 11 changed to the *trans* configuration. Thus, only four *cis*/*trans* isomers—*c*6,*t*8; *t*7,*c*9; *c*9,*t*11; and *t*10,*c*12—of CLA were formed in these reactions. The remaining *cis*/*trans*, *cis*,*cis,* and *trans*,*trans* forms of 6,8; 7,9; 9,11; and 10,12 CLA isomers were also synthesized by configurational isomerization of the originally synthesized isomers with I_2 (17).

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Abbreviations: DMOX, 4,4′-dimethyloxazoline; GC-EIMS, gas chromatography-electron ionization mass spectrometry; GLA, γ-linolenic acid.

EXPERIMENTAL PROCEDURES

A mixture of CLA FFA, CLA (FAME), and GLA were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Pure CLA isomers (*c*9,*t*11-18:2, *t*10,*c*12-18:2, *c*9,*c*11-18:2, and *t*9,*t*11-18:2) were obtained as FFA from Matreya Inc. (Pleasant Gap, PA). Acetonitrile and hexane were UV grade. Ethyl ether was anhydrous. BF_3 in methanol (catalog no. 3-3021) was obtained from Supelco (Bellefonte, PA); hydrazine hydrate (10217-52-4) and ethylene glycol (29,323-7) were obtained from Sigma-Aldrich (Milwaukee, WI). Solvents were distilled in glass grade.

Partial hydrogenation was carried out using published principles (13). Specifically, 0.5 g of GLA was stirred with 100 mL of 10% hydrazine hydrate in methanol for 2.5 h at 45°C. The solution was diluted with 100 mL H_2O and then acidified with 40 mL 6 N HCl. Partially hydrogenated FFA were extracted three times with 50 mL ether/petroleum ether (1:1). The combined extracts were dried over Na_2SO_4, concentrated to dryness with argon, and then conjugated (14) by addition of 75 mL of 6.6% KOH in ethylene glycol and heated under N_2 purge for 2 h at 150–160°C. The product was acidified with 6 N HCl until the pH was strongly acidic. FFA were extracted into ethyl ether/petroleum ether (1:1). FFA were methylated in 14% BF_3/methanol as previously described (18). The procedure of Eulitz *et al.* (16), i.e., the catalytic application of iodine and UV light, was used to prepare solutions that contained all the geometric (*cis/trans*) isomers of 6,8; 7,9; 9,11; and 10,12 CLA. *Cis/trans* geometries of the synthetic products in sample test portions were confirmed by GC-FTIR (19). Derivatives of 4,4′-dimethyloxazoline (DMOX) were prepared as described previously (3).

GC-FID. GC was performed by using a Hewlett-Packard 5890A instrument under the following conditions: column, 100 m × 0.25 mm i.d. CP-Sil 88 (Varian Analytical Supplies, Harbor City, CA) capillary; hydrogen carrier gas; helium makeup gas for the FID; temperatures (°C): injector 220, detector 280, column 75 for 2 min, then raised 5/min to 185 and held for 33 min, then raised at 4/min to 225. Samples were run in split (20:1) mode.

GC-electron impact (EI) MS. The GC-EIMS was performed using a gas chromatograph (Hewlett-Packard 5890, series II) coupled to a mass spectrometer (Autospec Q mass spectrometer) and a data system (OPUS 4000; Micromass, Manchester, United Kingdom). The GC-EIMS system utilized version 2.1 BX software. This system was used with a 50-m CP-Sil 88 capillary column as described previously (20). The GC-EIMS conditions were: splitless injection with helium sweep restored 1 min after injection; temperatures (°C): injector and transfer lines, 220; oven, 75 for one min after injection, then programmed at 20°C/min to 185, held there for 15 min, then programmed 4°C/min to 220, and held there for 45 min.

Ag^+ HPLC. Ag^+ HPLC separation of the CLA FAME was carried out using a Waters 2960 chromatographic system (Waters Associates, Milford, MA), equipped with a photodiode array detector (Waters 996) operating between 200 and 300 nm, and a Millenium 3.20 chromatography manager. Single chromatograms of CLA isomers were extracted at 233 nm. Three ChromSpher 5 Lipids analytical silver-impregnated columns (each 4.6 i.d. × 250 mm stainless steel; 5 µm particle size; Chrompack, Bridgewater, NJ) were used in series. The columns were conditioned with 1% acetonitrile/hexane each day before starting the analysis of test portions. The mobile phase, 0.1% acetonitrile and 0.5% ethyl ether in hexane, was prepared fresh daily and introduced isocratically at a flow rate of 1.0 mL/min. The column was equilibrated with the elution solvent for 60 min before starting test portion injections. The column head pressure was 1000 ± 50 psi. Typical injection volumes were 5–15 µL. Peaks were isolated by trapping using two semipreparative Ag^+ HPLC columns (10 i.d. × 250 mm stainless steel; 5 µm particle size; Chrompack) in series, with a 0.18% acetonitrile in hexane mobile phase, at 3 mL/min.

RESULTS AND DISCUSSION

Figure 1 shows a GC/FID chromatogram for FAME obtained after partial hydrogenation of GLA. The presence of methyl stearate (18:0) represents complete hydrogenation. The *cis*-6, -9, and -12 monoenes and the *cis,cis*-6,9, -6,12, and -9,12 nonconjugated dienes are also present in addition to unreacted GLA. It was advantageous to reduce as much of the GLA as possible because, during conjugation, it forms all-*trans,* fully conjugated octadecatrienoic acids, and the FAME of these compounds interfere with *cis/trans* CLA FAME in the Ag^+ HPLC chromatography. Figure 2 shows the GC-FID chromatogram obtained following the conjugation reaction. The stearate and the 18:1 FAME as well as the 6,12-18:2 FAME are unaffected by the isomerization with alkali. The *cis/trans* CLA produced in this reaction elutes between *ca.* 43 and 44.5 min. A new peak representing partially conjugated trienes is apparent. Fully conjugated triene FAME are also produced in small amounts, but the retention times (64–68 min) for these compounds are not included. This procedure, as well as the conjugation, was found to be highly reproducible. Figure 3 shows chromatograms obtained, after conjugation, by GC-EIMS of

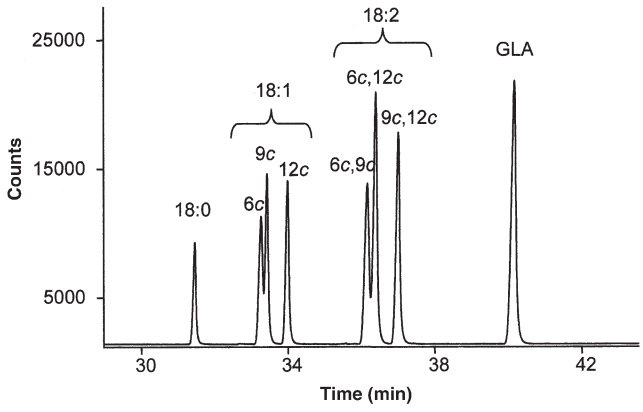

FIG. 1. Partial GC-FID chromatogram of methylated reaction product of γ-linolenic acid (GLA) partially hydrogenated with hydrazine. Peaks include the fully hydrogenated FAME, methyl stearate, 18:0, partially hydrogenated 18:1 and 18:2, and unreacted GLA.

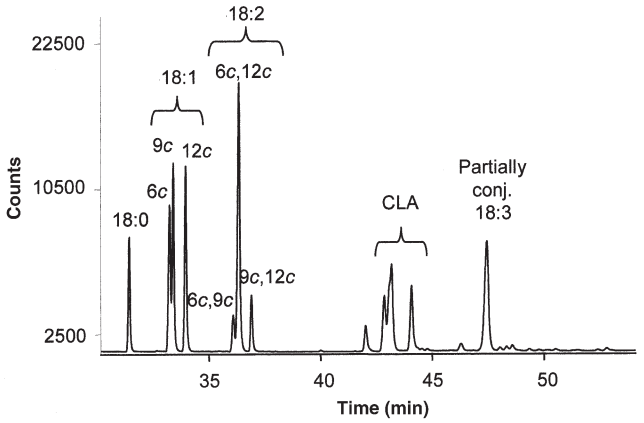

FIG. 2. Partial GC-FID chromatogram of the methylated reaction product of GLA after conjugation and remethylation. In addition to the peaks identified in Figure 1, CLA FAME and partially conjugated trienes are also present, although GLA itself is fully reacted. Unlabeled peaks were not identified. For abbreviation see Figure 1.

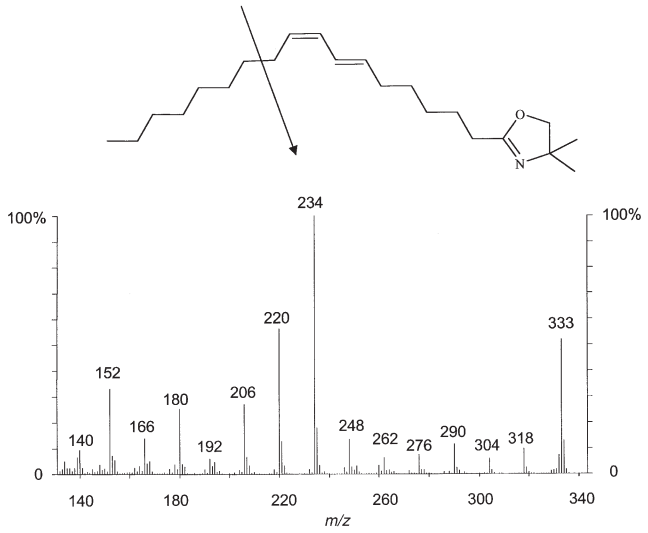

FIG. 4. Extended electron impact MS (EIMS), *m/z* 130–340, for the 7,9-18:2 DMOX. The indicated cleavage accounts for the base *m/z* at 234. For abbreviation see Figure 3.

the DMOX derivatives, using *m/z* values of 333 (A), 234 (B), and 220 (C) as masses selected for detection. Identifications of the 7,9-, 9,11-, and 10,12-CLA DMOX isomers have been described in detail (1,3,4,18). The *m/z* 333 trace in Figure 3A shows the molecular ion of CLA-DMOX. Ions at *m/z* 234 (Fig. 4) and 220 (Fig. 5) have been selected by the principle that the carbon chain allylic to the double bond is the favored radical site in the electron impact MS system (20). The *m/z* 234 ion is allylic for the 7,9 isomer (Fig. 3B) and the ion at *m/z* 220 is allylic for the 6,8 isomer (Fig. 3C). As indicated in the figure, the 6-*cis*,8-*trans* CLA elutes first, followed by the *trans*-7,*cis*-9 and the *cis*-9,*trans*-11 and *trans*-10,*cis*-12. This is consistent with longer retention times for longer equivalent chain lengths for these compounds. The *cis/trans* geometric configuration of all identified isomers has been confirmed by GC-FTIR.

The *cis*/*trans* assignments are made using the reported observation that when a *cis* bond shifts into conjugation, it converts (99%) to the *trans* configuration, and if a bond does not move, it retains its configuration (15). Thus, the *cis*-9 bond becomes *trans*-8 in the *cis*-6,*trans*-8 compound, and the *cis*-6 double bond becomes *trans* when it moves to the 7-carbon in the *trans*-7,*cis*-9 compound. As indicated above, extended mass spectra, *m/z* 110–340, for the *trans*-7,*cis*-9 and *cis*-6,*trans*-8 isomers are shown in Figures 4 and 5, respectively. Carbons in the chain allylic to the conjugated diene system are favored radical sites and produce more abundant fragmentation than other positions in the chain for the *trans*-7,*cis*-9 and *cis*-6,*trans*-8 isomers.

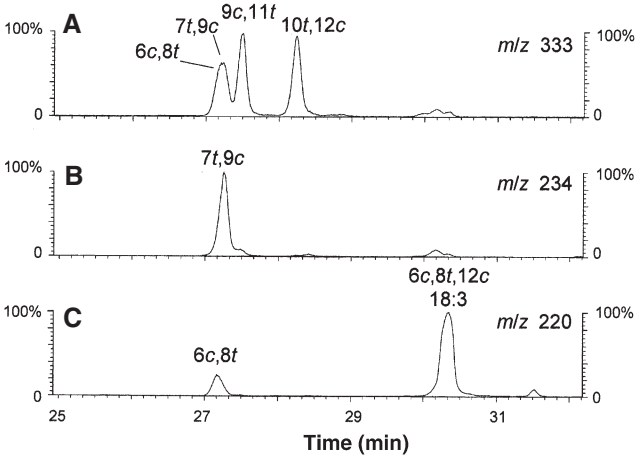

FIG. 3. Limited mass searches of partially hydrogenated, conjugated 4,4′-dimethyloxazoline (DMOX) derivatives of GLA for (A) *m/z* 333, the parent ion for DMOX 18:2 conjugated dienes; (B) *m/z* 234, the allylic cleavage of the 7,9-18:2 DMOX; and (C) *m/z* 220, the allylic cleavage of the 6,8-18:2 DMOX. For abbreviations see Figures 1 and 2.

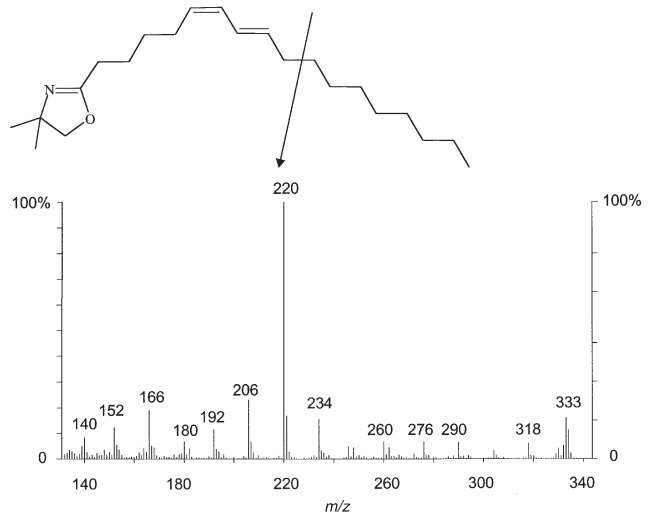

FIG. 5. Extended EIMS, *m/z* 130–340, for the 6,8-18:2 DMOX. The indicated cleavage accounts for the base *m/z* at 220. For abbreviations see Figures 3 and 4.

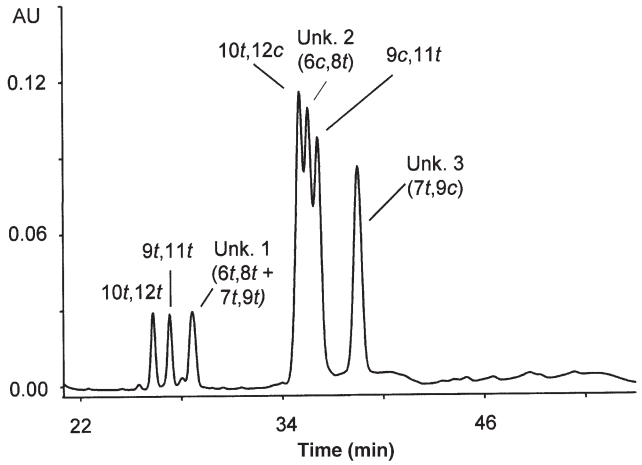

FIG. 6. Partial silver ion (Ag^+) HPLC chromatogram for partially hydrogenated, conjugated GLA FAME. References for the *c*9,*t*11 and *t*10,*c*12 were commercially available for their identification. Unknown peaks are identified in this work; see text. For abbreviation see Figure 1.

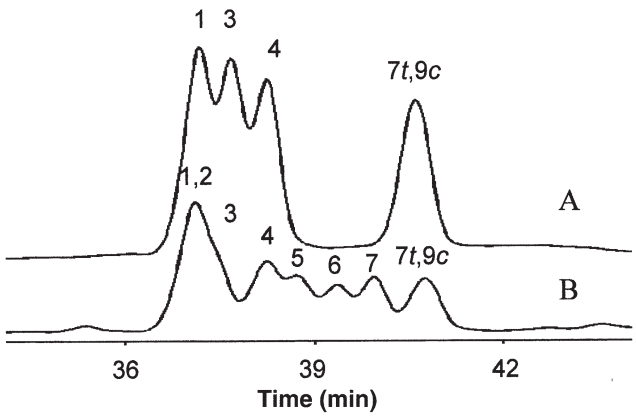

FIG. 8. Trace A: Ag^+ HPLC for partially hydrogenated, conjugated GLA FAME; trace B: partially hydrogenated, conjugated GLA FAME treated with I_2. Peak identifications: 1, *t*10,*c*12; 2, *c*10,*t*12; 3, *c*6,*t*8; 4, *c*9,*t*11; 5, *t*9,*c*11; 6, *c*7,*t*9; 7, *t*6,*c*8.

These abundant fragment ions make it difficult to measure a loss sequence of 12, 14, and 12 amu that facilitates assignment of the positions of the double bonds in the carbon chain for DMOX derivatives. A more thorough explanation has been reported (18,20).

Assigning the Ag^+ HPLC peaks that are indicated in Figure 6 to isomers that give rise to the GC peaks shown in Figure 3 was accomplished by trapping the peak, Unknown 3. This procedure was followed by GC-EIMS of the DMOX derivatives. Unknown 3 was found to be the 7,9-isomer by GC-EIMS. The purity of this trapped peak, 16 mg, was 98% by Ag^+ HPLC at 233 nm, and 70$^+$% by GC-FID. There was only one other major compound by GC, *viz*., *cis*-6-18:1. Figure 7 shows all four 7,9

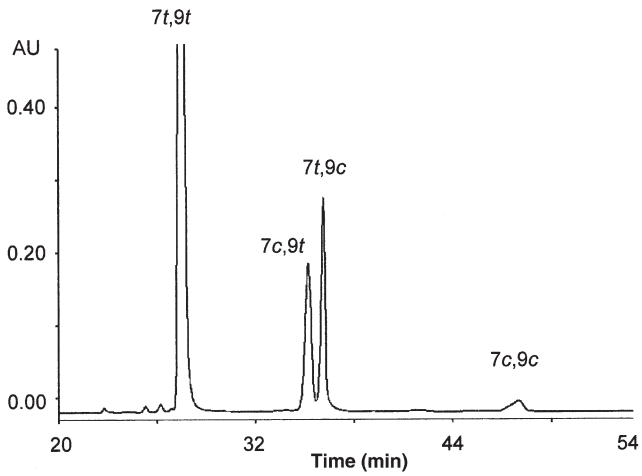

FIG. 7. Ag^+ HPLC for all four *cis/trans* isomers of 7,9-18:2 FAME, produced by the reaction of *trans* 7,*cis* 9-CLA with I_2.

cis/trans isomers obtained by isomerization with I_2 (17). Part of what constitutes the peak labeled Unknown 1 in Figure 6 is thus identified as *t,t*-7,9-CLA. Isomerization with I_2 of the trapped solution that did not contain the *t*7,*c*9 yielded the information that part of the peak labeled Unknown 1 was due to *t,t*-6,8 and that the peak labeled Unknown 2 was *c*6,*t*8. The partial Ag^+ HPLC chromatograms of the partially hydrogenated and conjugated methylated reaction product of GLA (A), and the same product isomerized with I_2 (B) are shown in Figure 8. As indicated in Figures 7 and 8, respectively, the elution order of the 7,9-isomers on Ag^+ HPLC is *t*7,*t*9, *c*7,*t*9, followed by *t*7,*c*9 and finally *c*7,*c*9 and the order of the 6,8 elution is *t*6,*t*8, followed by *c*6,*t*8 and *t*6,*c*8, and finally *c*6,*c*8.

Several techniques were needed to identify the components that gave Ag^+ HPLC responses. The identification of these peaks has now been made. The starting materials for this synthesis are readily available, and we have found all the procedures that we used to be readily reproducible. We believe that it will now be easy for other researchers to obtain a working reference, albeit in a mixture, of the *t*7,*c*9-CLA for future analytical research.

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Quantitative Characterization of Phospholipids in Milk Fat *via* ^{31}P NMR Using a Monophasic Solvent Mixture

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ABSTRACT: The phospholipids (PL) occurring in both ewe and cow milk fat globule membrane were identified and quantitatively determined using ^{31}P NMR spectroscopy with inverse gated decoupled sequences, which allowed a rigorous quantitative analysis. A strict relation between amount and distribution of PL and type of feeding was found. The method was calibrated over a mixture of PL standards. A recently introduced solvent constituted by a monophasic dimethylformamide/triethylamine/guanidinium hydrochloride solvent mixture was used. Compared to the traditional chloroform/methanol/water-EDTA solvent, the new solvent mixture shows very similar accuracy and precision from a quantitative point of view. The monophasic solvent overcomes the partition problems related to a biphasic system, and slightly enlarges the range of ^{31}P NMR chemical shifts, thus improving the resolution. In addition, the new solvent apparently displays a lower chemical shift dependence on the various PL concentrations. The limit of the method is mainly determined by the formation of adducts between triethylamine and some PL, namely, PE, monomethylphosphatidylethanolamine, phosphatidylethanolamine plasmalogens, and some lyso-PL. However, the new ^{31}P NMR signals arising from these adducts could be easily quantified in the determination of PE.

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The enormous number of scientific articles written in recent years that refer to phospholipids (PL) is symptomatic of the great importance that this class of molecules has in the medical, biological, biotechnological, and industrial fields (1). Indeed, PL are essential components of all vegetable and animal cells, and their implication in many biological processes has been demonstrated: A variation in their composition can be, for instance, related to serious pathologies such as cancer or Alzheimer's disease (2,3).

The most important sources of PL are soybean lecithin and, to a smaller extent, egg yolk lecithin. Commercial interests for these PL mixtures are mainly due to their emulsifying properties. These properties are strongly related to the composition of the PL mixtures, which, in turn, depends on the native biomass. Because of an increasing demand for biosurfactants for pharmaceutical and cosmetic applications, a growing interest in recovering PL from other kinds of biomass such as milk or byproducts of the dairy industry (buttermilk, for instance) has been observed. PL occur in milk mainly as constituents of the milk fat globule membranes (MFGM), even if small amounts of them can also be found in milk plasma as lipoprotein particles, which originate from membranes, microvilli, cytoplasmic organelles, and microbial and white blood cells (4).

PL extracted from the biological sources are usually identified using TLC followed by fluorescence detection or HPLC techniques equipped with spectrophotometric (UV-vis-diode array detector), refractive index, or evaporative light scattering detectors (5–7). In the last two decades, owing to the easy access to high-field NMR spectrometers, ^{31}P NMR spectroscopy has also become a competitive technique.

In the case of ^{31}P NMR PL analysis, the choice of solvent becomes a crucial factor since these molecules easily give self-association phenomena (generally bilayers arranged in lamellar liquid crystals) in both polar (water) and apolar (several oils) solvents, which produce a chemical shift (C.S.) anisotropy pattern in the ^{31}P NMR spectrum.

After Meneses and Glonek's pioneering work (8), the biphasic chloroform/methanol/water-EDTA solvent system came into common use in one-dimensional (1-D) and also two-dimensional (2-D) ^{31}P NMR analysis of PL (9,10). However, recently it has been demonstrated that significant variations in the C.S. of the PL signals can occur because of their dependence on the relative volume ratio of the three solvents, although good reproducibility can be obtained through careful sample preparation (11). C.S. variations dependent on PL concentration were also observed (9).

A recently introduced monophasic solvent (12,13), which is a mixture of dimethylformamide (DMF), triethylamine (Et_3N), and guanidinium hydrochloride (GH^+), was suggested as a means to avoid the problems mentioned previously, but no quantitative measurements were performed. Here, after a quantitative calibration for the NMR analysis on a mixture of standard PL and a comparison with the $CHCl_3/CH_3OH/H_2O$-EDTA solvent, the new solvent was used to characterize the PL in ewe and cow milk cream both qualitatively and quantitatively by ^{31}P NMR spectroscopy.

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Abbreviations: at, acquisition time; C.S., chemical shift; 1-D, one-dimensional; 2-D, two-dimensional; D1, delay time; DMF, dimethylformamide; DMPE, dimethylphosphatidylethanolamine; EPLAS, phosphatidylethanolamine plasmalogens; Et_3N, triethylamine; GH^+, guanidinium hydrochloride; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MFGM, milk fat globule membrane; MMPE, monomethylphosphatidylethanolamine; nOe, nuclear Overhauser effect; PG, phosphatidylglycerol; PL, phospholipids; SM, sphingomyelin; T_1, spin lattice relaxation time.

MATERIALS AND METHODS

Materials. All the organic solvents (acetone, benzene-d_6, chloroform, DMF, methanol, Et_3N, and trimethylphosphate), Na_4-EDTA, EDTA free acid, CsOH, the GH^+ reagent, and the PL standards of PC, PE, PS, PI, PG, PA, LPC, LPE, MMPE, DMPE, EPLAS, and SM (see list of abbreviations in Table 1) were purchased from Sigma-Aldrich S.r.l. (Milano, Italy) and used as received.

Milk cream was kindly supplied by a local dairy industry (F. Podda S.p.A, Sestu, Cagliari, Italy). The unpasteurized milk was obtained from ewes and cows in the month of February, preheated at 35°C, and centrifuged at 6000 rpm to obtain the milk cream.

The fat content of milk cream, tested with the classic butyrometric method, was 45 ± 2% for cow milk cream and 67 ± 2% for ewe milk cream (errors are reported in terms of SD).

Sample preparation. As mentioned previously, the analysis of PL from all biological sources requires a lipid extraction. Since the milk cream is essentially constituted of TG (about 98 wt%) and approximately only 1 wt% of PL, a deoiling procedure was performed (1) to enrich the samples in the PL fraction. Acetone, in which only apolar lipids dissolve, was added to the milk cream. Typically, 6 g of milk cream was dissolved in 420 mL of acetone. After centrifugation (15 min at 2500 rpm), the insoluble residue was collected. A Folch *et al.* (14) extraction procedure followed: The residue was homogenized in 20 times wt/vol (g/mL) of chloroform/methanol 2:1 (vol/vol) mixture. After filtration, the liquid extract was washed once in the same volume of a 0.1 N Na_4-EDTA/0.1 N NaCl solution (to avoid the presence of divalent cations) in a separatory funnel and allowed to separate overnight. Then the lower (organic) phase was collected, dried with anhydrous Na_2SO_4, filtered, and evaporated in a rotary evaporator at 37°C.

A new solvent mixture for NMR measurement was prepared by mixing 10 mL of DMF, 3 mL of Et_3N, and 1 g of GH^+, according to Reference 12. The $CHCl_3/CH_3OH/H_2O$-EDTA solvent was prepared, according to Reference 9, by mixing two reagents: (A) $CHCl_3$ containing 5% benzene-d_6 and TMP (1.31 μmol/mL), used respectively for internal deuterium field-frequency stabilization and as a C.S. and quantification reference; and (B) CH_3OH containing 0.2 M aqueous Cs-EDTA at pH 6 (4:1 vol/vol). The aqueous Cs-EDTA was generated by titrating EDTA free acid with CsOH to pH 6. The final analytical medium was obtained by adding 1 mL of reagent B to 2 mL of reagent A.

NMR measurements. NMR experiments were performed with a Bruker Avance 300 (7.05 T) spectrometer, equipped with a multinuclear probe operating at 121.495 MHz for the ^{31}P nucleus. The experiments were carried out at 25°C using a standard variable-temperature control unit with an accuracy of ±0.5°C. Solution (2.5 mL) was placed inside NMR sample tubes with an external diameter of 10 mm.

The ^{31}P NMR C.S. and spin-lattice relaxation time (T_1) values of most PL in the new NMR solvent mixture already have been reported (12). Here, the T_1 of several PL standards, particularly PS and SM, for which the T_1 were not known, were measured by the standard inversion recovery sequence 180-τ-90-at-D1 (where at and D1 are the aquisition time and the delay time between two consecutive pulses, respectively) by acquiring the partially relaxed spectra at 12–14 different τ values. T_1 values for SM and PS were found to be 0.74 ± 0.02 s and 0.69 ± 0.01 s, respectively. For the other PL, T_1 values very close to those previously reported were determined. Using the same sequences, the T_1 values of TMP, PG, PE, and PC were also detected in the $CHCl_3/CH_3OH/H_2O$-EDTA solvent: $^{TMP}T_1$ = 14.81 ± 0.01, $^{PG}T_1$ = 2.46 ± 0.01, $^{PE}T_1$ = 2.73 ± 0.01, and $^{PC}T_1$ = 2.09 ± 0.02.

For the quantitative analysis, the conditions adopted were such that the sum of at and D1 was more than five times T_1 (at + D1 > T_1). ^1H-decoupled ^{31}P NMR spectra were acquired by exploiting an inverse gated pulse sequence to suppress the nuclear Overhauser effect (nOe) and by using a 90° pulse (14.5 μs) for both solvents. An at = 0.8 s and a D1 = 7 s were chosen for the DMF/Et_3N/GH^+ solvent, whereas an at = 1.7 s and a D1 = 75 s were used for the $CHCl_3/CH_3OH/H_2O$-EDTA solvent. Usually, 3,000 scans were performed to achieve an optimal signal-to-noise ratio in the biological samples.

The quantitative analysis was carried out through an iterative fitting of the spectra (assuming a Lorentzian shape for the ^{31}P

TABLE 1
List of Abbreviations, Origins, and Formula Weights (FW) of the Phospholipid (PL) Standards

Acronym	Name	Origin	FW
PC	1,2-Dioctadecanoyl-*sn*-glycero-3-phosphocholine	Synthetic	790.2
DMPE	1,2-Dioctadecanoyl-*sn*-glycero-3 phospho(dimethylaminoethanol)	Synthetic	720.0
MMPE	1,2-Dihexadecanoyl-*sn*-glycero-3 phospho(methylaminoethanol)	Synthetic	706.0
PE	1,2-Di[(*cis*)-9-octadecenoyl]-*sn*-glycerophosphoethanolamine	Synthetic	744.0
PG	1,2-Dioctadecanoyl-*sn*-glycero-3-phospho-*rac*(1-glycerol)	Synthetic	796.1
PA	1,2-Dioctadecanoyl-*sn*-glycero-3-phosphate	Synthetic	727.0
LPE	L-α-Lysophosphatidylethanolamine (contains primarily stearic and palmitic acids)	Egg yolk	—
LPC	L-α-Lysophosphatidylcholine	Egg yolk	—
SM	Sphingomyelin (contains primarily stearic and nervonic acids)	Bovine brain	—
PI	L-α-Phosphatidylinositol (contains primarily stearic and arachidonic acids)	Bovine liver	—
PS	1,2-Dihexadecanoyl-*sn*-glycero-3-phospho-L-serine	Synthetic	758.0
EPLAS	Ethanolamine phosphatides (contains approx. 60% plasmalogen)	Bovine brain	—

NMR signals) to get the peak areas by use of the program Microcal™ Origin™ from Microcal Software (Northhampton, MA).

The ^{31}P NMR signal of PC was chosen as an internal reference (0 ppm) for the C.S. scale in the DMF/Et_3N/GH^+ solvent (0.37 ppm with respect to 85% orthophosphoric acid), whereas TMP were the internal reference for the C.S. scale in the $CHCl_3$/CH_3OH/H_2O-EDTA solvent (1.97 ppm with respect to 85% orthophosphoric acid).

RESULTS AND DISCUSSION

Qualitative analysis of standard PL. The DMF/Et_3N/GH^+ solvent mixture was tested with commercial standards of PC, PE, DMPE, SM, PI, and PG to ascertain the validity of a quantitative analysis performed through the ^{31}P NMR method. The PG and DMPE PL are absent in milk cream and were used as internal standards. PG was added to the milk cream to evaluate the efficiency of the extraction procedure (see below), whereas DMPE was added prior to the NMR experiment for the quantitative data analysis. Thus, the number of ^{31}P nuclei, i.e., the number of moles, belonging to each PL class was determined with reference to the weighed amount of the synthetic standard DMPE (see Table 1).

A sample containing about 1.0–1.5 mg of six different PL standards was dissolved in the DMF/Et_3N/GH^+ solvent. Figure 1A shows the ^1H-decoupled ^{31}P NMR spectra of this sample, where nine NMR signals can be observed.

In previous papers SM, when dissolved in the traditional $CHCl_3$/CH_3OH/H_2O-EDTA biphasic solvent, was reported to give a single NMR signal (at 11.75 T) (8), whereas by using the monophasic DMF/Et_3N/GH^+ solvent, a 7-T magnet gave a sufficient resolution (13) to display two signals at 0.846 and 0.833 ppm, respectively, as in the present work. No justification has been suggested. It should be noted that the standard SM from bovine brain used here contains primarily nervonic and stearic acids (see Table 1). Therefore, the two ^{31}P NMR signals are likely to arise from different SM species, one giving rise to the signal at the highest fields (0.833 ppm), containing C_{18} FA species (indeed, it is improbable that unsaturations produce a resolvable shift alteration) and another (0.846 ppm) containing C_{24} FA species. This deduction is also supported by the fact that a standard sample of SM from chicken egg yolk containing mainly palmitic acid displays two signals in the same solvent, one with very low intensity at 0.846 ppm (a very low amount of C_{24} occurs) and another with high intensity, attributed to the SM molecules having the C_{16} N-acyl chain, at 0.826 ppm. However, this point was not investigated further.

The two unknown signals (indicated as "U" at 0.17 and 0.19 ppm in Fig. 1A) were assigned on the basis of the following experiment. The monophasic solvent of the sample of Figure 1A was evaporated and the recovered PL were then dissolved in $CHCl_3$/CH_3OH (2:1 vol/vol). The ^{31}P NMR spectrum reported in Figure 1B, where only six ^{31}P NMR signals occur, was obtained. Evidently, the two unknown signals arise from some interaction of the PL with one or more components of the new solvent mixture. All the PL standards were separately

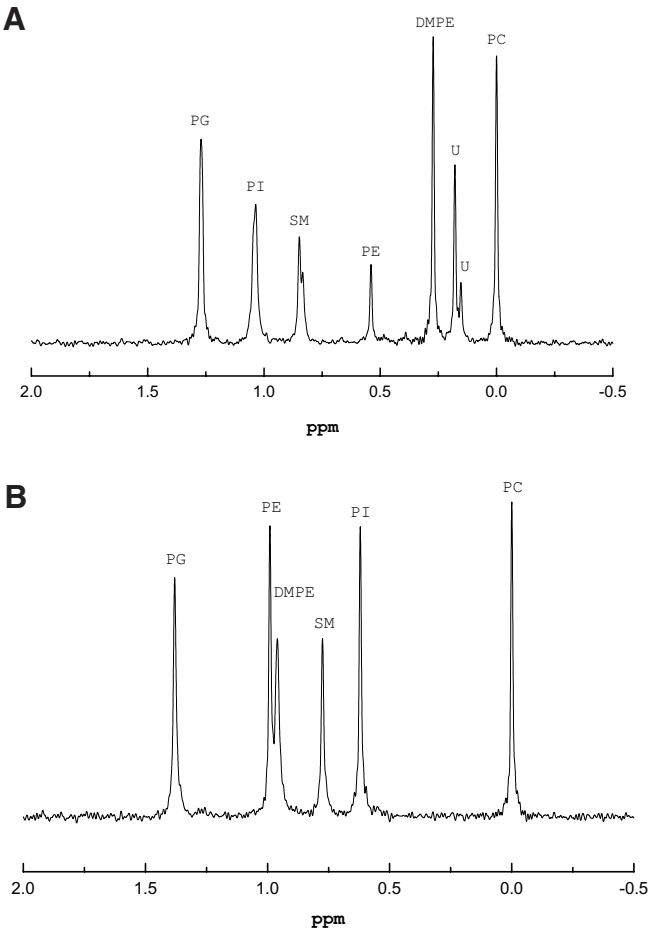

FIG. 1. ^1H-decoupled ^{31}P NMR spectra of phospholipid (PL) standards: (A) dissolved in the dimethylformamide/triethylamine/guanidinium hydrochloride (DMF/Et_3N/GH^+) solvent, (B) redissolved in $CHCl_3$/CH_3OH. U, unknown signal; see Table 1 for other abbreviations.

checked in DMF and/or in Et_3N, either in the presence or in the absence of GH^+. These analyses allowed us to establish that the solvent Et_3N interacts with MMPE and PE PL species. In Figure 2 the ^1H-decoupled ^{31}P NMR spectrum of the pure standard of PE dissolved in DMF/Et_3N is reported. This spectrum displays three signals. The spectrum of pure MMPE dissolved in DMF/Et_3N showed the same pattern, but the two high-field NMR signals were smaller. PC, DMPE, and PG PL showed only a single ^{31}P NMR signal in both solvents. As already mentioned, SM gives two signals. In addition, it was noted that in the case of PE and MMPE, the signal areas varied with time. These experimental findings suggest the formation of different adducts in which one or more molecules of Et_3N are linked to the amine protons of the polar head of PE and MMPE *via* hydrogen bonds. This interaction gives the two high-field NMR signals. However, the binding equilibrium is obtained through a rather slow process since variations in the signal areas were still observed 3 d after sample preparation. The amount of adducts formed by MMPE was smaller than that formed by PE as a result of the sterical hindrance of the methyl group and, for the same reason, the DMPE did not form any adduct, at least to an appreciable extent.

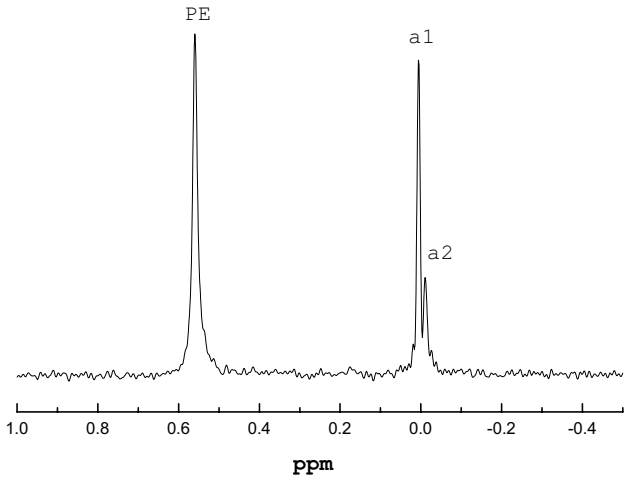

FIG. 2. ^{1}H-decoupled ^{31}P NMR spectrum of PE dissolved in DMF/Et_3N/GH^+. The ppm scale does not refer to any standard. a1, adduct 1; a2, adduct 2; see Table 1 and Figure 1 for other abbreviations.

Thus, the two unknown high-field NMR signals in the spectrum of Figure 1A can be substantially assigned to PE adducts (named a1 and a2 in Figs. 2 and 4) with the Et_3N solvent. If significant amounts are present, MMPE adducts can be quantified similarly to PE adducts. In milk fat this PL can reasonably be neglected (see below). EPLAS is likely to behave similarly, but the available standard for PE was impure (about 40 wt%); thus, only one "extra" resonance was assigned to this PL class (see the signal indicated as a3, at 0.23 ppm, in the inset of Fig. 4).

Qualitative analysis of standard lyso-PL. The ^{1}H-decoupled ^{31}P NMR spectrum of the LPC standard, dissolved in the DMF/Et_3N/GH^+ solvent, displays two signals, and the ratio of their areas is about 80:20. Similarly, two signals can be detected in the $CHCl_3$/CH_3OH (2:1 vol/vol) solvent, but the area ratio is about 97:3. It is well known that the lyso-PL easily undergo acyl migration processes (15). In water, the isomer composition at equilibrium is approximately 90 wt% for the 1-acyl isomer and 10 wt% for the 2-acyl isomer. Thus, the high-field resonance signal can be assigned to the 2-acyl lyso-PL. The difference in the ratios between the isomers in the two different solvents could be explained by considering that the rate of migration in lyso-PL strongly depends on the pH, and it occurs faster as the solvent is more basic (or more acidic).

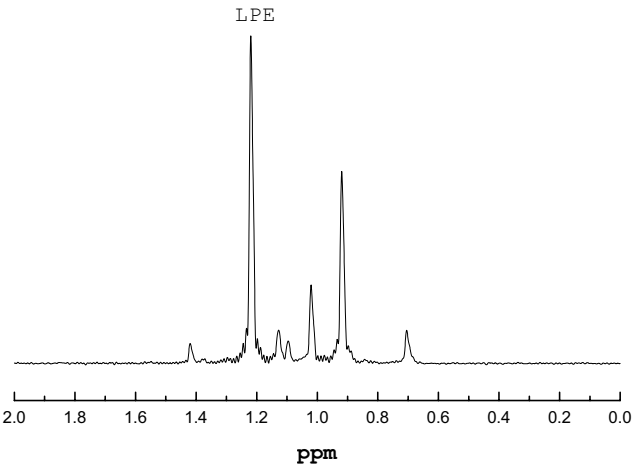

FIG. 3. ^{1}H-decoupled ^{31}P NMR spectrum of lysophosphatidylethanolamine (LPE) dissolved in DMF/Et_3 N/GH^+. See Table 1 and Figure 1 for other abbreviations.

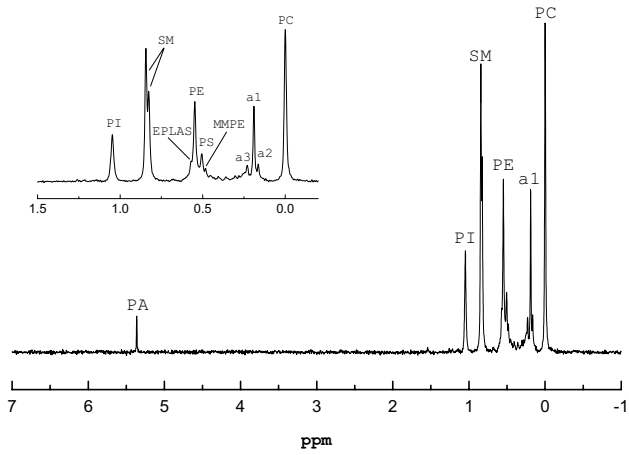

FIG. 4. ^{1}H-decoupled ^{31}P NMR spectra of a sample of a PL mixture obtained from ewe milk fat globule membrane (MFGM). a3, adduct 3; see Table 1 and Figures 1 and 2 for other abbreviations.

LPE displays only one signal in the $CHCl_3$/CH_3OH solvent and at least seven different NMR signals in the DMF/Et_3N/GH^+ solvent, as shown in Figure 3. As in the case of PE, it can be suggested that all the new resonances are caused by the interaction between Et_3N and the two acyl isomers of LPE, although no traces of the 2-acyl isomer were detected in the $CHCl_3$/CH_3OH solvent. Again, it can be suggested that the polarity of this DMF/Et_3N/GH^+ solvent mixture favors either acyl migration, as in the case of LPC, or other association–dissociation phenomena. However, this point was not investigated further since the analysis of lyso-PL is not essential in milk cream (they occur to a very small extent; see the following).

Quantitative analysis. An iterative fitting of spectra to evaluate the peak areas (see the Materials and Methods section) was used to perform a rigorous quantitative analysis of the NMR spectra.

Table 2 reports the C.S. and the experimental and calculated weights of two samples of PL standards dissolved in the DMF/Et_3N/GH^+ and in the $CHCl_3$/CH_3OH/H_2O-EDTA solvents obtained from NMR spectra acquired with the conditions described previously (see the Materials and Methods section). The errors are reported in terms of SD on the basis of five different NMR spectra recorded for each sample. The PI and SM standards were not pure PL, being mixtures of biological origin (see Table 1), thus their M.W. could not be calculated. The errors on the calculated weights of the standards ranged between 1 and 4 wt%. These determinations were performed in other samples containing variable amounts of the various PL standards. The accuracy of the calculated weight was always better than 5 wt%.

TABLE 2
Chemical Shifts (C.S.) and Quantitative Analysis Results of the PL Standard Samples[a]

DMF/Et_3N/GH^+				
PL	C.S.[b] (ppm)	P_{exp} (mg)	$mol_{calc}*10^{-6}$	P_{calc} (mg)
PG	1.27	1.596	2.06 ± 0.03	1.64 ± 0.03
PE	0.56	1.480	1.91 ± 0.07	1.42 ± 0.05
PC	0	1.422	1.84 ± 0.02	1.45 ± 0.01
DMPE[c]	0.27	1.330	—	—
PI	1.04	1.659	1.96 ± 0.03	—
SM	0.85	1.110	1.23 ± 0.03	—
$CHCl_3$/CH_3OH/H_2O-EDTA				
PL	C.S[d] (ppm)	P_{exp} (mg)	$mol_{calc}*10^{-6}$	P_{calc} (mg)
PG	0.49	1.382	1.76 ± 0.05	1.40 ± 0.03
PE	0.02	1.368	1.77 ± 0.03	1.32 ± 0.05
PC	–0.09	1.254	1.66 ± 0.04	1.31 ± 0.03
PI	–0.35	1.490	1.65 ± 0.07	—
SM	–0.85	0.813	1.10 ± 0.04	—

[a]Errors are reported in terms of SD. DMF, dimethylformamide; Et_3N, triethylamine; GH^+, guanidinium hydrochloride. See Table 1 for other abbreviations.
[b]PC is used as a C.S. reference.
[c]DMPE is used as a quantification reference.
[d]TMP is used as a C.S. and quantification reference.

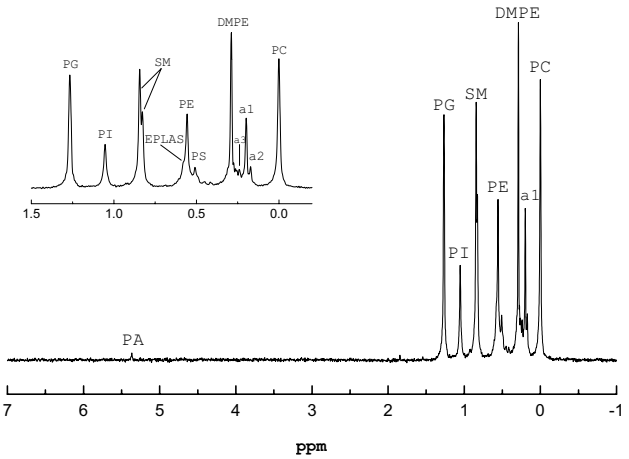

FIG. 5. ^1H-decoupled ^{31}P NMR spectra of a PL mixture obtained from ewe MFGM, where PG and DMPE were added as internal standards. See Table 1 and Figure 4 for abbreviations.

In particular, we noted that quantitative analysis based on the $CHCl_3$/CH_3OH/H_2O-EDTA solvent using TMP as the internal standard was notably affected by errors (>20%) when the NMR experimental settings suggested by Meneses and Glonek (8) and Glonek and Merchant (9) were applied (D1 = 1.86 s, 45° pulse). On the contrary, in the same condition very low errors (≤2%) were detected when expressing results in terms of mol%. These findings are undoubtedly due to the partial relaxation of the TMP ^{31}P nucleus under these conditions as a consequence of its long T_1 (see the Materials and Methods section).

MFGM PL analysis. Figure 4 shows the ^1H-decoupled ^{31}P NMR spectrum of PL of a ewe milk cream sample dissolved in the DMF/Et_3N/GH^+ solvent. The line width of each signal ranged between 1 and 2 Hz, and the resonances of all the main PL classes were well resolved (except for EPLAS and PE ^{31}P NMR signals, which were close to coalescence). On the basis of the qualitative analysis described above, the NMR signals between 0.1 and 0.4 ppm were assigned to adducts formed by PE (a1 and a2 signals in Fig. 4) and by EPLAS (a3 signal in Fig. 4).

As shown in Figure 4, PG and DMPE were not found in milk. Therefore, 2 mg of PG was added to the milk cream before its dissolution in acetone (see the Materials and Methods section) to obtain the extraction yield, whereas 1 mg of DMPE was added prior to the NMR analysis and used as an internal standard for the quantitative evaluation. Figure 5 shows the ^1H-decoupled ^{31}P NMR spectra of another sample of ewe milk (spectra of cow milk displayed the same features), where the two PG and DMPE standards were added. As in the case of the standard samples, the signals of the major PL classes were well resolved. On the basis of the PG addition, it was estimated that the yield from the extraction procedure of PL from MFGM was in the range of 45–50% for all the samples analyzed.

LPC, MMPE, and LPE species were not considered in the quantitative analysis since they gave many NMR signals that fell within a small, crowded region of the ^{31}P NMR spectra. It should be noted that LPC, MMPE, and, in particular, LPE occurred to a very small extent in the milk cream samples. Indeed, their NMR signals could hardly be detected, as shown in the inset of Figure 4. Hence, they may be neglected without causing significant errors when determining the distribution of the main PL.

PL classes in natural samples occur as a mixture of various molecular species differing in acyl side chain composition, and the precise M.W. is unknown. Each ^{31}P NMR signal contains the contribution of different molecular formulas and only the number of ^{31}P nuclei, that is, the number of PL moles, can be determined. The data in Table 3 show the results of the quantitative analysis performed on cow and ewe MFGM, respectively. These data refer to the average values obtained from three different samples for each type of milk. As expected, the most abundant PL were SM, PE, and PC in both types of milk. The same classes of PL were detected and the total amounts of PL were rather similar (the difference was within 2%). Some significant differences were noticed within the PC, PI, PS, and EPLAS PL classes. However, it should be noted that the PL content can be affected by the age, breed, diet, and stage of lactation of the animal (16).

In the data reported in Table 3, the milk cream samples were obtained from the dairy industry and could not be related to any specific feeding, age, or lactation stage.

Indeed, in the case of ewe milk, for which an analytical investigation as a function of the stage of lactation is in progress with six selected animals (same race, age, starting stage of lactation, and feeding), the amount and distribution of PL was shown to be strictly related to the type of feeding. Table 4 shows the variations in PL obtained from the milk cream of the six ewes, sampled before and 1 wk after introducing soybean lecithin into their diet. A significant increase in the total amount

TABLE 3
C.S. and Quantitative Analysis Results of PL in Cow and Ewe Milk Cream Samples[a]

		Cow milk cream		Ewe milk cream	
PL	C.S. (ppm)	mol*10^{-6} × 1 g	mol%	mol*10^{-6} × 1 g	mol%
PA	5.37	0.02	0.5	0.02	0.5
PI	1.05	0.55	14.0	0.36	9.4
SM	0.85[b]	1.05	26.8	1.09	28.3
EPLAS	0.58[b]	0.18	4.6	0.25	6.5
PE	0.55[b]	1.01	25.8	1.06	27.5
PS	0.51	0.06	1.5	0.15	3.9
PC	0	1.05	26.8	0.92	23.9
Total		3.92	100	3.85	100

[a]See Tables 1 and 2 for abbreviations.
[b]C.S. of the downfield signal. The quantitative analysis is based on the area of all the NMR signals assigned to the considered species.

of PL, more than 60% in terms of moles, was observed. Except for PA and EPLAS, less significant variations were found in the distribution of PL species in the cream.

SM was still the most abundant PL, but a decrease of 3 mol% was calculated. Indeed, SM does not occur in soybean lecithin, yet its content increased 50%.

Turning our attention to the validity of the method, a good precision of quantitative analysis was achieved for the most important PL in the milk cream samples. Moreover, without entering into questions on the mechanism that relates feeding and milk composition, in this context it is worth observing the ability of this NMR method to quantify PL in the submicromolar range and their variations due to feeding changes. Furthermore, this method is not time-consuming, especially when compared with the 2-D NMR approach. Indeed, 6 h was sufficient to achieve an optimal signal-to-noise ratio.

It is worth mentioning that the new solvent mixture slightly enlarged the C.S. range, overcame the problems related to a biphasic solvent, and apparently produced a lower C.S. dependence on PL concentration. Moreover, from a quantitative point of view, this solvent showed precision and accuracy very similar to those obtained using the $CHCl_3/CH_3OH/H_2O$-EDTA solvent. Although the quantitative analysis became less straightforward in the presence of adducts, with a careful determination of the deconvolution parameters a good performance could still be obtained, as shown for PE. Finally, the DMF/Et_3N/GH^+ solvent, together with an extremely easy preparation of the samples for the NMR analysis and the possibility of recovering the PL (12), seems suitable to distinguish between SM species with different N-acyl chains.

In conclusion, the results reported here demonstrate, in spite of the limits previously described, that the DMF/Et_3N/GH^+ solvent allows an accurate quantitative analysis of the most important PL classes in milk cream. Indeed, it can be taken into account as an alternative choice to the traditional $CHCl_3/CH_3OH/H_2O$-EDTA solvent system, especially in natural mixtures where plasmalogens and lyso-PL are minor components or totally absent, such as milk cream or, in general, products and by-products of the dairy industry.

TABLE 4
Effect of Ewe Feeding on the PL Distribution and Total Amount[a]

	Before lecithin feed		After lecithin feed	
PL	mol*10^{-6} × 1 g	mol%	mol*10^{-6} × 1 g	mol%
PA	0.01	0.4	0.05	1.3
PI	0.24	9.8	0.36	9.0
SM	0.87	35.5	1.30	32.7
EPLAS	0.03	1.2	0.12	3.0
PE	0.64	26.1	1.03	25.9
PS	0.08	3.3	0.15	3.8
PC	0.58	23.7	0.97	24.4
Total	2.45	100	3.98	100.1

[a]See Tables 1 and 2 for abbreviations.

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Combined Effects of EFA Deficiency and Tumor Necrosis Factor-α on Circulating Lipoproteins in Rats

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ABSTRACT: Both tumor necrosis factor-α (TNF-α) and EFA deficiency (EFAD) have been established as causes of marked perturbations in lipid and lipoprotein metabolism. Excessive levels of circulating TNF-α can coexist with EFAD in various clinical disorders such as cystic fibrosis and type I diabetes. The present study therefore aimed to investigate their combined effects on lipid profile and lipoprotein composition by administering TNF-α to EFAD rats. Lipoprotein lipase (LPL), the rate-limiting enzyme in TG catabolism, was also measured in epididymal adipose tissue. EFAD, after a 4-wk period, induced significant increases in plasma TG (80%, $P < 0.001$), total cholesterol (TC, 27%, $P < 0.025$), and HDL-cholesterol (HDL-C, 62%). Two hours after the administration of TNF-α, a further rise in TG (43%, $P < 0.05$) was noted in controls, but not EFAD animals. TC and HDL-C were unaffected by TNF-α treatment. In addition, TNF-α modified lipoprotein–lipid composition. VLDL and HDL_2 derived from EFAD rats were depleted in apolipoprotein (apo) E and apo A-II, and enriched in apo A-I 2 h after TNF-α administration. Finally, TNF-α decreased adipose tissue LPL activity in both control and EFAD animals. The TNF-α-induced inhibition was more marked in EFAD rats. The present results demonstrated that TNF-α can amplify or antagonize the effects of EFAD on lipid profile, lipoprotein composition, and LPL activity. These data also suggest that the host's nutritional status is a determining factor for the modulating effect of TNF-α on lipid metabolism.

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The pivotal role of tumor necrosis factor-α (TNF-α) in immune and inflammatory reactions, endotoxic shock, cachexia, and the regulation of cell growth has been clearly established (1–3). This pluripotent cytokine is implicated in several human diseases and induces a variety of metabolic and physiologic disturbances in response to infection (4,5). Among its numerous biological effects, TNF-α results in sustained hypertriglyceridemia, caused by enhanced hepatic lipogenesis, and the inhibition of lipoprotein lipase (LPL) activity (6–9).

Nutrient deficiencies or imbalances are also capable of causing profound metabolic derangements. A considerable body of evidence has implicated EFA deficiency (EFAD) as one of the major factors involved in the wasting that accompanies invasive and chronic diseases (10–12). Not only does EFAD impair growth and provoke anatomical and degenerative changes in the kidney, lung and liver, but, as with TNF-α, it can also induce metabolic derangements (10–12). EFAD in humans and animal models is accompanied by disturbances in lipoprotein concentration, composition, size, and metabolism (13–16). Like TNF-α, EFAD increases hepatic lipid synthesis and reduces the clearance of circulating lipoproteins *via* a decline in LPL activity (14,17).

Therefore, TNF-α and EFAD independently share the ability to trigger marked alterations in metabolism, particularly the development of hypertriglyceridemia. Moreover, both factors—excessive TNF-α and EFAD—coexist in various chronic disease states, including type I diabetes, cystic fibrosis, and inflammatory bowel disease (18–21). A significant positive correlation was also noted between TNF-α and plasma TG levels in EFAD patients with cystic fibrosis (20). Since EFAD and TNF-α characterize many diseases, we elected to determine whether they have additive or synergistic effects on lipid profile and lipoprotein composition. We also investigated the combined influence of EFAD and TNF-α on LPL activity, which is known to be highly modulated.

EXPERIMENTAL PROCEDURES

Animals and diets. Male Sprague–Dawley rats (80 g) were purchased from Charles River Breeding Laboratories (Montréal, Québec, Canada). The rats were allowed free access to water and food. After 1 wk of acclimatization, the rats were randomly divided into two groups: half (EFAD group) were assigned to an EFA-deficient diet (ICN Biochemicals, Cleveland, OH), and the other half were pair-fed (equicaloric) with a standard chow diet. The composition of the diets has been detailed previously (14). Pair-feeding was carried out to make up for decreased food intake in EFAD animals. Briefly, the EFAD diet contained only traces of n-3 family and 1.27% n-6 family compared to 9.87 and 48.14%, respectively, in normal diet. Saturated FA (97.68% vs. 22.39% in controls) compensated for the reduced levels of PUFA. However, normal and EFAD diets displayed a similar composition regarding sucrose, starch, casein, fiber, minerals, vitamins, DL-methionine, and choline. The rats were maintained at 22°C with a fixed lighting schedule (7 A.M. to 7 P.M.). Body weight and food intake were recorded three times weekly to monitor

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Abbreviations: apo, apolipoprotein; CE, cholesteryl ester; EFAD, EFA deficiency; FC, free cholesterol; HDL-C, HDL-cholesterol; IDL, intermediate-density lipoprotein; LPL, lipoprotein lipase; PL, phospholipid; PR, protein; TC, total cholesterol; TNF-α, tumor necrosis factor-α.

growth, and, as anticipated from the pair-feeding, no differences were observed in food consumption. After 4 wk on their respective diet, the overnight-fasted animals were injected with 25 μg of TNF-α in 0.5 mL of 0.9% saline or saline alone, *via* the jugular vein under anesthesia. The human recombinant TNF-α compound had a specific activity of 5×10^7 U/mg (kindly provided by Genentech, San Francisco, CA). All animal experiments were approved by the animal ethics committee from Hôpital Sainte-Justine, Montréal, Québec, Canada.

Isolation of lipoproteins. TNF-α affects the gastrointestinal tract and leads to anorexia (1–3). Its administration thus may disturb intestinal fat transport and thereby plasma lipid and lipoprotein concentrations. Therefore, all animals were fasted at the time of sampling to eliminate fat intake as a confounding variable and its potential influence on lipoprotein metabolism.

Blood samples were collected from the aorta at the time of sacrifice, 2 h after TNF-α administration, in tubes containing 1 mg EDTA/mL. Specimens were separated immediately by low-speed centrifugation ($700 \times g$ for 20 min) at 4°C. Lipoproteins were isolated by conventional discontinuous density gradient ultracentrifugation as previously described (14,22). Briefly, an initial centrifugation was performed to remove chylomicrons ($41,000 \times g$ for 30 min) by using a Beckman L5-65 ultracentrifuge with a Ti-50 rotor. Subsequently, VLDL, intermediate-density (IDL), and LDL lipoproteins were isolated at densities of 1.006, 1.019, and 1.063 g/mL, respectively, at $100,000 \times g$ for 18 h at 5°C. The separation of HDL subpopulations was performed at 100,000 $g \times 48$ h at the following densities: 1.125 g/mL for HDL_2 and 1.21 g/mL for HDL_3. The lipoprotein fractions were washed at their equilibrium density and dialyzed against 0.15 M NaCl, 0.001 M EDTA, pH 7.0.

Lipid and lipoprotein analysis. Plasma concentrations of total cholesterol (TC), free cholesterol (FC), and TG were measured enzymatically using a commercial kit (Boehringer Mannheim, Montréal, Canada) as reported previously (14,22). Cholesteryl esters (CE) were calculated as the difference between total and unesterified cholesterol × 1.7. Lipoprotein–protein (PR) was quantified according to Lowry *et al.* (23) with BSA as a standard. Phospholipids (PL) were determined by the Bartlett method (24). HDL-cholesterol (HDL-C) was measured after precipitation of VLDL and LDL with phosphotungstic acid (25). Apolipoprotein (apo) content of plasma lipoproteins was qualitatively assayed using SDS (26) as described previously (13,14). The gels were stained for 1 h with Coomassie blue and destained in 7% acetic acid. The bands for apo were identified by comparison with the mobility of apo standards and by standards of different M.W. Densitometric estimation was performed despite the potential differences in apo chromogenicity, since our studies are mostly comparative. We assumed that chromogenicity would affect to the same extent the two control and EFAD animal groups with and without TNF-α treatment.

Preparation of tissue for assay of lipoprotein lipase. Epididymal tissue was weighed and homogenized in ice-cold acetone using the Polytron homogenizer. The homogenates were centrifuged at $2,000 \times g$ for 10 min at 4°C, and the supernatants were discarded. The residues were re-extracted three times with 50 mL of ice-cold acetone and twice with diethyl ether. The defatted preparations were dried at 0°C under nitrogen, and designated "acetone powder." The defatted preparations were suspended in 0.025 M of NH_3-HCl buffer, pH 8.1, containing heparin (2 IU/mL). The clear supernatants obtained after centrifugation ($1,600 \times g$ for 10 min at 4°C) were used for LPL studies. LPL activity was assayed after the addition of serum as described previously (14) and was calculated as the difference between the total activity and the lipolytic activity remaining after the addition of 1 M NaCl in the assay mixture.

Statistical analysis. All values were expressed as the means ± SE. Statistical differences were determined by using ANOVA with *post hoc* Bonferroni test, with a P value of ≤0.05 considered significant.

RESULTS

Plasma FA composition. Striking alterations were noted in the FA profile of EFAD animals compared with controls. PUFA were markedly decreased while n-7 and n-9 families were increased (Table 1). The extensive changes led to an elevation of the commonly used indices for EFA deficiency (20:3n-9/20:4n-6 and 16:1n-7/18:2n-6).

Plasma lipids. As illustrated in Figure 1, higher lipid and lipoprotein concentrations were noted in the plasma of EFAD rats relative to controls. A significant increase in TG (80%, $P < 0.001$) and TC (27%, $P < 0.025$) was observed in the EFAD group compared with the control group. The raised TC levels were primarily associated with elevated concentrations of HDL-C (62%, $P < 0.025$).

A different TG response resulted from the injection of TNF-α in EFAD compared with control animals. TNF-α treatment led to a marked rise in TG in control (~160%, $P < 0.01$) compared with EFAD animals (48%, $P < 0.01$).

As shown in Figure 1, there was a tendency for the plasma cholesterol to be somewhat increased in EFAD animals compared with control animals. Following TNF-α treatment, TC moderately decreased in control and EFAD groups.

Lipid and apo composition of lipoproteins. The composition of the lipoprotein classes obtained with sequential ultracentrifugation is shown in Table 2. The VLDL fraction of EFAD rats was significantly enriched in TG and lower in protein when compared with VLDL of control rats. Changes were also observed in the IDL fraction, including a rise in PL

TABLE 1
Plasma FA Families and Ratios[a]

	Control	EFAD
Saturated, %	35.05 ± 0.43	39.51 ± 1.28
Total PUFA, %	50.05 ± 1.95	17.69 ± 5.34*
PUFA/saturated	1.43 ± 0.07	0.58 ± 0.03*
16:1n-7/18:2n-6	0.07 ± 0.01	1.38 ± 0.15*
20:3n-9/20:4n-6	0.02 ± 0.004	1.00 ± 0.15*

[a]Values represent means ± SE for six animals/group *$P < 0.01$. EFAD, EFA deficiency.

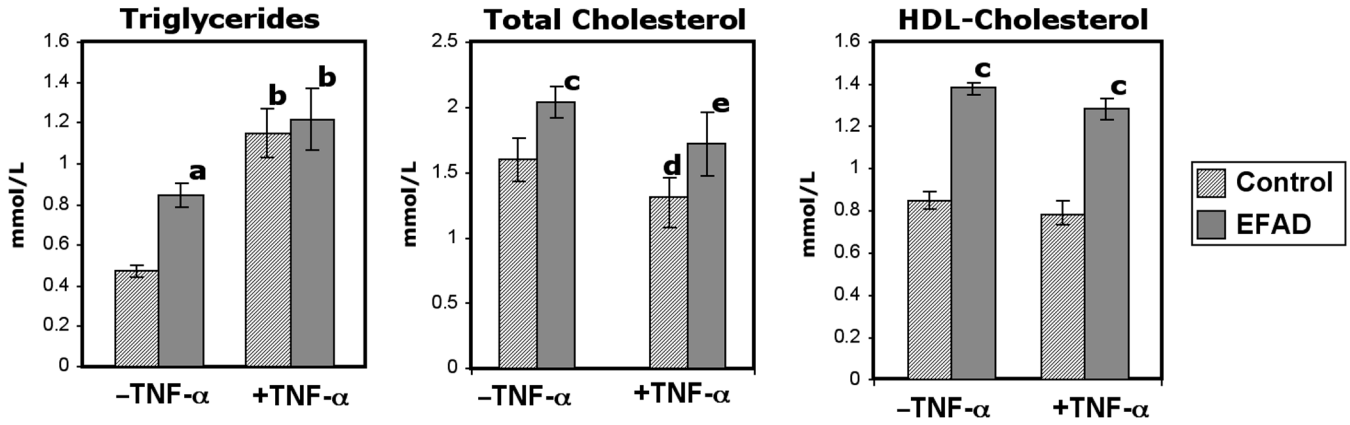

FIG. 1. The effect of tumor necrosis factor-α (TNF-α) on plasma TG, total cholesterol, and HDL-cholesterol in control and EFA deficient (EFAD) rats. Data are means ± SE of $n = 6$/group. [a]$P < 0.001$ vs. control (–TNF-α); [b]$P < 0.01$ vs. control (–TNF-α) and EFAD (–TNF-α); [c]$P < 0.025$ vs. control (–TNF-α); [d]$P < 0.025$ vs. EFAD (–TNF-α); [e]$P < 0.05$ vs. control (+TNF-α).

TABLE 2
Chemical Composition of Lipoproteins[a]

	Composition (mol %)					Ratio		
Lipoprotein	TG	CE	FC	PL	PR	TG/PL	CE/PR	(TG+CE)/(FC+PL+PR)
VLDL								
CONT	55.0 ± 2.0	6.3 ± 1.3	3.3 ± 0.5	13.3 ± 0.06	22.0 ± 2.7	3.71 ± 0.46	0.30 ± 0.06	1.61 ± 0.12
CONT+TNF	64.0 ± 0.7[a]	7.0 ± 0.5	2.7 ± 0.2	15.0 ± 0.4	20.8 ± 0.3[a]	4.29 ± 0.17	0.65 ± 0.04[a]	2.50 ± 0.03[a]
EFAD	63.8 ± 1.4	5.0 ± 0.1	3.0 ± 0.4	13.3 ± 0.2	15.0 ± 1.8	4.82 ± 0.10c	0.35 ± 0.04	2.22 ± 0.15[c]
EFAD+TNF	70.2 ± 1.1[b]	4.8 ± 0.7	2.5 ± 0.2	13.0 ± 0.2	9.2 ± 0.5[b]	5.41 ± 0.16[b]	0.54 ± 0.09[b]	3.05 ± 0.08[b]
IDL								
CONT	39.8 ± 4.2	15.3 ± 4.2	5.0 ± 0.6	14.5 ± 0.6	25.0 ± 0.7	2.78 ± 0.38	0.62 ± 0.18	1.24 ± 0.03
CONT+TNF	42.7 ±1.4	12.5 ± 1.1	3.7 ± 0.2	15.8 ± 0.8[a]	25.7 ± 1.8	2.74 ± 0.19	0.51 ± 0.08	1.26 ± 0.07
EFAD	40.3 ± 1.6	14.8 ± 2.1	6.5 ± 0.3	16.8 ± 0.5	21.8 ± 0.2	2.41 ± 0.06[b]	0.68 ± 0.10	1.22 ± 0.04
EFAD+TNF	48.5 ± 2.4[b]	6.8 ± 2.8[b]	6.8 ± 0.7	7.8 ± 1.2[b]	29.8 ± 1.7[b]	6.85 ± 1.18	0.24 ± 0.13	1.27 ± 0.11
LDL								
CONT	14.3 ± 2.8	32.3 ± 3.7	4.5 ± 0.3	18.8 ± 1.1	30.5 ± 1.5	0.78 ± 0.19	1.08 ± 0.16	0.87 ± 0.04
CONT+TNF	16.2 ± 0.3	31.6 ± 0.4	2.6 ± 0.1[a]	18.4 ± 0.5	32.2 ± 0.7	0.83 ± 0.05	1.00 ± 0.07	0.88 ± 0.03
EFAD	9.8 ± 0.7	33.0 ± 1.5	4.3 ± 0.2	18.9 ± 1.0	34.0 ± 2.7	0.52 ± 0.05	0.99 ± 0.11	0.76 ± 0.05
EFAD+TNF	9.3 ± 1.9	29.3 ± 2.2	4.2 ± 0.6	22.7 ± 1.3[b]	34.0 ± 3.5	0.43 ± 0.25	0.92 ± 0.13	0.65 ± 0.06
HDL_2								
CONT	0.49 ± 0.05	30.5 ± 0.65	1.5 ± 0.29	21 ± 0.65	46.5 ± 1.2	0.023 ± 0.002	0.66 ± 0.02	0.45 ± 0.015
CONT+TNF	0.74 ± 0.7[a]	31.7 ± 0.49	0.99 ± 0.18	23.7 ± 0.9	43.5 ± 0.77	0.031 ± 0.003	0.73 ± 0.02	0.48 ± 0.015
EFAD	0.21 ± 0.01	28 ± 0.41	0.99 ± 0.01	25.01 ± 0.75	45.3 ± 0.48	0.008 ± 0.005	0.62 ± 0.01	0.4 ± 0.006
EFAD+TNF	0.23 ± 0.02	28.3 ± 0.41	1.7 ± 0.17[b]	26.2 ± 0.53	43.5 ± 0.86	0.009 ± 0.008	0.65 ± 0.02	0.4 ± 0.007
HDL_3								
CONT	0.46 ± 0.09	23 ± 0.8	0.82 ± 0.07	14.00 ± 0.7	61.3 ± 1.45	0.032 ± 0.007	0.38 ± 0.02	0.31 ± 0.015
CONT+TNF	0.54 ± 0.12	23.03 ± 0.69	0.87 ± 0.17	17.2 ± 0.4[a]	58.0 ± 0.82	0.032 ± 0.008	0.39 ± 0.016	0.31 ± 0.001
EFAD	0.25 ± 0.01	23.3 ± 0.25	0.78 ± 0.04	17.5 ± 0.85	58.02 ± 0.7	0.015 ± 0.002	0.4 ± 0.005	0.31 ± 0.005
EFAD+TNF	0.18 ± 0.01	23.02 ± 0.53	1.22 ± 0.12[b]	21.5 ± 0.22[b]	54 ± 0.61[b]	0.008 ± 0.004	0.42 ± 0.014	0.30 ± 0.008

[a]Data are means ± SE ($n = 6$/group). CE, cholesteryl ester; FC, free cholesterol; PL, phospholipid, PR, protein; CONT, control; TNF, tumor necrosis factor; IDL, intermediate-density lipoprotein. [a]$P < 0.05$, vs. control; [b]$P < 0.05$, vs EFAD; [c]$P < 0.05$, vs. control.

and a decrease of proteins. HDL_2 and HDL_3 fractions were noted to have a relative drop of TG accompanied by an elevation of PL in EFAD compared with controls.

The administration of TNF-α produced several important modifications in lipoprotein composition (Table 2). An increased proportion of TG characterized the VLDL fraction of TNF-α treated animals ($P < 0.005$). A lower percentage of PR was also detected at 2 h post TNF-α administration regardless of the VLDL origin. These alterations led to greater ratios of TG/PL, CE/PR and (TG + CE)/(FC + PL + PR) at 2

h following the injection of TNF-α (data not shown). The mass ratio of core (TG + CE) to surface (FC + PL + PR) constituents can be used to make inferences on the size of spherical lipoproteins; lighter and larger particles are relatively enriched with core components when compared to the denser and smaller populations. Thus, the calculated values of these ratios indicate that VLDL particles were larger in EFAD, and that TNF-α led to even larger VLDL particles in the EFAD group 2 h after its administration.

In the IDL fraction, an increased PL content was noted 2 h after the injection of TNF-α in control rats. In contrast, both CE and PL were decreased, and TG and PR were increased in TNF-α-EFAD-treated rats.

With regard to the LDL fraction, the most important changes in control and EFAD groups after TNF-α administration were the decrease in FC and increase in PL, respectively (Table 2). Alterations were also observed in HDL_2 and HDL_3 fractions following TNF-α treatment, i.e., a rise in TG and FC in control and EFAD rats, respectively.

Apo distribution in isolated VLDL is illustrated in Figure 2. The 12.5% SDS-PAGE analysis showed an enrichment in the lower M.W. apo E and apo C isoforms in EFAD rats compared with the controls. Following the administration of TNF-α, apo A-IV appeared distinctly at 2 h in control animals only. Moreover, apo E isoforms with higher M.W. were reduced and apo E isoforms with lower M.W. were increased 2 h after the TNF-α treatment.

Apo changes were also noted in HDL subfractions on 15% SDS-PAGE (Figs. 3 and 4). HDL_2 in EFAD rats was enriched in apo A-II when compared with controls. Thus, a substantial reduction of apo A-I/A-II ratio in HDL_2 was noted in EFAD rats (Table 3). The administration of TNF-α led to a higher proportion of apo E and apo A-I in control animals. However, a different apo E profile was apparent in EFAD HDL_2 2 h after TNF-α administration. A deficient content in apo E, along with an enriched proportion of apo A-I, characterized the HDL_2 particles of EFAD rats.

With regard to the HDL_3 fraction, the relative content of apo A-I increased with TNF-α treatment (Fig. 4). As seen in Table 4, apo E and apo A-II increased in control rats.

Adipose tissue LPL activity. LPL is the rate-limiting enzyme in the removal process of TG from the circulation. Its activity was therefore measured to determine whether it could account for the hypertriglyceridemia observed. As illustrated in Figure 5, LPL activity was significantly decreased in EFAD rats compared with control rats. TNF-α decreased adipose tissue LPL activity in both control and EFAD groups of animals. However, the TNF-α-induced inhibition of LPL activity was more marked in EFAD rats (54.2%, $P < 0.025$) compared with control rats (20.3%).

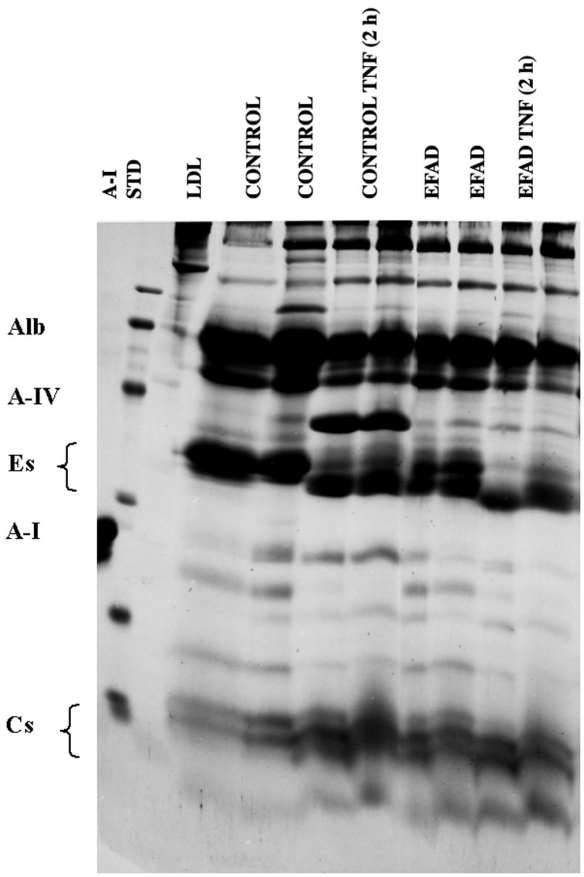

FIG. 2. SDS-PAGE (12.5% gels) of VLDL apolipoproteins (apo). The location of apo species (A-IV, Es, A-I, Cs) from EFAD rats and control rats was identified by comparison with rat apo LDL, purified apo A-I and M.W. standards (STD): phosphorylase B (97,400), BSA (66,200), ovalbumin (Alb; 45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). Apo profile was examined 2 h after TNF-α administration. See Figure 1 for other abbreviations.

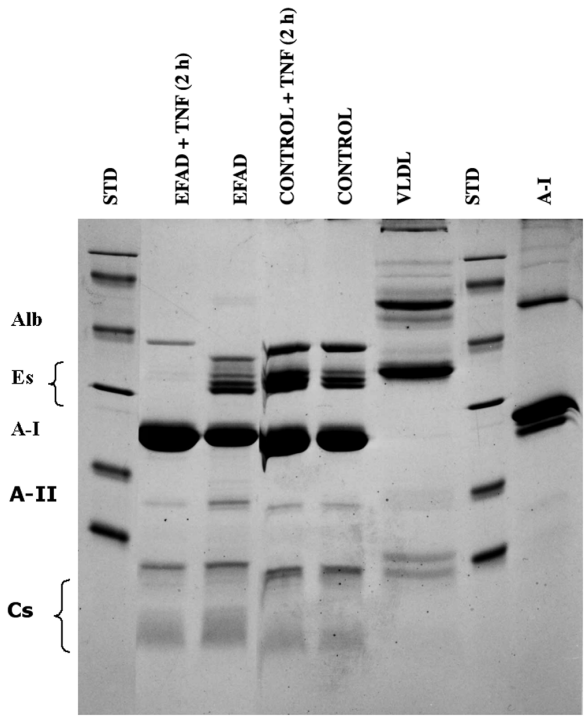

FIG. 3. Apo pattern of isolated HDL_2 from EFAD rats and control rats on 15% SDS-PAGE. Apo species were identified by comparison with rat apo VLDL, purified apo A-I, and M.W. standards as indicated in Figure 2. See Figures 1 and 2 for abbreviations.

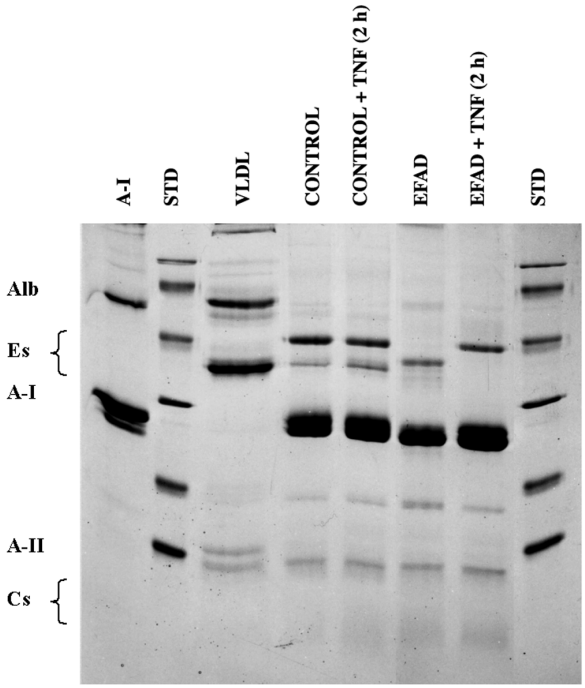

FIG. 4. Apo pattern and densitometric distribution of isolated HDL_3 from EFAD rats and control rats on 15% SDS-PAGE. The experimental details are included in the legend of Figure 2. See Figures 1 and 2 for abbreviations.

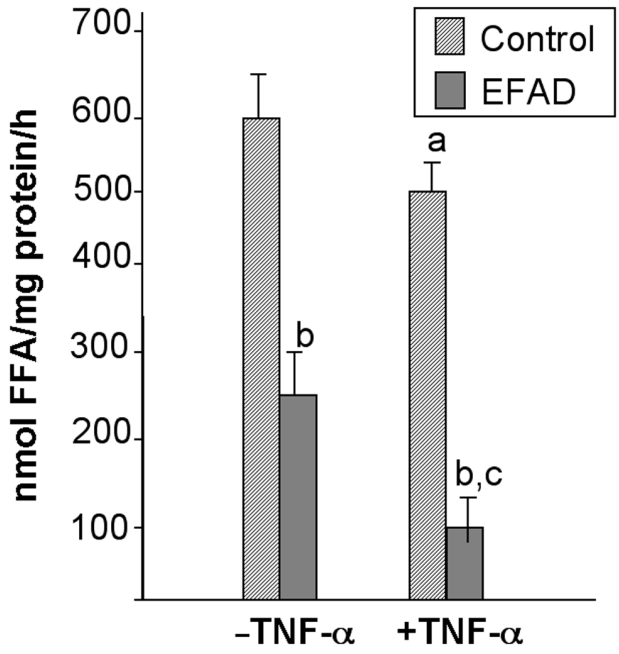

FIG. 5. The effect of TNF-α on epididymal lipoprotein lipase activity in control and EFAD rats. Data represent means ± SE for $n = 6$/group. [a]$P < 0.05$ vs. control (–TNF-α); [b]$P < 0.02$ vs. control (–TNF-α); [c]$P < 0.025$ vs. control (+TNF-α). See Figure 1 for abbreviations.

TABLE 3
Densitometric Evaluation of the Apo Pattern of Isolated HDL_2[a]

	Apo (%)			
Rats	E	A-I	A-II	A-I/A-II
–TNF-α				
Control	15.5	82.6	1.9	43.5
EFAD	13.9	82.3	3.8	21.6
+TNF-α				
Control	18.5	80.5	1.0	80.5
EFAD	1.5	97.7	0.7	139.5

[a]After separation by SDS-PAGE, HDL_2-apolipoproteins (apo) were used for the densitometric estimation of apo distribution. Values represent the means of two experiments carried out in duplicate. The variability between the specimens did not exceed 5–8%. See Table 1 for other abbreviation.

TABLE 4
Densitometric Evaluation of the Apo Pattern of Isolated HDL_3[a]

	Apo (%)			
Rats	E	A-I	A-II	A-I/A-II
–TNF-α				
Control	7.7	90.0	2.6	39.6
EFAD	12.4	82.9	4.7	17.6
+TNF-α				
Control	9.8	88.3	1.9	46.0
EFAD	16.3	81.4	2.3	35.8

[a]After separation by SDS-PAGE, HDL_3-apo were used for the densitometric estimation of apo distribution. Data represent the means of two experiments carried out in duplicate. The variability between the specimens did not exceed 5–8%. See Tables 1 and 3 for abbreviations.

DISCUSSION

Disturbances in lipoprotein metabolism occur in sepsis, inflammatory disorders, and malnutrition, as well as in a variety of other clinical conditions. These metabolic derangements are often attributed to obscure, multifactorial causes. Such alterations in lipid handling have been reported to be associated with EFAD, or to be mediated by proinflammatory cytokines. EFAD and increased plasma TNF-α levels contribute to the abnormalities in lipid and lipoprotein metabolism in cystic fibrosis (13,20,21). In the present investigation, experiments were designed to decipher the independent and combined effects of EFAD and TNF-α on lipoprotein concentration and composition in rats. By measuring plasma lipid levels and lipoprotein profile, we observed that each factor (EFAD or TNF-α) independently induced hypertriglyceridemia. However, EFAD condition attenuated the responses to TNF-α 2 h after its administration, when compared with the control animals. These results confirm that both EFAD and TNF-α induce hypertriglyceridemia but that their effects are not additive.

To study the dose-response effect of TNF-α on plasma lipid concentration, lipoprotein composition, and LPL activity, various concentrations of the cytokine were administered to control and EFAD rats in preliminary experiments (results not shown). The dose of 25 μg per animal was found to be optimal for producing hypertriglyceridemia and LPL activity inhibition. Our findings corroborate data previously obtained by Feingold and Grunfeld (6), and provided us with the effective TNF-α dose to be injected to experimental rats.

Striking differences were observed between control and EFAD groups regarding TNF-α-induced hypertriglyceridemia. TNF-α raised plasma TG levels significantly after 2 h in control rats, while the EFAD group experienced only a moderate TG increase. These results demonstrate that EFAD exerts an attenuating effect on the hypertriglyceridemia induced by TNF-α.

It is well recognized that some of the actions of cytokines are mediated by prostaglandins, and that prostaglandin synthesis inhibitors can prevent these effects (25–28). Feingold *et al.* (29) have shown that TNF-α increased lipolysis, resulting in an increase in circulating FFA levels. This stimulates hepatic TG production, thereby contributing to the TNF-α-induced hyperlipidemia. The addition of indomethacin, a well-known inhibitor of prostaglandin synthesis, prevented the TNF-α-induced increase in lipolysis. It might be possible that EFAD in our animal model inhibited the conversion of arachidonic acid to prostaglandins and other eicosanoids, important modulators of many physiological functions (30). Conceivably, the altered eicosanoid status resulting from EFAD depressed responses to TNF-α, thus moderating the expression of hypertriglyceridemia. Is it that EFAD brings about hypertriglyceridemia independently of changes in lipolysis? Alternatively, may we consider that TNF-α produces maximal hypertriglyceridemia because it blunts TG hydrolysis or increases TG production to a maximal possible degree, such that background hypertriglyceridemia in the EFAD rat does not impact the response to TNF-α? Additional studies are necessary to delineate the mechanisms by which EFAD exerts its effects.

In vitro studies have clearly established that TNF-α reduces LPL activity in cultured fat cells by inhibiting the synthesis of this enzyme (1,31,32). *In vivo* experiments have confirmed these observations, demonstrating that TNF-α induced an increase in circulating lipids by impairing adipose tissue LPL activity (8,9). On the other hand, other observations indicate that TNF-α may also increase serum TG levels *in vivo* by stimulating hepatic lipogenesis and VLDL production, rather than by inhibiting adipose tissue LPL activity and TG clearance (6,7,33). In our study, TNF-α produced hyperlipidemia by decreasing LPL activity, as measured in adipose tissue. However, we cannot exclude the potential role of hepatic lipogenesis in the observed hypertriglyceridemia, particularly under the influence of TNF-α. Nevertheless, adipose tissue LPL activity was markedly decreased in EFAD animals compared with controls after TNF-α administration. The EFAD condition thus does not resemble the situation obtained in diabetic rats, in which TNF-α treatment did not result in a further decrease in LPL activity (32).

Numerous studies have dealt with the effects of cytokines on TG metabolism (2,3,6–9,29,31–33). However, few laboratories have investigated the modulation of cholesterol metabolism by cytokines. Feingold *et al.* have reported the stimulation of hepatic cholesterol synthesis in mice by both TNF and interleukin-1 (34). In addition, this group has demonstrated increased serum cholesterol levels in parallel with an approximately twofold increase in hepatic HMG-CoA reductase activity in C57Bl/6 mice treated with TNF-α and interleukin-1β (35). Our data disclosed a limited effect of TNF-α on cholesterol levels in control rats, and no effect was observed in EFAD rats. The variability in animal species and experimental conditions may explain these differences.

Our results support the hypothesis that dietary factors can influence the mechanism by which TNF-α stimulates plasma TG elevation. In sucrose-fed rats, Feingold *et al*. (33) have shown that TNF-α administration did not increase plasma FFA or glycerol levels, indicating that TNF-α did not stimulate lipolysis under these dietary conditions. However, TNF-α did produce an increase in plasma TG levels. These observations indicate that in sucrose-fed animals, the TNF-α-induced hypertriglyceridemia is not dependent on adipose tissue lipolysis, but rather on *de novo* FA synthesis. Further experiments will be required to delineate other possible mechanisms, in addition to LPL activity, responsible for the TG rise in EFAD rats, in which elevated values of FFA were associated with hypertriglyceridemia (14).

As mentioned previously, changes in lipid metabolism as well as circulating lipoproteins levels and composition were induced by TNF-α and EFAD. In addition to lipogenesis and lipolysis mostly evoked, other potential mechanisms may be responsible for these cytokine-mediated abnormalities, including hepatic VLDL production and apo content, alterations in the activity of HMG-CoA reductase, cholesterol 7α-hydroxylase and LCAT and HDL composition (13–15,17,23,33,36–40).

In our study, TNF-α produced various modifications in lipoprotein–lipid composition and apo moiety. VLDL and HDL_2 derived from EFAD rats were depleted in apo E and apo A-II and enriched in apo A-I 2 h after TNF-α administration. The current results thus demonstrate that TNF-α can modulate apo composition. However, additional investigation is needed to examine the effects of EFAD and TNF-α on the composition and metabolism of the *d* 1.05 g/mL lipoprotein fraction that is enriched in apo E in rat. Another study has demonstrated that the lymphokine interferon-γ inhibited apo E production in human macrophages by posttranslational mechanisms, which included increased intracellular degradation and/or inhibition of secretion (41). Whether TNF-α specifically modifies the degradation of apo E, or its secretion by macrophages or numerous peripheral tissues, remains to be elucidated. Furthermore, additional studies are needed to comprehend the role of TNF-α in the expression of other apo.

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Antagonism of Croton Oil Inflammation by Topical Emu Oil in CD-1 Mice

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ABSTRACT: Emu oil is derived from the emu (*Dromaius novaehollandiae*), which originated in Australia, and has been reported to have anti-inflammatory properties. Inflammation was induced in anesthetized CD-1 mice by applying 50 μL of 2% croton oil to the inner surface of the left ear. After 2 h, the area was treated with 5 μL of emu, fish, flaxseed, olive, or liquified chicken fat, or left untreated. Animals were euthanized at 6 h postapplication of different oils, and earplugs (EP) and plasma samples were collected. Inflammation was evaluated by change in earlobe thickness, increase in weight of EP tissue (compared to the untreated ear), and induction in cytokines interleukin (IL)-1α and tumor necrosis factor-α (TNF-α) in EP homogenates. Although reductions relative to control (croton oil) were noted for all treatments, auricular thickness and EP weights were significantly reduced (–72 and –71%, respectively) only in the emu oil-treated group. IL-1α levels in homogenates of auricular tissue were significantly reduced in the fish oil (–57%) and emu oil (–70%) groups relative to the control group. The cytokine TNF-α from auricular homogenates was significantly reduced in the olive oil (–52%) and emu oil (–60%) treatment groups relative to the control group. Plasma cytokine levels were not changed by croton oil treatment. Although auricular thickness and weight were significantly correlated with each other (r = 0.780, $P < 0.003$), auricular thickness but not weight was significantly correlated with cytokine IL-1α (r = 0.750, $P < 0.006$) and TNF-α (r = 0.690, $P < 0.02$). These studies indicate that topical emu oil has anti-inflammatory properties in the CD-1 mouse that are associated with decreased auricular thickness and weight, and with the cytokines IL-1α and TNF-α.

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Although several patents have been issued based on the various biological properties of emu oil, only two studies using rodents as experimental models have reported on the anti-inflammatory properties of emu oil in peer-reviewed journals (1,2). Neither study evaluated tissue cytokine levels as biomarkers of inflammation. Two other studies looked at the anti-inflammatory and wound-healing properties of emu oil when applied topically (3,4).

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Abbreviations: EP, ear plug; IL, interleukin; TNF-α, tumor necrosis factor-α.

Emu oil studies. Emu oil is derived from both retroperitoneal and subcutaneous adipose tissue sites. One study in CD-1 mice found that the auricular swelling induced by applying 50 μL of 2% croton oil was significantly reduced 6 h after the application of 5 μL of emu oil, when compared to the control and the porcine oil groups (1). In this study, inflammation was measured by the weight difference between left (inflamed) and right (noninflamed) earplugs (EP). A second study, using both female out-bred Wistar and Dark Agouti rats with adjuvant-induced polyarthritis, revealed significant reductions in paw swelling (up to 84%) and arthritis score (up to 70%) upon exposure to emu oil (2). Although this anti-inflammatory effect of emu oil has now been reported in these two separate studies, the role of proinflammatory cytokines such as interleukin (IL)-1α and tumor necrosis factor-α (TNF-α) has not been investigated. Since it is well established that acute and/or chronic inflammation is mediated by expression of a host of proteins including the cytokines, it is hypothesized that the anti-inflammatory effect of emu oil may be associated with decreased levels of certain proinflammatory cytokines (5–7).

Role of FA in inflammatory responses. Particularly germane to the present communication are the reported findings that exposure to oils enriched in the n-9 FA, oleic acid, and the n-3 FA, such as α-linolenic acid, EPA, and DHA, is associated with anti-inflammatory activity when compared to the proinflammatory actions of the n-6 FA, linoleic acid (8,9). Moreover, both of these review articles describe several studies indicating that the anti-inflammatory activities of the n-9 and, in particular, the n-3 FA, are associated with reductions in levels of cytokines IL-1α, TNF-α, IL-6, and IL-1β. The FA composition of emu oil was unremarkable, especially when

TABLE 1
Content[a] of Major FA (%)

	16:0	18:0	18:1	18:2	18:3	20:5	22:6	Other
Emu oil	20	11	49	15	ND	ND	ND	5
Fish oil	16	3	21	3	ND	18	12	27
Flaxseed oil	5	4	20	13	58	ND	ND	0
Olive oil	8	3	84	4	ND	ND	ND	1
Liquified chicken fat	22	6	37	21	ND	ND	ND	14

[a]Values represent means of FA analyses performed in triplicate. ND, not detected (limits of detection <0.05%).

compared to other oils reported to have anti-inflammatory activity (Table 1). For example, emu oil contains significantly less of the reported anti-inflammatory FA oleic acid, α-linolenic acid, EPA, and DHA, than are found in olive oil, flaxseed oil and fish oil, respectively. Thus, it would appear that the anti-inflammatory properties of emu oil are probably not fully explained by the FA profile.

Model justification. Compared to other animal models, CD-1 mice were found to be highly susceptible to induction of cutaneous inflammation following exposure to croton oil (4,10), lipopolysaccharide (11), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (12), and petroleum distillates (13). High levels of inflammation were found when compounds were applied topically or administered as dietary component (14–17). Studies done with other mouse strains such as Balb/C or C57BL6/J did not produce as significant an inflammatory reaction to croton oil, as with the CD-1 mice (18–20). In addition, the CD-1 mouse strain lacks T4 cells, part of the natural anti-inflammatory defense mechanisms of the body to causative agents. Because of this, the inflammatory response to exogenous agents was more pronounced in the CD-1 strain than in other strains of mice.

Goals of the study. (i) Compare the anti-inflammatory properties of emu oil with other oils also reported to reduce inflammation (21–23); (ii) determine whether reported anti-inflammatory properties of these oils were associated with effects on proinflammatory cytokines. The role of various cytokines in the inflammatory process is well established (5,6,24). Cytokines are large groups of locally acting proteins involved in cell signaling during immune responses. The cytokines IL-1α, IL-1β, TNF-α, and IL-6 are often designated as proinflammatory cytokines. They are predominantly produced by circulating monocytes and tissue macrophages and mediate the host response to inflammatory stimuli.

EXPERIMENTAL PROCEDURES

Animals. Seven-week-old male CD-1 mice (Charles River Laboratories, Wilmington, MA) with a mean body weight of 25 to 28 g were housed in standard polycarbonate cages (33 × 23 × 12 cm) under controlled conditions of temperature (22 ± 0.5°C), RH (50%), and 12:12 light/dark cycle. Mice had free access to water and rodent chow (Ralston Purina, St. Louis, MO) and were allowed to adapt to laboratory housing for 1 wk before the commencement of the study. The care and treatment of the experimental animals conformed to the guidelines of the Institutional Animal Care and Use Committee of University of Massachusetts Lowell and *Guide for Care and Use of Laboratory Animals* (25).

Different treatment protocols. In this study, 60 mice were randomly assigned to six groups of 10, with the following designation: Group 1 = control (croton oil), Group 2 = emu oil, Group 3 = fish oil, Group 4 = flaxseed oil, Group 5 = olive oil, Group 6 = liquified chicken fat. Emu oil was obtained from LB Processors, LLC (Chapmansboro, TN) and the remaining oils were provided by ACH (Memphis, TN). These choices of oils for study were based on previous reports indicating that these oils possessed anti-inflammatory properties (23,26). Animals were anesthetized at the beginning of the study, with a combination of ketamine (100 mg/mL) (Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (20 mg/mL) (Bayer, Shawnee Mission, KS) at a dosage of ketamine/xylazine, 87 mg/kg/13 mg/kg body wt, delivered intramuscularly (27)

Induction and evaluation of inflammation. Croton oil (2%) was prepared for topical administration by addition of 20 μL of croton oil (catalog no. 6719; Sigma, St. Louis, MO) to 1-mL acetone. Fifty microliters of this preparation was slowly applied to the inner surface of the left pinna of the anesthetized mouse. Two hours later, 5 μL of emu, fish, flaxseed, or olive oil or liquified chicken fat was applied to the inflamed site with a Hamilton microliter syringe. The control group was left untreated. Six hours after application of oils to the site, blood was collected from anesthetized mice and plasma samples were prepared. Animals were then sacrificed using carbon dioxide gas for determination of auricular thickness, EP weight, and cytokine levels in auricular homogenates. Pilot experiments indicated that this schedule was optimal for determining the effect of different anti-inflammatory treatments. Thickness measurements of the left auricles were taken using a micrometer caliper (Control Co., Friendswood, TX) as reported by Lopez *et al.* (1). The final measurement was taken at 6 h posttreatment with emu, fish, flaxseed, or olive oil or liquified chicken fat. At the 6-h time point, the animals were euthanized using carbon dioxide. Uniform-sized auricular tissue EP were punched from both the auricles using a biopsy punch and weighed. The weight difference between the untreated right and the treated left EP indicated the magnitude of swelling posttreatment, as well as the reduction response.

Evaluation of cytokines. Exposed and unexposed EP were harvested and weighed at time of sacrifice, immersed immediately in liquid nitrogen, and stored at −80°C for further analysis. Levels of IL-1α and TNF-α mouse cytokines from plasma and tissue samples were measured by ELISA. Frozen ears were prepared for cytokine analyses as described by Wang and Stashenko (7). Frozen ear tissue samples were ground using a pre-cooled sterile mortar and pestle and the tissue fragments dispersed in 800 μL lysis buffer consisting of 100 μg/mL BSA (fraction V; Sigma), 100 μg/mL Zwittergent-12 (Boehringer Mannheim, Indianapolis, IN), 50 μg/mL gentamycin (Life Technologies, Rockville, MD), 10 mM HEPES buffer (Life Technologies), 1 μg/mL aprotinin (Sigma), 1 μg/mL leupeptin (Sigma), and 0.1 μM EDTA (Sigma) in RPMI 1640 (Mediatech, Herndon, VA). This process releases the cytokines from the cells. The supernatant containing the cytokines was collected after centrifugation and stored at −80°C until assayed. Mouse cytokine assays were carried out using commercially available ELISA kits obtained from the following sources: IL-1α (Endogen, Cambridge, MA; sensitivity 6 pg/mL) and TNF-α (3 pg/mL) from BioSource International (Camarillo, CA). Results were expressed as pg cytokine/mg tissue.

FA analyses of oils. For FA analyses of the various oils, a 300-μL aliquot of oil was mixed with 5 mL of methanol con-

taining 0.2% BHT. Ten milliliters of chloroform was added, and the sample vortexed for 30 s. After addition of 1.0 mL of 0.15 M NaCl, the mixture was vortexed again and centrifuged at 500 × *g* for 10 min. The top aqueous layer was discarded, and the bottom organic layer was stored at −80°C under N_2 in a glass vial with a Teflon-lined cap. Prior to analyses, samples were evaporated to dryness under N_2 and esterified as previously described (28) with Instant Methanolic HCl kit (Alltech-Applied Science, Deerfield, IL). The FAME profile was determined on a Hewlett-Packard model 5890 gas–liquid chromatograph, with a DB-23 column (30 m column length, 0.25 μm film thickness, helium carrier gas) (J&W Scientific, Folsom, CA).

Statistical analyses. Sigma Stat software (Jandel Scientific, San Rafael, CA) was used for all statistical evaluations. A one-way ANOVA was used to analyze all data. When statistical significance was found by ANOVA, the Student–Newman–Keuls separation of means was used to determine group differences. Correlations (*r*) between auricular thickness, EP weights, and IL-1α and TNF-α were performed using Pearson's product-moment correlation coefficient. To determine whether auricular thickness was correlated with levels of cytokines, the different oil treatments were combined to provide a sufficient sample size and greater range of values, as previously described (29). All values were expressed as mean ± SD, and statistical significance was set at the minimum $P < 0.05$ (30).

RESULTS

To optimize the time of inflammatory induction, treatment duration, and cytokine responses, a preliminary study was conducted in 10 CD-1 animals. From this preliminary study it was found that the inflammatory reaction to 2% croton oil application reached its peak at 2 h posttreatment, as reported by Lopez *et al.* (1). From other studies (21–23) it has been demonstrated that fish, flaxseed, and olive oils can elicit an anti-inflammatory response. The maximal anti-inflammatory responses for different oils were observed at 6 h following topical application of the oils. Although previous studies of inflammation have been reported in other strains such as Balb/c or C57BL6/J, these strains do not produce as significant an inflammatory response to croton oil as compared to the CD-1 animals (18).

The thickness and weight differences seen in this present study between the various groups are shown in Table 2. Compared to the untreated control group, auricular thickness reductions were statistically significant ($P < 0.05$) only in the emu group (−72%), although nonsignificant reductions occurred for fish oil (−50%), flaxseed oil (−50%), olive oil (−40%), and liquified chicken fat (−28%) groups.

Auricular weight reductions were maximum with the emu oil group (−70%), which again were statistically significant ($P < 0.05$), although reductions were also noted for fish oil (−54%), flaxseed oil (−46%), olive oil (−44%), and liquified chicken fat (−25%) that were not statistically significant (Table 2).

EP tissue concentrations of the two different cytokines IL-1α and TNF-α as measured by ELISA method are shown in Table 3. For IL-1α, statistically significant reductions were noted for the emu oil (−70%), and fish oil (−57%) groups ($P < 0.05$), with lesser reductions observed for olive oil (−49%), flaxseed oil (−43%), and liquified chicken fat (−24%) that were not statistically significant (Table 3). EP tissue levels of TNF-α were significantly reduced in emu (−60%) and olive oil (−52%) ($P < 0.05$)-treated groups, with lesser reductions observed for fish oil (−36%), flaxseed oil (−34%), and liquified chicken fat (−28%) (Table 3).

Plasma levels of both IL-1α and TNF-α showed no consistent pattern of response to any of the various treatments (data not shown). Whereas there was a significant association between EP thickness and weight ($r = 0.780$, $P < 0.003$) (Fig. 1), only EP thickness was significantly correlated with TNF-α ($r = 0.690$, $P < 0.02$) (Fig. 2) and IL-1α ($r = 0.750$, $P < 0.006$) (Fig. 3).

TABLE 2
Thickness and Weight Differences[a] of Ears in Mice Treated with Various Oils 2 h After Croton Oil Application and 6 h After Oil Treatment

Treatment	Thickness (mm)	Weight (mg)
Control	0.285 ± 0.023[a]	24.44 ± 6.45[a]
Emu oil	0.081 ± 0.009[b]	7.22 ± 1.45[b]
Fish oil	0.143 ± 0.013[a,b]	11.22 ± 1.84[a,b]
Flax oil	0.143 ± 0.025[a,b]	13.27 ± 4.13[a,b]
Olive oil	0.171 ± 0.026[a,b]	13.78 ± 1.76[a,b]
Liquified chicken fat	0.205 ± 0.022[a,b]	18.08 ± 2.00[a,b]

[a]Values are mean ± SD, $n = 10$. Values in a column not sharing a common superscript roman letter are significantly different at $P < 0.05$.

DISCUSSION

Application of emu oil at 2 h after croton oil exposure significantly reduced the degree of inflammation in the auricles of CD-1 mice, in agreement with previous work (1). In the present study, in addition to the comparison of auricular thickness and weights, the possible relationship between the cytokines IL-1α and TNF-α and the anti-inflammatory process in response to various treatments was investigated.

On the basis of the preliminary study and the current study conducted in CD-1 mice, it was demonstrated that auricular tissue levels of both IL-1α and TNF-α were the predominant

TABLE 3
Ear Plug Tissue Concentrations[a] of IL-1α and TNF-α in Mice Treated with Various Oils 2 h After Croton Oil Application and 6 h After Oil Treatment

Treatment	IL-1α (pg/mg)	TNF-α (pg/mg)
Control	307.2 ± 35.02[a]	79.25 ± 15.53[a]
Emu oil	92.3 ± 12.18[b]	31.74 ± 3.62[b]
Fish oil	132.2 ± 19.65[b]	50.67 ± 10.17[a,b]
Flax oil	173.9 ± 40.95[a,b]	52.61 ± 7.14[a,b]
Olive oil	155.9 ± 27.38[a,b]	38.27 ± 5.23[b]
Liquified chicken fat	227.7 ± 23.13[a,b]	56.85 ± 6.19[a,b]

[a]Values are mean ± SD, $n = 10$. Values in a column not sharing a common superscript roman letter are significantly different at $P < 0.05$. IL, interleukin; TNF-α, tumor necrosis factor-alpha.

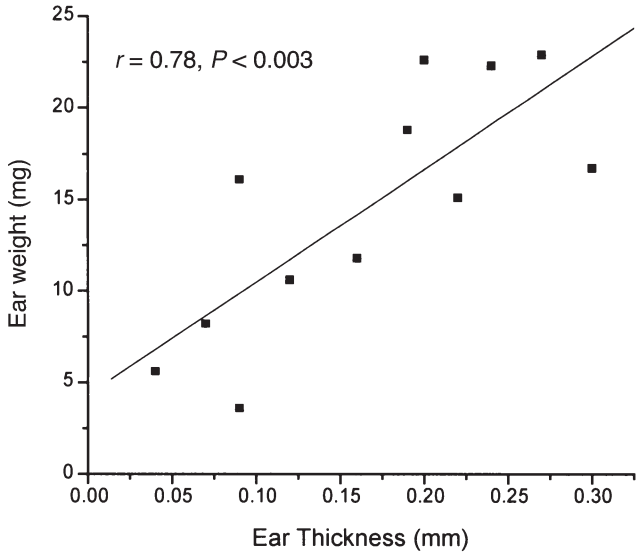

FIG. 1. Correlation between ear plug thickness and weight for all treatments.

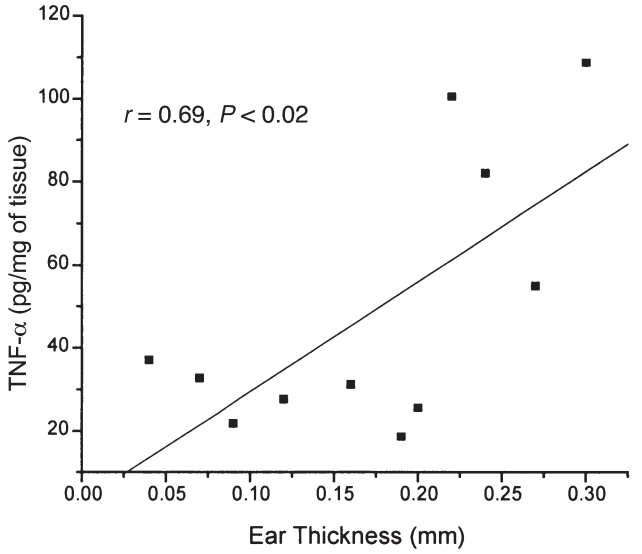

FIG. 2. Correlation between ear plug thickness and tumor necrosis factor-α (TNF-α) for all treatments.

cytokines responsible for this inflammatory induction, and the anti-inflammatory action of emu oil was directed toward these cytokines. The specific pathway for this action is not clearly identified, and it may depend on the levels and time of release.

It is noteworthy to look at this anti-inflammatory phenomenon from the viewpoint of the FA constituents of the different oils. The different oils used in this study contained either n-3 or n-9 PUFA. It has been reported that fish oil and other oils rich in n-3 or n-9 FA inhibit macrophage migration, an anti-inflammatory effect mediated through restriction of cytokine production (8,9). The n-3 FA also are known to decrease the levels of proinflammatory cytokines IL-1α, IL-6, and TNF-α and to increase the anti-inflammatory cytokine

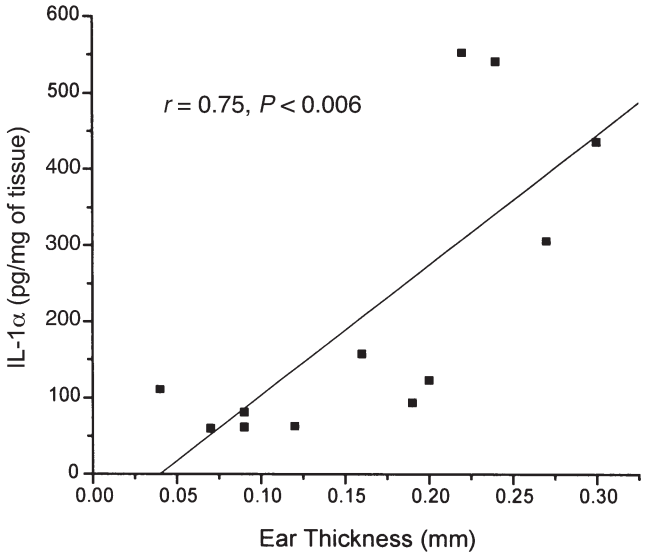

FIG. 3. Correlation between ear plug thickness and interleukin (IL)-1α for all treatments.

IL-2 (21). *In vitro* studies with n-3 FA reveal that one potential mechanism for the beneficial effects of these oils is associated with the suppression of cell-mediated immune responses (22).

Other dietary studies, which investigated the inflammatory response in the ears of mice sensitized with 2,4-dinitro-1-fluorobenzene, revealed immunosuppressive effects as measured by the degree of ear swelling in mice consuming a diet rich in DHA but not EPA. The effect of DHA but not EPA was also associated with reductions in the expression of IL-6, IL-β, and IL-2 mRNA. Fish oil contains variable amounts of EPA and DHA along with other FA, and thus it is not clear whether the reduced inflammatory response with fish oil noted in the present study was due to EPA, DHA, or both.

Although not statistically significant, the reduction in inflammation by the flaxseed oil-treated mice demonstrated in this study is in agreement with other studies of oils enriched in α-linolenic acid (21–23). But, in contrast to these dietary studies, in this study the different oils were applied topically. Further application of these findings will depend on studies done in human subjects.

Whereas it was possible from this study to demonstrate that emu oil's anti-inflammatory properties were associated with reductions in proinflammatory cytokines, it does not appear to be the result of its FA composition. Emu oil is enriched in monounsaturated FA but not to the extent of olive oil, and although it has low levels of the proinflammatory n-6 FA, linoleic acid (8,9), it is essentially devoid of the anti-inflammatory n-3 FA.

One report (3) does suggest that emu oil's anti-inflammatory activity may reside in its non-TG component, sometimes called the unsaponifiable fraction. However, the nature of the active ingredient(s) remains unresolved.

Results from these studies lead us to conclude that in CD-1 mice, topical emu oil is relatively more anti-inflammatory

than other oils or chicken fat. Direct measurements of decreases in thickness and weights of ear tissue treated with emu oil support this finding. In addition, the cytokine evaluations suggest one of the many pathways of the anti-inflammatory action.

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Dietary CLA and DHA Modify Skin Properties in Mice

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ABSTRACT: This study investigated the influence of PUFA on the properties of mouse skin. Mice (3 wk old) were given free access to oils high in linoleic acid, CLA, or DHA for 4 wk. At the end of the experiment, their skins were compared by both biochemical and histological methods. No significant differences in lipid and collagen contents were detected among treatments, although the FA composition in the skin was altered depending upon the FA composition of the supplemented oils. Electron microscopy revealed that the subcutaneous tissue layers in the CLA and DHA groups were significantly thinner than that in the high linoleic acid group, whereas no differences in the thickness of dermis layers were observed among the three groups. These results suggest that skin properties in mice are readily modified by dietary FA sources within 4 wk of dietary oil supplementation.

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Three layers, i.e., epidermis, dermis, and subcutaneous tissue, make up the skin. The epidermis has a role in protection from the external environment including bacteria and UV radiation. The dermis is made of collagen and elastin fibers, whereas the subcutaneous tissue (hypodermis) includes fat cells surrounded by collagen fibers. These layers are thought to maintain the elasticity, flexibility, and springiness of the skin. Collagen is present in higher amounts in the skin than in other organs (1), and lipids are also stored in the skin as adipose tissue. At present, the occurrence of obesity is gradually increasing due to excessive energy intake. Consequently, many people are trying to reduce their body weight and adipose tissue mass. However, rapid body weight loss or fat mass loss can lead to rough and moistureless skin similar to that induced by aging and UV radiation (2,3). Linoleic acid (LA) is an n-6 PUFA that is metabolized to arachidonic acid and eicosanoids. The importance of LA is confirmed by deficiency because ingestion of a fat-free diet induced growth depression, cornification of the skin, and kidney lesions in rats (4). This suggests that LA is one of the important factors in maintaining healthy skin. On the other hand, excessive LA may promote inflammation and allergic responses in various organs including the skin (5,6).

DHA is an n-3 PUFA and is reported to decrease skin inflammation and local cutaneous levels of leukotriene B_4 (LTB_4) (7). Dietary DHA is readily incorporated into the skin (8). A diet rich in n-3 PUFA, i.e., EPA (20:5n-3) and DHA, has been shown to lower plasma levels of prostaglandin E_2 (PGE_2) and LTB_4, thus lowering the inflammatory response (7,9). Dietary n-3 PUFA decreased UV radiation-induced immunosuppression and reduced susceptibility to UV radiation-induced skin tumors (10,11).

CLA is the generic term representing the positional and geometrical isomers of LA that are found mainly in food items produced from ruminant animals. CLA possesses properties that alter lipid metabolism (12–14). CLA decreases the weight of epididymal white adipose tissue and mesenteric adipose depots, whereas it increases the weight of the liver and spleen (15,16). CLA also blocks the desaturation of n-6 PUFA (17), thus decreasing arachidonic acid and PGE_2 (14). Furthermore, CLA inhibits the growth of cancer cells and induces apoptosis in the liver, adipose tissues, and cultured epithelial cells (14). However, no information is available concerning the effect of CLA on skin conditions.

The purpose of the present study was to compare the effect of LA, CLA, and DHA oils on skin properties such as collagen and lipid contents, FA composition, and thickness of the skin collagen layers and subcutaneous tissue layers using both biochemical and histological methods.

MATERIALS AND METHODS

Animals. Female mice (3 week old, Sea:ddY strain, purchased from Seac Yoshitomi, Fukuoka, Japan) were kept at 24°C on a 12-h dark/light cycle (9:00 A.M.–9:00 P.M.) and had free access to a commercial powder diet (MF; Oriental Yeast, Tokyo, Japan) and water. After 5 d of acclimation, mice were divided into three groups of 10 mice each according to their body weights. All mice consumed *ad libitum* the commercial diet mentioned above and drinking water. In all cages, a bottle separate from the water bottle and containing one of the oils

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Abbreviations: HLA, high linoleic acid; 17-HDoHE, 17-hydroxydocosahexaenoic acid; LA, linoleic acid, 18:2 n-6; LTB_4, leukotriene B_4; PGE_2, prostaglandin E_2.

was prepared. The mice had free access to these bottles, which contained high levels of LA (HLA), CLA, or DHA for 4 wk. Bottles containing oils were shaded by aluminum foil, and mice licked oil through a nozzle of the bottle. The bottle containing each oil was weighed weekly, and the amount of oil ingested was determined by measuring the disappearance of the oil from the preweighed bottle. The HLA group was used as a control and given HLA safflower oil (Rinoru Oil Mills, Nagoya, Japan), which contained 71.8% LA. Similarly, the DHA group was given an oil containing 70.0% DHA (DHA-70G; Nippon Chemical Feed, Hakodate, Japan). Mice in the CLA group were given an oil containing 70.8% CLA (Rinoru Oil Mills). This oil contained 32.4% 9*c*,11*t*-CLA and 33.3% 10*t*,12*c*-CLA. To prevent oxidation, 4 mg/g of (±)-α-tocopherol (Sigma Aldrich Japan, Tokyo, Japan) was added to each oil. The FA composition of these oils is shown in Table 1. Experimental procedures followed the guidance for Animal Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

Collection of skin tissues. At the end of the experiment, mice were killed by an overdose of sodium pentobarbital given intraperitoneally. Then, their whole skin (with hair) was stripped. Skin sheets (1 × 1 cm) were cut from the back of all mice for histology. Half of the remaining skin was used for collagen analysis, and the other half was used for FA analysis. After weighing, the skin was kept at –30°C until analysis.

Determination of collagen content. Skin samples were finely chopped and hydrolyzed in 20 mL of 6 N HCl for 24 h at 20°C, and then kept for 10 h at 120°C with a block heater (ALB-121; Asahi Technoglass, Tokyo, Japan). The hydrolyzed sample was filtered and adjusted to 100 mL with distilled water. The sample (1 mL) was evaporated with a centrifugal evaporator (CVE-3100; Eyela, Tokyo, Japan), and evaporated samples were diluted with distilled water. A 0.2-mL sample was mixed with 0.4 mL isopropanol, followed by mixing with 0.2 mL of oxidant solution (75% wt/vol chloramine T and acetate/citrate buffer, pH 6.0, were mixed at a ratio of 3:1). The mixed sample solution was kept for 4 min at room temperature (17–20°C). After the addition of 2.6 mL Ehrlich's reagent solution, the sample was incubated at 60°C for 25 min. After being cooled with water for 2–3 min, the sample solution was diluted with 6.6 mL isopropanol. Hydroxyproline concentration in the sample solution was determined using a spectrophotometer at 560 nm (18). The total collagen content was calculated by multiplication of hydroxyproline content by 7.25 (19) because skin collagen contains 13.3% hydroxyproline.

TABLE 1
FA Composition of Experimental Oils[a]

Oil	HLA (%)	CLA (%)	DHA (%)
16:0	6.5	6.6	2.7
18:0	2.5	2.5	1.1
18:1	17.1	16.7	1.6
18:2 (LA)	71.8	1.5	0.1
18:3	0.7	NG	0.1
CLA	NG	70.8	NG
9*c*,11*t*	NG	32.4	NG
10*t*,12*c*	NG	33.3	NG
9*c*,11*c*	NG	1.2	NG
10*c*,12*c*	NG	1.1	NG
t,*t*	NG	2.8	NG
20:0	0.3	0.3	0.4
20:1	0.3	NG	0.5
20:4	NG	NG	3.0
20:5	NG	NG	3.8
22:6 (DHA)	NG	NG	70.0
Other	0.8	1.6	16.7

[a]Abbreviations: LA, linoleic acid; HLA, high LA; NG, negligible; *c, cis*; *t, trans*.

Determination of total lipid content. Total lipid content was analyzed gravimetrically by the modified Folch *et al.* method (20). Briefly, a chloroform/methanol (1:2, vol/vol) solution was added to the skin sample and homogenized in a centrifugal tube. The mixture was centrifuged for 10 min at 850 × *g* to separate it into two distinct phases, and the lower phase was collected. The chloroform/methanol solution was again added to the upper phase followed by homogenization and centrifugation. Both fractions were filtered and mixed with KCl solution (0.9% wt/vol). After 5 min, the samples were centrifuged at 1250 × *g* for 10 min. Then the lower phase was added to a methanol/water (1:1 vol/vol) solution; after 5 min, it was centrifuged at 1250 × *g* for 10 min. After the volume of the lower phase was adjusted to 20 mL with chloroform, a portion (5 mL) was placed in a screw-capped glass vial and evaporated. Air in the screw-capped glass vial was replaced by gaseous nitrogen, and the resulting extracts were stored at –80°C until FA analysis (21–23).

FA analysis. FAME were prepared and their composition was measured by GC (HP5890II; Hewlett-Packard) according to Kamegai *et al*. (24).

Observation of skin layers. The skin samples were processed following a conventional procedure (25). Specimens were fixed in 3% glutaraldehyde diluted with phosphate buffer (pH 7.5) at 4°C. The fixed samples were soaked in 10% NaOH for 5 d and then in distilled water for 3 d. Samples were immersed in 1% tannic acid for 2 h, and then soaked in 2% osmium tetroxide solution for 1 h. Skin samples were dehydrated through a graded series of ethanol washes. Specimens were placed in *t*-butyl alcohol and freeze-dried (TIS-U-DRY; FIS Systems International, New York, NY). Samples were laid on aluminum holders and coated with Pt-Pd (Eiko IB-3 Ion Coater, Eiko, Japan), and examined under an ss-550 scanning electron microscope (Shimadzu) at the Center of Advanced Instrumental Analysis, Kyushu University. The thicknesses of the dermis and subcutaneous tissue layers on the photomicrograph were randomly measured at five points of each mouse sample. The boundary of these layers was determined by characteristics for the dermis and subcutaneous tissue. The dermis and subcutaneous tissue were defined as the layer composed only of collagen fibers and the layer having hollows surrounded by collagen fiber, respectively. Lipids in the fresh tissue before NaOH treatment filled the hollows.

Measurements of adipocyte size and number. The skin samples were fixed in 3% glutaraldehyde diluted with phosphate buffer (pH 7.5) at 4°C. The sample was immersed in 1% tannic acid for 0.5 h and soaked in 2% osmium tetroxide solution for 1 h. The remainder of the procedure was as described above. The cell size and number of the adipocytes in the subcutaneous tissue layer were measured. The adipocyte size was determined along the major axis of the cell. The numbers of adipocytes with diameters >10 μm were counted in 1 mm^2 and divided into groups with 10-μm intervals.

Statistical methods. Data were analyzed by one-way ANOVA. When significant effects were found, the three dietary groups were compared by Tukey's test. Significant difference was set at $P < 0.05$. The results are shown as means ± SEM.

TABLE 2
FA Composition of Total Skin

Group	HLA (%)	CLA (%)	DHA (%)
14:0	1.42 ± 0.08	0.80 ± 0.04	2.02 ± 0.11
16:0	15.6 ± 0.53	18.9 ± 0.60	26.3 ± 0.41
16:1	2.94 ± 0.37	1.97 ± 0.30	3.57 ± 0.37
18:0	3.32 ± 0.35	6.01 ± 0.42	6.03 ± 0.27
18:1	26.1 ± 0.53	30.3 ± 0.89	28.5 ± 0.81
18:2 (LA)	47.4 ± 1.50	18.4 ± 1.85	25.0 ± 0.67
18:3	0.65 ± 0.03	0.44 ± 0.09	0.82 ± 0.06
CLA	0.30 ± 0.06	17.2 ± 2.07	0.06 ± 0.02
9*c*,11*t*	0.20 ± 0.09	9.01 ± 1.07	0.06 ± 0.02
10*t*,12*c*	NG	6.73 ± 0.81	NG
c,*c*	NG	0.56 ± 0.06	NG
t,*t*	0.10 ± 0.03	0.93 ± 0.15	NG
20:0	0.18 ± 0.01	1.01 ± 0.24	0.35 ± 0.04
20:1	0.52 ± 0.06	1.21 ± 0.19	0.98 ± 0.08
20:2	0.28 ± 0.02	0.25 ± 0.02	0.25 ± 0.01
20:3	0.14 ± 0.01	0.22 ± 0.04	0.21 ± 0.01
20:4	0.71 ± 0.14	1.69 ± 0.21	1.08 ± 0.10
20:5	0.01 ± 0.01	0.10 ± 0.04	0.35 ± 0.04
22:5	0.01 ± 0.01	0.24 ± 0.04	0.20 ± 0.02
22:6 (DHA)	0.48 ± 0.08	1.29 ± 0.21	4.29 ± 0.66
Σ Saturated	20.5 ± 0.86	26.7 ± 0.23	34.7 ± 0.67
Σ Unsaturated	79.5 ± 0.86	73.3 ± 0.23	65.3 ± 0.67

[a]See Table 1 for abbreviations.

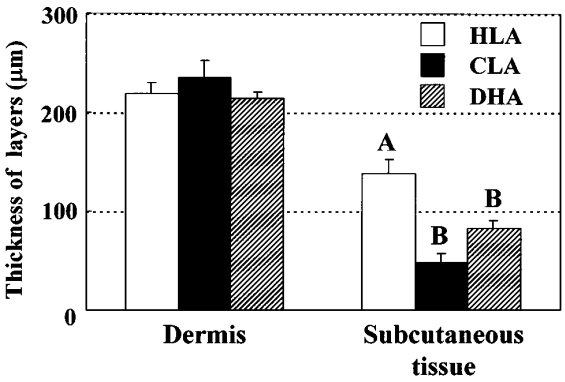

FIG. 1. The thickness of the dermis and subcutaneous tissue layers of mice administered linoleic acid (LA; $n = 9$), CLA ($n = 9$), or DHA ($n = 8$). Values are means ± SEM. Groups with different letters are significantly different ($P < 0.05$).

RESULTS

There were no significant differences [$F(2,27) = 1.138$, $P > 0.05$] in daily food intake among the HLA, CLA, and DHA groups (5.20 ± 0.06, 5.40 ± 0.12, and 5.16 ± 0.17 g, respectively). There was also no difference in daily oil intake [$F(2,27) = 0.536$, $P > 0.05$], which was 1.12 ± 0.02, 1.16 ± 0.04, and 1.16 ± 0.04 g in the HLA, CLA, and DHA groups, respectively. The total collagen concentration (mg/g tissue) in the skin of the HLA, CLA, and DHA groups was 30.3 ± 2.4, 34.2 ± 5.5, and 44.5 ± 4.3, respectively. The level for the DHA group tended to be higher than that for the other two groups, but the effect was not significant [$F(2,12) = 2.964$, $P = 0.0899$]. The total lipid content of the skin did not differ [$F(2,12) = 0.228$, $P > 0.05$] among treatments. The values (mg/g tissue) were 25.8 ± 6.4 in the HLA group, 23.5 ± 4.1 in the CLA group, and 27.8 ± 2.3 in the DHA group.

The FA composition of the skin was clearly altered depending on the PUFA administered (Table 2). In the HLA group, the LA level was the highest followed by oleic acid (18:1n-9) and palmitic acid (16:0n-9). In the CLA and DHA groups, the three major FA (i.e., oleic acid, palmitic acid, and LA) were present similarly to the HLA group. A large amount

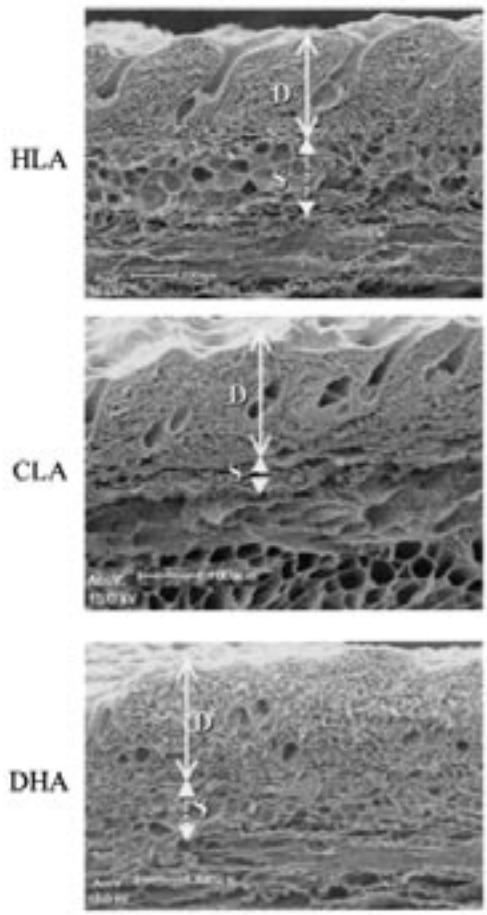

FIG. 2. Representative scanning electron microscopic photographs of skin of mice administered linoleic acid, CLA, or DHA. Abbreviations: D, dermis layer; S, subcutaneous tissue layer. Bars indicate 100 μm.

of CLA was found in the CLA group, whereas the CLA level was very low in the other two groups. The DHA group had the highest concentration of skin DHA.

Figure 1 shows the thicknesses of the dermis and subcutaneous layers; representative scanning electron microscopic photographs are shown in Figure 2. No significant [$F(2,23) = 3.422$, $P > 0.05$] difference in the thickness of the dermis layers was detected among the groups. On the other hand, the subcutaneous tissues were significantly thinner [$F(2,23) = 3.422$, $P < 0.05$] in the CLA group and thickest in the LA group.

The frequency of adipocytes 10–20 μm in diameter was highest in the CLA and DHA groups, whereas the frequency of cells in each group ranging from 10 to 60 μm was constant in the HLA group (Fig. 3). The percentage of cells 10–20 μm in diameter vs. the percentage in the remaining groups was 18.1 ± 2.9 and 81.9 ± 2.9 in the HLA group, 55.4 ± 10.9 and 44.6 ± 10.9 in the CLA group, and 63.5 ± 10.7 and 36.5 ± 10.7 in the DHA group. Electron micrographs of the adipocytes in each group are shown in Figure 4. The numbers of adipose cells did not differ [$F(2,26) = 3.369$, $P > 0.05$] among the HLA (376 ± 58), CLA (214 ± 34), and DHA (508 ± 151) groups. However, the number of adipocytes in the CLA group was the lowest, and that in the DHA group was 2.3 times higher than that in the CLA group.

DISCUSSION

The skin is the largest organ in the body and has a wide surface area. Subcutaneous tissue plays a role in the flexibility between skin and the internal body. Excessive fat accumulation is associated with several diseases. Dietary CLA reduces the accumulation of adipose tissue in the abdomen of experimental animals (16,26,27). In the present experiment, the total lipid contents of the skin were not affected by oil sources. However, the electron microscope clearly revealed that the subcutaneous tissue was thinner in the CLA group than in the other two groups. This fact suggests that CLA reduced the adipose tissue in the skin subcutaneous tissue as previously observed in the abdomen. Adipocytes in the CLA and DHA groups were smaller than those in the HLA. The frequency of adipocytes that were 10–20 μm in width was greater than those of other sizes in the CLA and, in particular, the DHA groups. Furthermore, adipocytes between 20 and 50 μm in width in the HLA group were swollen and huddled. This suggests that CLA and DHA might prevent the accumulation of lipid in adipocytes of the subcutaneous tissue compared with HLA. Belzung *et al.* (28) reported that DHA limited the hypertrophy of fat. The number of adipocytes was lowest in the CLA group and highest in the DHA group. In addition, there was a greater frequency of cells between 10 and 20 μm in diameter. These results could explain the thinner layer of subcutaneous tissue in the CLA group.

The FA composition of the skin was also altered by oil sources, and was dependent on the FA composition of the ingested oil. About half of the FA in the HLA group was LA. In contrast, the DHA content in the DHA group was <5% of the total FA. These results suggest that incorporation of LA into the skin occurs more easily than incorporation of DHA. Katan *et al.* (29) reported that incorporation of DHA was erratic and lower. They suggested that different n-3 FA were incorporated with different efficiencies, possibly because of interconversions or different affinities of the enzymatic pathways involved. On the other hand, Carine *et al.* (8) reported that the amounts of DHA incorporated were higher in the liver and remaining carcass, including skin. CLA was clearly incorporated into the skin lipid because ~17% of CLA was detected in the skin. Bee (30) estimated that the transfer efficiency of dietary CLA isomers was 41–52% for the back fat of sows; incorporation and uptake efficiency seemed to be selective, with the highest values found for 9*c*,11*t*-CLA. The efficient uptake of 9*c*,11*t*-CLA was confirmed in the present study. The CLA source used contained 32.4% 9*c*,11*t*-CLA and 33.3% 10*t*,12*c*-CLA. However, the composition of the skin was 1.3 times higher in 9*c*,11*t*-CLA than in 10*t*,12*c*-CLA. On the other hand, the thinner subcutaneous tissue in the CLA group could not be explained by the efficiency of incorporation. According to

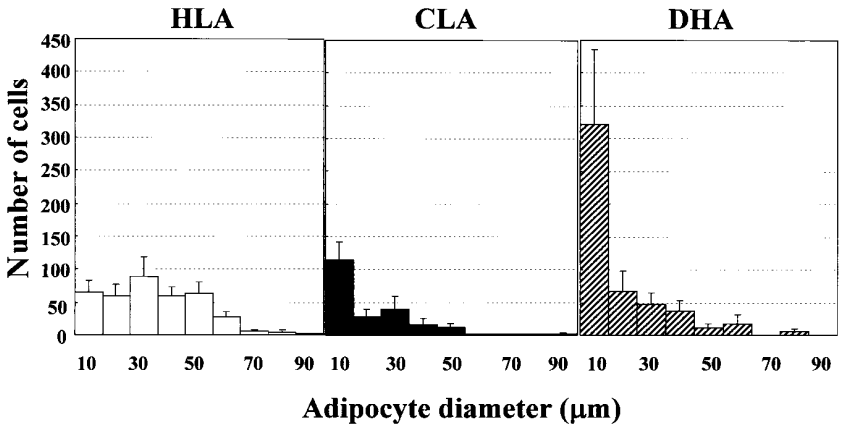

FIG. 3. Frequency distribution of adipocyte size in the subcutaneous tissue of mice administered linoleic acid (LA; $n = 10$), CLA ($n = 9$), or DHA ($n = 10$). Cell size was measured along the major axis of the adipocytes. Values are means ± SEM.

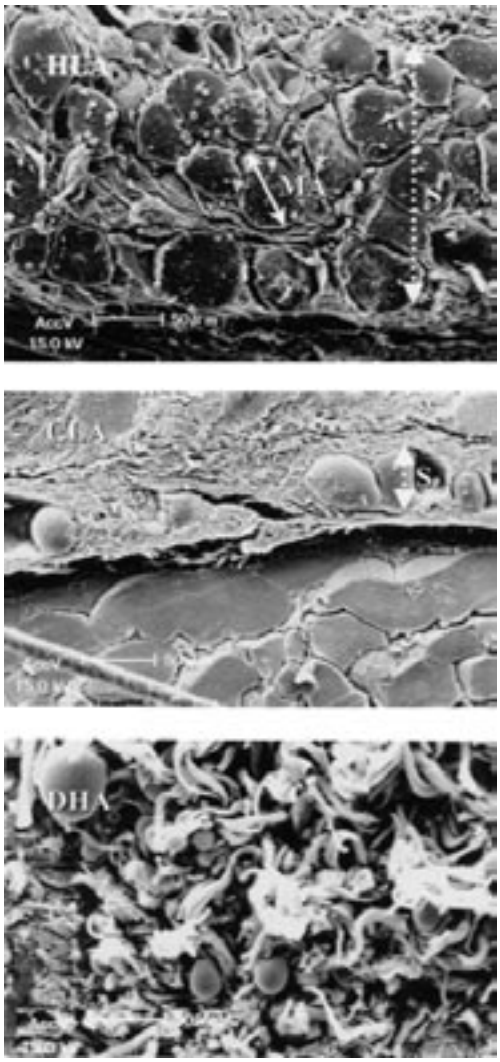

FIG. 4. Representative scanning electron microscopic photographs of the subcutaneous tissue layer of mice administered linoleic acid (LA), CLA, and DHA. Abbreviations: S, subcutaneous tissue layer, MA, major axis. From the top, bars indicate 50, 50, and 20 μm, respectively.

Brown *et al*. (31), the antiobesity actions of CLA isomers may be due to inhibition of lipogenesis by the 10*t*,12*c* isomer. Even in the HLA and DHA groups, CLA was detected in the skin. This was explained by the action of gut microflora because Chin *et al*. (32) reported that 9*c*,11*t*- and 10*c*,12*t*-CLA were produced in conventional but not germ-free rats fed LA.

When excess LA is given, the skin of mice becomes inflamed and an allergic reaction develops (5). However, skin containing CLA is more resistant to cancer (33). The present results suggest a new possibility to improve characteristics and properties of the skin by ingested oil. The metabolisms of LA, CLA, and DHA share common enzymes such as Δ6 desaturase, elongase, and Δ5 desaturase (14,34). Thus, CLA and DHA in the skin may inhibit LA metabolism, and this metabolism decreases production of arachidonic acid and eicosanoids. EPA is synthesized from α-linolenic acid and is also synthesized from DHA *via* β-oxidation (35). In addition, DHA has been shown to lower LTB_4 (36). DHA is metabolized to 17-hydroxydocosahexaenoic acid (17-HDoHE) by 15-lipoxygenase, and the products of 17-HDoHE inhibit proinflammatory LTB_4 generation by the 5-lipoxygenase enzyme (36). It is therefore possible that ingestion of CLA and DHA prevents inflammatory disorders and allergic reactions in the skin by reducing the action of eicosanoids.

Because DHA has six double bonds, it is easily oxidized and changed to a lipid peroxide. As a result, the surrounding cells or tissues are injured. However, the DHA group tended to contain more collagen than the other groups, suggesting that orally ingested DHA did not injure the skin collagen. DHA might stimulate synthesis of ascorbic acid (37), and then antioxidation may be promoted by ascorbic acid (38). Because ascorbic acid is essential for the synthesis of collagen, it is possible that increases in ascorbic acid enhance the skin collagen content (39).

In summary, the present data suggest that ingested LA, CLA, or DHA altered the FA composition of the skin, the thickness of subcutaneous tissue, and the size of the adipocytes. Skin properties can be modified by the combination of several PUFA sources.

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Protective Action of CLA Against Oxidative Inactivation of Paraoxonase 1, an Antioxidant Enzyme

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ABSTRACT: The effect of CLA on paraoxonase 1 (PON1), one of the antioxidant proteins associated with HDL, was investigated for its protective action against oxidative inactivation as well as its stabilization activity. When *cis*-9 (*c*9),*trans*-11 (*t*11)-CLA and *t*10,*c*12-CLA were examined for their protective activity against ascorbate/Cu^{2+}-induced inactivation of PON1 in the presence of Ca^{2+}, two CLA isomers exhibited a remarkable protection (E_{max}, 71–74%) in a concentration-dependent manner (50% effective concentration, 3–4 μM), characterized by a saturation pattern. Such a protective action was also reproduced with oleic acid, but not linoleic acid. Rather, linoleic acid antagonized the protective action of CLA isomers in a noncompetitive fashion. Additionally, the two CLA isomers also protected PON1 from oxidative inactivation by H_2O_2 or cumene hydroperoxide. The concentration-dependent protective action of CLA against various oxidative inactivation systems suggests that the protective action of CLA isomers may be mediated through their selective binding to a specific binding site in a PON1 molecule. Separately, the inactivation of PON1 by *p*-hydroxymercuribenzoate (PHMB), a modifier of the cysteine residue, was also prevented by CLA isomers, suggesting the possible existence of the cysteine residue in the binding site of CLA. The *c*9,*t*11-CLA isomer seems to be somewhat more effective than *t*10,*c*12-CLA in protecting against the inactivation of PON1 by either peroxides or PHMB, in contrast to the similar efficacy of these two CLA isomers in preventing ascorbate/Cu^{2+}-induced inactivation of PON1. Separately, CLA isomers successfully stabilized PON1, but not linoleic acid. These data suggest that the two CLA isomers may play a beneficial role in protecting PON1 from oxidative inactivation as well as in its stabilization.

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CLA is a natural substance found mainly in the fat of ruminants and dairy products (1). Studies of animals have shown that CLA inhibits carcinogenesis (2–4), lowers body fat (5–8), decreases atherogenesis (9–11), prevents the catabolic effects of immune stimulation (12,13), and exhibits antidiabetic action (14). Its biological activity has been attributed to two major CLA isomers, *cis*-9 (*c*9), *trans*-11 (*t*11)-octadecadienoic acid (15) and *t*-10,*c*-12-octadecadienoic acid (16,17). To date, no convincing data are available concerning the antiatherogenic action of CLA; CLA inhibited atherogenesis in rabbits and hamsters (9–11), whereas it promoted the formation of fatty streaks in a mouse model (18). Among blood lipids, LDL-cholesterol was either decreased (9–11) or unaffected (19) by CLA feeding. Such inconsistent results might be ascribed to differences in the animal model as well as feeding regimens. Nevertheless, an interesting finding is that *t*10,*c*12-CLA decreased levels of HDL-cholesterol and non-HDL-cholesterol, whereas *c*9,*t*11-CLA had no such effect (20). Such an effect of *t*10,*c*12-CLA may be similar to that of *trans* 18:1, which lowered plasma HDL-cholesterol in hamsters (21). Thus, the antiatherogenic effect of CLA is thought to be isomer-specific.

Originally, CLA was thought to prevent the oxidation of LDL because it demonstrated antioxidant capacity (22). However, this idea was disproved by a lack of protective action of CLA against metal-catalyzed oxidation of lipid membranes (23). Rather, a recent report (24) showed that *t*10,*c*12-CLA enhanced the level of biomarkers of lipid peroxidation in the urine of healthy humans, and there was no evidence concerning the prooxidant action of *c*9,*t*11-CLA. Because CLA belongs structurally to the family of linoleic acid, it can compete with linoleic acid in the pathway of phospholipid synthesis (15). Recently, CLA was observed to reduce early aortic atherosclerosis in hypercholesterolemic hamsters to a greater extent than linoleic acid, possibly through changes in LDL oxidative susceptibility (10,25). From this, it was suggested that the incorporation of CLA into LDL membranes could contribute to a decrease in the atherogenicity of the LDL membranes subject to the oxidative condition (26). For this purpose, *c*9,*t*11-CLA could be more beneficial than *t*10,*c*12-CLA because the former is incorporated preferentially into membranes, and cleared less efficiently into muscle (15,27). The actual availability of the CLA isomers might clarify the practical effect of each one on the atherogenicity of LDL.

Previously, it was reported that HDL-associated proteins were crucial for maintaining the function of HDL and in preventing LDL from being atherogenic (28). In particular, paraoxonase 1 (PON1) was suggested to exert antiatherogenicity through its antioxidant action and to contribute to the

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Abbreviations: *c, cis*; EC_{50}, 50% effective concentration; E_{max}, maximal effect%; PHMB, *p*-hydroxymercuribenzoic acid; PON1, paraoxonase1; ROS, reactive oxygen species; *t, trans*.

prevention of coronary heart disease (29,30). However, PON1 was observed to lose such activity under oxidative conditions during Cu^{2+}-mediated oxidation of lipoproteins or during exposure to peroxides (31,32). Recently, PON1 was found to be very susceptible to a Cu^{2+}-catalyzed oxidation system employing ascorbate as reductant (33). Therefore, the preservation of PON1 activity in an *in vivo* system may be important in preventing the formation of oxidized LDL. An earlier study showed that phospholipids, in combination with apolipoprotein A-1, were required for the optimal activity of PON1 (34). In addition, some phospholipids were observed to stimulate PON1 activity (35). Interestingly, dietary oleate, but not linoleate, enhanced HDL-associated PON1 activity (36). Similarly, it is conceivable that the intake of CLA, geometrically resembling oleic acid rather than linoleic acid, may lead to the increase in the level of PON1. It is of interest to determine whether or how CLA would affect PON1 under oxidative stress. Here, the beneficial effects of two CLA isomers on PON1 were examined in terms of their protective actions against oxidative inactivation, as well as the stability of PON1.

MATERIALS AND METHODS

Materials. All materials including phenyl acetate were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. H_2O_2 (30%) was from Junsei Chemical (Tokyo, Japan). *t*10,*c*12-octadecadienoic acid (*t*10,*c*12-CLA, ≥98%) and *c*9,*t*11-octadecadienoic acid (*c*9,*t*11-CLA, ≥96%) were obtained from Cayman Chemical (Ann Arbor, MI). Calcium chloride or cupric sulfate was of analytical grade. In these experiments, HEPES buffer was passed through Chelex 100 resin before use.

Assay of PON1. PON1 activity toward phenyl acetate was measured by adding enzyme solution to 0.5 mL of 50 mM Tris buffer, pH 7.4, containing 1 mM $CaCl_2$ and 10 mM phenyl acetate; the rate of generation of phenol was determined as described previously (35–37). One unit of enzyme activity was expressed as 1 μmol product/min.

Purification of PON1. PON1 was purified from human plasma by (pseudo) affinity chromatography using Cibacron Blue 3GA, DEAE Sephacel chromatography, Sephacryl S-200-HR gel chromatography (Sigma Chemical), and finally, affinity chromatography using concanavalin-A sepharose with a slight modification of the published procedures (37–39); the identification of PON1 from human plasma was reported previously (39). The purified enzyme with a M.W. of ~45 kDa, homogeneous in SDS-PAGE analysis, possessed a specific activity of ~1018 and 0.552 μmol/(min·mg protein) in the hydrolysis of phenyl acetate and paraoxon, respectively.

Protection by CLA isomers against ascorbate/Cu^{2+}-induced oxidative inactivation of PON1. The ascorbate/Cu^{2+}-induced oxidative inactivation of PON1 was performed using a slight modification of a procedure reported previously (33). PON1 (0.5 U) was incubated with 0.5 mM ascorbate and 1 μM Cu^{2+} in 0.1 mL of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C for 10 min; an aliquot (20 μL) was taken for an assay of the remaining activity. Similarly, the oxidative inactivation of PON1 by 0.5 mM ascorbate and 1 μM Cu^{2+} was performed in the presence of each CLA isomer at various concentrations (1–30 μM). Separately, the effects of other FA such as oleic acid or linoleic acid on ascorbate/Cu^{2+}-induced inactivation of PON1 were examined by incubating PON1 (0.5 U) with 0.5 mM ascorbate and 1 μM Cu^{2+} in the presence of each FA at 10 μM.

Inhibitory effect of linoleic acid on protective action of CLA isomers against ascorbate/Cu^{2+}-induced inactivation of PON1. The oxidative inactivation of PON1 by 0.5 mM ascorbate and 1 μM Cu^{2+} was performed in the presence of CLA isomers at various concentrations (1–30 μM) in 0.1 mL of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C for 10 min. Separately, the protective actions of each CLA isomer against ascorbate/Cu^{2+}-induced inactivation of PON1 were examined in the presence of linoleic acid (5 or 10 μM) under the incubation conditions described above.

Prevention by CLA isomers of oxidative inactivation of PON1 by H_2O_2 or cumene hydroperoxide. PON1 (0.5 U) was incubated with 2 mM H_2O_2 or cumene hydroperoxide in 0.1 mL of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C for 30 min, and an aliquot (20 μL) was taken to assay the remaining activity. Separately, the oxidative inactivation of PON1 by each hydroperoxide was performed in the presence of each CLA isomer at various concentrations (1–10 μM) as described above.

Preventive action of CLA isomers against the inactivation of PON1 by p*-hydroxymercuribenzoate (PHMB).* PON1 (0.5 U) was incubated with 50 μM PHMB in the presence of each CLA isomer at various concentrations (3–100 μM) in 0.1 mL of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C; 30 min later an aliquot (20 μL) was taken to assay the remaining activity.

Stabilizing effect of CLA isomer on the PON1 activity during a lengthy incubation. PON1 (0.5 U) was incubated with *c*9,*t*11-CLA or *t*10,*c*12-CLA at various concentrations (3–10 μM) in 0.1 mL of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} and 50 μM BHT for 12 h at 38°C; then, an aliquot (20 μL) was taken to assay the remaining activity. Separately, the effect of linoleic acid on the stability of PON1 was examined by performing the same experiment in 50 mM HEPES buffer (pH 7.4) containing linoleic acid (10 μM).

Other analyses. The 50% effective concentrations (EC_{50}) of protective FA were expressed as the concentration of each FA to achieve a half-maximal protection percentage (*E*max), which was observed under the above conditions. The inhibition of PON1 activity by FA was carried out by incubating PON1 (4 U/mL) with each FA in 0.5 mL of 50 mM Tris buffer (pH 7.4) containing 10 mM phenyl acetate.

RESULTS

It was reported previously (31,32) that PON1 was highly susceptible to oxidants as well as PHMB, a cysteine modifier.

Because either oxidant-inactivated PON1 or PHMB-induced PON1 was observed to be less effective than native PON1 in preventing LDL oxidation (31,32), it was supposed that the protection of PON1 from the oxidative inactivation might be important for the maintenance of antioxidant action of PON1 against LDL oxidation. Earlier (22,36), the intake of a diet rich in C_{18} FA such as oleic acid or linoleic acid was shown to affect the activity of PON1 associated with HDL. In this regard, it is intriguing to determine how CLA, another C_{18} FA affects the activity of PON1. First, the possible effect of CLA isomers on the activity of PON1 was examined and compared with that of other C_{18} FA such as oleic or linoleic acid. When the inhibitory effect of C_{18} FA on PON1 activity was investigated in 50 mM Tris buffer, pH 7.4, containing 10 mM phenyl acetate (Table 1), both oleic acid and CLA isomers (10 or 100 μM) weakly inhibited PON 1 activity (21–26%), whereas linoleic acid at 10 μM showed a remarkable inhibition (~68%) under the conditions used. Thus, CLA isomers were found to mimic oleic acid rather than linoleic acid in the inhibition of PON1 activity.

Protective action of C18 FA against ascorbate/Cu^{2+}-induced inactivation of PON1. Next, these C_{18} FA were examined for their protective action against ascorbate/Cu^{2+}-induced oxidative inactivation of PON1. After a 10-min exposure to 0.5 mM ascorbate/1 μM Cu^{2+} in 0.1 mL of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C, the activity of PON1 decreased to ~8% of control. When each FA (10 μM) was included in the above inactivation conditions, it was observed (Table 2) that oleic acid and two CLA isomers had a remarkable protective effect, whereas stearic acid was weakly protective (12.6%). In contrast, linoleic acid showed negligible activity (<5%). In a further study (Fig. 1), two CLA isomers showed a concentration-dependent protection with a maximal protection (E_{max}, 71–74%), slightly smaller than the E_{max} value (~82%) of oleic acid. In comparison, there was no significant difference in either the E_{max} or EC_{50} value (3–4 μM) between the two CLA isomers, indicative of a similar protective efficacy of the two isomers against ascorbate/Cu^{2+}-induced inactivation of PON1.

Taken together, these data indicate that CLA, despite its structural similarity to linoleic acid, appears to resemble oleic acid, rather than linoleic acid, in protecting PON1 from oxidative inactivation. In an attempt to determine the possible opposing action between CLA and linoleic acid, the concentration-dependent protective action of CLA was examined in the presence of linoleic acid as an inhibitor of PON1. Figure 2 indicates that the protective action of the *c*9,*t*11-CLA isomer at 3, 10, and 30 μM is diminished by ~43, 45, and 42%, respectively, in the presence of linoleic acid at a concentration as low as 5 μM.

A similar but greater inhibition was expressed by 10 μM linoleic acid. Interestingly, the inhibitory degree of linoleic acid was similar over the range of concentrations (3–30 μM) of *c*9,*t*11-CLA, indicative of a noncompetitive antagonism

TABLE 1
Inhibitory Effect of Each FA on Paraoxonase 1 (PON1) Activity[a]

FA	Concentration (μM)	Inhibition (%)
Linoleic acid	10	67.7 ± 3.6
*c*9,*t*11-CLA	10	21.7 ± 3.2
	100	25.6 ± 3.4
*t*10,*c*12-CLA	10	23.5 ± 2.9
	100	24.6 ± 5.6
Oleic acid	10	21.8 ± 2.6
	100	23.7 ± 2.8

[a]PON1 (4 U/mL) was incubated with 10 mM phenyl acetate in the presence of each FA at room temperature in 0.5 mL of Tris buffer (pH 7.4) containing 2 mM Ca^{2+}. The formation of phenol was monitored at 270 nm to determine the remaining activity. Data are expressed as the mean ± SD of triplicate sets; *c, cis*; *t, trans*.

TABLE 2
Protective Action of Each FA Against the Inactivation of PON1 by Ascorbate/Cu^{2+}[a]

FA	Concentration (μM)	Protection (%)
Oleic acid	10	82.0 ± 3.1
*c*9,*t*11-CLA	10	71.8 ± 4.6
*t*10,*c*12-CLA	10	68.5 ± 5.1
Stearic acid	10	12.6 ± 3.7
Linoleic acid	10	<5

[a]See Figure 1 legend for details. See Table 1 for abbreviations.

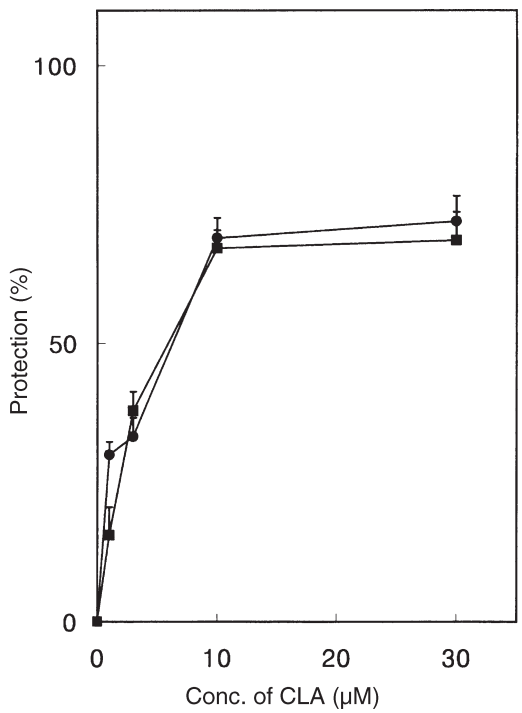

FIG. 1. Protective action of CLA isomers against the inactivation of paraoxonase 1 (PON1) by ascorbate/Cu^{2+}. PON1 (0.5 U) was incubated with 0.5 mM ascorbate and 1 μM Cu^{2+} in the presence of each CLA isomer at 38°C in 0.1 mL of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} for 10 min; the remaining activity was determined as described in the Materials and Methods section. Protection was expressed as a percentage of the activity lost under the inactivation conditions and restored by the FA. Data are expressed as the mean ± SD (bar) of triplicate sets; *c, cis*; *t, trans*. ■, *t*10,*c*12-CLA; ●, *c*9,*t*11-CLA.

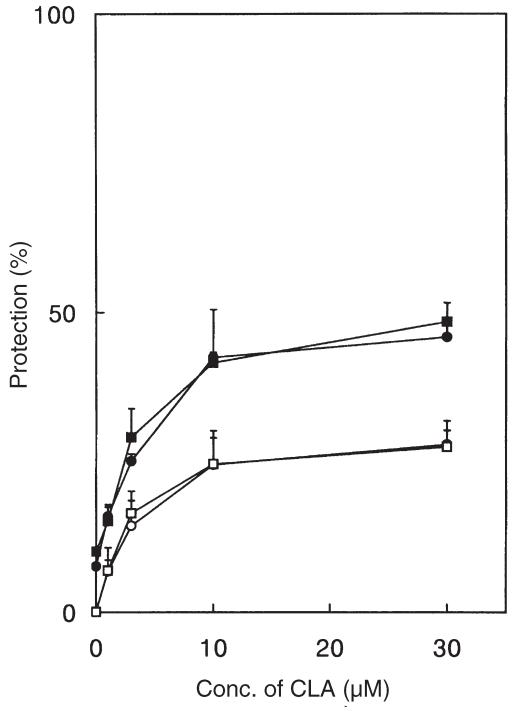

FIG. 2. Inhibitory effect of linoleic acid on the protective effect of CLA isomers against ascorbate/Cu^{2+}-induced inactivation of PON1. The protective activity of CLA isomers against ascorbate/Cu^{2+}-induced inactivation of PON1 was tested in the presence of 5 or 10 μM linoleic acid under the incubation conditions described in Figure 1. Data are expressed as the mean ± SD (bar) of triplicate sets. ■, *t*10,*c*12-CLA (5 μM); ●, *c*9,*t*11-CLA.(5 μM); □, *t*10,*c*12-CLA (10 μM); ○, *c*9,*t*11-CLA (10 μM). See Figure 1 for abbreviations.

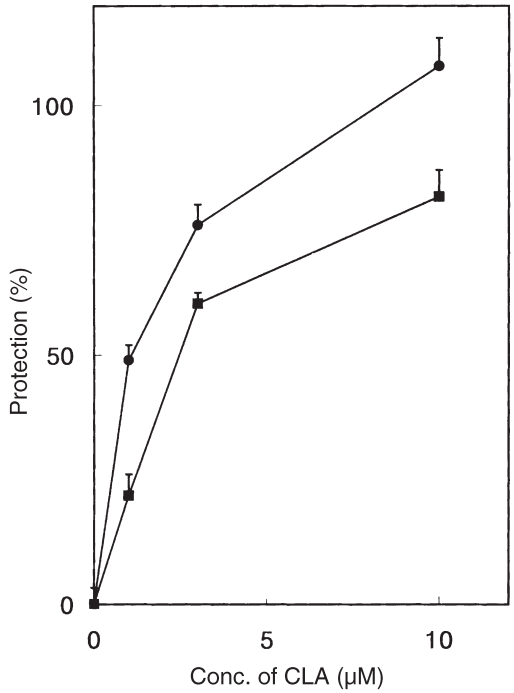

FIG. 3. Protective action of CLA isomers against the inactivation of PON1 by hydrogen peroxide. PON1 (0.5 U) was incubated with 2 mM hydrogen peroxide in the presence or absence of each CLA isomer at 38°C in 0.1 mL of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} for 10 min; the remaining activity was determined as described in the Materials and Methods section. Data are expressed as the mean ± SD (bar) of triplicate sets. ■, *t*10,*c*12-CLA; ●, *c*9,*t*11-CLA. See Figure 1 for abbreviations.

between *c*9,*t*11-CLA and linoleic acid. A similar result was also observed for the antagonism between *t*10,*c*12-CLA and linoleic acid.

Prevention by CLA isomers against inactivation of PON1 by hydrogen peroxide or cumene hydroperoxide. Subsequently, we examined the protective action of CLA isomers against the inactivation of PON1 by other oxidation systems such as H_2O_2 or cumene hydroperoxide. First, the protective effect of CLA isomers on H_2O_2-induced (2 mM) inactivation was investigated. Part (34–38%) of the PON1 activity was lost after a 30-min exposure to 2 mM H_2O_2. The inclusion of CLA isomers in the above incubation mixture prevented the loss of PON1 activity as shown in Figure 3; *c*9,*t*11-CLA and *t*10,*c*12-CLA at 10 μM were 105 and 82% protective, respectively, against H_2O_2-induced inactivation of PON1. The concentration-dependent profile indicates that *c*9,*t*11-CLA was more effective than *t*10,*c*12-CLA over the range of concentrations used, although the EC_{50} values of the two CLA isomers appeared to be similar.

Next, the effect of CLA isomers on cumene hydroperoxide–induced oxidative inactivation of PON1 was investigated. Cumene hydroperoxide (2 mM) alone caused ~40% inactivation of PON1 after a 30-min incubation (Su, N.-D., and Sok, D.E., unpublished data). When the cumene hydroperoxide-induced inactivation of PON1 was examined in the presence of CLA isomers (Fig. 4), *c*9,*t*11-CLA and *t*10,*c*12-CLA demonstrated a concentration-dependent protection against the oxidative inactivation; *c*9,*t*11-CLA and *t*10,*c*12-CLA at 10 μM were ~90 and 58% protective, respectively. This suggests that *c*9,*t*11-CLA is somewhat more efficient than *t*10,*c*12-CLA in protecting PON1 from the peroxide-mediated oxidative inactivation.

Prevention by CLA isomers against PHMB-induced inactivation of PON1. Because PHMB, a modifier of cysteine residue, was reported to inactivate PON1 strongly (31), the possible protective effect of CLA isomers on the PHMB-induced inactivation of PON1 was investigated. After a 30-min exposure to 50 μM PHMB in 0.1 mL of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C, the activity of PON1 decreased to ~6% that of control. When each FA was included in the above inactivation condition (Fig. 5), a concentration-dependent protection against PHMB-induced inactivation of PON1 was expressed by *c*9,*t*11-CLA and *t*10,*c*12-CLA with E_{max} values of 75.9 and 62.0%, respectively. Additionally, the EC_{50} values of the two CLA isomers were estimated to be 6–8 μM. From this, it is suggested that the binding site of CLA, implicated in the prevention against PHMB-induced inactivation, might somewhat overlap that of the CLA responsible for the protection against the oxidative inactivation of PON1. However, the higher EC_{50} value of CLA in preventing PHMB-induced inactivation, compared with that in preventing ascor-

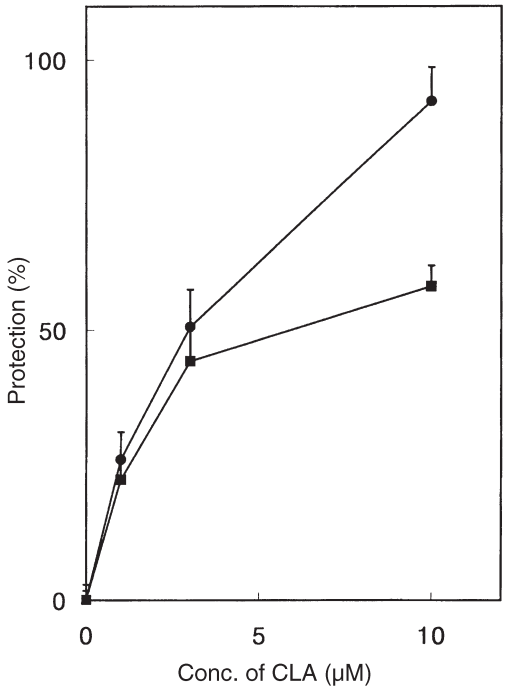

FIG. 4. Protective action of CLA isomers against the inactivation of PON1 by cumene hydroperoxide. PON1 (0.5 U) was incubated with 2 mM cumene hydroperoxide in the presence or absence of each CLA isomer at 38°C in 0.1 mL of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} for 10 min; the remaining activity was determined as described in the Materials and Methods section. Data are expressed as the mean ± SD (bar) of triplicate sets. ■, *t*10,*c*12-CLA; ●, *c*9,*t*11-CLA. See Figure 1 for abbreviations.

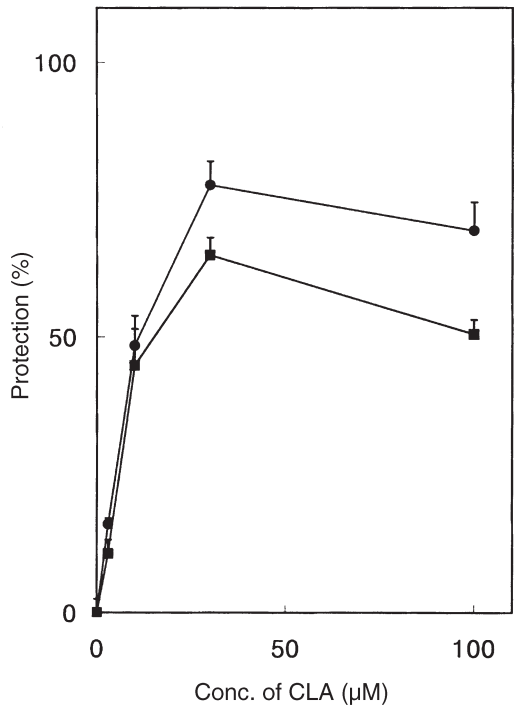

FIG. 5. Protective action of CLA isomers against *p*-hydroxymercuribenzoate (PHMB)-induced inactivation of PON1. PON1 (0.5 U) was incubated with PHMB (50 μM) in the presence or absence of each CLA isomer at various concentrations (0–30 μM) in 0.1 mL of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} at 38°C for 30 min; the remaining activity was determined as described in the Materials and Methods section. Data are expressed as a mean ± SD (bar) value of triplicate sets. ■, *t*10,*c*12-CLA; ●, *c*9,*t*11-CLA. See Figure 1 for other abbreviations.

bate/Cu^{2+}-induced oxidative inactivation, suggests that CLA may have a lower affinity toward the binding site including the cysteine residue. Again, *c*9,*t*11-CLA was more efficient than *t*10,*c*12-CLA in preventing PHMB-induced inactivation. Together, these data indicate that *c*9,*t*11-CLA seems to be more efficient than *t*10,*c*12-CLA in preventing peroxide- or PHMB-mediated inactivation of PON1.

Stabilizing effect of CLA isomers on the deactivation of PON1. Here, the stabilizing effect of CLA isomers on PON1 activity during a 12-h incubation at 38°C in buffer containing 50 μM Ca^{2+} was examined. Although PON1 lost ~53% of its activity in the absence of CLA isomers (Fig. 6), the inclusion of each CLA isomer prevented the deactivation of PON1 in a concentration-dependent manner (0.3–10 μM). An almost complete stabilization was accomplished by both CLA isomers at 10 μM, and there was no difference in the stabilizing effect of the two CLA isomers. In contrast, linoleic acid at 1 or 10 μM produced no remarkable stabilization.

DISCUSSION

PON1, an A-esterase associated mainly with HDL, exerts a preventive action against LDL oxidation (31,32), possibly contributing to its antiatherogenicity (40). Earlier studies showed, however, that PON1 activity decreased in atherosclerotic apolipoprotein E-deficient mice (41) and in patients with coronary heart disease (42). The loss of PON1 activity in an *in vivo* system might be explained in part by the reactive oxygen species (ROS) mechanism as suggested from previous reports (31–33) that PON1 was susceptible to oxidative inactivation by hydrogen peroxide or copper ion. In particular, PON1 was highly sensitive to the Cu^{2+}/ascorbate system employing Cu^{2+}-catalyzed oxidation (33). Additionally, alkyl hydroperoxides such as lipid hydroperoxide (32) caused a partial inactivation. These results indicate that some amino acid residues in the active site of PON1 may be susceptible to oxidative modification. The candidate amino acid residues might include residues of histidine, responsible for the catalysis of PON1, or tryptophan, necessary for substrate binding (43). An additional candidate could be the cysteine residue as suggested from the decreased activity of PHMB- or iodoacetamide-modified PON1 (31).

Because the loss of PON1 activity is related to a decrease in its antioxidant activity, the preservation of PON1 activity in an *in vivo* system is important for the maintenance of the antioxidant function of HDL under oxidative stress. Such a result can be obtained by the use of effective antioxidants or selective ligands. Although some polyphenols were reported to preserve the activity of PON1 during LDL oxidation (32),

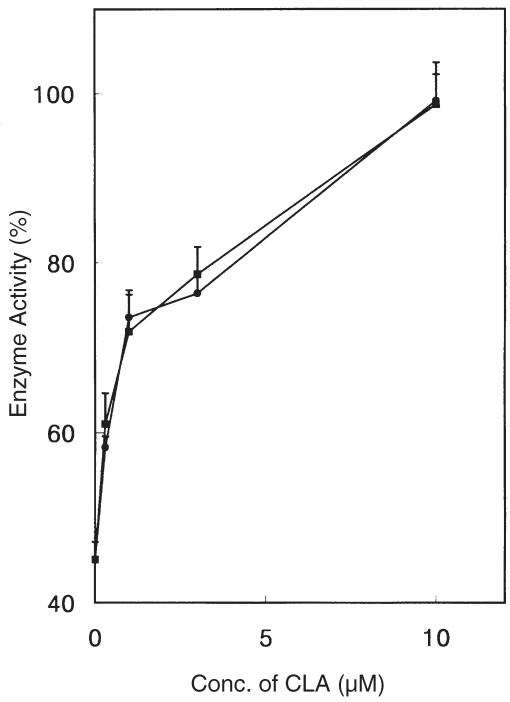

FIG. 6. Stabilizing effect of FA on PON1 during a lengthy incubation. PON1 (0.5 U) was incubated with each FA in 0.1 mL of 50 mM HEPES buffer (pH 7.4) containing 50 μM Ca^{2+} for 12 h at 38°C; then, an aliquot (20 μL) was taken to assay the remaining activity. Data are expressed as the mean ± SD (bar) of triplicate sets. ■, *t*10,*c*12-CLA; ●, *c*9,*t*11-CLA. See Figure 1 for abbreviations.

quercetin was not effective in protecting against Cu^{2+}/ascorbate-induced oxidative inactivation of PON1 (33).

The present data provide evidence that CLA isomers can successfully protect PON1 from oxidative systems such as ascorbate/Cu^{2+}, H_2O_2, or cumene hydroperoxide. The concentration-dependent effect of CLA, showing a saturation pattern, on various oxidative inactivation systems supports the notion that the action of CLA may be announced through the selective binding of CLA to PON1. Earlier, the interaction of lipids with PON1 was suggested because of the stimulating effect of phospholipids on PON1 activity (35). In addition, PON1 was shown to associate with HDL through direct binding of its N-terminal with HDL phospholipids (38). These data suggest the existence of multiple binding sites for lipids in the PON1 molecule. More importantly, CLA isomers, despite a structural similarity to linoleic acid, resemble oleic acid in the prevention of oxidative inactivation of PON1. Moreover, such a relation was reproduced in the inhibition as well as the stabilization of PON1. This might support the view that the binding site for CLA is distinct from that for linoleic acid, which was found to inhibit PON1 competitively at its active center (Su, N.D., and Sok, D.-E., unpublished data).

Further support may be gained from the noncompetitive inhibition of CLA activity by linoleic acid. It is worth noting that *c*9,*t*11-CLA was somewhat more effective than *t*10,*c*12-CLA in preventing the inactivation of PON1 by PHMB or hydroperoxides, in contrast to the similar efficacy of the two CLA isomers in preventing ascorbate/Cu^{2+}-induced inactivation of PON1. Thus, the two CLA isomers may be distinguished by a specific binding site, containing a cysteine residue, of PON1. This is an interesting observation because the cysteine residue is known to be crucial for the antioxidant action of PON1. All of these results provide positive support for the idea that intake of CLA would be beneficial for the preservation of PON1 under oxidative stress in an *in vivo* system. For this purpose, *c*9,*t*11-CLA would be preferred because *t*10,*c*12-CLA was prooxidative under physiologic conditions (24). Oleic acid, a monounsaturated FA, also demonstrated a similar protective effect against ascorbate/Cu^{2+}-induced inactivation of PON1. However, *c*9,*t*11-CLA may have an advantage over oleic acid because *c*9,*t*11-CLA does not induce obesity, in contrast to oleic acid, which induced the accumulation of TG (44).

Although the mechanism for the antiatherogenic effect of CLA remains unclear, some part of the antiatherogenic effect may be due to the protective action of CLA against oxidative inactivation of HDL-bound PON1. Nevertheless, the actual effect of CLA itself is questioned because most of CLA administered is incorporated into membranes or metabolized; thus, the physiologic concentration of CLA could be too low. Instead, conjugated linoleoylated phospholipids in the HDL membrane could be responsible for the protection of PON1 as suggested from a separate finding that oleoylated phospholipids expressed a remarkable protection against oxidative inactivation of PON1 (Su, N.D., and Sok, D.-E., unpublished data). Alternatively, the antiatherogenic effect of CLA may be mediated through an alteration in the lipid composition of the HDL membrane (10); the replacement of PUFA by *c*9,*t*11-CLA could lead to a decrease in the oxidative susceptibility of lipoprotein membranes as was observed in the greater beneficial effect of CLA vs. linoleic acid on HDL membranes in hypercholesterolemic hamsters (10,25). Additionally, the stabilization effect of CLA on PON1 activity may also contribute to the preservation of PON 1 activity in an *in vivo* system. Future study of the possible synergy between *c*9,*t*11-CLA and oleic acid (45) in exerting antiatherogenicity might prove to have an application in the prevention of atherosclerosis.

ACKNOWLEDGMENT

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Vaccenic Acid (*t*11-18:1) Is Converted to *c*9,*t*11-CLA in MCF-7 and SW480 Cancer Cells

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ABSTRACT: The aims of this study were to determine whether vaccenic acid (VA; *t*11-18:1) is converted to *c*9,*t*11-CLA in human mammary (MCF-7) and colon (SW480) cancer cell lines and whether VA influences cell viability and other CLA-bioresponsive markers. When cells were incubated in the presence of VA at concentrations of 5 to 20 μg/mL, both VA and *c*9,*t*11-CLA increased in cellular lipids in a dose-dependent manner. After 4 d of incubation of SW480 and MCF-7 cells with VA (20 μg/mL), *c*9,*t*11-CLA increased from undetectable levels to 8.57 and 12.14 g/100 g FAME in cellular lipids, respectively. VA supplementation for 4 d at 5, 10, and 15 μg/mL had no effect on cell growth, whereas 20 μg/mL significantly ($P < 0.05$) reduced cell growth in both cell lines. VA (20 μg/mL) treatment induced DNA fragmentation and significantly ($P < 0.05$) depleted cytosolic GSH levels in the SW480 cell line after 4 d of incubation, suggesting that apoptosis was the mode of cell death induced by VA. Both VA and *c*9,*t*11-CLA reduced ($P < 0.05$) total ras expression in SW480 cells. ^{14}C-Arachidonic acid uptake into the MG fraction was significantly increased ($P < 0.05$) in both cell lines while uptake into the phospholipid fraction decreased in response to VA. VA treatment significantly ($P < 0.05$) increased 8-epi-prostaglandin $F_{2\alpha}$ in both cell lines. The data indicate that growth suppression and cellular responses of both cells lines are likely mediated by VA desaturation to *c*9,*t*11-CLA *via* Δ^9-desaturase.

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Cancer cells derive biologically important FA from either *de novo* synthesis or the host circulation (1). The end products of *de novo* synthesis are palmitoleate and oleate, which may also derive from palmitate and stearic acid, respectively, by Δ^9-desaturase (2). Analysis of the FA composition of cellular lipids clearly shows an altered balance of saturated to monounsaturated FA in tumors compared with nonneoplastic cells (3). In particular, increased proportions of oleic acid were found in experimental tumors (4–6), hepatoma cell lines (7), and in virally transformed cell lines (8,9), reflecting possible increased expression or activity of Δ^9-desaturase.

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Abbreviations: AA, arachidonic acid (20:4); HRP, horseradish peroxidase; LA, linoleic acid (18:2); MG, monoacylglyceride; PBST, PBS containing 0.1% (by vol) Tween 20; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PL; phospholipid; TG, triacylglyceride; VA, vaccenic acid (*t*11-18:1).

A large amount of experimental data has shown that tumor cell growth can be modulated by individual FA (10,11). CLA is a group of PUFA that have been highly publicized recently as a result of their potent anticarcinogenic properties observed at low dietary levels in animal models of carcinogenesis (reviewed in Ref. 12). The mechanism by which CLA exerts its anticancer activity is attributed in part to a series of events marked primarily by changes in composition of cellular lipids, inhibition of Δ^9-desaturase, modulation of arachidonic acid (AA) distribution and metabolism, changes in eicosanoid production, activation of nuclear transcription factors critical for lipid metabolism, induction of apoptosis, and modulation of the cell cycle (reviewed in Ref. 13). Dietary CLA consists primarily of the *c*9,*t*11-CLA isomer of ruminant-derived fats, in particular milk and beef fat (14,15). Vaccenic acid (VA; *t*11-18:1), the predominant *trans* monounsaturated FA in milk fat, is formed by ruminal biohydrogenation of linoleic acid (LA) and occurs in milk fat at levels up to five times that of *c*9,*t*11-CLA (16,17). Endogenous synthesis of CLA from VA represents the primary source of CLA in milk fat of lactating cows (18). Santora and coworkers (19) reported that VA is desaturated to CLA in mice. Rats fed CLA-enriched butterfat accumulated more total CLA in their tissues than those consuming synthetic *c*9,*t*11-CLA, suggesting that the availability of VA in the butterfat served as a precursor for endogenous synthesis of CLA (20). This was confirmed in a recent study wherein feeding VA (2% of diet, w/w) elicited a biological response in a rat mammary tumor model, reducing the total number of premalignant lesions in the rat mammary gland by approximately 50% in carcinogen-treated rats (21). Although CLA is present at relatively low concentrations in human adipose tissue, bile, duodenal juice, breast milk, and serum lipids (22–24), specific dietary intervention trials markedly increased the CLA content in human milk (25), plasma (26), and adipose tissue (27). Together, these observations suggest that increasing the amount of VA in the diet may enhance the pool of CLA in human tissues.

Previously, we demonstrated that CLA uptake into MCF-7 human mammary cancer cells was more proficient from milk fat than from synthetic *c*9,*t*11-CLA, suggesting possible formation of CLA from VA present in the milk fat by a Δ^9-desaturase enzyme (28). The present study evaluated the possible bioconversion of VA to CLA in the MCF-7 (breast) and SW480 (colon) human cancer cell lines. The relative effects

of VA and *c*9,*t*11-CLA on cell growth were compared over a range of concentrations after 4 d of incubation. Time- and dose-experiments were performed to determine the effects of VA on FA composition in both cell lines. Whether CLA was synthesized from VA in amounts sufficient to modulate known bio-responsive markers was examined. We previously found that the growth-suppressive effects of CLA isomers in MCF-7 and SW480 cell lines may be due to alterations in AA distribution among cellular lipids, an altered prostaglandin (PG) profile, lipid peroxidation (29) and to stimulation of an apoptotic signal transduction pathway (30). In this report, we examined the effects of VA on AA uptake and conversion to eicosanoid classes in MCF-7 and SW480 cell lines as well as its ability to modulate ras expression and induce apoptosis in the SW480 cell line.

MATERIALS AND METHODS

Cell culture. MCF-7 and SW480 cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Culture media and supplements were purchased from Sigma-Aldrich Ireland Ltd. (Dublin, Ireland). Both cell lines were maintained in DMEM supplemented with FBS (5% by vol), 0.2 mM L-glutamine, 1 mM HEPES, and 1 unit/mL penicillin and streptomycin. The MCF-7 cells required an additional supplement of 10 mM sodium pyruvate. Cells were grown in Falcon™ (BD Biosciences, Oxford, United Kingdom) T-75 cm^2 flasks and maintained as previously described (31).

Bioconversion of VA to CLA. Cells were seeded in T-25 cm^2 flasks at a density of 5×10^5/flask and cultured for 24 h, allowing the cells to attach to the substratum. The medium was then replaced with medium containing VA (Sigma-Aldrich Ireland Ltd.) at 5, 10, or 20 μg/mL. Control flasks were supplemented with an equivalent volume of ethanol (≤1% by vol). After 24 h and 4 d of incubation, cells were harvested and total cellular lipids were extracted from cell pellets using the method of Bligh and Dyer (32), resuspended in 1 mL of chloroform, and stored at –20°C prior to GLC analysis.

FA analysis. FAME of cellular lipids were prepared using base-catalyzed methanolysis by incubating extracts with tetramethylguanidine as described by Shanta *et al.* (33). FAME were analyzed by GLC, using a Varian 3500 GLC fitted with an FID and a SUPELCOWAX 10 capillary GLC column (Supelco Inc., Bellefonte, PA) (60 m × 0.32 mm i.d., 0.25 μm film thickness). GLC conditions were exactly as previously described (34).

Viability experiments. Cells were seeded in 6-well plates, and the MCF-7 and SW480 cells were seeded at densities of 1×10^5/well and 5×10^4/well, respectively. Cells were cultured for 24 h to allow the cells to attach to the substratum. The medium was then replaced with medium containing VA (99% pure from Sigma-Aldrich Ireland Ltd.) or the pure *c*9, *t*11-CLA isomer (95% pure from Natural ASA, Hovdebygda, Norway) at varying concentrations from 5 to 25 μg/mL dissolved in ethanol. Control wells were supplemented with equivalent volumes of ethanol. After 4 d of incubation, cells were harvested in the presence of PBS containing 0.25% (by vol) trypsin. Viable cell numbers were quantified using the trypan blue exclusion (0.4% by vol) assay.

Uptake of ^{14}C-AA and conversion to eicosanoids. Cells were seeded in T-25 cm^2 flasks at a density of 2×10^5/flask and grown to 90% confluency. The medium was then replaced with medium containing ^{14}C-AA at 0.2 μCi along with VA (20 μg/mL) or an equivalent volume of ethanol. After 24 h of incubation, cells were harvested to determine uptake of ^{14}C-AA. Total cellular lipids were extracted from cell pellets and then separated into triacylglyceride (TG), monoacylglyceride (MG) and phospholipid (PL) fractions as described (29). An aliquot of each fraction was counted in a Beckman LS6500 scintillation counter. Eicosanoids were extracted twice with ethyl acetate from medium acidified to pH 3.0 with 0.1 N HCl as described (29). Eicosanoid extracts were dried under nitrogen, redissolved in ethyl acetate, and separated using normal-phase TLC (29). Bands of PGE_2, $PGF_{2\alpha}$, and PGD_2 were removed from TLC plates and placed in vials for counting by liquid scintillation. The isoprostane, 8-epi-$PGF_{2\alpha}$, was extracted from media as described (35) and a competitive horseradish peroxidase (HRP) enzyme-linked immunoassay kit (BIOXYTECH 8-Isoprostane assay system; *Oxis* Research, Portland, OR) was used to quantify 8-epi-$PGF_{2\alpha}$ levels according to the manufacturer's instructions.

DNA laddering. Cells were seeded in T-75 cm^2 flasks at a density of 1×10^6 cells/flask and incubated for 24 h. The medium was then replaced with fresh medium containing VA (20 μg/mL) or ethanol control as described above and incubated for 4 d. Adherent and floating/loosely attached cells were collected and processed separately. Apoptotic DNA was extracted and precipitated using the Suicide-Track DNA Laddering Isolation Kit (Oncogene Research Products, Boston, MA) according to the manufacturer's instructions. DNA was analyzed by electrophoresis in a 1.5% agarose gel at 50 V (constant voltage) for 4.5 h. The gel was then stained for 0.5 h with ethidium bromide (0.5 μg/mL). DNA fragments were visualized and photographed using the ImageMaster VDA documentation system from Amersham Biosciences (Little Chadfort, Buckinghamshire, United Kingdom).

Measurement of reduced GSH. Cells were seeded in T-75 cm^2 flasks at a density of 1×10^6 cells/flask and incubated for 24 h. The medium was then replaced with fresh medium containing VA (20 μg/mL) and incubated for 4 d, after which both floating and adherent cells were collected and pooled. Cells were resuspended in a PBS/PMSF buffer containing 10 mM sodium phosphate buffer (pH 7.2), 100 mM NaCl, 0.2 mM PMSF, 0.1 mM leupeptin, and 0.2 μg/mL aprotinin (Sigma-Aldrich Ireland Ltd.), sonicated on ice, and centrifuged at 100,000 × *g* for 20 min at 4°C. Activity of GSH in the cytosolic fraction was measured according to the method of Hissen and Hilf (36). Briefly, the supernatant fraction (100 μL) was diluted in 1.8 mL phosphate-EDTA buffer (0.1 M sodium phosphate, 0.005 M EDTA, pH 8) and mixed with

100 μL *o*-phthalaldehyde (10 μg/mL). Samples were incubated at 25°C for 15 min, and fluoresence was detected at 420 nm following activation at 350 nm. The glutathione concentration of the samples was determined from a standard curve and expressed relative to the protein content, as determined using the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, Hertfordshire, United Kingdom).

Total ras expression. SW480 cells were seeded at 5×10^6 cells/150 cm^2 flask and were cultured for 24 h to allow the cells to attach to the substratum. The medium was then replaced with medium containing either *c*9,*t*11-CLA or VA (20 μg/mL). An equivalent volume of ethanol was added to the control flasks. Quercetin was used as a positive control. After 24 h of incubation, the cells were harvested using PBS containing 0.25% (by vol) trypsin. The pellets were washed twice in ice-cold PBS. To assess total ras, a lysis buffer [containing 10 mM sodium phosphate buffer (pH 7.2), 100 mM NaCl, 10 mM sodium deoxycholate, 1 mM PMSF, 1% (vol/vol) Triton X-100, 0.1 mM leupeptin, and 0.2 μg/mL aprotinin] was added to the cell pellets, which were then sonicated on ice for 10 min. The lysates were concentrated using Microcon filters (Millipore B.V., Cork, Ireland) and the protein content determined using the Bio-Rad protein assay. Lysates were electrophoresed using 12% (by vol) polyacrylamide gel (70 μg of protein per well). The separated proteins were transferred onto Hybond ECL membrane (Amersham) in a Trans-blot Electrophoretic transfer cell (Bio-Rad). Blots were stained with Ponceau S solution to ensure transfer of proteins was complete and to determine whether an equivalent amount of protein was loaded in each lane. The blots were destained with PBS containing 0.1% (by vol) Tween 20 (PBST). The blots were blocked with 5% (by vol) nonfat dry milk dissolved in PBST. Blots were then incubated with anti-ras monoclonal antibody diluted 1:40 (Oncogene Science, Manhasset, NY) in PBST containing 0.5% nonfat dry milk. Blots were washed extensively in PBST and reincubated with a HRP-linked secondary antibody (Amersham) diluted 1:2000 in PBST containing 0.5% (wt/vol) nonfat dry milk. The blots were then thoroughly washed in excess PBST and probed with the Super Signal detection system (Pierce, Rockford, IL) and exposed to autoradiography films (Amersham) according to the manufacturer's instructions. Densitometry (using NIH Image software) was performed on Ponceau S scans and autoradiographed.

Statistical analysis. Data represent three independent experiments performed in triplicate. Student's *t*-test was used to determine significance between treatments.

RESULTS

Effect of VA uptake on cellular lipids. To determine if VA was bioconverted to *c*9,*t*11-CLA, MCF-7 and SW480 cells were incubated with increasing concentrations of VA (5, 10, and 20 μg/mL), and total cellular lipids subsequently were analyzed by GLC. Neither CLA nor VA was detectable in control untreated SW480 or MCF-7 cells. Incubation of the SW480 colon cell line with increasing concentrations of VA resulted in accumulation of VA and *c*9,*t*11-CLA in a dose-dependent manner to a maximum of 25.11 and 7.10 g/100 g FAME, respectively, following treatment with 20 μg/mL for 24 h (Table 1). The apparent percentage bioconversion of VA to *c*9,*t*11-CLA [g CLA·100^{-1} g FAME/(g CLA·100^{-1} g FAME + g VA·100^{-1} g FAME) × 100] at 5, 10, and 20 μg/mL was 33.8, 28, and 22%, respectively. As shown in Table 2, there was no further accumulation of VA after 4 d. SW480 cells treated with 20 μg/mL VA accumulated 20.58 g/100 g FAME VA after 4 d, which was lower than the level observed after 24 h. Bioconversion to *c*9,*t*11-CLA was increased at this concentration to 29.4% (Table 2). The accumulation of VA caused perturbations in other FA. Treatment with 20 μg/mL VA for 24 h and 4 d resulted in respective reductions of 32 and 38% for palmitic (16:0), 47 and 41% for palmitoleic (16:1), 43 and 44% for stearic (18:0), 44 and 40% for oleic (18:1), and 35 and 29% for LA (18:2), respectively. AA (20:4) was reduced by 32% after 24 h, but no effect was observed after 4 d following treatment with 20 μg/mL VA.

In general, the MCF-7 mammary cell data were similar to

TABLE 1
FA Composition of Total Cellular Lipids from SW480 Cells Incubated in the Presence of Vaccenic Acid (5–20 μg/mL) for 24 h

FA	Untreated controls	VA (5 μg/mL)	VA (10 μg/mL)	VA (20 μg/mL)
		g FA/100 g FAME		
14:0	1.81 ± 0.09	1.74 ± 0.40	2.28 ± 0.24	1.49 ± 0.16
16:0	19.90 ± 1.15	17.79 ± 2.02	17.03 ± 1.86	13.26 ± 0.89*
16:1	3.23 ± 0.16	4.04 ± 2.14	2.57 ± 0.41	1.71 ± 0.06*
18:0	13.67 ± 0.34	10.96 ± 0.41*	10.04 ± 0.82*	7.77 ± 0.29*
18:1	28.04 ± 0.90	21.76 ± 1.86	18.92 ± 0.99*	15.72 ± 0.79*
18:1[a]	0	7.25 ± 1.24*	12.43 ± 0.71*	25.11 ± 2.86*
18:2	5.76 ± 0.22	4.46 ± 0.30*	4.50 ± 0.23*	3.74 ± 0.22*
18:2 (CLA)	0	3.71 ± 0*	4.89 ± 0.23*	7.10 ± 0.86*
20:4	8.54 ± 0.07	7.03 ± 0.64	6.51 ± 0.37*	5.80 ± 0.11*
Others	19.03 ± 2.5	21.26 ± 5.04	20.83 ± 4.03	18.28 ± 5.40

[a]Vaccenic acid (VA; *t*11-18:1). *Denotes results that are significantly different from untreated cells ($P < 0.05$).

TABLE 2
FA Composition of Total Cellular Lipids from SW480 Cells Incubated in the Presence of VA (5–20 μg/mL) for 4 d

FA	Untreated controls	VA (5 μg/mL)	VA (10 μg/mL)	VA (20 μg/mL)
		g FA/100 g FAME		
14:0	2.50 ± 0.44	2.25 ± 0.20	1.90 ± 0.02	1.48 ± 0.26
16:0	23.90 ± 1.13	23.68 ± 0.42	19.50 ± 0.50*	14.78 ± 0.36*
16:1	2.32 ± 0.26	2.01 ± 0.15	1.70 ± 0.01*	1.38 ± 0.14*
18:0	18.19 ± 0.30	14.60 ± 1.20*	12.26 ± 0.33*	10.17 ± 0.43*
18:1	21.26 ± 1.11	17.34 ± 0.29*	15.40 ± 0.39*	12.73 ± 0.42*
18:1 (VA)	0	6.75 ± 0.17*	12.69 ± 0.44*	20.58 ± 0.44*
18:2	5.95 ± 0.16	5.10 ± 0.32*	4.68 ± 0.28*	4.20 ± 0.09*
18:2 (CLA)	0	3.86 ± 0.20*	5.96 ± 0.37*	8.57 ± 0.48*
20:4	7.18 ± 0.50	6.21 ± 0.21*	6.42 ± 0.29*	7.13 ± 0.29
Others	18.67 ± 2.90	18.43 ± 0.26	19.49 ± 1.09*	18.95 ± 0.46

[a]For abbreviation see Table 1. *Denotes results that are significantly different from untreated cells ($P < 0.05$).

the SW480 colon cell data. As shown in Tables 3 and 4, the concentrations of VA and *c*9,*t*11-CLA in cellular lipids increased proportionately with VA treatment. MCF-7 cells treated with 20 μg/mL VA for 24 h accumulated VA and *c*9,*t*11-CLA to 18.98 and 12.09 g/100 g FAME, respectively. The apparent percentage bioconversion of VA at 5, 10, and 20 μg/mL to *c*9,*t*11-CLA was 38, 39.2, and 38.9% after 24 h, respectively. After 4 d, the levels of VA had fallen to 15.53 g/100 g FAME, and this was accompanied by a 44% level of bioconversion to *c*9,*t*11-CLA. Even though bioconversion had increased after 4 d, levels of *c*9,*t*11-CLA did not, suggesting further metabolism by desaturation and elongase enzymes. Levels of bioconversion were higher in MCF-7 cells compared with SW480 cells. Treatment of MCF-7 cells with 20 μg/mL VA for 24 h and 4 d resulted in respective reductions of 47 and 26% for myristic (14:0), 39 and 35% for palmitic (16:0), 33 and 48% for palmitoleic (16:1), 39 and 24% for stearic (18:0), 14 and 35% for oleic (18:1), and 18 and 27% for AA (20:4). LA was decreased by 22% after 24 h, but no changes were observed after 4 d.

The effect of VA and c*9,*t*11-CLA on cell viability.* Effects of incubation with VA and *c*9,*t*11-CLA on growth of MCF-7 and SW480 cells after 4 d of incubation were determined. Both cell lines were sensitive to the antiproliferative effect of the *c*9,*t*11-CLA isomer as previously reported (29). All *c*9,*t*11-CLA concentrations significantly lowered ($P < 0.05$) cell numbers in both cell lines (Fig. 1A, 1B). In the MCF-7 cell line, there were no significant differences between the final cell numbers ($27.6–26.4 \times 10^4$) obtained for the 5, 10, and 16 μg/mL *c*9,*t*11-CLA treatments (Fig. 1A). The 20 and 25 μg/mL treatment significantly lowered ($P < 0.05$) cell number by 56 and 61%, respectively, which were not significantly different from each other. The two highest *c*9,*t*11-CLA concentrations (20 and 25 μg/mL) had a significantly greater antiproliferative effect on cell growth when compared with concentrations of 5–16 μg/mL. In the MCF-7 cell line, VA supplementation for 4 d at concentrations less than 20 μg/mL had no effect on cell growth, whereas supplementation with 20 and 25 μg/mL VA significantly reduced ($P < 0.05$) growth by 30 and 41%, respectively. In the SW480 cell line, incubation with CLA at concentrations of 5–25 μg/mL decreased cell growth to a similar level (49–52%) (Fig. 1B). Similarly,

TABLE 3
FA Composition of Total Cellular Lipids from MCF-7 Cells Incubated in the Presence of VA (5–20 μg/mL) for 24 h

FA	Untreated controls	VA (5 μg/mL)	VA (10 μg/mL)	VA (20 μg/mL)
		g FA/100 g FAME		
14:0	4.43 ± 0.15	3.96 ± 0.30	3.51 ± 0.18*	2.33 ± 0.08*
16:0	25.78 ± 0.46	25.29 ± 0.49	22.89 ± 0.69*	15.69 ± 0.25*
16:1	6.85 ± 0.28	6.21 ± 0.24	5.72 ± 0.25	4.60 ± 0.11*
18:0	18.71 ± 2.87	16.23 ± 1.98	13.28 ± 0.65*	11.49 ± 0.49*
18:1	20.19 ± 0.74	19.10 ± 0.49	18.76 ± 1.04	17.33 ± 0.44*
18:1 (VA)	0	5.87 ± 0.52*	10.77 ± 0.80*	18.98 ± 0.90*
18:2	3.34 ± 0.27	2.85 ± 0.24*	2.73 ± 0.09*	2.59 ± 0.07*
18:2 (CLA)	0	3.63 ± 0.45*	6.95 ± 0.21*	12.09 ± 0.30*
20:4	4.73 ± 0.28	4.23 ± 0.10	4.13 ± 0.13*	3.89 ± 0.29*
Others	15.98 ± 2.30	12.64 ± 0.17	11.25 ± 0.75	10.99 ± 0.85

[a]For abbreviation see Table 1. *Denotes results that are significantly different from untreated cells ($P < 0.05$).

TABLE 4
FA Composition of Total Cellular Lipids from MCF-7 Cells Incubated in the Presence of VA (5–20 μg/mL) for 4 d

FA	Untreated controls	VA (5 μg/mL)	VA (10 μg/mL)	VA (20 μg/mL)
		g FA/100 g FAME		
14:0	3.17 ± 0.20	2.88 ± 0.10	2.78 ± 0.44	2.34 ± 0.08*
16:0	26.82 ± 0.80	24.79 ± 0.57*	21.89 ± 0.58*	17.44 ± 1.09*
16:1	4.37 ± 0.32	2.71 ± 0.13*	2.44 ± 0.14*	2.29 ± 0.33*
18:0	17.87 ± 0.24	15.91 ± 0.29*	14.93 ± 0.26*	13.51 ± 0.45*
18:1	19.95 ± 0.60	17.69 ± 0.96	15.25 ± 0.71*	12.92 ± 1.52*
18:1 (VA)	0	5.31 ± 0.19*	8.34 ± 0.62*	15.53 ± 2.00*
18:2	4.08 ± 0.41	4.25 ± 0.35	4.21 ± 0.56	4.06 ± 0.39
18:2 (CLA)	0	4.87 ± 0.28*	8.64 ± 2.03*	12.14 ± 1.50*
20:4	7.09 ± 0.80	6.81 ± 0.18	6.83 ± 0.28	5.18 ± 1.04*
Others	16.63 ± 0.78	14.76 ± 0.82	14 .66 ± 1.91	14.58 ± 1.41

[a]For abbreviation see Table 1. *Denotes results that are significantly different from untreated cells ($P < 0.05$).

incubation with VA at a concentration of 20 μg/mL significantly decreased cell growth in the SW480 cell line. The MCF-7 mammary cell line was more sensitive to the antiproliferative effects of VA and *c*9,*t*11-CLA.

Effect of VA on incorporation of ^{14}C-AA into cellular lipid fractions. To examine whether cellular AA distribution was altered by VA, we investigated the effect of VA (20 μg/mL) on incorporation of ^{14}C-AA into cellular lipid fractions. Levels of ^{14}C-AA uptake into PL, TG, and MG were 64, 26, and 7%, respectively, in control MCF-7 cells (Fig. 2A), which are similar to levels of incorporation previously reported (29). ^{14}C-AA uptake into the MG fraction was significantly ($P < 0.05$) increased by 10% in MCF-7 cells treated with VA. Levels of uptake into PL, TG, and MG were 77, 20, and 3%, respectively, in control SW480 cells (Fig. 2B), which are similar to levels previously reported (29). ^{14}C-AA uptake into the MG fraction was significantly ($P < 0.05$) increased by 8% in SW480 cells treated with VA, whereas uptake into the PL fraction was significantly ($P < 0.05$) decreased by 17%.

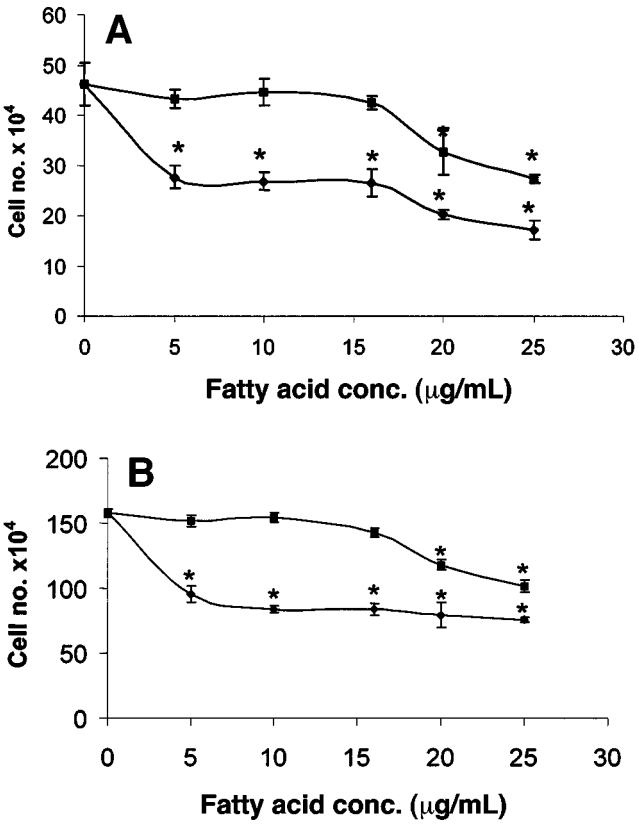

FIG. 1. MCF (A) and SW480 (B) cell number following treatment with varying concentrations of *c*9,*t*11-CLA (◆) and vaccenic acid (VA) (■) for 4 d. *$P < 0.05$ relative to control. Error bars represent SD.

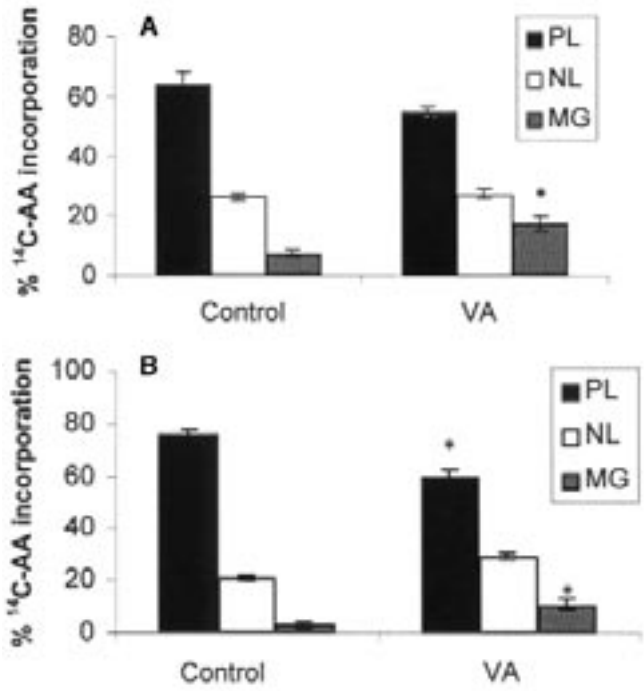

FIG. 2. Percentage of ^{14}C-arachidonic acid (AA) incorporation into phospholipids (PL), triacylglyceride (TG) and neutral lipids (NL), and monoacylglyceride (MG) following 24-h treatment of MCF-7 cells (A) and SW480 cells (B) with vaccenic acid (20 μg/mL) . *$P < 0.05$ relative to control. Error bars represent SD.

Effect of VA on PG and 8-epi- $PGF_{2\alpha}$ synthesis. The effects of VA on enzymatic conversion of AA to PG (PGD_2, PGE_2, and $PGF_{2\alpha}$) and on oxidation to 8-epi- $PGF_{2\alpha}$ were examined. Following incubation of both cell lines with VA (20 μg/mL), negligible effects of ^{14}C-AA conversion to ^{14}C-PGD_2, -PGE_2, and -$PGF_{2\alpha}$ were observed (Fig. 3). We previously reported that the *c*9,*t*11-CLA isomer at 16 μg/mL significantly decreased ^{14}C-AA conversion to ^{14}C-PGE_2 while increasing conversion to ^{14}C-$PGF_{2\alpha}$ (29). We have also examined the effect of a range of *c*9,*t*11-CLA concentrations (5, 10, 16, and 20 μg/mL) on conversion to ^{14}C-PGE_2 and found that only 16 and 20 μg/mL *c*9,*t*11-CLA significantly decreased ^{14}C-PGE_2 levels (Fig. 4). Therefore, it is plausible that bioconversion of VA did not achieve a *c*9,*t*11-CLA concentration high enough to alter PG synthesis. VA did significantly increase ($P < 0.05$) the levels of the isoprostane 8-epi-$PGF_{2\alpha}$, a biomarker of lipid peroxidation. The *c*9,*t*11-CLA isomer also has been shown to increase the levels of 8-epi-$PGF_{2\alpha}$ in both cell lines (29).

Effect of VA on apoptosis in SW480 cells. The formation of distinct DNA fragments of oligonucleosomal size (180–200 bp) is a biochemical hallmark of apoptosis in many cells (37) and is observed as a DNA ladder in agarose gels. Evidence was sought to determine if the antiproliferative action of VA induced a specific pattern of chromatin cleavage into oligonucleosomes. After cells had been treated with VA for 4 d, the media and PBS wash of the monolayer, both of which may contain apoptotic cells that have floated loose from the monolayer, were collected separately from the attached cells in the monolayer. VA treatment exhibited the characteristic ladder on electrophoresis of DNA extracted from the floating cells (Fig. 5A). A small amount of DNA laddering was observed in the control cells, which would be expected as a small percentage of cells die normally in culture. No DNA laddering was observed in DNA extracted from adherent cells (Fig. 5B). The effect of VA treatment on levels of cytosolic GSH was evaluated. Diminished GSH levels have been observed in apoptotic cells and have been associated with cytochrome c release from the mitochondria (38). SW480 cells were treated with VA (20 μg/mL) as described earlier, and after 4 d all cells were collected and the cytosolic fraction was prepared. VA significantly ($P < 0.05$) reduced GSH levels by 15% (Fig. 5C).

Effect of VA and c*9,t11-CLA on ras expression*. Molecules that inhibit ras localization to cell membranes are potential cancer therapeutic agents (39). The *ras* oncogene encodes a protein whose GTPase activity cannot be stimulated and that leaves ras in an active GTP-bound form on the membrane switching on nuclear transcription factors controlling cell proliferation *via* a cascade of kinase-driven phosphorylation events (40). Figure 6 shows representative example of Western blot analysis of membrane *ras* p21 in cells treated with *c*9,*t*11-CLA or VA for 4 d. Quercetin was used as a positive control. The duplet present was identified as nonfarnesylated ras at 21 kDa and farnesylated ras at 23 kDa. The upper and lower bands were measured using densitometry. Quercetin decreased total ras after 4 d by 58%. After 4 d of incubation, the *c*9,*t*11-CLA and VA significantly ($P < 0.05$) reduced total *ras* expression by 23 and 45%, respectively.

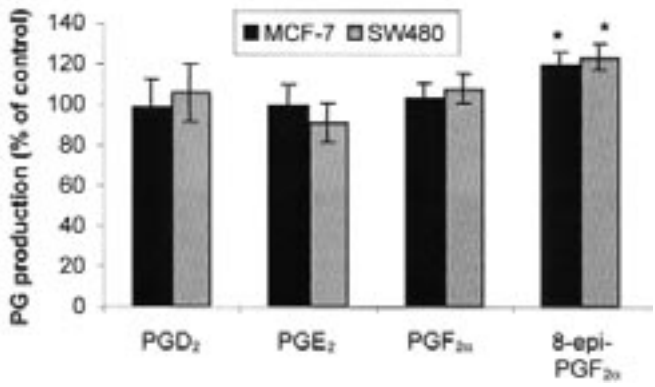

FIG. 3. Prostaglandin (PG) production in MCF-7 cells and SW480 cells following treatment with VA (20 μg/mL). *$P < 0.05$ relative to control. See Figure 1 for other abbreviation. Error bars represent SD.

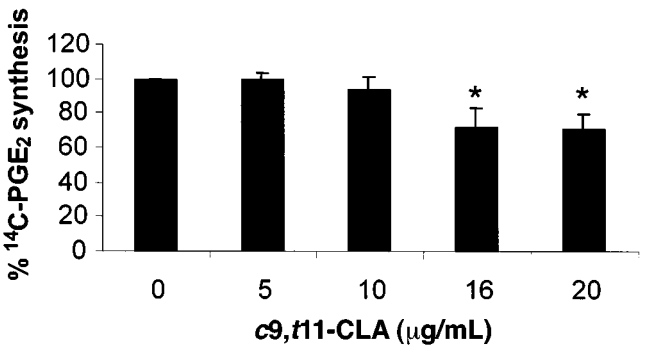

FIG. 4. Percentage of ^{14}C-prostaglandin E_2 (PGE_2) synthesis in SW480 cells following treatment with *c*9,*t*11-CLA (5–25 μg/mL). *$P < 0.05$ relative to control. Error bars represent SD.

DISCUSSION

Parodi (41) was the first to propose that VA could be converted to CLA in humans based on the observation that a Δ^9-desaturase enzyme from rat liver microsomes has been shown to produce *c*9,*t*11-CLA from VA (42,43). Santora *et al.* (19) reported and quantified the desaturation of VA to the *c*9,*t*11-CLA isomer in mice fed purified diets. Based on concentrations of VA and *c*9,*t*11-CLA in the total carcass, 11.4% of dietary VA and 50.8% of stored VA was desaturated. The CLA produced from VA desaturation was found only in TG, suggesting that bioconversion occurred in the adipose tissue. Salminen *et al.* (44) provided evidence to suggest that CLA in human serum was in part derived from the bioconversion of dietary *trans*-FA but provided no quantitative estimate of desaturation. Emken *et al.* (45) originally found no evidence for desaturation of VA in the plasma lipids of men given deuterium-labeled VA with the limit of detection used in the study. However, when one sample from this study was reanalyzed, it was demonstrated that VA was converted to *c*9,*t*11-

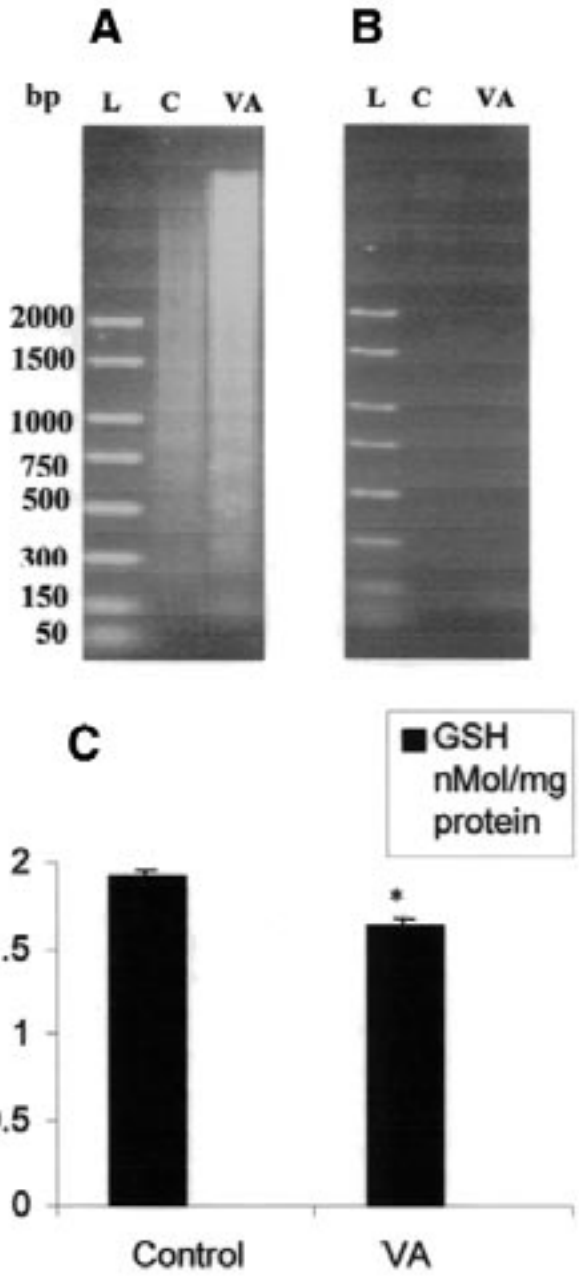

FIG. 5. Effect of vaccenic acid (VA) (20 μg/mL) on apoptosis in SW480 cells. (A) Floating cells collected after 4 d of incubation; (B) adherent cells collected after 4 d of incubation. For panels A and B, L = marker lane containing DNA fragments ranging from 50 to 2000 base pairs and C = control cells treated with ethanol. (C) Effect of VA on GSH levels in SW480 cells. *$P < 0.05$ relative to control. Error bars represent SD.

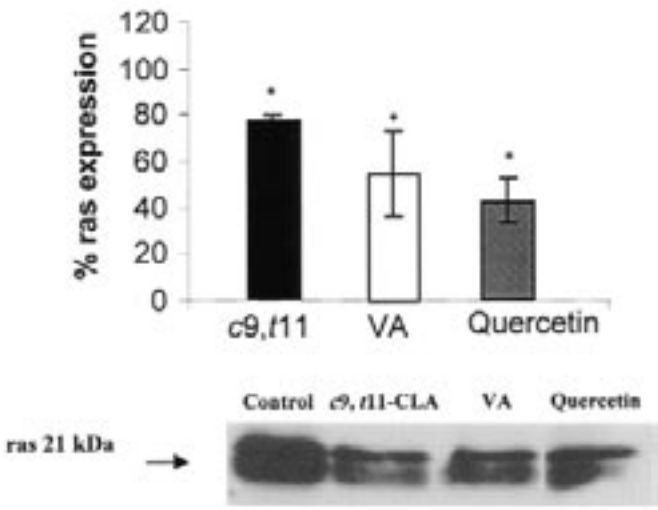

FIG. 6. Western blot and densitometry result for total ras expression in SW480 cells following treatment with vaccenic acid (VA) (20 μg/mL) for 4 d. Densitometric quantification was normalized using Ponceau S scans. Values are expressed as percentage of control (100%). *$P < 0.05$ relative to control. Error bars represent SD.

CLA, at a CLA enrichment of 30% presumably *via* the Δ^9-desaturase reaction (46). Consistent with this assumption is the recent study showing that the concentrations of CLA and CLA metabolites increased proportionately in the liver and mammary gland of rats fed an increasing dietary VA concentration (21).

This study clearly demonstrates that VA is incorporated into the cellular lipids of MCF-7 and SW480 cancer cells in a dose- and time-dependent manner and that these cells have the capability to convert a portion of this VA to *c*9,*t*11-CLA. Both cell lines preferentially synthesized *c*9,*t*11-CLA, as indicated by the marked decrease in oleic acid and palmitoleic acid. FA data were presented as percentage of total FA and not as actual amounts of lipid, as the effect of VA on cellular lipid was not determined. It is possible that as the mass of VA and CLA increases, the other FA maintain a constant mass but decrease in proportion. Our calculation for bioconversion does not take into account the fraction of VA that may be oxidized or the fraction of CLA that may be further metabolized by β-oxidation or by elongation/desaturation. In our study, the percentage of VA (20 μg/mL) bioconverted to *c*9,*t*11-CLA was greater (44%) in the MCF-7 cell line than in the SW480 cell line (29.4%) after 4 d of incubation in the presence of VA (20 μg/mL). Although bioconversion differences between cell lines can be ascribed to differences in rates of CLA metabolism and/or VA oxidation, another possible explanation may be that there are differences in level of expression and/or activity of Δ^9-desaturase between the two cell lines. The MCF-7 cell line recently has been reported to express relatively high levels of Δ^9-desaturase (47). Unlike the MCF-7 cell line, in which the percentage bioconversion to *c*9,*t*11-CLA remained similar throughout 24 h and 4 d, the dose-dependent decrease in percentage bioconversion in the SW480 cell line suggests product inhibition may have occurred. Δ^9-Desaturase is regulated by PUFA at the level of transcription and mRNA stability (2). CLA has been shown to reduce hepatic Δ^9-desaturase mRNA levels in mice, (48), Δ9-desaturase mRNA expression and its activity in 3T3-L1 adipocytes (49), and activity but not expression in the HepG2 human hepatoblastoma cell line (50). These effects were due to the *t*10,*c*12-isomer. However, Choi *et al.* (47) recently reported that both the *t*10,*c*12- and *c*9,*t*11-CLA isomers have a direct inhibitory effect on Δ^9-desaturase activity in the MCF-7 cell line.

VA elicits a biological response *in vivo,* reducing mammary gland premaligant lesions in carcinogen-treated rats (21). There is evidence that other monounsaturated FA can exhibit antiproliferative action on cancer cells and that effects are independent of conversion to CLA by Δ^9-desaturase. VA (8.4 μg/mL), in either the *cis* or *trans* form, significantly reduced growth of HT-29 human colon cancer cells by 17% when compared with control cells that were supplemented with an equimolar concentration of stearic acid after 9 d (51). A study carried out in our laboratory showed that oleic acid inhibited growth of SW480 cells by 20% when compared with control cells (data not shown). The present study demonstrates that incubation with VA at a concentration of 25 μg/mL inhibits the growth of human MCF-7 and SW480 cancer cells by up to 41 and 36%, respectively, after 4 d. VA treatment induced apoptosis in SW480 cells, as indicated by DNA fragmentation. Studies suggest that oxidative stress in general and lipid peroxidation in particular are involved in both initiation and mediation of apoptosis (52). The isoprostane 8-epi-$PGF_{2\alpha}$, a biomarker of lipid peroxidation, was increased and GSH was reduced following VA treatment. Diminished GSH levels have been observed in apoptotic cells and are associated with cytochrome c release from the mitochondria (38). We previously reported that a mixture of the *t*10,*c*12- and *c*9,*t*11-CLA isomers lowered the expression of the anti-apoptotic bcl-2 protein, decreased cytosolic GSH levels, increased accumulation of cytochrome c in the cytosol, activated caspase 9 and 3, and caused DNA fragmentation (30). This study suggests that growth inhibition by VA and *c*9,*t*11-CLA in SW480 cells may also be mediated in part by reduced expression of ras oncoprotein. The decrease in total ras expression following 4 d of treatment of SW480 cells with VA and *c*9,*t*11-CLA suggests that they may inhibit a ras signaling pathway.

It was apparent from the data presented that, with the exception of SW480 cells treated with 20 μg/mL VA for 4 d, AA (g/100 g FAME) decreased in cells treated with VA, possibly as a result of oxidation. A decrease in AA as a result of oxidation would be consistent with the observation that VA increased isoprostane 8-epi-$PGF_{2\alpha}$ in both cell lines (Fig. 3). The return to basal levels in SW480 cells treated with 20 μg/mL VA for 4 d may reflect possible antagonism of AA metabolism by CLA and/or its conjugated metabolites. The growth-inhibitory effects of VA observed in this study were associated with alterations in AA uptake into cellular lipid fractions. In MCF-7 cells, ^{14}C-AA uptake was increased into the MG fraction following treatment with VA. The pattern of incorporation was similar to that previously observed in MCF-7 cells treated with *c*9,*t*11-CLA (29). However, a different pattern was observed in SW480 cells, where VA treatment, unlike *c*9,*t*11-CLA, increased ^{14}C-AA uptake into the MG fraction at the expense of uptake into the PL fraction (29). Although partitioning of MG between metabolic pathways leading to PL or TG synthesis was not studied, the preferential incorporation of AA into the MG fraction in cells suggests that acylglycerol synthesis pathways in cells may be influenced by exogenous FA. There is evidence that in enterocytes the metabolic fate of MG is related to compartmentalization of multiple acyl CoA synthetase genes (53). Further studies are needed to understand the biochemical significance of possible separate AA pools for acyl-CoA-dependent enzymes in anabolic reactions leading to PL and TG synthesis. We propose that VA may have biological activity independent of its conversion to CLA. If only biosynthesized *c*9,*t*11-CLA was influencing AA uptake, it would be expected that ^{14}C-AA uptake would have increased into TG. These changes in AA uptake following VA treatment did not alter the PG profile, as was previously observed with *c*9,*t*11-CLA. This suggests that reduced availability of AA cannot solely account for reduced PG production. CLA isomers have been shown to inhibit the oxygenation of AA by PG H synthase (54). CLA and VA may have different modulatory effects on this enzyme.

The human Δ^9-desaturase gene has been isolated, sequenced, and shown to be expressed in human skin, adipose, liver, and brain tissue (55). Expression of the human Δ^9-desaturase gene and enzyme activity was demonstrated recently in MCF-7 cells (47). Whereas little is known about the expression of desaturase mRNA in normal colon, it is of interest that Δ^9-desaturase mRNA was found to be overexpressed in human colonic tumors (56). Based on the findings in this study, we hypothesize that intakes of ruminant/dairy fats with a FA composition consisting of VA and CLA may be beneficial to health in terms of chemoprevention because of not only CLA but also VA content.

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Transfer of Arachidonic Acid from Lymphocytes to Macrophages

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ABSTRACT: The incorporation and oxidation of arachidonic acid (AA) by rat lymphocytes (LY), the transfer of AA from LY to rat macrophages (Mϕ) in co-culture, and the subsequent functional impact on Mϕ phagocytosis were investigated. The rate of incorporation of [1-^{14}C]AA by untreated-LY and TG (thioglycolate treated)-LY (TG-LY) was 158 ± 8 nmol/10^{10} LY per h for both untreated-LY and TG-LY. The oxidation of AA was 3.4-fold higher in TG-LY as compared with untreated cells. LY from TG-injected rats had a 2.5-fold increase in the oxidation of palmitic (PA), oleic (OA), and linoleic (LA) acids. After 6 h of incubation, [^{14}C] from AA was distributed mainly into phospholipids. The rate of incorporation into total lipids was 1071 nmol/10^{10} cells in untreated-LY and 636 nmol/10^{10} cells in TG-LY. [^{14}C]AA was transferred from LY to co-cultured Mϕ in substantial amounts (8.7 nmol for untreated and 15 nmol per 10^{10} for TG cells). Exogenously added AA, PA, OA, and LA caused a significant reduction of phagocytosis by resident cells. Mϕ co-cultured with AA-preloaded LY showed a significant reduction of the phagocytic capacity (about 40% at 35 μM). LY preloaded with PA, LA, and OA also induced a reduction in phagocytic capacity of co-cultured Mϕ. TG treatment abolished the AA-induced inhibition of phagocytosis in Mϕ co-cultured with TG-LY. Therefore, the transfer of AA between leukocytes is a modulated process and may play an important role in controlling inflammatory and immune response.

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Glucose and glutamine are partially oxidized by macrophages (Mϕ) (1–3). These substrates can generate acetyl-CoA that is channeled into *de novo* lipid synthesis. Ultimate products may include FA, cholesterol (CHOL), and phospholipids (PL) (4). Lipids can be exported to the extracellular medium when Mϕ are maintained in culture (5). This phenomenon is not restricted to Mϕ. In fact, lymphocytes (LY) export lipids to the incubation medium, and this process is increased by concanavalin A stimulation (5).

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Abbreviations: AA, arachidonic acid; CEST, cholesterol ester; CHOL, cholesterol; LA, linoleic acid; LY, lymphocytes; Mϕ, macrophages; ME, methyl esters; OA, oleic acid; PA, palmitic acid; PGE_2, prostaglandin E_2; PKC, phosphokinase C; PL, phospholipids; PLA_2, phospholipase A_2, PLC, phospholipase C; resident-Mϕ, peritoneal macrophages from untreated rats; SA, specific activity; TG, thioglycolate; TG-LY, mesenteric lymph node lymphocytes from rats injected with thioglycolate; TG-Mϕ, peritoneal macrophages from rats injected with TG (TG-elicited macrophages); untreated-LY, mesenteric lymph nodes lymphocytes from untreated rats.

Mϕ transfer FA (6), CHOL (7), and PL (8) to co-cultured LY. Radioactivity from labeled arachidonic acid (AA), oleic acid (OA), linoleic acid (LA), and palmitic acid (PA) is transferred from peritoneal resident-Mϕ (peritoneal macrophages from untreated rats) to LY in co-culture (6) and is subsequently incorporated into the PL fraction of the acceptor cells (in excess of 80% of the FA investigated). AA is quantitatively the most important FA transferred by Mϕ to LY. Thioglycolate-elicited Mϕ (TG-Mϕ) are also able to transfer AA to LY in the same amount (6).

TG-Mϕ are characterized by increased phagocytic capacity, plasma membrane turnover, and high activity of lysosomal enzymes (3) compared to resident Mϕ of tissues and organs, which present low functional activities of these parameters (3,9). However, after recruitment by inflammatory or immune stimuli, these cells express different secretory and endocytic properties (9,10). Studies on phagocytosis, respiratory burst, and metabolism of TG-Mϕ indicate that the effects of TG are partially due to changes in glucose, glutamine, and FA metabolism (3,11,12). TG injection, however, also affects the metabolism and function of LY (13). LY obtained from TG-injected rats show increased proliferative activity, protein synthesis, and glutaminolysis (13).

We have now investigated whether AA can be transferred from LY to Mϕ and whether TG injection modulates this process. We determined [^{14}C]-AA incorporation by LY, the distribution of the [^{14}C] into LY lipid fractions, and the transfer of radioactivity to resident and TG-Mϕ in co-culture. The effects of exogenously added AA on Mϕ phagocytosis and of AA-preloaded LY on phagocytic capacity of co-cultured Mϕ also were examined.

MATERIALS AND METHODS

Chemicals. Solvents and May-Giemsa stain were obtained from Merck (Darmstadt, Germany). FA, lipid standards, and antibiotics were purchased from Sigma (St. Louis, MO). RPMI 1640 medium and heat-inactivated (56°C for 30 min) FCS were obtained from GIBCO (Life Technologies, Inc., Rockville, MD). [1-^{14}C]AA (20:4n-6; specific activity (SA): 55 mCi/mmol); [U-^{14}C]PA (16:0; SA: 850 mCi/mmol); [1-^{14}C]OA (18:1n-9; SA: 50 mCi/mmol), and [1-^{14}C]LA (18:2n-6, SA: 58 mCi/mmol) were purchased from NEN (Du Pont, Boston).

Preparation of LY and Mϕ. The Ethical Committee of the Institute of Biomedical Sciences, University of São Paulo, ap-

proved the experimental procedure used in this study. Untreated Mϕ (resident-Mϕ) were collected from the peritoneal cavity of rats as previously described (14). TG-Mϕ were collected in a similar manner 4 d after an intraperitoneal injection of 3 mL of a 40 g/L sterile Brewer's TG broth solution (Merck). LY were obtained from untreated and TG-injected rats (untreated-LY and TG-LY, respectively) by pressing the mesenteric lymph nodes through a wire mesh (6). Mϕ and LY pellets were resuspended in medium to achieve a final suspension of an appropriate number of cells per volume for each experiment.

Time-course incorporation of [^{14}C]AA by LY in culture. Untreated- and TG-LY (8.2×10^5 cells/mL) were cultivated in 12.5 mL RPMI 1640 medium containing 0.04 μCi/mL [^{14}C]AA (corresponding to 0.727 nmol/mL of AA) for 6 h at 37°C in an atmosphere of 5% CO_2 (vol/vol) in a Microprocessor CO_2 Incubator (Lab-Line, Melrose Park, IL). At the end of each incubation period, cells were collected, centrifuged, and washed using PBS. Cells were resuspended in 50 μL ethanol, and the total radioactivity incorporated was determined by using Ecolume scintillating cocktail (ICN Biochemicals, Costa Mesa, CA) in a Beckman-LS 6000 IC liquid scintillation counter.

Incorporation of [1-^{14}C]AA into lipid fractions of cultured LY. Untreated- and TG-LY (1.0×10^7 cells in 2 mL per well) were cultivated during 6 h in medium containing [^{14}C]AA (0.2 μCi/mL corresponding to 4 nmol/mL of AA). After this period, cells and medium were collected. LY were washed to remove any remaining [^{14}C]AA and resuspended in 0.5 mL PBS. The total lipids from cells and medium were then extracted by a modification of the method of Folch *et al.* (15,16) and chromatographed using TLC as previously described (6).

Oxidation of AA, PA, OA, and LA by LY. Untreated- and TG-LY were incubated for 6 h in medium containing 0.2 μCi per well of [^{14}C]-labeled AA, PA, OA, or LA complexed to albumin as previously described (14). The $^{14}CO_2$ produced from [^{14}C]-labeled FA was collected, and the radioactivity was determined in a Beckman counter.

Transfer of [1-^{14}C]AA from LY to Mϕ in co-culture. Untreated- and TG-LY were preloaded with [^{14}C]AA (0.2 μCi/mL) under the same conditions described above. After 6 h, LY were collected and washed with medium to remove the excess [^{14}C]-labeled AA. Concomitantly, resident-Mϕ and TG-Mϕ (1.0×10^7 cells in 2 mL per well) were preincubated for 1 h under sterile conditions to promote Mϕ adhesion. Adhered Mϕ were then washed twice. For [1-^{14}C]AA transfer, resident-adhered Mϕ were co-cultured with labeled untreated-LY (1.0×10^7 cells) in 2 mL medium, whereas TG-adhered Mϕ were co-cultured with labeled TG-LY. For this purpose, labeled LY were added to wells in an insert system (12 mm, 0.4 μm; Millipore Corp., Bedford, MA) that avoids the contact between LY and Mϕ but cannot restrict the exchange of medium between both compartments. After 3 h of co-culture, the medium was transferred to Eppendorf® tubes and centrifuged ($15{,}000 \times g$ for 1 min). LY were collected from the inserts. The plates with the labeled adhered Mϕ were then washed twice with 1 mL PBS, and Mϕ were scraped off and collected. The radioactivity was then determined in samples of Mϕ and LY, and in the medium of co-culture. The lipids from each sample were extracted and separated by TLC. Each lipid band was analyzed as described above.

Phagocytic activity of adhered peritoneal Mϕ. An aliquot of 100 μL of peritoneal suspension containing resident- or TG-Mϕ (2×10^5 of total cells) was placed onto a glass coverslip and left to adhere for 20 min at room temperature. The coverslips were washed with PBS and incubated in RPMI medium. The effect of increasing concentrations of AA, PA, OA, and LA was evaluated on Mϕ phagocytosis. Phagocytosis was also evaluated in Mϕ after being co-cultured with LY preloaded with the FA for 3 h. After incubation in the presence of the FA or co-culture with FA-preloaded LY, the coverslips containing Mϕ were washed and incubated with opsonized zymosan as previously described (17). The percentage of phagocytosis was then determined (18).

Effect of AA, PA, OA, and LA on phagocytic activity of Mϕ. Adhered Mϕ (2×10^5 cells) were incubated for 6 h in medium containing various concentrations of AA, PA, OA, and LA (0, 20, 35, 50, and 75 μM). After that, cells were washed with PBS to remove the FA in excess. Cells were then incubated in 1 mL RPMI medium for 3 h to allow comparison with the results of Mϕ that were co-cultured with LY for the same period. Mϕ were then incubated with opsonized zymosan (17).

Effect of AA-, PA-, OA-, and LA-preloaded lymphocytes on phagocytic activity of co-cultured Mϕ. Untreated- and TG-LY (2×10^5 cells) were treated in advance with AA, PA, OA, and LA (0, 20, 35, 50, and 75 μM) in 1 mL RPMI medium. After 6 h of incubation, LY were washed several times with PBS. The co-culture was initialized after the addition of preloaded LY to the plates containing the adhered Mϕ and incubated for 3 h. For phagocytosis assay, nonadhered cells (LY) were removed and adhered Mϕ were incubated with zymosan (17).

Analysis of plasma membrane integrity and morphology of LY and Mϕ submitted to the experimental conditions described above. Viability of LY and Mϕ treated with FA was evaluated using a flow cytometer and *Cell Quest* software (FACScalibur; Becton Dickinson, San Jose, CA). Morphology of the cells was analyzed (× 1000) by using an optical microscope.

Statistical analysis. The differences between resident-Mϕ and TG-Mϕ cells and FA-treated and untreated cells, and the direct effect of AA on phagocytosis compared with the effect of AA-preloaded LY were assessed by using ANOVA, and the significance level was set for $P < 0.05$.

RESULTS

The morphology and viability of both LY and Mϕ were unmodified by the addition of FA and the experimental procedure imposed (data not shown).

The rate of incorporation of [1-^{14}C]AA by untreated- and TG-LY was linear over a period of 6 h (Fig. 1). The rate of incorporation was 158 ± 8 nmol/10^{10} LY/h for both untreated- and TG-LY.

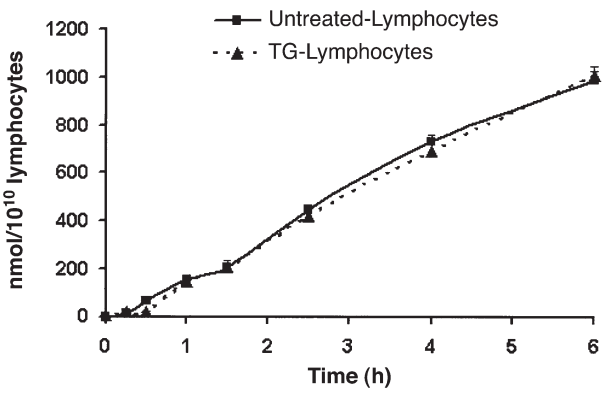

FIG. 1. Incorporation of [1-^{14}C]arachidonic acid by untreated and TG-LY during 6 h in culture. Results are expressed as nmol per 10^{10} LY as mean ± SEM of three determinations. The incorporated radioactivity was determined after 0, 15, and 60 min and 1 h 30 min, 2 h 30 min, 4 h, and 6 h. After each period, cells were collected and washed, and the radioactivity was determined. LY, lymphocytes; TG, thioglycolate; untreated-LY, LY from noninjected rats; TG-LY, LY obtained from rats injected with TG.

After 6 h of incubation, [^{14}C] from AA was distributed into various lipid fractions, but PL was quantitatively the most important destination (about 90% for both untreated- and TG-LY) (Fig. 2A). The incorporation of AA into total lipids was 1071 nmol/10^{10} cells in untreated LY and 636 nmol/10^{10} in TG-LY. Therefore, TG treatment of rats prior to LY harvest induced a reduction of 41% in the incorporation of AA into the total lipid fraction (Fig. 2A).

The oxidation of AA was 3.4-fold higher in TG-LY than in untreated LY (440 nmol per 10^{10} LY over 6 h compared to 130 nmol) (Table 1). For comparison, the oxidation of [^{14}C]-labeled PA, OA, and LA by untreated and TG-LY was also evaluated. The results of the FA oxidation by untreated LY, expressed as nmol per 10^{10} LY in 6 h, were (mean of seven determinations) 333, 327, and 367 for PA, OA, and LA, respectively. LY from TG-injected rats demonstrated a 2.5-fold increase in the oxidation of these FA ($P < 0.05$).

After 3 h in co-culture, LY transferred a considerable amount of ^{14}C from AA to Mϕ lipid fractions (Fig. 3). The radioactivity was mainly found in PL (99% in untreated, and 56% in cells obtained from TG-injected rats). The amount transferred was modulated by TG treatment. The transfer of AA into lipid fractions of the acceptor cells was increased from 8.7 in untreated to 15 nmol per 10^{10} cells obtained from TG-injected rats. The increment in radioactivity was mainly found in FFA, cholesterol esters (CEST), and methyl ester (ME) fractions.

The remaining radioactivity of co-cultured LY was found mainly in the PL fraction (Fig. 4). There was only a slight re-

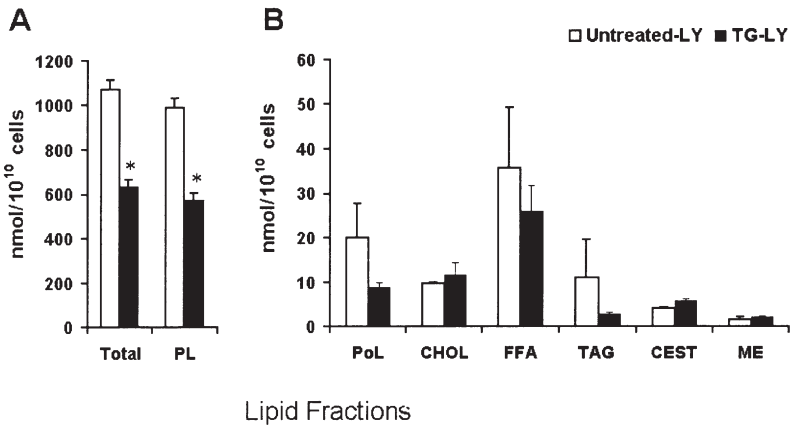

FIG. 2. Incorporation of [1-^{14}C]arachidonic acid into lipid fractions of LY from untreated and TG-treated rats after 6 h in culture. Results are expressed in nmol/10^{10} cells as mean ± SEM of six determinations from three experiments. LY were cultivated with 0.2 μCi/mL of [1-^{14}C]arachidonic acid. After 6 h, cells were washed and lipids were extracted. Lipid fractions were separated by TLC, and the radioactivity of each fraction was then determined. PL, phospholipids; PoL, polar lipids; CHOL, cholesterol; CEST, cholesterol ester; ME, methyl ester; Total, sum of all fractions; *($P < 0.05$) due to TG injection. For other abbreviations see Figure 1.

TABLE 1
$^{14}CO_2$ Production[a] from [U-^{14}C]Palmitic, [1-^{14}C]Oleic, [1-^{14}C]Linoleic, and [1-^{14}C]Arachidonic Acids by Untreated and TG-Lymphocytes After 6 h Incubation

	PA	OA	LA	AA
Untreated-LY	333.0 ± 115.1	327.1 ± 36.4	367.4 ± 107.1	130.0 ± 32.7
TG-LY	881.4 ± 63.9*	829.1 ± 55.1*	933.2 ± 82.3*	439.5 ± 53.5*

[a]Results are expressed as the mean ± SEM of seven experiments, in nmol per 10^{10} LY in 6 h. For details see the Materials and Methods section. PA, palmitic acid; OA, oleic acid; LA, linoleic acid; AA, arachidonic acid; TG-LY, lymphocytes (LY) from thioglycolate-injected rats. *($P < 0.05$) due to thioglycolate injection.

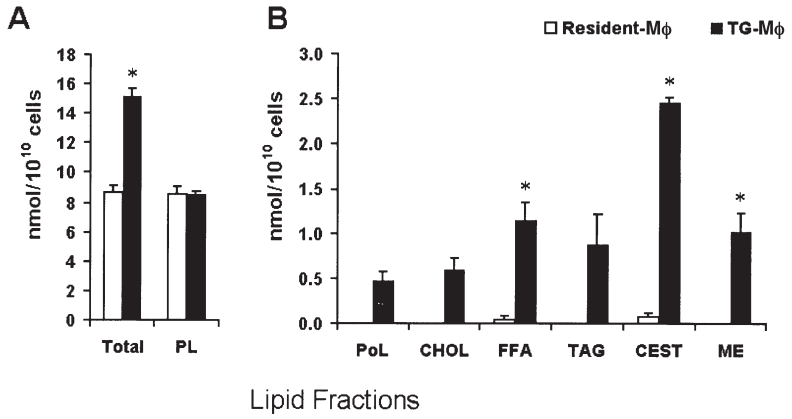

FIG. 3. Transfer of radioactivity to lipid fractions of macrophages (Mϕ) from [1-^{14}C]arachidonic acid preloaded LY after 3 h in co-culture. Resident and TG-elicited Mϕ were co-cultivated with [1-^{14}C]arachidonic acid-preloaded lymphocytes. Results are expressed in nmol per 10^{10} cells as mean ± SEM of six determinations from three experiments. LY were cultivated with 0.2 μCi/mL of [1-^{14}C]arachidonic acid for 6 h. Afterward, labeled LY were washed and co-cultivated with Mϕ for 3 h. After this period, lipids from Mϕ were extracted and chromatographed, and the radioactivity of each lipid fraction was determined. Resident-Mϕ, macrophages from noninjected rats; TG-Mϕ, Mϕ obtained from rats injected with TG; for other abbreviations see Figures 1 and 2.

duction of radioactivity in LY during co-culture. Part of the radioactivity lost from LY (especially from FFA, CHOL, and PL) (Fig. 4) was found in the medium of the co-culture (Fig. 5). This fact does not exclude the possibility that the incorporated AA can be metabolized, exported to the medium, and be reincorporated by LY.

Addition of AA to the medium caused a significant reduction of phagocytosis by resident Mϕ (as assessed by ANOVA). This reduction was 54% at 35 μM and about 33% at 50 and 75 μM (Fig. 6). PA, OA, and LA also caused a significant decrease of phagocytic capacity by resident Mϕ. Although the phagocytic capacity of TG-Mϕ was higher than that of resident-Mϕ, it was not modulated by the four FA studied.

Mϕ co-cultured with AA-preloaded LY showed a significant reduction of phagocytic capacity, as compared to Mϕ co-cultured with untreated LY; there was a slight decrease at all concentrations of AA and these decreases were significant at 35 and 75 μM (reduction of about 40%) (Fig. 6). PA-, LA-, and OA-preloaded LY also induced a reduction on phagocytic capacity of co-cultured Mϕ. The higher inhibition was induced by OA (64% at 50 μM) (data not shown).

DISCUSSION

Previous studies have shown that TG treatment stimulates LY metabolism (13). In fact, the oxidation of AA was higher in

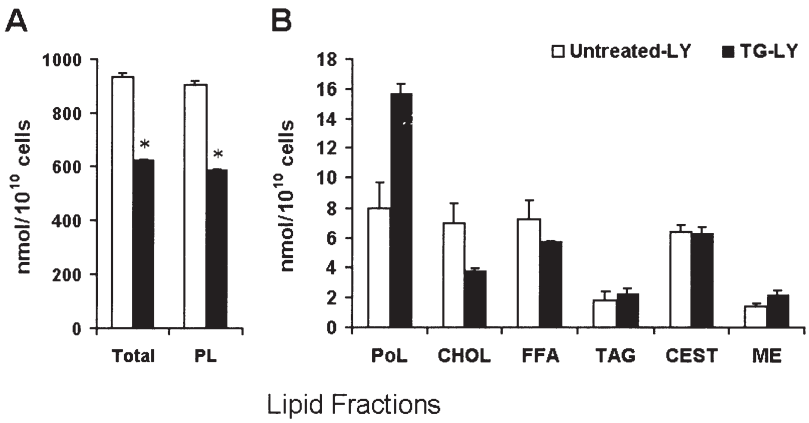

FIG. 4. Remaining radioactivity in LY after being co-cultured with Mϕ for 3 h. Untreated-LY and TG-lymphocytes were used. Results are expressed in nmol/10^{10} cells as mean ± SEM of six determinations from three experiments. LY were cultivated with 0.2 μCi/mL of [1-^{14}C]arachidonic acid. After 6 h, labeled LY were washed and co-cultivated with macrophages during 3 h. After this time, lipids from LY were extracted and chromatographed, and the radioactivity of each lipid fraction was determined. For abbreviations see Figures 1 and 2.

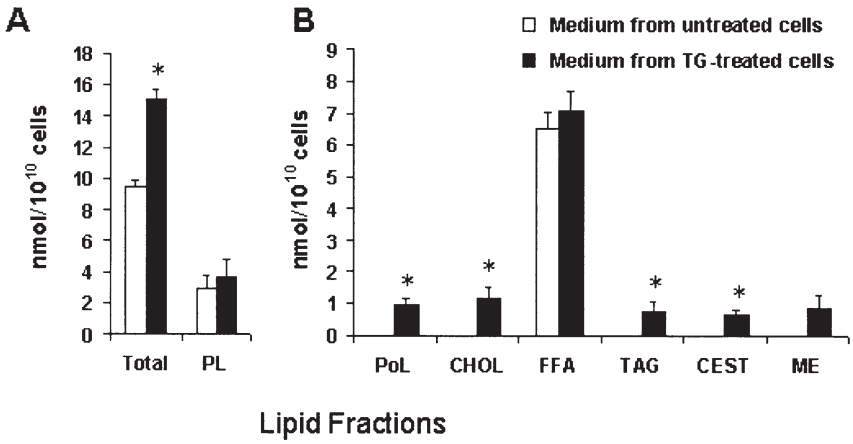

FIG. 5. Distribution of the radioactivity in the lipid fractions of the medium of [1-^{14}C]arachidonic acid-preloaded LY co-cultured with Mϕ. Untreated-LY and TG-LY were used. Results are expressed in nmol/10^{10} cells as mean ± SEM of six determinations from three experiments. LY were cultivated with 0.2 μCi/mL of [1-^{14}C]arachidonic acid. After 6 h, labeled LY were washed and co-cultivated with Mϕ for 3 h. After this time, lipids from the medium were extracted and chromatographed, and the radioactivity of each lipid fraction was determined. Medium from untreated cells = medium of cultures with untreated LY; medium from TG-treated cells = medium of cultures with LY and Mϕ from TG-injected rats. For other abbreviations see Figures 1 and 2.

TG-LY than in untreated cells (3.4-fold) (Table 1). This may explain the reduction of [^{14}C]AA incorporation into lipid fraction over 6 h (Fig. 2A).

LY can both oxidize and incorporate exogenously added FA. TG treatment appears to accelerate oxidation at the expense of incorporation of exogenously added AA (Table 1, Fig. 2). Evidence is also presented herein that LY can export part of the previously accumulated lipid to the medium (Fig. 5) over a period of 3 h in culture, leading to a significant decrease of intracellular [^{14}C]-lipid content (Fig. 4). Others have reported the export of FA by LY. Sanderson *et al.* (19) showed that T LY release AA extracellularly when stimulated by the calcium ionophore A23187 or by monoclonal antibodies (especially anti-CD2).

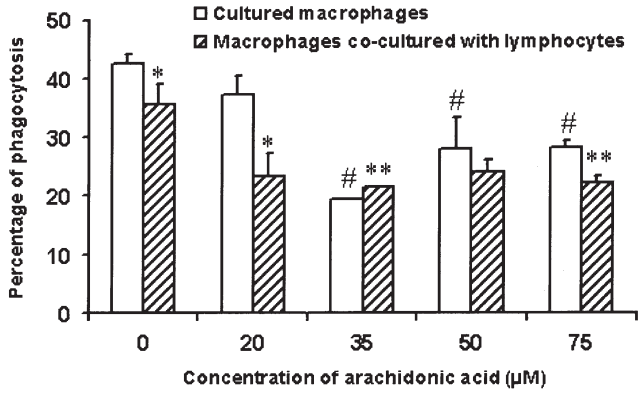

FIG. 6. Effects of arachidonic acid on Mϕ phagocytosis and of arachidonic acid-preloaded LY on co-cultured adhered Mϕ phagocytosis. Resident Mϕ were treated with different concentrations of arachidonic acid (0, 20, 35, 50, and 75 μM) for 6 h or co-cultured for 3 h with the respective LY preloaded with arachidonic acid (0, 20, 35, 50, and 75 μM). Afterward, Mϕ were incubated with opsonized zymosan particles for 40 min. Cells were stained with May-Giemsa, and the percentage of phagocytosis was determined. Results, expressed as the percentage of cells that incorporated three or more particles of zymosan, are reported as mean ± SEM of six determinations from three experiments. *$P < 0.05$ for comparison between Mϕ co-cultured with arachidonic acid-preloaded LY and Mϕ treated by arachidonic acid addition to the medium; #$P < 0.05$ due to the addition of arachidonic acid; **$P < 0.05$ for comparison between Mϕ co-cultured with arachidonic-preloaded LY and Mϕ cultured with untreated LY. For abbreviations see Figures 1 and 3.

A mechanism exists whereby LY transferred a significant proportion of [1-^{14}C]AA to co-cultured Mϕ *via* export to the extracellular medium (Fig. 3). The amount of [^{14}C]-AA transferred from LY to Mϕ was 0.8 and 2.4%, respectively, of the total AA content for untreated and TG-treated cells. The most important destination of transferred FA was macrophage PL. The incorporation of AA into PL can affect membrane fluidity, adhesion capacity, and Mϕ phagocytosis (20,21). PL such as PC and PS preferentially incorporate AA into the *sn*-2 position of the glycerol backbone (22). The ester bond at position *sn*-2 is cleaved by the action of phospholipase A_2 (PLA_2), releasing AA, which may then be acted on by cyclooxygenase or lipoxygenase enzymes to generate prostaglandins and thromboxanes (23). These eicosanoids regulate phagocytosis, facilitating the membrane fusion necessary for phagosome formation and consequent uptake of particles (23). An alternative fate for PC is the generation of DAG by the action of phospholipase C (PLC) (23). DAG may stimulate phosphokinase C (PKC), which is known to regulate superoxide production and phagocytosis by Mϕ (24).

Evidence is presented herein that exogenously applied or LY-transferred AA can inhibit the phagocytic capacity of opsonized zymosan by resident-Mϕ but not phagocytosis by TG-Mϕ. Phagocytosis of serum-opsonized zymosan by Mϕ is dependent on the complement receptor CR3 (25,26). AA can

be released by PLA_2 after induction by phagocytosis of opsonized zymosan in a process dependent on PLC and G protein (27); AA release is also related to a transient increase in intracellular calcium and activation of mitogen-activated protein kinases (MAPK) (28).

Bonney and Davies (29) showed an increase of prostaglandin E_2 (PGE_2) production by TG-Mϕ as compared to resident cells. The same authors indicated that TG-Mϕ also show a more prominent responsiveness to exogenous PGE_2 than resident-Mϕ. Our results are in agreement with previous studies (3,9) showing that TG-Mϕ have an increased phagocytic capacity compared to resident-Mϕ (an increase from 42 to 80%). TG treatment also induced an increase of phagocytosis by co-cultured TG-Mϕ (an increase from 36 to 83%). Evidence is presented herein that AA can inhibit the phagocytic capacity of opsonized zymosan by resident-Mϕ, but this FA does not affect phagocytosis by TG-Mϕ.

Previous *in vitro* studies by our group indicated that Mϕ transfer CHOL, PL, and FA to LY and this process modulated the rate of acceptor cell proliferation (7,8). In addition, Mϕ can transfer FA to islets of Langerhans and thus control insulin secretion (30). Evidence is presented herein that the transfer of FA occurs also from LY to Mϕ and modulates Mϕ phagocytic capacity. Therefore, there is a bidirectional transfer of AA between Mϕ and LY. Transcellular bidirectional AA transfer also occurs between alveolar epithelial cells and Mϕ in co-culture with a significant production of eicosanoids by both cell types after stimulation by the calcium ionophore A23187 (19).

There is evidence that the transfer of lipids between cells also occurs *in vivo*. [^{14}C]-Labeled CHOL and AA can be transferred from resident- and TG- Mϕ to various tissues and organs in rats (31). The physiological role of this lipid transfer on modulation of acceptor cell function (e.g., LY proliferation and cytokine production) *in vivo* still has to be determined.

Taken as a whole, AA can be incorporated, distributed into lipid fractions, oxidized, and exported by untreated- and TG-LY. AA also can be transferred from LY and modulates Mϕ phagocytic capacity. The same was found for PA, OA, and LA. These results support the proposition that the transfer of FA between leukocytes may play a role in regulating inflammatory and immune responses.

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Phospholipid Hydroperoxides Are Detoxified by Phospholipase A_2 and GSH Peroxidase in Rat Gastric Mucosa

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ABSTRACT: The aim of this study was to determine the metabolic fate of phospholipid hydroperoxides (PLOOH) in rat gastric mucosa. Here we report evidence concerning the mechanism for PLOOH detoxification in gastric mucosa homogenate. Analysis by the TLC blot technique showed that the gastric mucosa has the highest potential to eliminate 1-palmitoyl-2-linoleoyl-phosphatidylcholine hydroperoxides (PL-PtdChoOOH) compared with the intestinal mucosa and liver. Major products detected after incubation with gastric mucosa were the partially reduced linoleic acid hydroperoxides (LAOOH) and lysophosphatidylcholine, indicating the involvement of phospholipase A_2 (PLA_2) in the elimination pathway. Using unilamellar vesicles, we demonstrated that gastric mucosal PLA_2 does not distinguish between PLOOH and intact phospholipids. Although gastric mucosal PLA_2 activity efficiently eliminated excess amounts of PLOOH, the complete reduction of LAOOH was dependent on the supply of exogenous GSH. In a separate experiment, administration of egg yolk PtdChoOOH to rats for 6 d significantly elevated GSH peroxidase (GPx) activity in the gastric mucosa. We concluded that excess amounts of PLOOH are efficiently eliminated through the hydrolysis by PLA_2, and the subsequent reduction of FA hydroperoxide by GPx is the critical step for complete detoxification of oxidized phospholipids in the stomach.

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The gastrointestinal mucosa is constantly exposed to a variety of oxidants, including phospholipid hydroperoxides (PLOOH) derived from ingested foods. PLOOH have been actually detected in muscle foods and other lipid-containing diets (1). In addition, oxidative reactions in the gastrointestinal fluids during mastication and digestion can generate lipid hydroperoxides, including PLOOH, by the exposure of unsaturated lipids to catalytic heme and nonheme irons (2–5).

Lipid hydroperoxides are potentially toxic compounds, and their accumulation can trigger deleterious events that are associated with gut pathologies, such as inflammation and cancer (5–7). PLOOH are suggested to exert toxic effects through decomposition of their FA hydroperoxide moiety (8). The lipid radicals and other secondary products arising from homolytic cleavage of PLOOH are capable of modifying cell membranes and causing DNA and protein damage (9). Lipid hydroperoxides are also recognized as modulators for cellular redox balance, mediating specific cellular responses and gene expression (10–12). Accumulating evidence suggests that part of the dietary lipid hydroperoxides that escape from the breakdown process in the gut can enter the blood circulation and contribute to the pathogenesis of atherosclerosis (13–15).

Like other tissues, the gastrointestinal mucosa is equipped with defense mechanisms to eliminate peroxidized lipids. A defense mechanism against FA hydroperoxides in the gastrointestinal tract (GI tract) involves a specific form of GSH peroxidase, which is expressed mainly in the gastrointestinal epithelium, GI-GPx (16). On the other hand, little is known concerning the detoxification mechanism for PLOOH in the GI mucosa. Thus, the fate of PLOOH in the GI tract remains a subject of argument. In a previous work (17), we found that the antioxidant defense varies along the GI tract, and the gastric mucosa possesses the greatest potential to eliminate PLOOH compared with the small intestinal and large intestinal mucosa. Therefore, this study aimed to clarify the mechanism responsible for the efficient elimination of PLOOH in rat gastric mucosa. We used the fluorescent TLC blot technique (18) as an analytical tool because this technique is specific for detecting lipid hydroperoxides. In addition, we also conducted an animal study to estimate the physiologic effect of continuous intake of PLOOH on the mucosal antioxidant defense against peroxidized lipids.

EXPERIMENTAL PROCEDURES

Chemicals. 1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PL-PtdCho), linoleic acid (LA), GSH reductase, β-NADPH (tetrasodium salt), *tert*-butylhydroperoxide, and mercaptosuccinate were purchased from Sigma Chemical (St. Louis, MO). 1,2-Dilinolein and 9-anthryldiazomethane (ADAM) were obtained from Funakoshi (Tokyo, Japan). GSH (reduced form) was from the Peptide Institute (Tokyo,

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Abbreviations: ADAM, 9-anthryldiazomethane; DLOOH, dilinoleoylglycerol hydroperoxides; DPPP, diphenyl-l-pyrenylphosphine; EYPtdCho, egg yolk phosphatidylcholine; EYPtdChoOOH, egg yolk phosphatidylcholine hydroperoxides; GI, gastrointestinal; GPx, glutathione peroxidase; LA, linoleic acid; LAOH, linoleic acid hydroxides; LAOOH, linoleic acid hydroperoxides; LysoPtdCho, lysophosphatidylcholine; PHGPx, phospholipid hydroperoxide GPx; PLA_2, phospholipase A_2; PLOOH, phospholipid hydroperoxides; PL-PtdCho, 1-palmitoyl-2-linoleoyl-phosphatidylcholine; PL-PtdChoOH, 1-palmitoyl-2-linoleoyl-phosphatidylcholine hydroxides; PL-PtdChoOOH, 1-palmitoyl-2-linoleoyl-phosphatidylcholine hydroperoxides.

Japan). 5,5′-Dithiobis(2-nitrobenzoic acid) was purchased from Nacalai Chemicals (Kyoto, Japan). 1-Palmitoyl-2-linoleoyl-phosphatidylcholine hydroperoxides (PL-PtdChoOOH) and linoleic acid hydroperoxides (LAOOH) were prepared by enzymatic oxidation of PL-PtdCho and LA, respectively, using soybean lipoxygenase (19,20). Dilinoleoylglycerol hydroperoxide (DLOOH) was prepared by photooxidation of 1,2-dilinolein using methylene blue as a photosensitizer (21). Linoleic acid hydroxides (LAOH) were prepared by the reduction of LAOOH with $NaBH_4$ (22). Egg yolk phosphatidylcholine hydroperoxides (EYPtdChoOOH) were synthesized by photosensitized oxidation of egg yolk phosphatidylcholine (EYPtdCho) (21), which was purified from commercial egg by column chromatography (23). Solvents used for chromatographic analysis were of HPLC grade; all other chemicals and reagents were of analytical grade.

Mucosa homogenate preparation. Male Wistar rats, 6 wk old, weighing 180–220 g, were purchased from Charles River Japan (Kanagawa, Japan). Rats were maintained in accordance with the Guidelines for Animal Experimentation of The University of Tokushima. The rats were deprived of food for 20 h and killed by decapitation. Stomach, small intestine, large intestine, and liver were removed and rinsed with cold saline. Mucosa was obtained by scraping with a spatula, washed, and immediately frozen in liquid nitrogen. Mucosa and liver samples were stored at −80°C. Mucosa and liver samples (0.1–0.5 g) were homogenized in 6 vol (1.0–3.0 mL) of Tris-HCl buffer (0.1 M, pH 7.4, containing 0.135 M KCl) using a Potter-Elvehjem tissue homogenizer. The homogenate was sonicated three times for 1 min (Astrason Ultrasonic Processor) and centrifuged at 10,000 × *g* at 4°C for 15 min. The supernatant was collected and used for the assays. Protein concentration was determined by the method of Bradford (24), using BSA as a standard.

Hydroperoxide and homogenate incubations. A suspension of PL-PtdChoOOH (1.6 mM) was prepared by drying PL-PtdChoOOH solution in chloroform under a stream of nitrogen and then dispersing the residue in Tris-HCl buffer (0.1 M, pH 7.4, containing 0.135 M KCl) by vigorous mixing. Aliquots of this suspension (final concentration 0.5 mM) were mixed with mucosa homogenate or liver (final concentration 1 mg protein/mL) in a total volume of 0.32 mL and incubated at 37°C for 30 min. Incubation was terminated by adding 0.8 mL of methanol and 0.4 mL of chloroform containing 1 mM BHT. Lipids were extracted according to the method of Bligh and Dyer (25), and aliquots of the chloroform phase were taken for the analysis of residual PL-PtdChoOOH and reaction products.

Analysis of reaction products by TLC and TLC-blotting. Lipids extracted from the reaction mixture were concentrated and applied to TLC plates (silica gel 60 F254, 0.25 mm thick, Merck, Darmstadt, Germany). TLC analyses of polar and neutral lipids were done with the following solvent mixtures: chloroform/methanol/water (65:35:5, by vol) and hexane/isopropanol/acetic acid (70:30:1, by vol), respectively. General detection of lipids was done by the exposure of the TLC plate to iodine vapor. For the specific detection of residual PL-PtdChoOOH and other lipid hydroperoxides derived from their decomposition, we used the TLC blot technique (18). This method is based on the reaction of lipid hydroperoxides with diphenyl-1-pyrenylphosphine (DPPP) on the blotted membrane yielding a fluorescent DPPP oxide. Fluorescent spots on blotting membrane were visualized under UV irradiation using the fluorescent reader, Epi-light UV FA 500 (Aisin-Cosmos Institute, Tokyo, Japan).

Characterization of gastric mucosal PLA_2 activity toward unilamellar vesicles. To evaluate the activity and specificity of gastric mucosal PLA_2 activity toward PLOOH, we conducted experiments using unilamellar vesicles containing only PL-PtdCho or PL-PtdChoOOH and PL-PtdCho containing different concentrations of PL-PtdChoOOH as substrates. Unilamellar vesicles were prepared as described previously (26). Briefly, a chloroform solution of PL-PtdCho, PL-PtdChoOOH, or a mixture of PL-PtdCho containing 0.5–10% PL-PtdChoOOH was completely dried under a stream of nitrogen and *in vacuo* for 30 min. The residue was rapidly dispersed in Tris-HCl buffer (0.25 mM, pH 8.0) containing 0.25 M NaCl and 0.5 mM diethylenetriaminepentaacetic acid to prevent metal-catalyzed hydroperoxide breakdown. This suspension was then extruded through a 100-nm pore membrane to obtain unilamellar vesicles. For the incubation, an aliquot of gastric mucosa homogenate containing 5 mM $CaCl_2$ (final concentration: 0.5 mg protein/mL) was added to this suspension and incubated at 37°C for 30 min. The reaction was terminated by adding 1 mL of chloroform/methanol (1:1, vol/vol) and 0.25 mL of water. Methyl margarate (40 nmol) was added as an internal standard. The amount of LA was quantified by derivatization with ADAM. The ADAM derivative of LA was separated through a TSK-gel octyl-80Ts column (4.6 × 150 mm; Tosoh, Tokyo, Japan) and monitored by a fluorescence detector (RF-10Axl; Shimadzu, Kyoto, Japan) set at excitation wavelength 365 nm and emission wavelength 412 nm. Similarly, the amount of LAOOH was measured after reduction of the hydroperoxide with triphenylphosphine (1 mM in methanol) and derivatization with the ADAM reagent.

Effect of GSH and mercaptosuccinate on LAOOH reduction in gastric mucosa. The ability of gastric mucosa to reduce LAOOH to its hydroxide form (LAOH) was evaluated in the presence of GSH and mercaptosuccinate, a GPx inhibitor. For this purpose PL-PCOOH (final concentration, 1 mM) was incubated with the gastric mucosa homogenate (final concentration, 1 mg protein/min) at 37°C for 30 min in the presence of various concentrations of GSH or mercaptosuccinate. The reaction was terminated as described above, and the amounts of LAOOH and LAOH were determined according to the HPLC method described by Terao *et al.* (22). For analytical purposes, we used an HPLC system (Shimadzu LC 10AS) equipped with a silica 60 column (4.6 × 250 mm, TSK-gel; Tosoh). Elution was done in isocratic mode (hexane/isopropanol/acetic acid, 97.5:2.5:0.3, by vol) at a flow rate of 1.0 mL/min. LAOOH and LAOH were measured by the detection of conjugated dienes at 235 nm (Shimadzu SPD 10A).

Animal studies for the administration of EYPtdChoOOH. Male Wistar rats, 6 wk old, weighing 180–220 g, were purchased from Charles River Japan (Kanagawa, Japan) and divided into two groups (n = 5). The control group received EYPtdCho, and the second group received EYPtdChoOOH, both at a dose of 30 μmol/(rat·d) in 0.5 mL of water by intragastric intubation. Before each administration, rats were lightly anesthetized with ether. After 6 d of administration, rats were killed by decapitation, and the stomach and small intestine were removed as described above.

Determination of GPx activity in rat gastric and intestinal mucosa. GPx activity was determined using TBHQ as the substrate according to the method described by Flohé and Günzler (27). The mucosa homogenate was centrifuged at 10,000 × g for 15 min at 4°C, and the clear supernatant was mixed with Triton X-100 (final concentration 0.2%, vol/vol). The reaction was conducted in a cuvette warmed to 37°C. For the assay, 0.5 mL of phosphate buffer (0.1 M, pH 7.0), 0.1 mL GSH reductase (0.24 U), 0.1 mL of sample, and 0.1 mL of GSH (10 mM) were mixed in the cuvette and prewarmed for 10 min. Then 0.1 mL of NADPH (1.5 mM) was added and its consumption was monitored at 340 nm for 3 min.

Quantification of mucosal GSH content. Mucosal GSH content was determined by the disulfide reductase 5,5′-dithiobis(2-nitrobenzoic acid) recycling procedure described by Anderson (28). Briefly, 0.2 mL of the mucosa homogenate was mixed with 50 μL of 25% sulfosalicylic acid, vortexed, and centrifuged at 10,000 × g at 4°C for 15 min. An aliquot of the supernatant (0.1 mL) was used for the assay.

RESULTS

Elimination of PL-PtdChoOOH and formation of hydrolysis products by gastric mucosa. Figure 1A shows the result of TLC blot analysis for detecting residual PL-PtdChoOOH after incubation with gastric mucosa, small intestinal mucosa, large intestinal mucosa, and liver homogenate. The fluorescent spot of PL-PtdChoOOH disappeared completely after incubation with the gastric mucosa homogenate (Fig. 1A, lane 3). In contrast, PL-PtdChoOOH remained mostly intact after incubation with the small intestinal mucosa, large intestinal mucosa, and liver homogenate (Fig. 1A, lanes 4, 5, and 6, respectively). The disappearance of the PL-PtdChoOOH fluorescent spot on blotted membranes (Fig. 1A) was not due to metal-catalyzed breakdown of the hydroperoxides because the addition of a powerful metal chelator, Desferal, did not affect PL-PtdChoOOH elimination (data not shown).

The products formed after incubation of PL-PtdChoOOH were characterized as two main products: LAOOH (Fig. 1B) and lysophosphatidylcholine (LysoPtdCho) (Fig. 2). Both

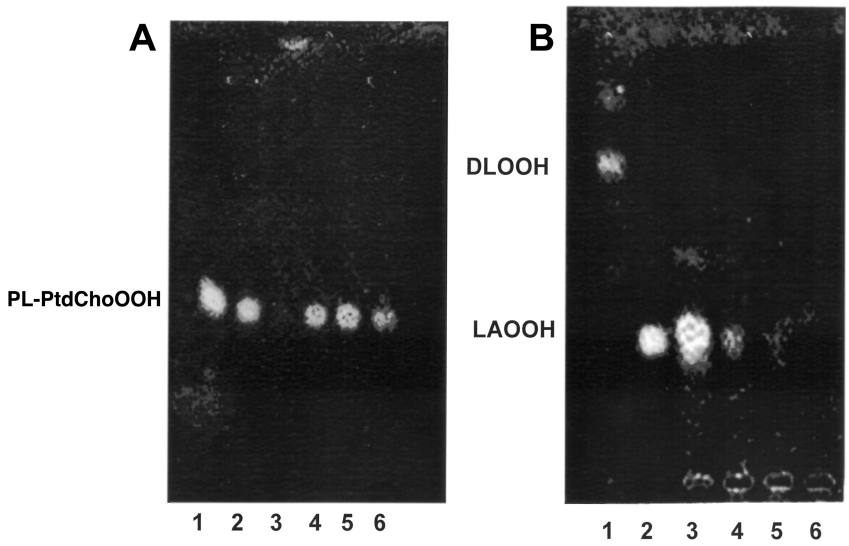

FIG. 1. TLC blot analysis of lipid hydroperoxides after incubation of PL-PtdChoOOH with rat gastric mucosa, small intestinal mucosa, large intestinal mucosa, or liver homogenate: (A) analysis of polar class of lipid hydroperoxides; (B) analysis of neutral class of lipid hydroperoxides. Incubations were conducted with PL-PtdChoOOH (final concentration 0.5 mM) in Tris-HCl buffer (0.1 M, pH 7.4, containing 0.135 M KCl) and mucosa or liver homogenate (final concentration 1 mg protein/mL) at 37°C for 30 min. Lipids were extracted, separated on TLC plates, and then analyzed by the blotting technique. Lanes A1: PL-PtdChoOOH standard, A2: control (incubation without homogenate), A3: gastric mucosa, A4: small intestinal mucosa, A5: large intestinal mucosa, A6: liver homogenate. Lanes B1: DLOOH standard, B2: LAOOH standard, B3: gastric mucosa, B4: small intestinal mucosa, B5: large intestinal mucosa, B6: liver homogenate. Abbreviations: PL-PtdChoOOH, 1-palmitoyl-2-linoleoyl-phosphatidylcholine hydroperoxides; DLOOH, dilinoleoylglycerol hydroperoxides; LAOOH, linoleic acid hydroperoxides.

products appeared in large amounts during the incubation of PL-PtdChoOOH with the gastric mucosa (Fig. 1B, lane 3; Fig. 2, lane 1). DLOOH, a product that could be formed by the action of phospholipase C on PL-PtdChoOOH, was not detected in the blotted membrane for any of the samples (Fig. 1B). These results indicate that the main pathway for PL-PtdChoOOH elimination is mediated by PLA_2 activity.

Reactivity of rat gastric mucosal PLA_2 toward PL-PtdChoOOH containing unilamellar vesicles. Figure 3 shows the activity of PLA_2 toward unilamellar PL-PtdCho or PL-PtdChoOOH vesicles. As shown in the velocity-substrate plots, the hydrolysis rate for either PL-PtdCho or PL-PtdChoOOH was essentially the same. Figure 4 shows the total amount of FA released (LA and LAOOH) from the PL-PtdCho vesicles containing different percentages of PL-PtdChoOOH (0–10%) after incubation with gastric mucosa. As can be observed, the total amount of FA released did not change with the increase of PL-PtdChoOOH in the vesicle; the relative percentage of LAOOH in relation to the total amount of LA did not increase more than the percentage of PL-PCOOH added. It is therefore concluded that under our experimental conditions, PL-PC and PL-PCOOH are equally hydrolyzed by gastric mucosal PLA_2.

Effect of GSH and mercaptosuccinate on LAOOH reduction by gastric mucosa. Figure 5 shows both the residual amount of PL-PtdChoOOH and the amount of LAOOH formed after incubation of PL-PtdChoOOH with gastric mucosa homogenate. The formation of LAOOH from PL-PtdChoOOH was visualized after incubation with gastric mucosa as shown in lane 2. Heat treatment of gastric mucosa homogenate before the incubation inhibited the clearance of PL-PtdChoOOH (Fig. 5, lane 3), suggesting the involvement of enzymes in a pathway eliminating PL-PCOOH. The addition of 5 mM GSH into the mucosa homogenate before the incubation resulted in the complete disappearance of the LAOOH spot on the blotted membrane (Fig. 5, lane 4). On the other hand, the addition of Ca^{2+} at the same concentration had no effect on the liberation or elimination of LAOOH (Fig. 5, lane 5). The effect of Ca^{2+} was tested to verify whether PLA_2 activity could be further increased, because rat gastric mucosal PLA_2 was reported to be Ca^{2+} dependent, displaying maximal activity at concentrations >1–2 mM (29).

Figure 6A shows the degree of LAOOH reduction to LAOH in the presence of increasing amounts of GSH, which is essential for the catalytic activity of GPx. Without the addition of GSH, <20% of LAOOH was reduced to LAOH. The addition of increasing amounts of GSH to the incubation mixture favored the conversion of LAOOH to LAOH in a dose-dependent manner. Complete reduction was achieved at doses

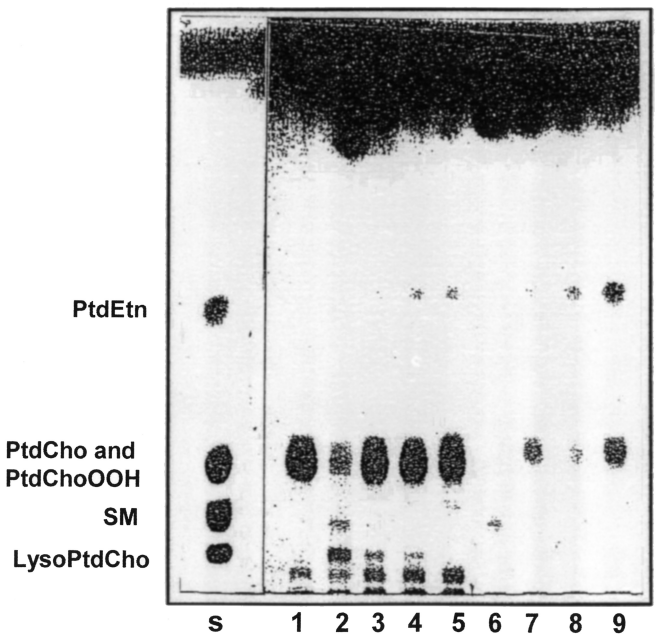

FIG. 2. TLC analysis of lipids after PL-PtdChoOOH incubation with rat gastric mucosa, small intestinal mucosa, large intestinal mucosa, or liver homogenate. Incubations were conducted as described in Figure 1. Lane S: standard lipid mixture, 1: control (incubation of PtdChoOOH without homogenate), 2: PtdChoOOH with gastric mucosa, 3: PtdChoOOH with small intestinal mucosa, 4: PtdChoOOH with large intestinal mucosa, 5: PtdChoOOH with liver homogenate, 6: gastric mucosa (no PtdChoOOH), 7: small intestinal mucosa (no PtdChoOOH), 8: large intestinal mucosa (no PtdChoOOH), and 9: liver homogenate (no PtdChoOOH). Abbreviations: PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; PtdChoOOH, phosphatidylcholine hydroperoxides; SM, sphingomyelin; and LysoPtdCho: lysophosphatidylcholine.

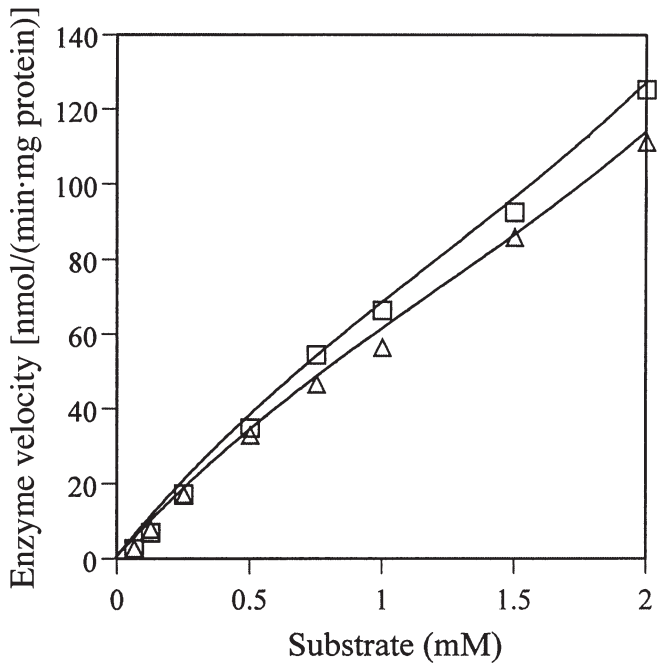

FIG. 3. Hydrolysis of PL-PtdCho or PL-PtdChoOOH unilamellar vesicles by gastric mucosal PLA_2. Unilamellar vesicles containing PL-PtdCho or PL-PtdChoOOH at different concentrations were incubated with gastric mucosa homogenate (0.4 mg protein/mL) in Tris-HCl buffer (0.25 mM, pH 8.0) in the presence of 0.25 M NaCl and 0.5 mM DTPA. Lipids were extracted, and hydrolysis rate was calculated from the amount of LA or LAOOH liberated. □: PtdCho, △: PtdChoOOH. Data are means ± SD, $n = 3$. Abbreviations: PL-PtdCho,1-palmitoyl-2-linoleoyl-phosphatidyl-choline; PL-PtdChoOH, 1-palmitoyl-2-linoleoyl-phosphatidyl-choline hydroxides; DTPA, diethylenetriaminepentaacetic acid; for other abbreviations see Figure 1.

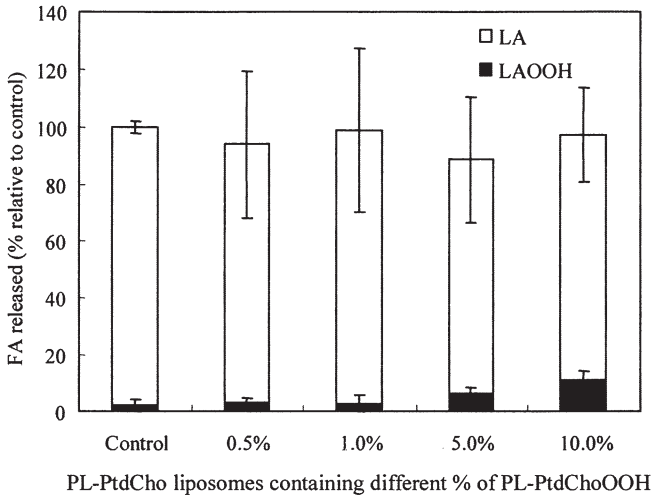

FIG. 4. Amount of LA and LAOOH detected after incubation of PL-PtdCho vesicles containing 0–10% PL-PtdChoOOH with gastric mucosa homogenate. PL-PtdCho unilamellar vesicles containing 0, 0.5, 1.0, 5.0, and 10.0% PL-PtdChoOOH were incubated with gastric mucosa homogenate (0.4 mg protein/mL) in Tris-HCl buffer (0.25 mM, pH 8.0) in the presence of 0.25 M NaCl and 0.5 mM DTPA. Lipids were extracted and LA or LAOOH was quantified by HPLC. Data are means ± SD, n = 4. LA, linoleic acid; see Figures 1 and 3 for other abbreviations.

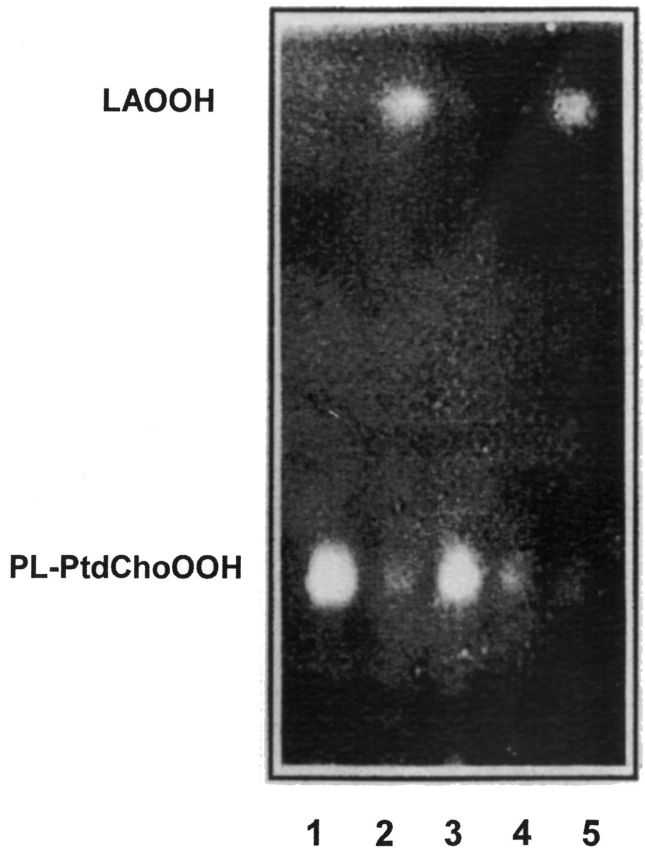

FIG. 5. TLC blot analysis of residual PL-PtdChoOOH and LAOOH after incubation of PL-PtdChoOOH and gastric mucosa homogenate under different conditions. PL-PtdChoOOH (final concentration 0.5 mM) in Tris-HCl buffer (0.1 M, pH 7.4, containing 0.135 M KCl) was incubated with gastric mucosa homogenate (final concentration 1 mg protein/mL) at 37°C for 30 min. Lanes 1: PL-PtdChoOOH standard, 2: control (no treatment), 3: heat treatment (mucosa homogenate was heated for 10 min at 100°C before incubation), 4: addition of 5 mM GSH and 5: addition of 5 mM $CaSO_4$. The blotted membrane is representative of at least three different experiments. See Figure 1 for abbreviations.

>1 mM GSH. An experiment in the presence of mercaptosuccinate, a specific inhibitor of GPx, was conducted to confirm that this reduction is mediated by a selenium-dependent GPx. This compound is reported to block the active site of GPx by binding selenium in competition with GSH (30). Figure 6B shows that mercaptosuccinate inhibited the reduction of LAOOH in a dose-dependent manner, therefore indicating that the reduction of LAOOH in gastric mucosa homogenate was mediated by a selenium-dependent GPx.

Effect of continuous EYPtdChoOOH administration on rat gastric mucosal lipid hydroperoxide-detoxifying system. To determine the effect of dietary excess PLOOH intake on the gastric mucosal lipid hydroperoxide-detoxifying system, rats were administered EYPtdChoOOH or EYPtdCho intragastrically at a daily dose of 30 μmol for six consecutive days. Bleeding and diarrhea were not observed in either group. Thus, this dose level of PtdChoOOH had no serious damaging effects on the gastrointestinal mucosa. Table 1 shows GPx activity and GSH content in the gastric mucosa and small intestinal mucosa after 6 d of EYPtdCho or EYPtdChoOOH administration to rats. No significant difference was observed between the EYPtdCho-treated group and EYPtdChoOOH-treated group in GPx activity of the small intestinal mucosa. In contrast, a significant increase in GPx activity was observed for the EYPtdChoOOH-treated group in the gastric mucosa. On the other hand, no significant difference was found in the GSH content between the EYPtdCho- and EYPtdChoOOH-treated groups in either gastric mucosa or small intestinal mucosa. In addition, our preliminary experiment showed that there were no significant differences in PLA_2 activity between these two groups [EYPtdCho-treated group; 12.1 ± 5.3 nmol/(min·mg protein); EYPtdChoOOH-treated group; 11.8 ± 3.4 nmol/(min·mg protein)]. These findings clearly show that the continuous introduction of small amounts of PtdChoOOH from the diet can increase GPx activity in the stomach but not in the intestine.

DISCUSSION

There are at least two possible mechanisms for the detoxification of PLOOH in biological systems. One mechanism is the direct reduction of hydroperoxides on phospholipids by the action of phospholipid hydroperoxide GPx (PHGPx) or by other types of GPx and GSH-*S*-transferase (31). Indeed, PHGPx seems to be the most important enzyme involved in PLOOH metabolism in hepatocytes (32). An alternative mechanism for PLOOH detoxification is the sequential action of PLA_2 and GPx (33,34). This mechanism involves the cleavage of FA hydroperoxide from PLOOH by PLA_2, followed by the reduction of the FA hydroperoxide by GPx. In this study, we obtained evidence for the involvement of the

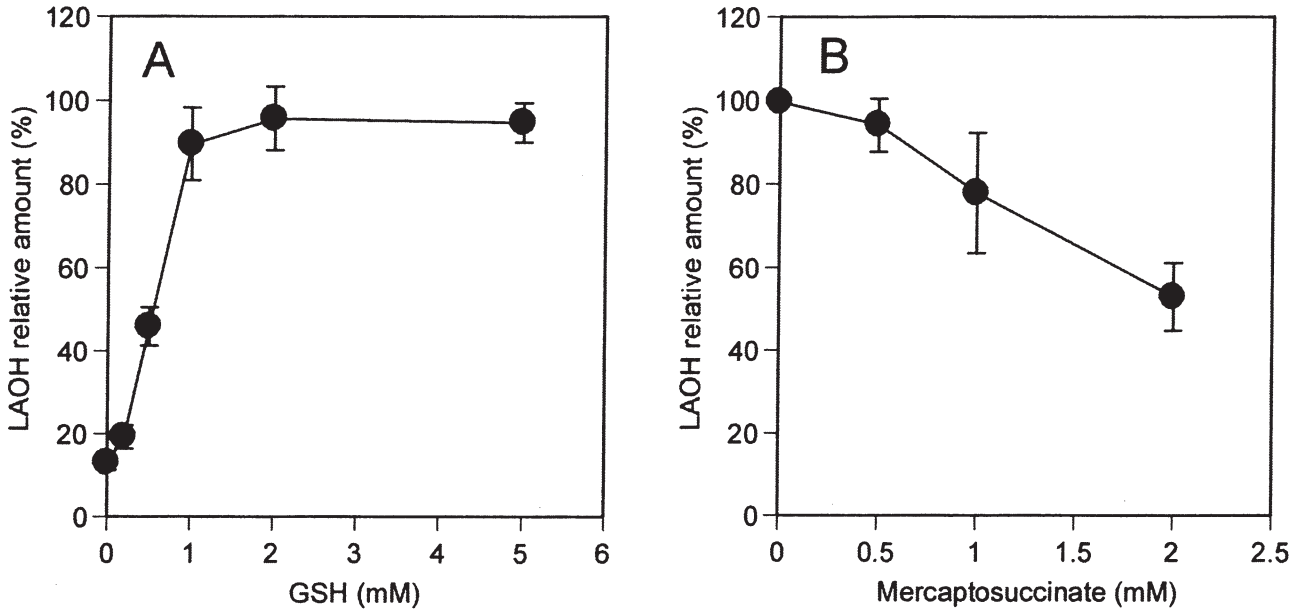

FIG. 6. Effect of GSH (A) and mercaptosuccinate (B) on LAOOH reduction in rat gastric mucosa. PL-PtdChoOOH (final concentration 1 mM) was incubated with gastric mucosa homogenate (final concentration 1 mg protein/min) at 37°C for 30 min in the presence of various concentrations of GSH or mercaptosuccinate. Incubations with mercaptosuccinate were conducted in the presence of 2 mM GSH. Data are mean ± SD, $n = 4$. See Figure 1 for abbreviations.

latter pathway in the elimination of excess amounts of PLOOH in the gastric mucosa.

Using the TLC blot technique for the specific detection of lipid hydroperoxides (18), we clearly demonstrated that excess amounts of PL-PtdChoOOH (0.5 mM) are efficiently eliminated in the gastric mucosa homogenate. The two major products were identified as LAOOH (Fig. 1B) and LysoPtdCho (Fig. 2), strongly indicating the involvement of PLA_2 in the PLOOH detoxification mechanism. The higher ability of the gastric mucosa over the small intestinal mucosa to eliminate PLOOH is likely related to the higher activity of PLA_2 in the gastric mucosa [gastric mucosa: 18.1 ± 17.3 nmol/(min·mg protein); small intestinal mucosa: 0.69 ± 0.35 nmol/(min·mg protein); $P < 0.05$]. The possibility of the involvement of another type of phospholipase, such as phospholipase C, on the elimination of PLOOH can be rejected because TLC and TLC-blotted membranes did not show the presence of other types of products such as DLOOH (Fig. 1B).

Earlier findings demonstrated that PLA_2 preferentially hydrolyzes peroxidized FA residues on membrane phospholipids and also displays enhanced activity toward peroxidized membranes (33–36). However, experiments using unilamellar vesicles containing PL-PtdCho or PL-PtdChoOOH as the substrate for gastric mucosal PLA_2 showed that the hydrolysis rates for both were essentially the same, i.e., in the concentration range of 0.1–2 mM (Fig. 3). Although the kinetics of the reaction did not obey the standard Michaelis–Menten equation, it can be suggested that gastric mucosal PLA_2 do not display higher reactivity toward PLOOH. A similar result was also obtained in the experiments using PL-PtdCho vesicles containing 0–10% PL-PtdChoOOH as the substrate. The hydrolytic activity of gastric mucosal PLA_2 was the same for PL-PtdCho vesicles without PL-PtdChoOOH or with 0.5–10.0 % of PL-PtdChoOOH (Fig. 4), strongly indicating that gastric mucosal PLA_2 does not display higher specificity toward PLOOH.

TABLE 1
Effect of Continuous Administration of EYPtdChoOOH on GPx Activity and GSH Concentrations in Rat Gastric Mucosa[a]

	Gastric mucosa		Small intestinal mucosa	
	PtdCho administration	PtdChoOOH administration	PtdCho administration	PtdChoOOH administration
GPx activity [nmol(min·mg protein)]	23.30 ± 4.98	41.02 ± 14.85*	14.94 ± 5.68	17.98 ± 3.74
GSH concentration (nmol/mg protein)	0.58 ± 0.07	0.60 ± 0.10	0.75 ± 0.20	0.67 ± 0.14

[a]Abbreviations: EYPtdChoOOH, egg yolk phosphatidylcholine hydroperoxides; GPx, glutathione peroxidase.
*Different from PtdCho administration, $P < 0.05$.

In a recent review, Nigam and Schewe (37) discussed in detail the difficulties of studying the role of PLA_2 in PLOOH metabolism. They also observed a lack of preference toward oxidized phospholipids for both secretory and cytosolic PLA_2. The differences observed between our results and those obtained by other groups can be related to the following: (i) differences in the type of PLA_2 used in the experiments; (ii) the type and composition of membranes; and (iii) the reaction conditions. Most of the studies used snake venom PLA_2, which is different from the pancreatic type of PLA_2 present in the rat stomach (38). No cholesterol is present in the vesicles used in our experiments, which differs from other works (33–36). It has been demonstrated that snake venom PLA_2 shows a preference for PL-PtdChoOOH over PL-PtdCho only when 25 mol% of cholesterol is incorporated into the liposomal membrane (36). This was explained by the fact that cholesterol makes the liposomal membrane pack more tightly, thus displacing the hydroperoxide moieties of PL-PtdChoOOH to the membrane surface where PLA_2 can easily access the substrate.

To perform a complete detoxification of PLOOH through the pathway involving PLA_2, the FA hydroperoxides liberated must be reduced to an inactive hydroxide form. Recent studies on the metabolism of FA hydroperoxides in the GI tract suggest that a gastrointestinal form of GPx (GI-GPx) constitutes the major enzyme against organic hydroperoxides (39,40). In fact, GI-GPx prevented transport of FA hydroperoxides in cultured intestinal cells and is believed to function as a barrier against food-borne hydroperoxide absorption (41–43). It is therefore likely that GI-GPx is one of the GPx enzymes that collaborate with PLA_2 for the complete detoxification of PLOOH. However, as could be visualized on TLC-blotted membranes, the elimination of PL-PtdChoOOH in the gastric mucosa generated high amounts of LAOOH (Figs. 1B and 5). Further analysis of LAOOH by HPLC revealed that only 20% of LAOOH was reduced to LAOH. This led us to investigate the factors affecting LAOOH reduction in gastric mucosa.

First, we observed that LAOOH was completely reduced by the addition of 5 mM GSH to the gastric mucosa homogenate, indicating an increase in the rate of either PHGPx or GPx operation. This observation supports the idea that an exogenous GSH supply is indispensable for the detoxification of excess lipid hydroperoxides in the intestinal mucosa (43,44). Although the GSH concentration required for the complete detoxification of LAOOH was relatively high, it has been reported that some foods can supply up to 2 mM of GSH to the GI tract (45). The method of absorption of GSH by the gastric mucosa remains unclear, although it is known that dietary GSH can be taken up by the epithelial cells of the intestinal tract for peroxide detoxification (46).

The observation of an inhibitory effect of mercaptosuccinate (29), a compound that blocks selenium at the active site of GPx, on LAOOH reduction indicates that a selenium-dependent GPx is the enzyme involved in PLOOH and/or LAOOH reduction. These data imply that the activity of selenium-dependent GPx is critical for the detoxification of excess amounts of PLOOH. Moreover, it points out the importance of dietary selenium in the overall PLOOH detoxification process. It has been reported that selenium supplementation of Caco-2 cells can increase GI-GPx protein fivefold, and the total GPx activity by a factor of 13 (41). On the other hand, selenium deficiency can decrease selenoproteins according to their hierarchical ranking. Classical glutathione peroxidase (cGPx) responds to a decreased selenium supply with a complete loss of protein and a marked reduction of mRNA levels, whereas GI-GPx remains detectable and its mRNA is stable.

The relative importance of PHGPx over PLA_2/GPx-mediated detoxification of PLOOH is a matter of controversy. In our previous work (17), we measured the activity of PHGPx and GPx in the gastrointestinal mucosa and found that the activity of PHGPx was more than 50 times lower than the activity of GPx in gastric mucosa. Considering the low activity of PHGPx and the high efficiency of PLA_2 to hydrolyze excess amounts of PL-PtdChoOOH, it is reasonable to conclude that in the gastric mucosa, the elimination pathway involving PLA_2 potentially contributes to detoxifying oxidized phospholipids. Nonetheless, it is possible that both pathways are working at the same time in the detoxification of PL-PtdChoOOH. Further work is necessary to clarify in depth the relative importance of each pathway.

Another interesting finding of our study was that the administration of EYPtdChoOOH to rats enhanced GPx activity in the gastric mucosa, but not in the intestinal mucosa. A similar effect on GPx activity was observed in a study conducted by Vilas *et al.* (47), who observed an increase in the specific activity of GPx in the gastric mucosa but not in the intestinal mucosa after the feeding of oxidized corn oil. Other groups (48,49) also observed an increase in GPx activity in the GI tract of animals after administration of oxidized lipids. Nevertheless, our study is the first to show an increase in gastric mucosal GPx activity after administration of PLOOH. We also found that treatment with PLOOH only slightly modified the activity of PLA_2 in the stomach.

The differences observed in the response of gastric and intestinal mucosa GPx to an exposure to PLOOH might be related to the differences in the tissue susceptibility to oxidative stress induced by peroxidized lipids from the diet, and also by the lower amount of intact peroxidized lipids that reach the intestine. Although the stomach is not a site of lipid absorption, the enhancement of GPx may protect it against dietary PLOOH. Because the lumen of the stomach is very acidic during digestion, PLOOH is probably decomposed as observed for TAG hydroperoxides and FA hydroperoxide (50,51). However, the introduction of the diet into the stomach is reported to allow increases in the pH (49); thus, PLOOH is present to some extent during digestion. Therefore, it is probable that gastric mucosal cells are in contact with some intact PLOOH, as well as their breakdown products, such as lipid-derived radicals, epoxyketones, and aldehydes. The observed increase in GPx activity may result from such interactions. PLOOH, or its decomposition products,

may cause changes in the cellular thiol redox balance, followed by the modulation of signaling pathways responsible for the transcriptional up-regulation of GPx. Indeed alterations in cellular redox state have been observed upon exposure of intestinal cells to lipid peroxides (10–12). Moreover in a recent study, Kelner *et al.* (52) showed that in humans, the GI-GPx promoter region is capable of responding to redox stress caused by *tert*-butylhydroperoxide. They sequenced the GI-GPx upstream promoter region and identified the presence of several transcription regulatory sites, such as for activator protein and nuclear factor (NF)κ-B. Identification of the mechanisms controlling GPx expression remains an area that requires extensive research.

In conclusion, we have shown clearly that PLOOH is eliminated efficiently in the gastric mucosa through the action of PLA_2. However, the complete detoxification of the FA hydroperoxide was dependent on the supply of GSH. The study of gastric mucosal PLA_2 substrate specificity showed that it apparently does not discriminate between oxidized and nonoxidized phospholipids. GPx activity in the gastric mucosa was activated upon PLOOH administration to rats. This elevation is likely to be involved in the protection of gastric mucosal cells against toxicity derived from peroxidized lipids.

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Properties of Lysophosphatidylcholine Acyltransferase from *Brassica napus* Cultures

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ABSTRACT: Acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT; EC 2.3.1.23) catalyzes the acyl-CoA-dependent acylation of lysophosphatidylcholine (LPC) to produce PC and CoA. LPCAT activity may affect the incorporation of fatty acyl moieties at the *sn*-2 position of PC where PUFA are formed and may indirectly influence seed TAG composition. LPCAT activity in microsomes prepared from microspore-derived cell suspension cultures of oilseed rape (*Brassica napus* L. cv Jet Neuf) was assayed using [1-^{14}C]acyl-CoA as the fatty acyl donor. LPCAT activity was optimal at neutral pH and 35°C, and was inhibited by 50% at a BSA concentration of 3 mg mL^{-1}. At acyl-CoA concentrations above 20 μM, LPCAT activity was more specific for oleoyl (18:1)-CoA than stearoyl (18:0)- and palmitoyl (16:0)-CoA. Lauroyl (12:0)-CoA, however, was not an effective acyl donor. LPC species containing 12:0, 16:0, 18:0, or 18:1 as the fatty acyl moiety all served as effective acyl acceptors for LPCAT, although 12:0-LPC was somewhat less effective as a substrate at lower concentrations. The failure of LPCAT to catalyze the incorporation of a 12:0 moiety from acyl-CoA into PC is consistent with the tendency of acyltransferases to discriminate against incorporation of this fatty acyl moiety at the *sn*-2 position of TAG from the seed oil of transgenic *B. napus* expressing a medium-chain thioesterase.

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Elucidation of the mechanisms of lipid biosynthesis in oleaginous crops provides a valuable foundation for developing metabolic engineering strategies to alter the FA composition of seed oil. In developing oilseeds, the biosynthesis of membrane phospholipids is closely linked to TAG biosynthesis and involves reactions catalyzed by an assortment of membrane-bound acyltransferases (1–4). The specificity properties of the acyltransferases can influence the types and relative amounts of FA moieties that are ultimately incorporated into TAG. In the *sn*-glycerol-3-phosphate pathway leading to TAG, acyl-CoA:lysophosphatidate acyltransferase (LPAAT; EC 2.3.1.51) catalyzes the acylation of lysophosphatidate to generate phosphatidate and CoA (1,4). Studies with microsomes from developing seeds of *Brassica napus* have indicated that the resident LPAAT discriminates against unusual FA moieties including erucoyl (22:1)- (5,6) and lauroyl (12:0)-CoA (7). The decreased specificity of endogenous LPAAT for 12:0-CoA was believed to be the reason for decreased incorporation of 12:0 at the *sn*-2 position of TAG from *B. napus* genetically engineered to express a medium-chain thioesterase from *Umbellularia californica* (8,9). Increased incorporation of 12:0 at the *sn*-2 position of *B. napus*, however, was achieved through coexpression of the medium-chain thioesterase from *U. californica* and a 12:0-CoA-preferring LPAAT from *Cocos nucifera* (10).

There are other possible routes for the incorporation of FA moieties at the *sn*-2 position of TAG. Membrane-bound acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT; EC 2.3.1.23) catalyzes the acyl-CoA-dependent acylation of lysophosphatidylcholine (LPC) to produce PC and CoA (1,11). In developing seeds of oleaginous crops such as *B. napus*, LPCAT activity may affect the incorporation of FA at the *sn*-2 position of PC where PUFA are formed (1). *sn*-1,2-DAG, derived from PC *via* the reverse reaction of CDP-choline:1,2-DAG cholinephosphotransferase (EC 2.7.8.2), may be used as substrate in the acyl-CoA-dependent biosynthesis of TAG (12–15). PC can also serve as a source of *sn*-2 FA moieties in the acyl-CoA-independent biosynthesis of TAG *via* donation of the FA moiety to *sn*-1,2-DAG (16). The resulting LPC product could then serve as substrate for LPCAT. As well, in studies with developing safflower (*Carthamus tinctorius*) seed, there is evidence for acyl-exchange between the FA at the *sn*-2 position of PC and the acyl-CoA pool (1,17–20). Phospholipase A_2, which catalyzes the hydrolysis of the *sn*-2 FA from PC, may also have a role in generating LPC (21,22) for utilization by LPCAT. Thus, the activity level and the specificity properties of LPCAT may influence the FA composition of seed oil. Studies on microsomal LPCAT have been restricted mainly, however, to developing safflower seeds (1,23,24). Bernerth and Frentzen (6) reported that *B. napus* LPCAT was capable of discriminating against 22:1-CoA, but no data were presented. A deeper understanding of the role of oilseed LPCAT in storage lipid biosynthesis could be realized through the availability of molecular probes, but there have been no reports on the purification of the plant enzyme to homogeneity or the identification of an encoding cDNA. Given the global importance of *B. napus*, the current study has focused on examining the properties of microsomal LPCAT from microspore-derived cell suspension cultures of this oilseed crop. Until now, this enzyme

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Abbreviations: 12:0, lauroyl; 16:0, palmitoyl; 18:0, stearoyl; 18:1, oleoyl; 22:1, erucoyl; ACBP, acyl-CoA binding protein; LPAAT, acyl-CoA:lysophosphatidate acyltransferase; LPCAT, acyl-CoA:lysophosphatidylcholine acyltransferase.

activity has been essentially uncharacterized in *B. napus*. In addition to providing fundamental knowledge on the properties of *B. napus* LPCAT, our characterization studies suggest that, along with LPAAT specificity, LPCAT specificity also may represent a barrier to the incorporation of medium-chain FA at the *sn*-2 position of TAG.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Lauric acid (59 Ci mol^{-1}), [1-^{14}C]oleic acid (56 Ci mol^{-1}), and [1-^{14}C]stearic acid (54 Ci mol^{-1}) were obtained from Amersham Biosciences Inc. (Brie d'Urfé, Québec, Canada). [1-^{14}C]Palmitic acid (56 Ci mol^{-1}) was from NEN Life Science Products, Inc. (Boston, MA). Acyl-CoA were synthesized from radiolabeled FA using acyl-CoA synthetase (25). Merck silica gel 60H, used for preparing TLC plates, was from VWR Canlab (Mississauga, Ontario, Canada). Ecolite^{TM} (+) biodegradable scintillant was from ICN Biomedicals, Inc. (Irvine, CA). Dye reagent concentrate for protein assays was from Bio-Rad (Hercules, CA). HPLC-grade solvents were from BDH, Inc. (Toronto, Ontario, Canada). All other lipids and biochemicals were of the highest purity available and were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada).

Cell culture and preparation of microsomes. The microspore-derived cell suspension culture of *B. napus* L. cv Jet Neuf was maintained essentially according to Orr *et al.* (26). The culture was grown in 125-mL Erlenmeyer flasks on a rotary shaker (150 rpm) at 25°C under constant light with an intensity of about 30 μmol m^{-2} s^{-1}. One-third of the mass of cells was routinely transferred to fresh medium at 2-wk intervals. The remainder of the cells, obtained after 2 wk of culture, was washed with water over a nylon sieve and blotted with filter paper to remove excess water; the fresh weight was then determined. Cells were ground in 4 vol of grinding buffer (10 mM MOPS-NaOH, pH 7.2, 0.4 M sorbitol, 0.5 mM EDTA) using a chilled mortar and pestle. The homogenate was centrifuged at 3000 × *g* for 20 min at 4°C and the resulting pellet discarded. The supernatant was filtered through glass wool to remove the lipid layer and centrifuged at 20,000 × *g* for 30 min at 4°C. The resulting pellet was discarded, and the supernatant was filtered through glass wool. The supernatant was then centrifuged at 100,000 × *g* for 1 h at 4°C, and the resulting pellet was resuspended in a volume of grinding buffer equivalent to 1/20 of the original mass of cells. The resuspended microsomes were divided into 100-μL aliquots, which were frozen with liquid N_2 and stored at –80°C.

Enzyme assays. The assay for LPCAT activity was adapted from previously described methods (24,27). Assays were conducted in a volume of 0.7 mL in 10-mL glass tubes at 30°C. Unless indicated otherwise, the standard reaction mixture consisted of 80 mM Tris-HCl, pH 7.5, 0.21 M sorbitol, 0.13 mM EDTA, 100 μM [1-^{14}C]oleoyl-CoA (0.5 Ci mol^{-1}), 75 μM 18:1-LPC, and about 25 μg of microsomal protein. Acyl-CoA was used to initiate the enzyme reaction, which was usually allowed to proceed for 10 min. The reaction was terminated by the addition of 3 mL chloroform/methanol (2:1, vol/vol). For zero reaction time controls, the solvent mixture was added to the reaction mixture before addition of microsomes. Phase separation was induced by the addition of 1.1 mL 0.9% (wt/vol) KCl. Following brief centrifugation, the upper aqueous phase was removed, and 1.5 mL of the lower organic phase was transferred to a 3-mL glass tube. The solvent was dried under a gentle stream of N_2 gas, and the sides of the glass were rinsed with a small volume of chloroform/methanol (2:1, vol/vol). Once completely dried, the samples were resuspended in 70 μL chloroform/methanol (2:1, vol/vol), and 50 μL was applied in 1.5-cm lanes to a 20 × 20 cm TLC plate coated with 0.5 mm silica gel 60H. Seventy microliters of PC solution (10 mg mL^{-1} in chloroform) was applied on top of the dried lipid extract to act as a carrier. A control lane with only PC was used to establish the migration distance of the phospholipid. The TLC plate was developed with one ascent of chloroform/methanol/NH_4OH (33:45:5, by vol). Based on the PC control, visualized with iodine vapor, corresponding radioactive PC spots in the adjacent lanes containing the reaction mixtures were scraped from the plate and radioactivity was determined in 5 mL Ecolite^{TM}(+) scintillant. A phosphoimaging system (Canberra-Packard Canada Ltd., Mississauga, Ontario, Canada) was used in preliminary experiments to confirm that radiolabeled PC was resolved from other lipid classes. The protein content of the microsomes was determined using the Bio-Rad protein microassay based on the Bradford method (28), using BSA as a standard.

RESULTS AND DISCUSSION

Effect of various parameters on LPCAT activity. The time course for production of PC, catalyzed by microsomal LPCAT under the buffer conditions of the standard assay, is depicted in Figure 1A. Production of PC was linear for up to about 20 min. To stay within the linear range of the time course, subsequent enzyme reactions for further characterization work were limited to 10 min. When assayed at the same temperature, the time course for production of PC catalyzed by solubilized microsomes containing LPCAT activity from developing safflower seed was shown previously to be linear for about 30 min (23). The effect of increasing protein content in the LPCAT reaction mixture is shown in Figure 1B. Enzyme activity was directly proportional to the microsomal protein content up to about 70 μg. Based on these results, the standard assay was routinely performed with about 25 μg of protein in the reaction mixture. The dependence of microsomal LPCAT activity on pH is shown in Figure 1C. Enzyme activity was maximal at pH 7.0. The neutral pH optimum is consistent with an earlier report on the effect of pH on microsomal LPCAT activity from developing safflower seeds (24). The effect of reaction temperature on microsomal LPCAT activity is shown in Figure 1D. Under the conditions of the assay, the enzyme exhibited an apparent temperature optimum of 35°C. Assays conducted for a longer period of time may affect enzyme stability and subsequently the apparent temperature optimum. Stymne and Stobart (20)

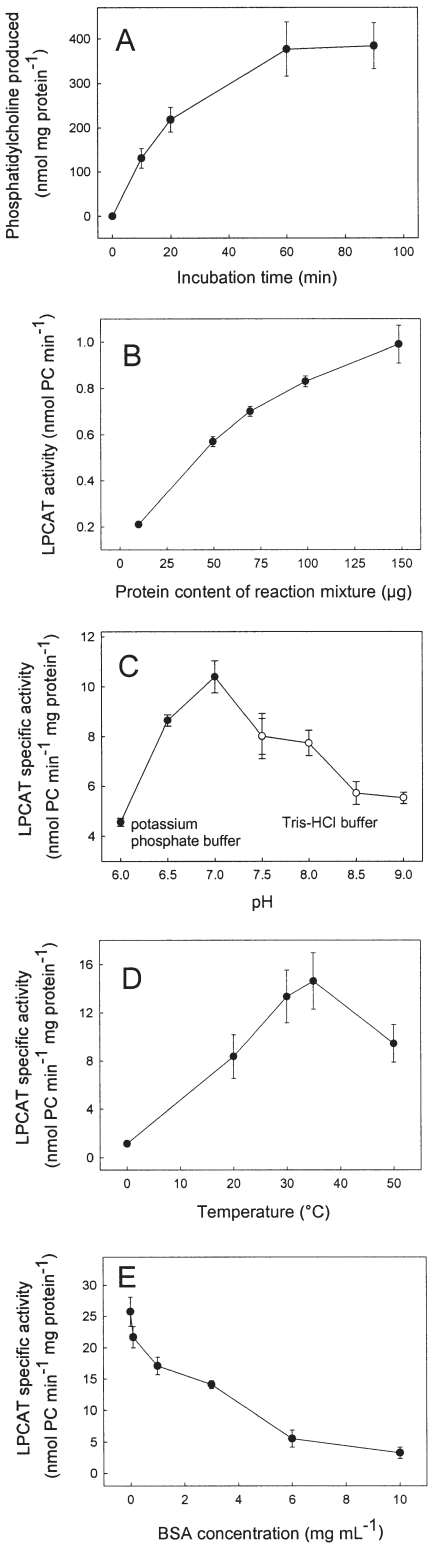

FIG. 1. Effect of various reaction conditions on microsomal acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT) activity. (A) Time course for the production of PC; (B) effect of the protein content of the reaction mixture; (C) effect of pH; the total concentration of buffer species was 80 mM; (D) effect of reaction temperature; (E) effect of BSA concentration. For (B) to (E), enzyme-catalyzed reactions were allowed to proceed for 10 min. Each data point represents the average of three determinations ± SEM.

observed increasing LPCAT activity up to 35°C in 1.5-min reactions conducted with safflower microsomes. The effects of higher temperatures on the rate of the enzyme-catalyzed reaction, however, were not evaluated in their investigations.

The effect of various concentrations of BSA on microsomal LPCAT activity is depicted in Figure 1E. BSA was inhibitory to microsomal LPCAT activity at all concentrations tested, with 50% inhibition occurring at a BSA concentration of approximately 3 mg mL^{-1}. Early work by Moreau and Stumpf (23), with microsomes from developing safflower seed, indicated that 1 mM BSA inhibited LPCAT activity at a pH value greater than 6. As well, in the presence of BSA, LPCAT exhibited maximum activity at pH 6. In contrast, in the absence of BSA, LPCAT activity was optimal at pH 8. Previous studies with microsomes of developing soybeans (*Glycine max*) (17) and safflower (19) have shown that the inclusion of BSA in the reaction mixture favors acyl-exchange at the *sn*-2 position of PC catalyzed by LPCAT. The reversible binding of acyl-CoA by BSA was presumed to shift the equilibrium toward removal of acyl groups from PC (19). BSA has been shown to have variable effects on the microsomal acyltransferases of storage lipid biosynthesis. For example, acyl-CoA:DAG acyltransferase (EC 2.3.1.20) in particulate fractions from microspore-derived embryos of *B. napus* was stimulated four- to fivefold at a BSA concentration of 3–4 mg mL^{-1} (29). DAG acyltransferase catalyzes the acyl-CoA-dependent acylation of *sn*-1,2-DAG to generate TAG (1). In studies with microsomes from developing safflower seeds, Bafor *et al.* (30) reported that inclusion of BSA in the reaction mixture altered the selectivity of LPAAT for acyl-CoA. Concentrations of BSA greater than 0.25 mg mL^{-1} caused LPAAT to have an increased preference for 18:2-CoA over acyl-CoA containing saturated acyl moieties.

Substrate specificity of LPCAT. The effect of increasing acyl-CoA concentration on microsomal LPCAT activity is shown in Figure 2A. At a concentration of 20 µM acyl-CoA, palmitoyl (16:0)-, stearoyl (18:0)-, and oleoyl (18:1)-CoA were utilized at the same rate by LPCAT. At higher concentrations of acyl-CoA, LPCAT was more active with 18:1-CoA. The enzyme was not active with 12:0-CoA at any of the thioester concentrations tested. The tissue concentration of acyl-CoA in developing seeds of *B. napus* has been determined to be in the range of 3-6 µM (31). With the assumption that the cell suspension cultures exhibit similar acyl-CoA concentrations, *B. napus* LPCAT would be equally effective using 16:0-, 18:0-, or 18:1-CoA in the direction of PC formation. In contrast, LPCAT activity from developing safflower microsomes has been shown to have a much greater specificity for 18:1-CoA in comparison to acyl-CoA composed of saturated FA at thioester concentrations of 20 µM or lower in the reaction mixture (23,24).

The effects of various concentrations (up to 10 µM) of 18:1-LPC on LPCAT activity are depicted in Figure 2B. The effect of higher concentrations of various molecular species of LPC, including 18:1-LPC, is shown in Figure 2C. Utilization rates for 16:0-, 18:0-, and 18:1-LPC were similar at all concentrations tested. From 10–50 µM LPC, 12:0-LPC was a less effective substrate than the other molecular species of LPC. The inability

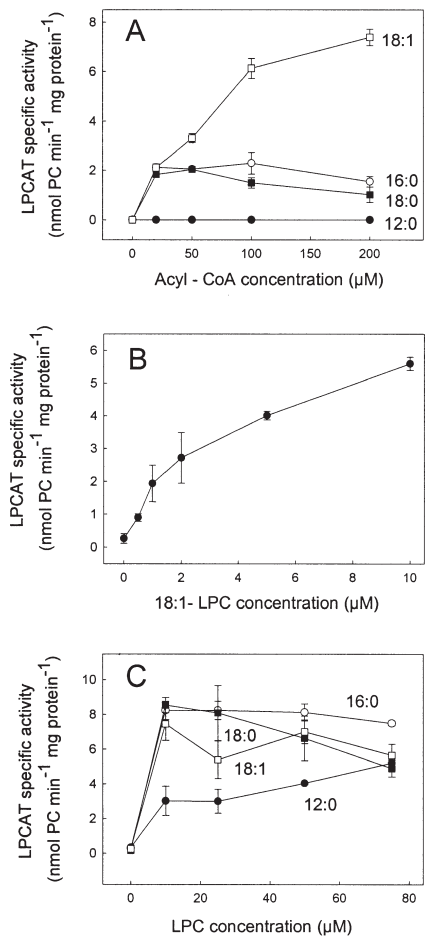

FIG. 2. Effect of substrate concentration on microsomal LPCAT activity. (A) Acyl-CoA specificity; reactions were initiated with [1-^{14}C]acyl-CoA; (B) Effect of 18:1 lysophosphatidylcholine (LPC) at concentrations up to 10 μM; (C) LPC specificity at LPC concentrations up to 75 μM. For B and C, reactions were initiated with [1-^{14}C]18:1-CoA. Reactions were allowed to proceed for 10 min. Each data point represents the average of three determinations ± SEM. For other abbreviation see Figure 1.

of *B. napus* LPCAT to utilize 12:0-CoA was consistent with studies using microsomes from developing safflower seed. Ichihara *et al.* (24) have also found that LPCAT activity in microsomes from developing safflower seeds utilized 16:0-, 18:0-, and 18:1-LPC with essentially equal effectiveness over a range of LPC concentrations. The safflower enzyme, however, utilized 12:0-LPC at a relatively lower rate than LPCAT activity in microsomes from the cell suspension cultures of *B. napus*. It is interesting to note that the ability of *B. napus* and safflower LPCAT activity to utilize 12:0-LPC is different from *B. napus* LPAAT activity from developing seeds, which has been shown to strongly discriminate against 12:0-lysophosphatidate (7).

The role of LPCAT activity in TAG biosynthesis in B. napus. Most research on plant LPCAT has involved assaying the enzyme activity in microsomes. There are a number of uncertainties in working with this heterogeneous assay system. Enzyme activity will depend on factors such as the protein and lipid content of microsomes, the critical micellar concentrations of different molecular species of acyl-CoA, and the presence of acyl-CoA binding proteins (ACBP). Nonetheless, investigations with microsomes have provided a number of insights into the action of LPCAT in developing oilseeds. Considerable research, mainly with developing safflower seed, has suggested that the reverse reaction of LPCAT may facilitate acyl-exchange at the *sn*-2 position of PC with the acyl-CoA pool, thereby creating new opportunities for the incorporation of PUFA into TAG (1). PC with polyunsaturated acyl groups is derived from FA desaturation at the level of PC (1,2,32). Endogenous ACBP may play a role in modulating lipid biosynthesis by altering the availability of acyl-CoA (33,34). Both soluble (33–35) and membrane-bound ACBP (36,37) have been identified and characterized in oilseeds. Soluble recombinant ACBP from *Arabidopsis thaliana* has been shown to protect [^{14}C]oleoyl-CoA against hydrolysis by acyl-CoA hydrolase (EC 3.1.2.14) (34). In the case of LPCAT, ACBP may have a role in reducing the concentration of free acyl-CoA such that acyl exchange is enhanced in similar fashion to the effect of BSA (19). As well, the existence of phospholipid:DAG acyltransferase (EC 2.3.1.158) (16) and phospholipase A_2 (21,22) activity in plants suggests that the forward reaction of LPCAT also could have a physiological role in reacylating LPC in a manner similar to that proposed for mammalian systems (38). Thus, LPCAT activity might participate indirectly in the exclusion of unusual FA from membranes by catalyzing the reacylation of LPC with non-unusual FA.

It has been suggested that the acyl-CoA selectivity properties of endogenous LPAAT activity are the reason for the decreased incorporation of 12:0 at the *sn*-2 position in *B. napus* expressing a medium-chain thioesterase (8). Indeed, earlier specificity studies with microsomal LPAAT from developing seeds of *B. napus* indicated that the enzyme could utilize 12:0-CoA, but with considerably less effectiveness than 18:1-CoA (7). Given the inability of *B. napus* LPCAT to accept 12:0-CoA and the possible reversibility of the cholinephosphotransferase-catalyzed reaction, we suggest that the endogenous LPCAT activity of *B. napus* also may have contributed to the decreased incorporation of 12:0 at the *sn*-2 position of *B. napus* genetically engineered to produce 12:0-CoA. Future metabolic engineering work aimed at further increasing the prevalence of 12:0 or 22:1 at the *sn*-2 position of TAG in seeds of *B. napus* should also take into consideration the activity of LPCAT. Further insights into the role of LPCAT activity in TAG biosynthesis will come from studies with transgenic plants in which the activity of this enzyme is altered through genetic engineering.

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Differences in CLA Isomer Distribution of Cow's Milk Lipids

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ABSTRACT: The uniqueness of ruminant milk lipids is based on their high concentration of CLA. Maximal CLA concentrations in milk lipids require optimal conditions of ruminal fermentation and substrate availability, conditions like those present in pasture-fed cows. Our previous work showed that farm management (indoor feeding vs. pasture feeding) markedly influenced the CLA concentration. In this study, the objective was to evaluate the influence of the farm management system as dependent on different locations. Milk samples from different locations (Thuringia and the Alps, representing diverse altitudes) were collected during the summer months and analyzed for FA profile and CLA isomer distribution. The proportion of PUFA and total CLA in milk fat was significantly lower in milk from indoor cows compared with the pasture cows in the Alps. The *trans*-11 18:1 in milk fat of Alpine cows was elevated, in contrast to lower values for *trans*-10 18:1. Milk from cows grazing pasture in the Alps was higher in EPA and lower in arachidonic acid than milk from indoor-fed cows. The proportion of *cis,trans/trans,cis* isomers of CLA was 10 times higher from the indoor cows than from the Alpine cows. In addition to the major isomer *cis*-9,*trans*-11, this difference also occurred for the *trans*-11,*cis*-13 isomer, which represented more than a fourth of the total CLA present in milk fat. This is the first report showing a special isomer distribution in the milk fat of cows living under very natural conditions. We hypothesize that the CLA isomer *trans*-11,*cis*-13 is formed in large quantity as a result of grazing mountain pasture, which is rich in α-linolenic acid.

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Cis-9,*trans*-11 octadecadienoic acid (rumenic acid) is the major CLA component in ruminant milk fat and meat fat (1,2). It has been shown that the isomers of CLA in ruminant lipids originate from incomplete biohydrogenation of PUFA (3,4) or that they are synthesized endogenously in the mammary gland from biohydrogenation derivatives (5,6).

The content of CLA in milk fat can vary widely. The underlying factors resulting in this variation are related predominantly to diet and to the farming methods for ruminants (7). Furthermore, the milk fat content of CLA is also related to animal variation (8–10). Our knowledge regarding the variation of isomer distribution in ruminant fat is limited. There are only a few publications dealing with this topic. Normally, after the overwhelmingly predominant CLA isomer *cis*-9,*trans*-11, the *trans*-7,*cis*-9 is the second-most prevalent CLA isomer in ruminant fat (1,11–15). It has been reported that this isomer represents as much as 40% of total CLA under special conditions (16).

Trans-10,*cis*-12 CLA seems to be exclusively rumen derived; and it accumulates under special dietary conditions (17). Its percentage in milk fat is generally very low. Kraft *et al*. (18) found, 5 d after an intraduodenal infusion of a CLA mixture, that milk fat decreased by 40%, indicating that the *trans*-10,*cis*-12 isomer is responsible for inhibition of milk fat synthesis.

In a dose-response experiment Baumgard *et al*. (19,20) confirmed our results when they fed the pure *trans*-10,*cis*-12 isomer. Milk fat from cows supplemented with the highest dose (14 g/d) contained more *trans*-10,*cis*-12 than *cis*-9,*trans*-11, resulting in a dramatically curvilinear reduction in milk fat yield.

The milk fat of cows grazed in the Alps is extraordinarily rich in total CLA, ranging from 1.92 to 2.87 g/100 g fat (21,22). With rising altitude, cows find pasture with a decreased proportion of grasses and an increase in dicotyledonous species, particularly *Compositae, Rosaceae,* and *Plantaginaceae* (23). Collomb *et al*. (24) correlated FA of milk fat with botanical families and individual plant species. The percentages of five plant species [*Leontodon hispidus, Plantago alpina, Aposeris foetida, Lotus corniculatus* (and *alpina*), and *Deschampsia cespitosa*] dominant in the Mountains and Highlands correlated negatively with the concentration of saturated FA (SFA). The percentages of three species [*Leontodon hispidus, Lotus corniculatus* (and *alpina*), and *Trifolium pratense*] correlated positively with the concentration of PUFA and with the concentrations of CLA and monounsaturated *trans* 18:1 FA in milk fat. The fat composition of these herbs must be the reason for the high total CLA content in the milk fat.

The aim of this study was to analyze the CLA-isomer distribution in milk fat from cows feeding at high altitudes in the Alps and compare that with the distribution in milk fat produced under intense farming practices.

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Abbreviations: Ag^+-HPLC, silver-ion HPLC; MUFA, monounsaturated FA; SFA, saturated FA; *t*VA, *trans*-vaccenic acid.

TABLE 1
Short Characterization of Cow Herds

Location	Farming	Altitude (m)	Milk yield (kg/yr)	Number of cows
1. Germany, Thuringia	Indoor-fed cows, silages and high concentrate rations, typical plain situation[a]	~200	>6000	>300
2. Germany, Thuringia	Organic farming, pasturing during the summer, only small amounts of concentrate[b]	~500	4000–5000	120–200
3. Switzerland, Alps	Summer pasturing without concentrate[c]	>1200, different places in Switzerland;	~4500	20–500
4. Switzerland, Alps	Summer pasturing without concentrate[c]	1275–2200, only L´Etivaz	~4500	30–50

[a]Most of the milk in Germany is produced under these conditions.
[b]About 5% of cows in Germany are stocked in organic farms.
[c]Summer pasturing of cows is practiced in the Alps regions of Switzerland, Austria, and Germany.

MATERIALS AND METHODS

Milk samples. Milk samples (n = 16) were collected in four locations during the summer months of the years 2000 and 2001. From each location, four samples of bulk milk from different cow herds were collected. There were significant differences in farm management among the four locations (Table 1).

Lipid extraction and preparation of FAME. Total milk lipids were extracted using a methanol/chloroform/water mixture (1:2:1, by vol) according to Folch *et al.* (25). For the preparation of FAME, $NaOCH_3$ was used. It completely converted esters to FAME, and the base-catalyzed methylation method prevented the isomerization of *cis*/*trans* conjugated bonds to *trans*/*trans* isomers and the formation of artifacts (26).

The resulting FAME were analyzed by two different GC procedures and by silver-ion HPLC (Ag^+-HPLC). The use of both methods was necessary to resolve all FA and CLA isomers (27–29). Additionally, identification of 18:1 isomers was accomplished by Ag^+-TLC before the second GC analysis.

A TAG 23:0 (Larodan Fine Chemicals AB, Malmö, Sweden) was used as an internal standard for quantification (~10% of total FAME). It was added before the methylation stage to ensure a representative sampling. Generally, milk fat contains negligible amounts of 23:0.

Analysis by GC and HPLC. (i) First GC analysis. During the first GC run, the quantification of most of the FA was realized using a gas chromatograph (Shimadzu 17A; Shimadzu, Kyoto, Japan) equipped with FID and automatic injection system (AOC-5000). Analyses were performed using a fused-silica capillary column (DB-225 MS; J&W Scientific, Folsom, CA; 30 m × 0.25 mm, i.d.; 0.2-μm film thickness) and H_2 as carrier gas.

This column was suitable for achieving a successful separation of milk fat FAME ranging from C_4 to C_{22} (including straight and branched structures) in a time-saving manner. Furthermore, it was possible to resolve *cis*-9,*trans*-11/*trans*-7,*cis*-9 CLA from other CLA isomers without interference from other FA. The detailed analysis of minor CLA isomers was achieved by Ag^+-HPLC.

(ii) Second GC analysis. The quantification of *cis* and *trans* isomers of 18:1 resulted from Ag^+-TLC separation followed by GC analysis. A fused-silica capillary column (CP-Sil 88; Chrompack, Middelburg, The Netherlands; 100 m × 0.25 mm, i.d.; 0.2-μm film thickness) was used. The system operated isothermally at 170°C. The results of the short column were combined with those of the better resolution of the 18:1 region using a 100-m CP Sil 88 column.

Ag^+-HPLC analysis. The distribution of CLA-isomers was established using a HPLC system (Shimadzu, LC10A) equipped with a solvent delivery system, an automatic sample injector with a 50-μL injection loop, a UV detector set at 234 nm, and three silver-impregnated ChromSpher 5 Lipids columns in series (each 4.6 mm i.d. × 250 mm stainless steel, 5-μm particle size; Varian-Chrompack). The isocratic mobile phase (0.1% acetonitrile and 0.5% diethylether in hexane) (15) was freshly prepared daily, stirred continuously, and pumped at a flow rate of 1.0 mL/min. Diethyl ether was used to prevent a drift in retention times. The usual injection volumes were 10–20 μL, representing <250 μg lipid. The identification of CLA isomers by Ag^+-HPLC was based on co-injection with commercial reference material (Matreya, Pleasant Gap, PA; Larodan Fine Chemicals AB, Malmö, Sweden) as well as a comparison of the elution order of CLA isomers with the existing literature (12,28).

In the first GC analysis, the main CLA isomer (*cis*-9,*trans*-11) co-eluted with both CLA isomers *trans*-7,*cis*-9 and *trans*-8,*cis*-10 (30). The HPLC areas for *trans*-7,*cis*-9 + *trans*-8,*cis*-10 + *cis*-9,*trans*-11 were added and used for calculation compared with the three isomer peaks from GC chromatogram:

$$\text{main CLA peak area}_{GC} = (t7,c9 + t8,c10 + c9,t11)\ \text{peak areas}_{HPLC} \quad [1]$$

The results were expressed as absolute values in mg/g fat using 23:0 as internal standard in the first GC analysis. The amounts of the other CLA isomers were calculated from their Ag^+-HPLC areas relative to the area of the main isomer *cis*-9,*trans*-11.

Statistical analysis. Results are expressed as means and SD. All data were analyzed by a one-way ANOVA followed by the Scheffé test. Differences were considered significant at $P < 0.05$. All analyses were performed with SPSS 10.0 (SPSS, Chicago, IL) software package.

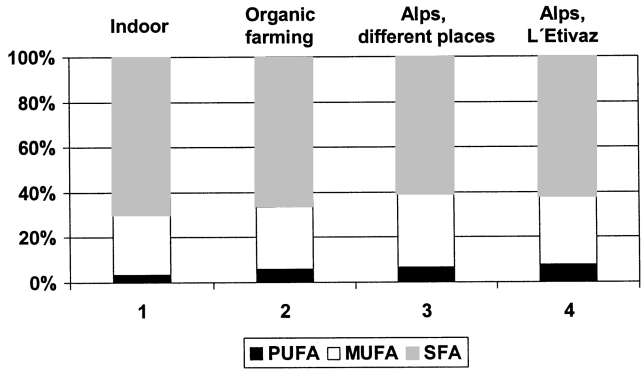


FIG. 1. Distribution of FA groups in milk fat of cows fed at different locations, farming practices, and altitudes.

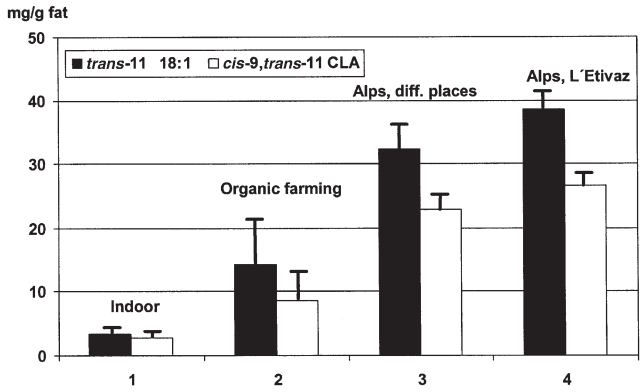


FIG. 2. Content of *cis*-9,*trans*-11 CLA and of *trans*-11 18:1 in milk fat of different origins (all differences between the groups are significant, $P < 0.05$, except between groups 3 and 4).

RESULTS

The results showed greater PUFA content in the milk fat from the pastured cows of the Alps compared with indoor cows (Fig. 1). The milk fat of indoor cows contained the highest proportion of SFA and the lowest of monounsaturated FA (MUFA). The total CLA was significantly lower in indoor cows (group 1) than Alpine cows (group 4) by a factor of 9. Among the *trans* isomers of 18:1, the *trans*-vaccenic acid (*t*VA; *trans*-11) was the most abundant FA (Table 2). Its proportion of total *trans* 18:1 varied significantly from one-fourth in the fat of indoor cows to about two- thirds in Alpine cows. The second-most prevalent *trans*-isomer was the combined *trans*-13/14 18:1 peak. The latter pair was not resolved under the GC conditions. Milk fat of pastured cows contained greater amounts of *t*VA (Fig. 2) and total *trans* 18:1 (Table 2) compared with indoor cows. In contrast, the percentage of the *trans*-10 isomer in pastured cows was significantly lower compared with indoor cows (Table 2). There were only small differences between the analyzed groups relating to the *cis*-isomers of 18:1. The milk fat of group 3 was significantly richer in *cis*-9 18:1. Similar to the *trans* isomers of 18:1, there were significant differences among the four groups mainly in branched-chain FA (*iso*- and *anteiso*-15:0, *iso*- and *anteiso*-17, as well as *iso*-14:0; Table 3). In the same way, more odd-numbered FA were found in the milk fat of Alpine cows (15:0, 17:0).

The content of 20:5 was significantly higher in the groups 2, 3, and 4. In contrast, 20:4 was significantly lower in these groups (Table 4). The most interesting differences were found for the CLA isomers (Table 5). The content of total CLA isomers, mainly the *cis*-9,*trans*-11 CLA, was significantly higher in group 4 than in group 1 by a factor of 10. The portion of *trans*-10,*cis*-12 CLA was very small (0.2%; or 2.3% of total CLA). The *trans*-7,*cis*-9 isomer, identified and quantified on the basis of the Ag^+-HPLC separations (Fig. 3), showed variations depending on the origin of the milk fat (1.1%; or 6.2%

TABLE 2
Isomers of 18:1 in Milk Fat (Values in mg/g fat)

Location	Germany, Thuringia		Switzerland, Alps	
group	1. Indoor cows	2. Organic farming	3. Different places	4. L'Etivaz
trans-4	0.08 ± 0.02[a]	0.18 ± 0.09[b]	0.12 ± 0.02[a,b]	0.12 ± 0.02[a,b]
trans-5	0.08 ± 0.01[a]	0.15 ± 0.05[b]	0.12 ± 0.02[a,b]	0.12 ± 0.02[a,b]
trans-6-8	1.03 ± 0.06[a]	2.13 ± 0.73[b]	1.95 ± 0.08[b]	2.18 ± 0.12[b]
trans-9	1.01 ± 0.02[a]	1.92 ± 0.45[b]	1.78 ± 0.07[b]	1.97 ± 0.07[b]
trans-10	2.87 ± 0.07[a]	2.04 ± 0.59[b]	1.68 ± 0.25[b]	1.78 ± 0.10[b]
trans-11	3.48 ± 0.08[a]	14.28 ± 6.68[b]	32.31 ± 4.18[c]	38.57 ± 3.41[c]
trans-12	1.27 ± 0.01[a]	3.21 ± 1.37[b]	1.90 ± 0.19[a,b]	2.46 ± 0.33[a,b]
trans-13	2.53 ± 0.16[a]	7.41 ± 3.78[b]	3.69 ± 0.35[a,b]	5.24 ± 0.64[a,b]
trans-15	0.95 ± 0.04[a]	3.15 ± 1.65[b]	1.94 ± 0.14[a,b]	2.33 ± 0.20[a,b]
trans-16	1.56 ± 0.07[a]	4.23 ± 1.86[b]	2.80 ± 0.22[a,b]	3.01 ± 1.53[a,b]
Σ*trans*-18:1	14.84 ± 0.04	38.70 ± 17.23	48.29 ± 4.73	57.77 ± 4.72
cis-9	182.82 ± 8.11[a]	182.61 ± 8.54[a]	205.65 ± 10.17[b]	175.15 ± 8.49[a]
cis-11	6.78 ± 0.17[a]	5.01 ± 0.37[b]	5.37 ± 0.31[b]	5.01 ± 0.28[b]
cis-12	1.19 ± 0.08[a]	2.43 ± 0.86[b]	0.68 ± 0.04[a]	0.86 ± 0.13[a]
cis-13	0.86 ± 0.06[a]	0.81 ± 0.09[a]	0.57 ± 0.07[b]	0.59 ± 0.07[b]
cis-15	0.73 ± 0.17[a]	2.46 ± 1.08[b]	1.45 ± 0.06[a,b]	1.75 ± 0.20[a,b]

[a]Values in a row not sharing a common superscript roman letter differ, $P < 0.05$.

TABLE 3
Branched FA in Milk Fat[a] (values in mg/g fat)

	Germany, Thuringia		Switzerland, Alps	
Group location	1. Indoor cows	2. Organic farming	3. Different places	4. L'Etivaz
iso-13:0	0.77 ± 0.02[a]	0.57 ± 0.17[a,b]	0.52 ± 0.09[b]	0.59 ± 0.09[a,b]
anteiso-13:0	0.25 ± 0.00[a]	0.39 ± 0.05[b]	0.54 ± 0.04[c]	0.54 ± 0.04[c]
iso-14:0	0.80 ± 0.02[a]	1.23 ± 0.10[a,c]	1.64 ± 0.09[b,c]	1.71 ± 0.29[b]
15:0	10.89 ± 0.18[a]	10.83 ± 0.61[a,b]	12.63 ± 0.21[a,b]	13.22 ± 1.48[b]
iso-15:0	2.16 ± 0.03[a]	2.58 ± 0.05[a,c]	3.47 ± 0.22[b,c]	3.32 ± 0.49[b]
anteiso-15:0	4.38 ± 0.02[a]	5.73 ± 0.41[b]	6.88 ± 0.45[c]	7.24 ± 0.59[c]
iso-16:0	2.36 ± 0.08[a]	2.70 ± 0.16[a]	2.87 ± 0.09[a]	2.91 ± 0.27[a]
17:0	5.93 ± 0.07[a]	5.88 ± 0.14[a]	7.49 ± 0.24[b]	6.94 ± 0.42[b]
iso-17:0	3.52 ± 0.07[a]	3.90 ± 0.36[a]	4.76 ± 0.15[b]	4.61 ± 0.37[b]
anteiso-17:0	4.81 ± 0.08[a]	4.89 ± 0.14[a]	5.07 ± 0.12[a]	5.01 ± 0.18[a]

[a]Values in a row not sharing a common superscript roman letter differ, $P < 0.05$.

of total CLA). The *trans*-7,*cis*-9 CLA isomer was identified by Yurawecz *et al*. (12) and reported to be the second-most abundant CLA isomer in normal commercial milk fat. The results of the indoor group (Table 5) were similar, thus confirming the previous report. The higher CLA content was associated mainly with higher levels of *trans*-11,*cis*-13 CLA, whereas the level of *trans*-7,*cis*-9 remained nearly constant (except group 4, Table 5).

TABLE 4
Distribution of FA in Milk Fat[a] (values in mg/g fat)

Location	Germany, Thuringia		Switzerland, Alps	
group	1. Indoor cows	2. Organic farming	3. Different places	4. L'Etivaz
4:0	37.88 ± 0.50[a]	37.26 ± 1.33[a]	35.29 ± 1.86[a]	35.59 ± 3.27[a]
6:0	26.92 ± 0.21[a]	25.56 ± 1.04[a,c]	21.62 ± 1.25[b]	22.57 ± 2.01[b,c]
8:0	13.93 ± 0.30[a]	13.47 ± 0.86[a]	10.49 ± 0.91[b]	11.25 ± 1.03[b]
10:0	30.50 ± 0.38[a]	27.81 ± 2.23[a,c]	20.93 ± 2.65[b]	23.00 ± 1.91[b,c]
10:1	2.95 ± 0.05[a]	2.76 ± 0.36[a]	2.56 ± 0.40[a]	2.78 ± 0.36[a]
12:0	33.54 ± 0.28[a]	28.23 ± 2.97[b]	22.17 ± 2.78[b,c]	23.81 ± 1.85[b,c]
12:1	0.81 ± 0.22[a]	0.68 ± 0.22[a]	0.60 ± 0.11[a]	0.67 ± 0.11[a]
13:0	1.20 ± 0.01[a]	0.87 ± 0.05[b,c]	0.72 ± 0.06[c]	0.80 ± 0.06[c]
14:0	94.66 ± 1.63[a]	87.75 ± 8.07[a,b]	79.36 ± 6.51[b]	82.80 ± 3.18[a,b]
14:1	10.28 ± 0.13[a]	10.65 ± 2.23[a]	8.05 ± 1.16[a]	8.93 ± 1.12[a]
15:0	10.81 ± 0.18[a]	10.83 ± 0.61[a,c]	12.63 ± 0.21[b,c]	13.22 ± 1.48[b]
16:0	273.46 ± 6.80[a]	226.23 ± 36.79[b]	207.38 ± 10.37[b]	219.44 ± 9.00[b]
16:1	20.57 ± 0.76[a]	14.13 ± 2.73[b]	12.95 ± 1.24[b]	13.48 ± 1.40[b]
17:0	5.93 ± 0.07[a]	5.88 ± 0.14[a]	7.49 ± 0.24[b]	6.94 ± 0.42[b]
17:1	0.05 ± 0.01[a]	0.12 ± 0.05[a]	4.82 ± 0.44[b]	4.71 ± 0.57[b]
18:0	75.34 ± 2.75[a]	106.44 ± 12.60[b]	98.37 ± 11.39[a,b]	86.34 ± 9.24[a,b]
18:2*t*9,*c*12	2.32 ± 0.06[a]	7.38 ± 4.37[b]	5.48 ± 0.51[a,b]	7.02 ± 0.83[b]
18:2n-6	16.33 ± 0.79[a]	15.18 ± 0.82[a]	11.93 ± 0.48[b]	13.05 ± 1.09[b]
18:3n-6	0.45 ± 0.02[a]	1.20 ± 0.69[b]	1.15 ± 0.09[a,b]	1.40 ± 0.13[b]
18:3n-3	3.31 ± 0.22[a]	8.61 ± 2.93[a,b]	11.67 ± 0.47[b]	13.02 ± 1.74[b]
18:4n-3	0.45 ± 0.02[a]	0.48 ± 0.14[a]	0.94 ± 0.38[a,b]	1.20 ± 0.12[b]
20:0	1.08 ± 0.07[a]	1.44 ± 0.09[a,b]	1.60 ± 0.12[b]	1.38 ± 0.18[a]
20:1n-9	0.56 ± 0.03[a,c]	0.63 ± 0.09[a]	0.44± 0.03[b,c]	0.42 ± 0.05[b]
20:3n-6	0.80 ± 0.03[a]	0.63 ± 0.16[a,b]	0.48 ± 0.04[b]	0.63 ± 0.04[a,b]
20:4n-6	1.15 ± 0.05[a]	0.75 ± 0.21[b]	0.56 ± 0.05[b]	0.68 ± 0.04[b]
21:0	0.15 ± 0.04[a]	0.42 ± 0.05[b]	0.39 ± 0.05[b]	0.37 ± 0.07[b]
20:5n-3	0.43 ± 0.04[a]	0.84 ± 0.27[b]	0.91 ± 0.10[b]	1.05 ± 0.12[b]
22:0	0.34 ± 0.05[a]	0.57 ± 0.05[b]	0.91 ± 0.09[c]	0.80 ± 0.09[c]
22:5n-3	0.63 ± 0.04[a]	1.02 ± 0.26[b]	1.01 ± 0.07[b]	1.13 ± 0.08[b]
24:0	0.24 ± 0.03[a]	0.36 ± 0.09[a]	0.69 ± 0.06[b]	0.68 ± 0.07[b]
∑SCFA	79.35 ± 0.94[a]	76.71 ± 2.36[a,b]	67.39 ± 2.45[b]	69.42 ± 6.24[b]
∑MCFA	479.64 ± 7.56[a]	400.98 ± 52.32[b]	367.39 ± 21.47[b]	389.03 ± 16.35[b]
∑LCFA	278.24 ± 6.93[a]	314.04 ± 25.47[a,b]	328.78 ± 20.37[b]	288.96 ± 16.80[a]

[a]Values in a row not sharing a common superscript roman letter differ, $P < 0.05$. SCFA, short-chain FA; MCFA, medium-chain FA; LCFA, long-chain FA.

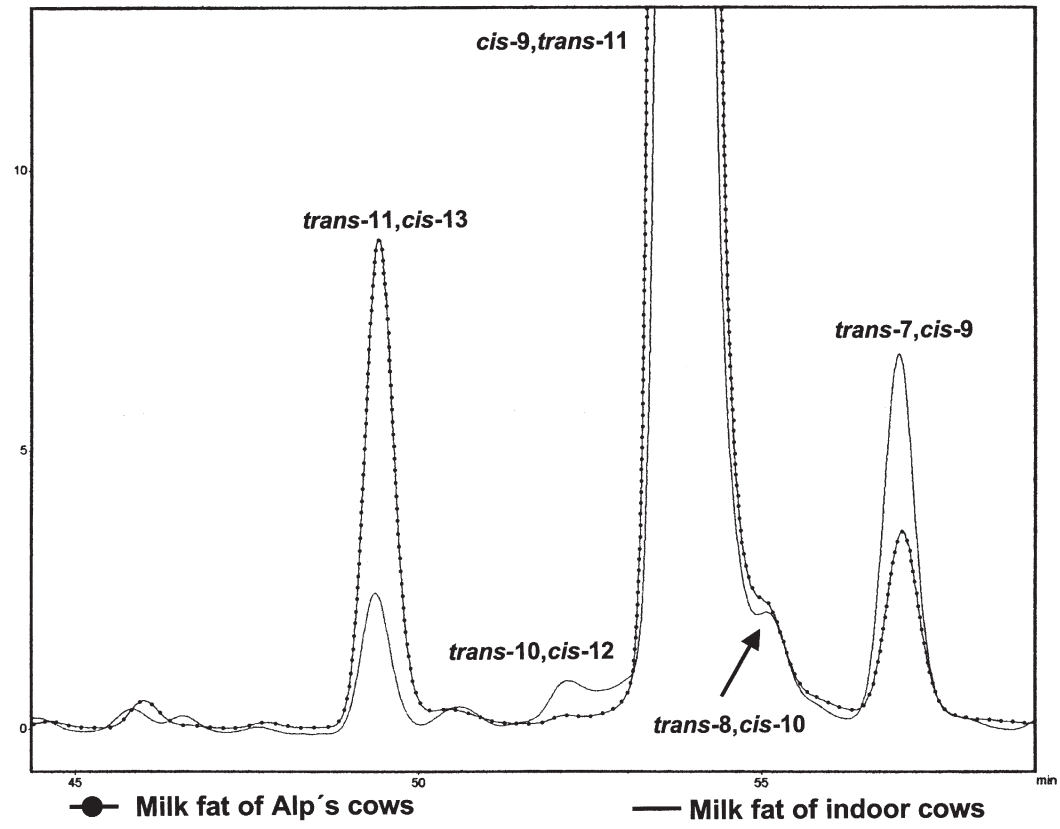


FIG. 3. Partial Ag^+-HPLC chromatogram of a milk fat from cows pastured in the Alps.

Ranking second, after the rumenic acid, was the *trans*-11,*cis*-13 CLA (from 2 to 8% of total CLA; Fig. 3). The amount of this FA per gram of fat was higher in group 4 than group 1 by a factor of 35.

DISCUSSION

FA composition of pasture. The CLA content of milk fat correlates with the FA concentration of the pasture. In a study

TABLE 5
Concentration[a] of Different CLA Isomers of the Milk Fat (values in mg/g fat)

Location	Germany, Thuringia		Switzerland, Alps	
group	1. Indoor cows	2. Organic farming	3. Different places	4. L'Etivaz
Isomers				
Σ*cis,trans/trans,cis*	3.14 ± 0.12[a]	10.15 ± 4.24[b]	24.97 ± 2.49[c]	29.84 ± 1.50[c]
11,13	0.07 ± 0.00[a]	0.85 ± 0.32[b]	1.49 ± 0.23[b]	2.49 ± 0.42[c]
10,12	0.08 ± 0.02[a]	0.06 ± 0.08[a]	0.05 ± 0.09[a]	0.07 ± 0.07[a]
9,11	2.76 ± 0.12[a]	8.72 ± 3.50[b]	22.94 ± 2.33[c]	26.7 ± 1.08[c]
7,9	0.22 ± 0.01[a]	0.20 ± 0.08[a]	0.29 ± 0.02[a,b]	0.37 ± 0.05[b]
Unidentified peaks	0.01	0.32	0.20	0.21
Σ*trans/trans*	0.35 ± 0.01[a]	1.52 ± 0.59[b]	0.93 ± 0.07[a,b]	1.31 ± 0.07[b]
12,14	0.05 ± 0.01[a]	0.30 ± 0.11[b]	0.22 ± 0.05[b]	0.32 ± 0.04[b]
11,13	0.09 ± 0.01[a]	0.52 ± 0.20[b,c]	0.37 ± 0.03[b]	0.57 ± 0.04[c]
10,12	0.05 ± 0.00[a]	0.11 ± 0.08[a]	0.07 ± 0.01[a]	0.08 ± 0.02[a]
9,11	0.08 ± 0.01[a]	0.40 ± 0.07[b]	0.12 ± 0.01[a,c]	0.17 ± 0.03[c]
8,10	0.03 ± 0.00[a]	0.05 ± 0.04[a]	0.05 ± 0.00[a]	0.06 ± 0.02[a]
7,9	0.03 ± 0.00[a]	0.12 ± 0.10[a,b]	0.07 ± 0.02[b]	0.08 ± 0.02[b]
Unidentified peaks	0.02	0.02	0.03	0.03
Σ*cis/cis*	0.06 ± 0.02[a]	0.21 ± 0.25[a]	0.03 ± 0.00[a]	0.04 ± 0.00[a]

[a]Values in a row not sharing a common superscript roman letter differ, $P < 0.05$.

including three different cow herds over a full year, the highest CLA content of milk fat was found during the summer months in the two herds grazing pasture (31). The primary FA in grass is α-linolenic acid; its content was ~50% of the total FAME (32).

The milk fat of Alpine cows had the highest PUFA content and the lowest SFA content (Fig. 1). The fodder of indoor cows consists mainly of fermented grass (silage: microbially biohydrogenated fat) and concentrates containing more SFA. Concentrates and silage contribute partially to the *de novo* synthesis of 16:0; the remainder originates from the uptake of preformed FA (33). Therefore, the milk fat of indoor cows is rich in SFA.

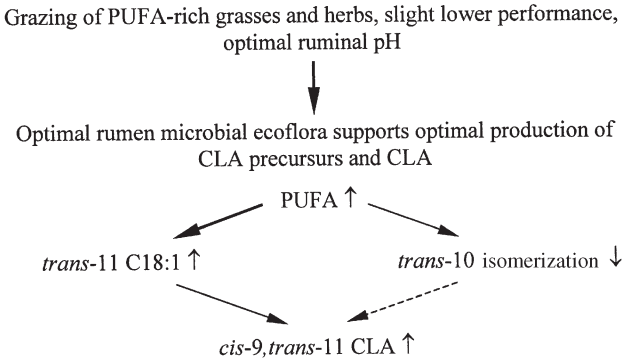
CLA and t*VA in milk fat.* Milk fat is the richest natural source of CLA. Under normal animal husbandry practice, it may contain 2.4 to 37.0 mg CLA/g fat (34). This corresponds exactly with the range for total CLA from our analysis (Fig. 2). Dhiman *et al*. (35) found that cows feeding only on pasture synthesize a CLA-richer milk fat than those grazing two-thirds or one-third pasture. Jeangros *et al*. (23) also observed an elevated CLA content in the milk of Alpine cows, which correlates with dicotyledons such as *Compositae, Rosaceae*, and *Plantaginaceae* occurring in mountain pasture. In connection with the results shown in Figure 2, it is of interest that green leaves of immature pasture plants contain more lipid extract than leaves from mature forage (36). Due to the short vegetation period, the meadows at higher altitude in the Alps are physiologically young. Furthermore, under the lower environmental temperatures typical of the highlands, plant tissues contain a higher percentage of α-linolenic acid (36). It has been suggested that feeding linseed oil (a rich source of α-linolenic acid) results in a large increase in the production of rumen *trans*-11 18:1, which can be used by the mammary gland for rumenic acid synthesis (5,37,38).

The low ruminal pH often found in high-performance cows fed concentrate-rich rations alters the microbial ecosystem to favor synthesis of *trans*-10 monoene or conjugated diene, or both (Table 3). FA with the *trans*-10 double bond inhibit mammary milk fat synthesis as well as the tissue synthesis of CLA from *t*VA by down-regulation of stearoyl-CoA desaturase 1 gene expression (39) and, accordingly, other unknown mechanisms. On the other hand, optimal ruminal fermentation in cows grazing herb-rich pasture (optimal pH, PUFA as substrate for *t*VA) minimizes the formation of *trans*-10 FA. The absence of this depressing agent maximizes the desaturation of *t*VA (40) (Fig. 4). Milk fat synthesized under these conditions is rich in CLA and relatively poor in *t*VA. Some authors described close correlations between *t*VA and *cis*-9,*trans*-11 CLA (40–42). Figure 2 shows a wide correlation between the precursor and the desaturated product. The ratio CLA/*t*VA ranged from 0.25 in group 1 to 0.52 in group 4. Evidently, a higher percentage of *t*VA is converted into *cis*-9,*trans*-11 CLA in the Alpine cows. The Δ9-desaturase acts very effectively in the mammary gland of Alpine cows (Fig. 4). More ineffective conversion ratios were found under experimental conditions using different oil supplements (43). Feeding a combination of fish oil and sunflower oil increased CLA in milk fat to >6% (mean), with individual cows exceeding 8% CLA. This was accompanied by *t*VA concentration >18%.

Piperova *et al*. (16) fed a ration supplemented with soybean oil. The cows produced a milk fat consisting of 15.6 g *t*VA/100 g FAME and 0.95 g CLA/100 g FAME, corresponding to a CLA/*t*VA ratio of 0.06. Morales *et al*. (44) confirmed that the apparent desaturation of *t*VA to CLA in the mammary gland is influenced by the oil source. Pasture oil seems to support an optimal conversion.

High rumen microbial activity in Alpine cows. Table 4 also demonstrates higher concentrations of such FA in the milk fat of pastured cows, which are unusual in plant oils. The results showed significant differences among the four groups, mainly in branched-chain FA (*iso*- and *anteiso*-15:0, *iso*- and *anteiso*-17:0, and *iso*-16:0). Furthermore, the results also support the hypothesis of a very active and specific rumen microbial ecoflora (Fig. 4). In organic dairy farming, with rations poorer in energy (starch) and richer in fiber, a more intensive activity of rumen bacteria has been suggested due to pasture feeding, as measured by a higher percentage of branched-chain FA in lipids (31,45).

CLA isomer distribution. The most interesting differences were found for the CLA isomers (Table 2). The content of *cis*,*trans*/*trans*,*cis* isomers showed marked differences from group 1 to 4 by a factor of 10. The proportion of *trans*-10,*cis*-12 CLA was very small (1 to 2% of *ct*/*tc*). Because mammals do not possess Δ12-desaturase, it follows that the *trans*-10,*cis*-12 CLA reported in ruminant milk fat and tissues originates from *trans*-10,*cis*-12 CLA that was absorbed from the intestine. Earlier publications showed that the *trans*-7,*cis*-9 is the second to most prominent CLA isomer *cis*-9,*trans*-11 in ruminant fat (11–14). The CLA isomer ranking depends on



Fatty acid distribution of milk fat	Ratio/Percentage
ratio of *trans*-C18:1/*cis*-9,*trans*-11	≈ 0.5
trans-11,*cis*-13	≈ 5% of total CLA
trans-10,*cis*-12	< 0.1% of total CLA

FIG. 4. Influence of optimal farming management on desaturation of *trans*-11 18:1 to *cis*-9,*trans*-11 CLA (*trans*-10 bonds inhibit Δ9-desaturase). Source: Reference 40.

the feeding regime. The chromatograms in Figure 3 show the impressive difference between indoor cows and Alpine cows in relation to the *trans*-11,*cis*-13 peak. Our results indicate that in the milk fat of pasture-fed cows, the second-most abundant CLA isomer is *trans*-11,*cis*-13 (Table 1). As mentioned above, plants growing under lower environmental temperatures such as in the Alps contain lipids with a higher percentage of α-linolenic acid (36), which could explain this result. α-Linolenic acid has been shown to be converted to *cis*-9,*trans*-11,*cis*-15 conjugated triene in the rumen (46). It is subsequently metabolized to *trans*-11,*cis*-15 18:2, and finally to octadecenoic acid (18:1 containing a *trans*-11, *trans*-15 or *cis*-15 bond, respectively). The pathway from *trans*-11,*cis*-15 (46, see above) to the second-most prominent isomer *trans*-11,*cis*-13 is unclear at this stage. The *trans*-11 double bond seems to be the most stable *trans*-bond found among the isomers of 18:1 and among the CLA isomers in ruminal fermentation. Thus, three different CLA isomers with a *trans*-11 double bond are generally possible: (i) *cis*-9,*trans*-11 (bacterial synthesis in rumen + tissue Δ9 desaturation), (ii) *trans*-11,*cis*-13 (bacterial origin), and (iii) *trans*-11,*trans*-13.

It is clear that *cis*-9,*trans*-11 and *trans*-11,*cis*-13 were the most abundant FA among the *cis,trans/trans,cis*-CLA, and the *trans*-11,*trans*-13 isomer was also the most abundant isomer among the *trans,trans*-CLA (Table 5). Thus, it could be shown by Ag^+-HPLC isomer analysis that milk fat synthesized under natural conditions contained, in addition to the major *cis*-9,*trans*-11 isomer, the *trans*-11,*cis*-13 isomer in a large quantity.

Overall, it can be concluded that the *trans*-11 double bond possesses a high stability during biohydrogenation of PUFA. We hypothesize that linolenic acid is an indirect precursor of *trans*-11,*cis*-13 CLA. More evidence could be provided by an infusion experiment using fistulated cows, infusing linolenic acid into the rumen and taking samples *via* duodenal fistula.

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Lipids and FA Analysis of Canine Prostate Tissue

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ABSTRACT: It is widely reported that an association exists between dietary fat intake and the incidence of prostate cancer in humans. To study this association, there is a need for an animal model where prostate carcinogenesis occurs spontaneously. The canine prostate is considered a suitable experimental model for prostate cancer in humans since it is morphologically similar to the human prostate and both humans and dogs have a predisposition to benign and malignant prostate disease. In this study, the FA and lipids profiles of the normal canine prostate tissue from nine dogs were examined. The total lipid content of the canine prostate tissue was 1.7 ± 0.5% (wet weight). The lipid composition analysis using TLC-FID showed that the two major lipid classes were phospholipids and TAG. Total FA, phospholipid, and TAG FA analysis showed that the major FA were palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2n-6), and arachidonic acid (20:4n-6). The n-3 FA were present at <3% of total FA and included α-linolenic acid (18:3n-3) (in total and TAG tissue FA), EPA (20:5n-3) (not in TAG), and DHA (22:6n-3) (not in TAG). The n-3/n-6 ratio was 1:11, 1:13, and 1:8 in total, phospholipid, and TAG FA, respectively. This study shows the canine prostate has a low level of n-3 FA and a low n-3/n-6 ratio. This is perhaps due to low n-3 content of the diet of the dogs. FA analysis of dogfoods available in Australia showed that the n-3 content in both supermarket and premium brand dogfoods was <3% (wet weight), and the n-3/n-6 ratio was low.

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Little is known about the etiology of prostate cancer, despite its prominence as the second leading cause of cancer mortality in men (1). In 1975, Armstrong and Doll (2) hypothesized that there was an association between dietary fat and death from prostate cancer. Since then, many epidemiological, animal, and cell culture studies have looked at this association; however, the results have been conflicting.

The body of animal data relevant to understanding carcinogenesis in the human prostate is also limited. This might be due to the substantial variation in the anatomy, biochemistry, and pathology of the prostate gland between mammals. Histologically, the prostate gland in humans is a rounded mass of smooth muscle and connective tissue filled with tubuloalveolar glands. It is divided into a central zone and a peripheral zone, where the central zone is shaped like an inverted pyramid and is characterized by larger and more irregular secretory acini than the acini in the peripheral zone (3). In most instances, malignant neoplasms of the prostate arise in the peripheral zone of the prostate gland (4). In the rat, the prostate has distinct separate lobes, each with a separate function and rats seldom develop prostate cancer spontaneously (5). The canine prostate is an androgen-dependent, bi-lobed gland composed of glandular and stromal elements that encircles the urethra of the male dog caudal to the neck of the urinary bladder (6). The canine prostate is considered a suitable experimental model since it is morphologically similar to the human prostate, and both humans and dogs have a predisposition to benign and malignant prostate disease (7,8). The dog is the only nonhuman species in which prostatic intraepithelial neoplasia (PIN) and invasive carcinoma are documented to spontaneously occur (9). PIN is an intermediate between benign epithelium and invasive carcinoma, and it is believed that PIN may be an intermediate step in the progression to prostatic cancer (9).

There is a need for a suitable animal model to study the association between fat intake and prostate cancer risk in humans. To our knowledge, there are no data in the literature on the FA profile of the canine prostate gland, even though it is the most suitable animal model to study carcinogenesis in humans. This study examines lipid and FA profile of canine prostate tissue from nine dogs.

MATERIALS AND METHODS

Prostates were collected from nine fresh canine cadavers at the Veterinary School, University of Melbourne, Melbourne, Australia. Tissues from intact male dogs, seven greyhounds, and two mongrels were collected, and samples were stored at –20°C until analysis. Lipids were extracted from approximately 0.5 g of each tissue using chloroform/methanol as described previously (10). One aliquot of each tissue lipids was converted to FAME for determination of the tissue total FA content by capillary GLC using a BPX-70 column (SGE, Melbourne, Australia). The column oven was programmed from 125°C for 3 min to 220°C at 8°C/min with helium as a carrier gas at a flow rate of 43 cm/s. FA were identified by comparison with standard mixtures of FAME and the results calculated using response factors derived from chromatograph standards of known composition (Nu-Chek-Prep, Elysian, MN). Another aliquot was used to separate phospholipids (PL) and TAG from neutral lipids using TLC, and then the PL and TAG fractions were converted to PL and TAG methyl esters for determination of the FA content as described

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Abbreviations: ALA, α-linolenic acid; PGHS, prostaglandin G/H synthase; PIN, prostatic intraepithelial neoplasia, PL, phospholipid.

above. The TAG fraction was collected and analyzed from only six of the nine canine prostates since three of the specimens had insufficient TAG for analysis. A third aliquot was analyzed using an Iatroscan MK-5 TLC-FID analyzer (Iatron Laboratories, Tokyo, Japan) to quantify the percentage of individual lipid classes in the tissue, namely PL, cholesterol, FFA, TAG, and cholesterol ester. Samples were analyzed in duplicate using silica gel Chromarods-S III (5 μm particle size) (Iatron Laboratories). Separation was achieved using a two-solvent system: the first of petroleum ether/diethyl ether/glacial acetic acid (60:15:0.1) and the second of petroleum ether/diethyl ether (56:4). After development, the rods were allowed to air dry for 2 min and then were dried in a Rod Dryer (Iatron Laboratories) at 120°C for 3 min to evaporate all remaining solvent. The rods were analyzed immediately and the peaks were quantified using a Chromatopac recorder (Shimadzu, Melbourne, Australia). Peaks were identified using a TLC standard (18-5-A; Nu-Chek-Prep, Inc.). A final aliquot was then subjected to TLC on silica gel G using a solvent system of petroleum ether/diethyl ether/glacial acetic acid (85:15:2) to confirm the lipids results obtained from TLC-FID analyses. For FA analysis of the dogfoods, lipids were extracted from approximately 5 g of 25 supermarket and five premium brand dogfoods using chloroform/methanol as described previously (10). An aliquot was converted to FAME for determination of the FA composition by capillary GLC, using 23:0 as an internal standard on a BPX-70 column (SGE).

Statistical analysis. Student's *t*-test was used to compare the differences between the groups. A P value of <0.05 was considered significant (95% confidence interval).

RESULTS AND DISCUSSION

The total lipid content of the prostate tissue was 1.7 ± 0.5%. The lipid composition analysis using TLC-FID showed that the two major lipid classes were PL and TAG at 56.3 ± 11.3 and 24.6 ± 12.2%, respectively (mean ± SD, n = 9); cholesterol, FFA, and cholesterol esters were 14.7 ± 3.1, 3.7 ± 2.0, and 2.0 ± 0.5%, respectively. These results were confirmed using conventional TLC analysis (Fig. 1). Total, PL, and TAG FA analysis showed that the canine tissues examined were rich in palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2n-6), and arachidonic acid (20:4n-6) (not in TAG analysis). They also contained α-linolenic acid (18:3n-3) (in total and TAG FA analysis), EPA (20:5n-3) (not in TAG analysis) and DHA (22:6n-3) (not in TAG analysis), as shown in Table 1.

Total n-6 PUFA content (%) in the canine tissue was 24 ± 5, 33 ± 3, and 7 ± 2, while the total n-3 content (%) was 2.2 ± 0.3, 2.5 ± 0.5, and 0.9 ± 0.2 in total, PL, and TAG FA analysis, respectively. The n-3/n-6 ratio was 1:11, 1:13, and 1:8 in total, PL, and TAG FA, respectively.

Recent studies have examined the relationship between dietary n-3 FA and the development of prostatic cancer. One study (11) examined prostatic levels of individual FA in relation to histopathological characteristics of cancer in men undergoing radical prostatectomy for localized disease. The results of the study showed that prostatic n-3 FA percentage was approximately 1.5- to 2.1-fold lower in cases than in control patients, and the prostatic n-3/n-6 FA ratio was approximately 2.2- to 3.3-fold lower in cases than in controls. Another case-control study (12) found a significant decrease in serum n-3/n-6 FA ratio in prostate cancer patients compared with

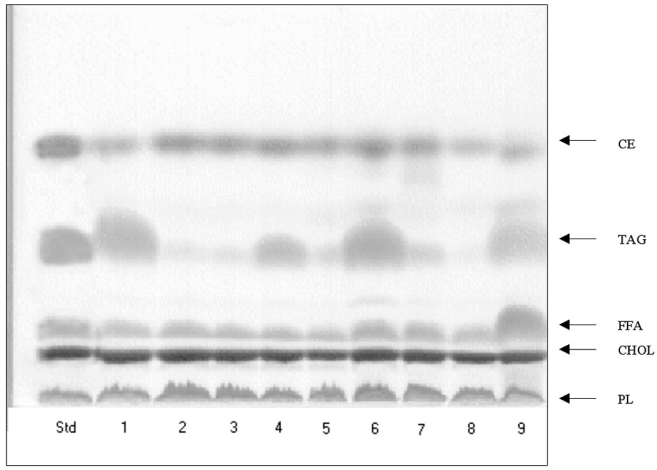

FIG. 1. TLC separation of canine prostate gland lipids on silica gel plate, with solvent system petroleum ether/diethyl ether/acetic acid (85:15:2, by vol). Std = TLC standard, numbers 1–9 represent canine prostate tissues 1–9. PL = phospholipids, CHOL = cholesterol, and CE = cholesterol ester.

TABLE 1
FA Composition of Total Lipids, Phospholipids (PL), and TAG in Canine Prostate (% of total FA)

FA	Total FA	Total PL	Total TAG
14:0	1.0 ± 0.8[a]	0.5 ± 0.4[a]	2.6 ± 0.2
16:0	11.5 ± 2.5	10.3 ± 1.8	18.9 ± 3.5
16:1	1.7 ± 1.4	—[b]	3.6 ± 0.6
17:0	1.5 ± 0.4	1.4 ± 0.4	0.9 ± 0.04
17:1	0.9 ± 0.4	—	0.7 ± 0.08
18:0	22.2 ± 12.4	24.8 ± 3.1	15.6 ± 6.4
18:1n-9	15.7 ± 9.8	10.4 ± 1.0	37.1 ± 8.0
18:2n-6	9.4 ± 1.6	10.7 ± 1.7	6.8 ± 1.7
18:3n-6	0.4 ± 0.1	0.4 ± 0.1	—
18:3n-3	0.4 ± 0.3	—	0.9 ± 0.2
20:0	0.5 ± 0.1	0.3 ± 0.04	0.4 ± 0.2
20:1	1.0 ± 0.3	1.1 ± 0.3	0.4 ± 0.2
20:2	1.2 ± 0.9	1.8 ± 0.9	0.2 ± 0.05
20:3n-6	1.1 ± 0.5	1.7 ± 0.3	—
20:4n-6	10.7 ± 4.3	17.8 ± 2.3	0.3 ± 0.1
22:0	0.5 ± 0.1	—	0.2 ± 0.09
22:1	0.2 ± 0.09	—	—
20:5n-3	0.3 ± 0.1	0.6 ± 0.1	—
22:4n-6	1.3 ± 0.4	2.1 ± 0.6	—
24:0	0.6 ± 0.2	0.5 ± 0.2	—
24:1	2.4 ± 1.2	0.2 ± 0.05	—
22:5n-3	1.0 ± 0.3	1.3 ± 0.3	—
22:6n-3	0.5 ± 0.1	0.7 ± 0.2	—

[a]Values are means ± SD of nine canine prostate tissues for total FA and PL and for six canine prostate tissues for TAG.
[b]The dash (—) indicates less than 0.02% of total FA.

controls. The present data show that dog prostate tissue contains a low level of n-3 FA and a low n-3/n-6 ratio. This is perhaps due to the diet of the dogs having low n-3 content.

FA analysis of dogfoods available in Australia showed that the proportion of n-3 in both supermarket and premium brand dogfoods was <3%, and that the n-3/n-6 ratio was low (Table 2). Therefore, increasing the n-3 intake may lead to an increase in the n-3 content and the n-3/n-6 ratio in the prostate and may be of importance to prostatic health.

Dietary n-3 PUFA also can affect eicosanoid synthesis, which may be important for prostate health. Prostaglandins and leukotrienes are derived from arachidonic acid, and these compounds can affect several aspects of tumor development, including cell proliferation, immune response, invasion, and metastasis (13). The n-3 PUFA competitively inhibit the incorporation of arachidonic acid into cellular membranes and interfere with the synthesis of prostaglandin E_2 (14,15). The first rate-limiting step in the synthesis of all prostaglandins from arachidonic acid is catalyzed by the enzyme prostaglandin G/H synthase (PGHS) (16). Two isoforms of the enzyme have been identified, PGHS-1 and PGHS-2 (16), and increased expression of PGHS-2 has been associated with various cancers in humans and animals (17,18). A study in dogs showed for the first time evidence of induction of PGHS-2 in prostate cancer, where 75% of dogs with prostatic adenocarcinoma were shown to have epithelial cell expression of PGHS-2, in contrast to normal dog prostate where no PGHS-2 activity was detected (19). A recent study has characterized the structure of the canine PGHS-2 and studied the regulation of its transcript protein and activity in canine prostatic adenocarcinoma. (20). The study suggested that PGHS-2 acts as an immediate early-response gene in prostatic epithelial cells.

Lipid analysis using TLC-FID of the canine prostate showed that PL (56%) was the major class followed by TAG (24%) and cholesterol (14%). To our knowledge, there are no data on the lipid distribution in the prostate in any other species, and it is not known if carcinogenesis of the prostate gland affects the lipid class distribution in the tissue. An early study by Campbell *et al.* (21) investigated the lipid patterns in canine prostatic serum and fluid. The study found that prostatic serum contained cholesterol (9.5%), cholesterol ester (40.8%), TAG (8.5%), FFA (5.9%), lysolecithin (6.1%), sphingomyelin (4.2%), lecithin (23.6%), and cephalins (1.2%). The prostatic fluid contained cholesterol (15.3%), cholesterol ester (26.9%), TAG (24.2%), FFA (27.4%), lysolecithin (0.4%), sphingomyelin (1.5%), lecithin (3.8%), and cephalins (0.5%) (21).

As mentioned earlier, dogs share similar prostate morphology to that of the human, but for practical reasons scientists developed an experimental animal model in rodents to study carcinogenesis of the prostate. All animal models require some form of initiating factor such as chemical, hormonal, or transgenic activation (22). Rodent studies suggest that dietary fat, particularly animal fat, increases both the incidence and the rate of growth of adenocarcinomas of the prostate (23–26). However, current animal research does not provide strong mechanistic evidence for the role of fat in prostate carcinogenesis (22). Development of an animal model in which prostatic carcinogenesis occurs spontaneously may offer advantages over the available models in studying the association between fat intake, FA quality, and prostate cancer.

TABLE 2
FA Composition of Supermarket and Premium Brand Dogfoods in Australia (% of Total FA)

FA	Supermarket (n = 25)	Premium (n = 5)
Saturated	38.0 ± 4.1[a]	36.1 ± 5.5[b]
Monounsaturated	38.5 ± 5.3	40.8 ± 11.0
Polyunsaturated	17.4 ± 5.1	30.6 ± 17.5
18:2n-6	14.8 ± 4.9	27.7 ± 15.2
18:3n-3	1.4 ± 0.6	2.9 ± 2.4
Long-chain n-3	0.3 ± 0.5	0.2 ± 0.4
n-6/n-3	9.6 ± 4.5	11.2 ± 6.1

[a]Values are means ± SD of 20 different dogfoods of supermarket brand and five premium dogfoods. Means within the same row bearing different roman superscripts are significantly different ($P < 0.05$).

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A Glucosylceramide with a Novel Ceramide and Three Novel Ceramides from the Basidiomycete *Cortinarius umidicola*

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ABSTRACT: A glucosylceramide with novel ceramide and three novel ceramide homologs were isolated from the basidiomycete *Cortinarius umidicola* and structurally characterized. The ceramide portion of the glucocerebroside consists of a rare (4*E*,8*E*)-9-methyl-4,8-sphingadienine sphingoid base. In contrast, the three ceramide homologs, while having the same sphingoid base, contain as FA residues 2-hydroxydocosanoic acid, 2-hydroxytricosanoic acid, and 2-hydroxytetracosanoic acid.

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In recent years, renewed attention has been paid to the constituents of basidiomycetes because of their possible medical usage (1). Antiviral, antibiotic, antiinflammatory, hypoglycemic, hypocholesterolemic, and hypotensive properties were ascribed to ingredients of such mushrooms (2). Sphingolipids, e.g., ceramides, cerebrosides, sphingomyelin, and gangliosides, are important building blocks of the plasma membrane of eukaryotic cells. Their function is to anchor lipid-bound carbohydrates to cell surfaces and to create an epidermal water permeability barrier, as well as to participate in antigen–antibody reactions and transmission of biological information (3). Some have shown antiulcerogenic, antihepatotoxic, antitumor, and immunostimulatory activities (4–6). In contrast to lower fungi, the glycolipids of higher fungi have been investigated less (7). The primary glycosphingolipids and ceramides isolated from higher fungi having an unsaturated sphingosine base (4,8-sphingadiene), saturated phytosphingosine, and 4-hydroxysphingosine have been investigated (8–11). *Cortinarius* (Cortinariaceae) is one of the largest genera, comprising hundreds of species and being widely distributed throughout the world (12). From the fruiting bodies of *Cortinarius* species, especially toadstools in Europe and Australia, a large number of toxins, pigments, cyclic polypeptides, bipyridyl compounds, anthraquinones, and triterpenoids have been isolated (13–16). In our continuing research on bioactive secondary metabolites of higher fungi in Yunnan, we investigated the glycosphingolipid and ceramide composition of fresh fruiting bodies of the mushroom *Cortinarius umidicola* Kauffm., which were grown under pine trees in the mountainous region near Kunming (Yunnan, P.R. China). Our investigation resulted in the isolation of a new glycosphingolipid containing a rare (4*E*,8*E*)-9-methyl-4,8-sphingadienine sphingoid base and three ceramides derived from an unusual 6-methyl-4-hydroxysphingosine, together with cerebrosides B and D (9,17,18). This report describes their isolation and structural elucidation.

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Abbreviations: CC, column chromatography; DEPT, distortionless enhancement by polarization transfer; HMBC, heteronuclear multiple bond correlation; LCB, long-chain base.

EXPERIMENTAL PROCEDURES

Chromatographic and instrumental methods. The m.p. were obtained on an XRC-1 apparatus (Sichuan University, Sichuan, P.R. China). Optical rotations were taken on a Horiba SEPA-300 automatic polarimeter (Horiba, Tokyo, Japan). The NMR spectra (^{1}H, ^{13}C, and 2-D NMR) were acquired on DRX-500 NMR instruments (Karlsruhe, Germany) at 500 MHz for ^{1}H and 125 MHz for ^{13}C NMR; tetramethylsilane was used as an internal standard and coupling constants were represented in hertz. Mass spectra were measured with a VG Autospec 3000 mass spectrometer (VG, Manchester, England). IR spectra were obtained in KBr pellets on a Bio-Rad FTS-135 IR spectrophotometer (Bio-Rad, Richmond, CA). GC–MS was performed with a Finnigan 4510 gas chromatograph–mass spectrometer (San Jose, CA) in the EI mode (ionizing potential, 70 eV) and a capillary column (30 m × 0.25 mm) packed with 5% phenyl/95% methylsilicone on 5% phenyl-dimethylsilicone (HP-5; Hewlett-Packard, Palo Alto, CA). Helium was used as carrier gas, column temperature was varied from 120 to 240°C with a slope of 5°C/min, and quantitative determination was based on the area of the GLC peaks.

Materials. Column chromatography (CC) was performed on silica gel (200–300 mesh; Qingdao Marine Chemical Ltd., Qingdao, P.R. China). Reversed-phase CC was carried out on a LiChroprep® RP-18 column (40–63 μm; Merck, Darmstadt, Germany). All solvents were distilled before use.

Fresh fruiting bodies of *C. umidicola* were collected in Kunming (Yunnan, P.R. China) in August 2002 and identified by X.H. Wang at the Kunming Institute of Botany, Chinese Academy of Sciences, where a voucher specimen (no. HKAS 41152) was deposited.

Extraction and isolation. The fresh fruiting bodies (25 kg) of *C. umidicola* were soaked in 95% ethanol at room tempera-

ture to destroy the enzymes. After filtration, the fruiting bodies were dried by air and finely crushed. The dried powders were extracted exhaustively with methanol (5 L × 3), then with chloroform/methanol (1:1, vol/vol; 5 L × 4) at room temperature. After concentrated to dryness *in vacuo*, the combined extracts were partitioned between water and ethyl acetate. The organic layer was concentrated under reduced pressure, affording a dark brown gum (120 g); the gum was dissolved in chloroform and then placed on a silica gel column eluted with petroleum ether containing increasing amounts of acetone. Twelve fractions were collected. Among them, the last fraction, eluted with acetone, was further chromatographed on a reversed-phase chromatography column (RP-18) eluting with MeOH/H_2O (9:1, vol/vol) to provide compound **1** (70 mg) and a mixture of **2** and **3** (118 mg). The fraction eluted with petroleum ether/acetone (1:10–1:9, vol/vol) was further purified on an RP-18 column eluting with MeOH/H_2O (95:5, vol/vol) to afford compounds **4**–**6** (mixture, 16.5 mg).

*(2*S*,3*R*,4*E*,8*E*)-1-(β-*D*-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxyhexadecanoyl]amino-9-methyl-4,8-heptadecadiene **1***. White amorphous powder (methanol), $[\alpha]_D^{20}$ +3.5° (*c* 0.15, $CHCl_3$). IR (KBr) ν_{max}: 3400 (OH), 2924, 2853, 2365, 2339, 1652 (HNC=O), 1544 (NH), 1077, 1035, 963, 721 (methylenes) cm^{-1}; negative FABMS *m/z* (relative intensity, %): 713 $[M]^-$ (100), 550 $[M - H - 162]^-$ (42), 488 (10), 456 $[M - 2H - COCH(OH)C_{14}H_{29}]^-$ (10), 443 $[M - NHCOCH(OH)C_{14}H_{29}]^-$ (7), 424 (13); ^1H and ^{13}C NMR spectra data are given in Table 1.

*Methanolysis of **1***. Compound **1** (12.8 mg) was refluxed with 3 mL 5% hydrochloride–methanol at 60°C for 6 h. Ten milliliters of cold water was poured into the reaction mixture, which was extracted three times with *n*-hexane (5 mL × 3). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue of the hexane phase was analyzed directly by GC–MS.

*Methyl (2*R*)-2-hydroxyhexanoate (**1a**)*. White solid, $[\alpha]_D^{20}$ –5.7° (*c* 0.18, $CHCl_3$). The retention time (t_R) of **1a** was 18.9 min. EI-MS (70 eV) *m/z* (relative intensity, %): 286 $[M]^+$ (2), 227 $[M - COOCH_3]^+$ (50), 125 (20), 111 (60), 103 (10), 97 (90), 90 (50), 83 (100), 69 (70), 57 (80), 55 (70), 43 (60).

*2-Acetoamino-1,3-diacetoxy-9-methyl-4,8-heptadecanediene (**1b**)*. The aqueous methanolic layer was neutralized with saturated Na_2CO_3, concentrated to dryness, and extracted with ether. The ether phase was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to afford a long-chain base (LCB), which was reacted with acetic anhydride/pyridine (1:1, vol/vol) at room temperature in 0.5 mL acetone overnight. The reaction mixture was diluted with water and then ex-

TABLE 1
^1H and ^{13}C NMR Spectral Data for Compound 1 in Pyridine-d_5[a]

Atom no.	^{13}C (multiplicity)	^1H (multiplicity, *J* in Hz)	^1H-^1H COSY	HMBC (selected)
Long-chain base				
1a	70.2 (CH_2)	4.68 (*m*)	H-1b, -2	H-2, -3, NH, 1″
1b		4.77 (*m*)	H-1a, -2	H-1″
2	54.6 (CH)	4.78 (*m*)	NH, H-1a, -1b, H-3	H-1, -3, -4
3	72.4 (CH)	4.75 (*m*)	H-2, -4	H-1, -2, -4
4	132.4 (CH)	5.94 (*dt*, 15.3)	H-3, -5	H-3, 6
5	132.0 (CH)	5.97 (*dd*, 15.3, 6.8)	H-4, -6	H-3, 7
6	33.1 (CH_2)	2.14 (*m*)	H-5, -7	H-4, 5
7	32.2 (CH_2)	2.14 (*m*)	H-6, -8	H-5, 8
8	124.2 (CH)	5.25 (*m*)	H-7	H-6, -7, -10, CH_3-18
9	135.6 (C)			H-7, CH_3-18
10	40.1 (CH_2)	2.00 (*m*)		H-8, CH_3-18
11–16	30.0–30.1 (CH_2)	1.25–1.41 (*br s*)		
17	14.1 (CH_3)	0.86 (*t*, 6.7)		
18	16.2 (CH_2)	1.61 (*s*)		H-8, -9, -10
NH		8.33 (*d*, 8.7)	H-2	H-1′
N-Acyl moiety				
1′	175.5 (C)			H-2, -2′, -3′, NH
2′	72.5 (CH)	4.57 (*dd*, 7.7)	H-3′	HN, H-3′, -4′
3′	35.7 (CH_2)	2.00 (*m*), 2.14 (*m*)	H-2′, -4′	H-2′, -4′
4′–15′	30.0–30.1 (CH_2)	1.25–1.31 (*br s*)		
16′-CH_3	14.4 (CH_3)	0.86 (*t*, 6.7)		
Sugar moiety				
1″	105.7 (CH)	4.88 (*d*, 7.9)	H-2″	H-1a, 1b, -2″
2″	75.1 (CH)	4.01 (*dd*, 9.0, 7.9)	H-1″, -3″	H-1″, -3″, -4″
3″	78.5 (CH)	4.20 (*t*, 9.0)	H-2″, -4″	H-1″, -2″
4″	71.5 (CH)	4.18 (*t*, 9.0)	H-5″, -3″	H-3″, -6″
5″	78.6 (CH)	3.88 (*ddd*, 9.0, 5.7, 2.5)	H-6″, -4″	H-3″, -4″, -6″
6″	62.7 (CH_2)	4.33 (*dd*, 11.5, 2.5)	H-5″	H-4″
		4.48 (*dd*, 11.5, 5.7)		

[a]HMBC, heteronuclear multiple bond correlation.

tracted with ethyl acetate. The residue of the organic phase was subjected to silica gel CC using petroleum ether/ethyl acetate (9:1, vol/vol) as eluent to produce a triacetate of the LCB (**1b**, 2.8 mg). EI-MS (70 eV) *m/z* (relative intensity, %): 423 $[M]^+$ (1), 381 $[M - Ac]^+$ (3), 363 $[M - CH_3COOH]^+$ (3), 303 $[M - 2CH_3COOH]^+$ (13), 144 $[AcOCH_2CHNHAc]^+$ (32), 102 (51), 84 (90), 55 (100).

1-O-*Methyl-D-glucopyranoside (**1c**)*. The remaining water layer was evaporated *in vacuo*. The residue was then chromatographed on the RP-18 column using methanol/water (1:9, vol/vol) to afford methyl glucopyranoside. $[\alpha]_D^{20}$ +76.0°(*c* 0.17, MeOH), negative FABMS *m/z* (relative intensity, %): 193 $[M - H]^+$ (100).

*(2*S*,3*R*,4*E*,8*E*)-1*-O-*(β-D-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxypalmitoyl]amino-9-methyl-4,8-hexadecadiene (= cerebroside B, **2**) and (2*S*,3*R*,4*E*,8*E*)-1*-O-*(β-D-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxyoctadecanoyl]amino-9-methyl-4,8-hexadecadiene (= cerebroside D, **3**)*. White amorphous powder (methanol), $[\alpha]_D^{20}$ +4.8° (*c* 0.18, $CHCl_3$). IR (KBr) ν_{max}: 3400 (OH), 2960, 1650 (HNC=O), 1540 (NH), 1100–1000, 720 cm^{-1}; negative FABMS *m/z* (relative intensity, %):755 $[M_1]^-$ (100), 726 $[M_2 - 1]^-$ (22), 592 $[M - H - 162]^-$ (38), 564 $[M - H - 162]^-$ (9); the NMR spectra of the mixture were consistent with those reported in the literature (3,11,12).

*(2*S*,3*S*,4*R*,2′*R*)-2-(2′-hydroxytetracosanoylamino)heptadecane-6-methyl-1,3,4-triol (**4**), (2*S*,3*S*,4*R*,2′*R*)-2-(2′-hydroxytricosanoylamino)heptadecane-6-methyl-1,3,4-triol (**5**) and (2*S*,3*S*,4*R*,2′*R*)-2-(2′-hydroxydocosanoylamino)heptadecane-6-methyl-1,3,4-triol (**6**)*. White amorphous powder (methanol), $[\alpha]_D^{20}$ −10.0° (*c* 0.18, $CHCl_3$). IR (KBr) ν_{max}: 3402 (OH), 1644, 1635, 1628, 1127, 1071, 1042, 721 cm^{-1}; negative FABMS *m/z* (relative intensity): 683 (83), 669 (75), and 655 (100), $[M]^-$ series. ^1H and ^{13}C NMR spectra data are given in Table 2.

*Methanolysis of **4–6***. The mixture of **4**, **5**, and **6** (11.3 mg) was refluxed with 3 mL 5% HCl–methanol at 60°C for 6 h. Ten milliliters of cold water was poured into the reaction mixture, which was extracted with *n*-hexane (5 mL × 3). The combined organic layer was dried over anhydrous Na_2SO_4. Concentration of the hexane phase yielded the mixture of FAME (3.4 mg), white solid, $[\alpha]_D^{20}$ −3.2° (*c* 0.09, C_5H_5N), which was analyzed by GC–MS. Result of the GC–MS analysis revealed the presence of three components. The most abundant one (49.2%) was characterized as methyl (2*R*)-2-hydroxytetracosanoate (**6a**), which displayed major ion peaks at *m/z* 398 $[M]^+$ and 339 $[M - COOCH_3]^+$; its t_R was 42.1 min. The second-most abundant one (47.8%) was identified as methyl (2*R*)-2-hydroxydocosanoate (**4a**), which displayed major ion peaks at *m/z* 370 $[M]^+$ and 311 $[M - COOCH_3]^+$; its t_R was 35.3 min. The minor one (3%) was identified as methyl (2*R*)-2-hydroxytricosanoate (**5a**). It displayed major ion peaks at *m/z* 398 $[M]^+$ and 339 $[M - COOCH_3]^+$, and its t_R was 38.7 min.

*2-Acetoamino-6-methyl-1,3,4-triacetoxyheptadecane (**4b**)*. The aqueous layer was neutralized with saturated $NaCO_3$, concentrated under reduced pressure to dryness, and extracted with ether. The ether phase was dried over anhydrous Na_2SO_4 and evaporated to afford LCB, which was reacted with acetic anhydride/pyridine (1:1, vol/vol) at room temperature overnight. The reaction mixture was diluted with water and then extracted with ethyl acetate. The residue of the organic phase was subjected to silica gel CC using petroleum ether/ethyl acetate (9:1, vol/vol) as eluent to produce a peracetate of the LCB (**4b**, 1.8 mg). EI-MS (70 eV) *m/z* (relative intensity, %): 485 $[M]^+$ (9), 425 $[M - CH_3COOH]^+$ (1), 365 $[M - 2CH_3COOH]^+$ (3), 305 $[M - 3CH_3COOH]^+$ (13), 245 $[M - 4CH_3COOH]^+$ (7).

TABLE 2
^1H and ^{13}C NMR Spectral Data for Compounds 4–6 in Pyridine-d_5[a]

Atom no.	^{13}C (multiplicity)	^1H (multiplicity, *J* in Hz)	^1H-^1H COSY (selected)	HMBC (selected)
Long-chain base				
1	62.1 (CH_2)	4.52 (*dd*, 10.6, 4.5) 4.43 (*dd*, 10.6, 5.2)	H-2	H-2, -3
2	53.0 (CH)	5.12 (*m*)	NH, H-2, -3	NH, H-1′, -1, -3
3	76.8 (CH)	4.40 (*dd*, 6.5, 4.0)	H-2, -4	H-1, -2, -4, -5
4	73.1 (CH)	4.35 (*m*)	H-3, -5	H-2, -3, -5, -6
5	35.8 (CH_2)	1.93 (*m*)	H-4, -6	H-3, -4, -6, CH_3-18
6	39.1 (CH)	1.69 (*m*)	H-7, H-3–19	H-4, -5, CH_3-18
7–16	29.6–32.2 (CH_2)	1.25–1.41 (*br, s*)		
17	14.3 (CH_3)	0.86 (*t*, 6.7)		
CH_3-18	11.2 (CH_3)	0.9 (*d*, 7.3)		
N-Acyl moiety				
1′	175.3 (C)			
2′	72.5 (CH)	4.63 (*dd*, 7.6, 4.0)	H-3′	HN, H-1′, -3′, -4′
3′	35.8 (CH_2)	2.24, 2.04 (*m*)	H-2′, -4′	H-2′, -4′
4′	25.9 (CH_2)	1.76 (*m*)	H-3′, -5′	H-2′, -3′
5′–21′*	29.6–32.2 (CH_2)	1.25–1.41 (*br, s*)		
22′**	14.3 (CH_3)	0.87 (*t*, 6.7)		
NH		8.58 (*d*, 8.8)	H-2	H-1′

[a]*21′ for **4,** 22′ for **5**, 23′ for **6**; ** 22′ for **4,** 23′ for **5**, 24′ for **6.** For abbreviation see Table 1.

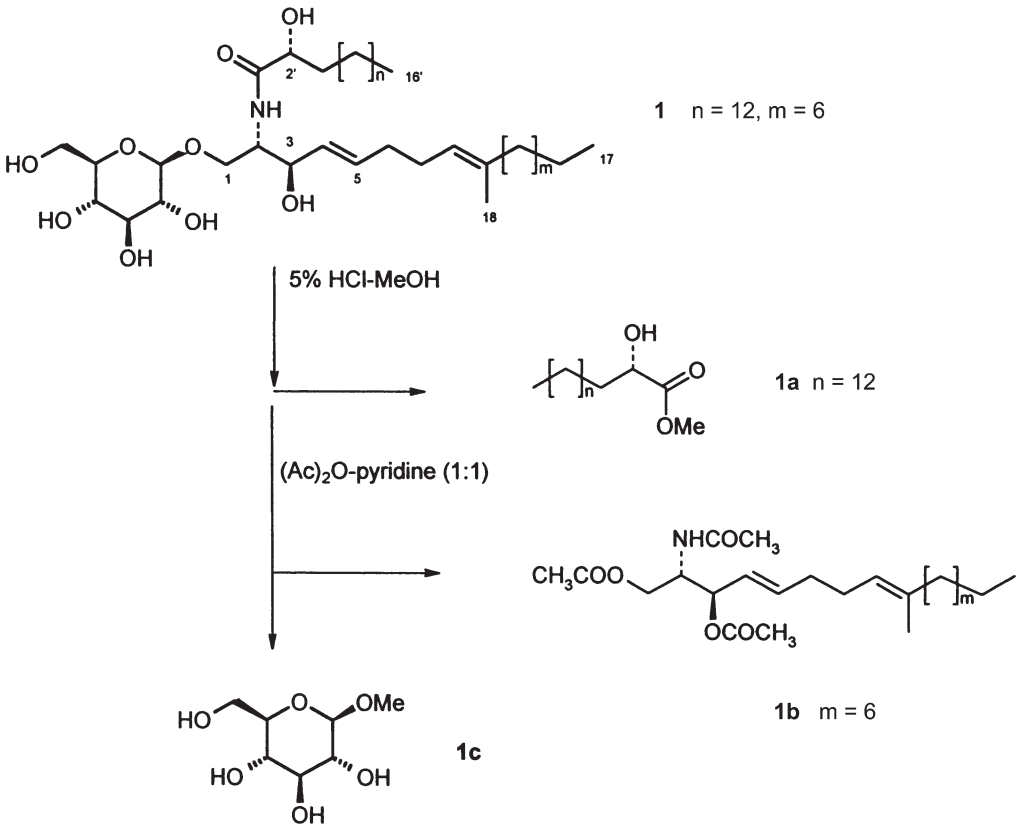
RESULTS AND DISCUSSION

The $CHCl_3$-soluble fraction of MeOH and $CHCl_3$/MeOH extract from the fruiting bodies of *C. umidicola* was subjected to repeated CC to yield compounds **1–6**. The structures of new compounds **1**, and **4–6** were elucidated as follows.

Compound **1** was isolated as an optically active amorphous solid ($[\alpha]_D^{20}$ +3.5°; *c* 0.15, $CHCl_3$). The IR spectrum displayed absorption bands of hydroxyl groups (3400 cm^{-1}, br.) and amide carbonyl (1652 cm^{-1}). Negative FABMS exhibited a molecular ion peak at *m/z* 713 ($[M]^-$) and the characteristic ion peak at *m/z* 550 ($[M - H - 162]^-$) with the loss of a hexose residue from the quasi-molecular ion. Its ^1H NMR spectrum presented an amide NH doublet at δ 8.33 (1H, *d*, *J* = 8.7 Hz; exchangeable with D_2O). The ^{13}C NMR (DEPT) spectrum of **1** showed the signals at δ 175.5 (C-1′) of a carbonyl carbon and δ 54.6 (C-2) of a methine carbon; both carbons were connected to amide nitrogen. The correlation peaks between NH and H-2 (δ 4.78, *m*) in ^1H-^1H COSY, and cross-peaks between NH and C-1′, H-2 and C-1′ in heteronuclear multiple bond correlation (HMBC) were observed. The existence of a methyl side chain and two long aliphatic chains in the molecule was provided by the presence of signals of three methyl groups [δ_C 14.1 (C-17), 14.4 (C-16′), 16.2 (CH_3-18); δ_H 0.86 (6H, *t*, CH_3-17, 16′), 1.61 (3H, *s*, CH_3-18)] and a complex region with overlapping signals characteristic of methylenes of long alkyl chain in the NMR spectra and with absorptions at 2924, 2853, and 721 cm^{-1} in the IR spectrum. All these analyses and comparisons of spectroscopic data with those of cerebrosides B and D strongly suggested the glycosphingolipid nature of **1** with the molecular formula $C_{40}H_{75}NO_9$ (11,12). From the reaction mixture from acidic methanolysis (Scheme 1), three fractions containing long-chain methyl esters (**1a**), triacetates of the sphingoid base (**1b**), and methyl glycosides (**1c**), respectively, were separated.

The sugar part of the molecular comprised a hexose unit that was attached at the C-1 position of the LCB, as indicated by the cross-peak between H-1″ (δ 4.88, 1H, *d*, *J* = 7.9 Hz) and C-1 (70.2) exhibited in the HMBC spectrum. The ^{13}C NMR spectrum displayed the signal of an anomeric carbon atom at δ 105.7, indicative of a β configuration of C-1″. The high-field chemical shift of proton H-5″ (δ 3.88, 1H, *ddd*, *J* = 9.0, 5.7, 2.5 Hz) identified the pyranose nature of the sugar moiety. The large coupling constants between H-2″ and H-3″, H-3″ and H-4″, and H-4″ and H-5″ (see Table 1), which evidenced the axial stereochemistry of all these protons, demonstrated the hexose as a glucose. The optical rotation of methyl glucoside ($[\alpha]_D^{20}$ +76.0°), separated from the methanolysis reaction mixture, was close to that of an authentic sample ($[\alpha]_D^{25}$ +77.3°), defining glucose as the D-isomer (19).

The cross-peak between H-2′ and H-3′ exhibited in ^1H-^1H COSY and correlations between NH and C-2′, H-3′, and the carbonyl carbon (C-1′) shown in the HMBC spectrum sug-



SCHEME 1

gested that the methylene of the fatty acyl chain is attached to an amide carbonyl only through an oxygenated methine carbon. Long-chain methyl α-hydroxy ester, one of the products of chemical degradation of **1**, was identified as methyl 2-hydroxyhexadecanoate (**1a**) by GC–MS analysis. It clearly revealed the length of the FA moiety of **1**. Moreover, the fragment ion at *m/z* 456 $[M - 2H - COCH(OH)C_{14}H_{29}]^+$, corresponding to the loss of the fatty acyl chain, and the ion at *m/z* 443 $[M - NHCOCH(OH)C_{14}H_{29}]^+$, corresponding to the loss of amide NH, along with the fatty acyl moiety displayed in negative FABMS, supported the GC–MS results. The optical rotation ($[\alpha]_D^{20}$ –5.7°; *c* 0.18, $CHCl_3$), which was very close to those of the methyl esters of 2-(*R*)-hydroxy FA reported earlier (20), identified the FAME as an *R*-isomer.

Apart from moieties of the sugar residue, FA, and the amide carbonyl group, the ^{13}C NMR spectrum exhibited signals at δ 72.4 (C-3) of oxygenated methine carbons and δ 70.2 (C-1) of an oxygenated methylene carbon, indicating the presence of 1,3-dihydroxy-sphinganines in LCB. This was also confirmed by the related correlation peaks shown in ^1H-^1H COSY and the HMBC spectrum. In addition, NMR showed the presence of two double bonds at δ_H 5.94 (1H, *dt*, *J* = 15.3 Hz, H-4), 5.97 (1H, *dd*, *J* = 15.3, 6.8 Hz, H-5), and 5.25 (1H, *m*, H-8) and δ_C 132.4 (C-4), 132.0 (C-5), 135.6 (C-8), and 124.2 (C-9). The correlation peaks between H-3 and H-4, H-4 and H-5, H-5 and H-6, and H-7 and H-8 in ^1H-^1H COSY, and the cross-peaks between H-2 and C-4, H-6 and C-4, H-3 and C-5, H-7 and C-5, H-8 and CH_3-18, H-10 and C-8, and H-7 and C-9 in the HMBC spectrum established the position of two double bonds at C-4 and C-8. It also indicated that C-9 was attached with a methyl side chain. The 4,5-alkene bond was found to be *trans*, as evidenced by the large vicinal coupling constants (*J* = 15.3 Hz). The *trans* geometry of 8,9 was established by the chemical shift of methyl (δ 16.2) attached to C-9 since the chemical shift of methyl carbons attached to a *trans* double bond appears at δ 15.4 ppm, whereas those of *cis* appear at 22.7 ppm (21). Thus, the sphingoid base of **1** was established to be (4*E*,8*E*)-9-methyl-4,8-sphingadienine (22). The EI-MS spectrum of **1b** exhibited characteristic ion peaks at *m/z* 423 $[M]^+$, 381 $[M - Ac]^+$, 363 $[M - CH_3COOH]^+$, 305 $[M - 2CH_3COOH]^+$, and 144 $[AcOCH_2CHNHAc + H]^+$, establishing **1b** as 2-acetoamino-6-methyl-1,3,4-triacetoxyheptadecane. The rotation of **1** ($[\alpha]_D^{20}$ +3.5°) is in accordance with those of natural (23) and synthetic analogs (9,24). This suggests that the absolute configuration of **1** at chiral centers C-2, -3, -2′ were *S, R*, and *R,* respectively, as for those of the analogs. Based on all the above facts, compound **1** was established as (2*S*,3*R*,4*E*,8*E*)-1-(β-D-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxyhexadecanoyl]amino-9-methyl-4,8-heptadecadiene.

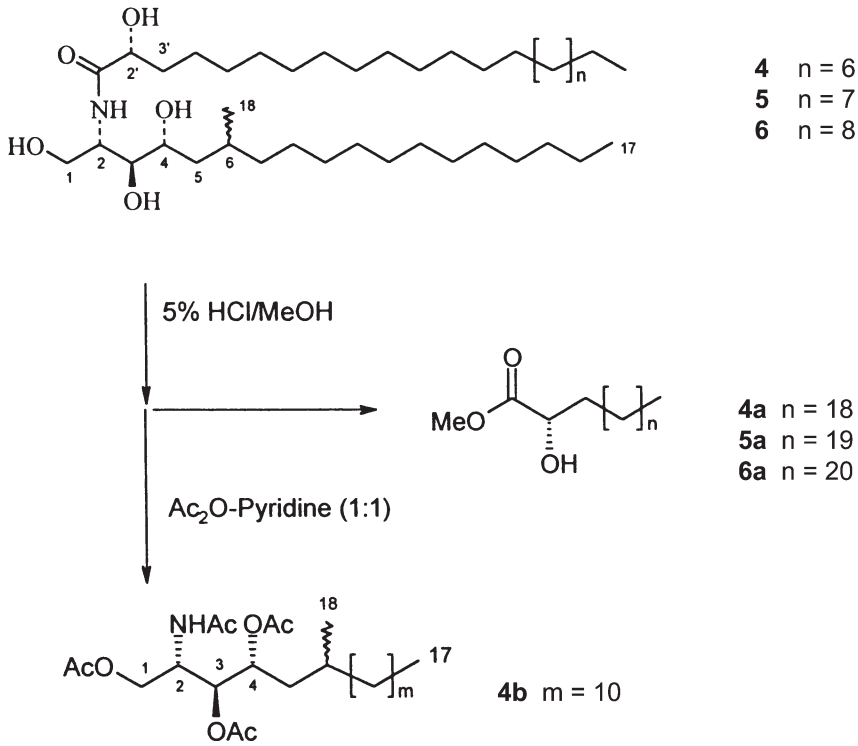
Several pseudomolecular ion peaks at *m/z* 655, 669, and 683 displayed in the negative ion fast atom bombardment mass spectra of compounds **4–6** proved to be a mixture of three congeners, in accordance with the molecular formula $C_{40}H_{81}NO_5 + nCH_2$ (*n* = 0–2). Its IR spectrum exhibited strong absorption bands for hydroxyl (3402 cm^{-1}, *br*), amide carbonyls (1644, 1635, 1628 cm^{-1}), and a long aliphatic chain (721 cm^{-1}). Resonance for two terminal methyl groups at δ 0.86 (6H, *t, J* = 6.7 Hz) and a branched methyl group at δ 0.90 (3H, *d*, *J* = 7.3 Hz, CH_3-18), together with the overlapped signals of methylenes at δ 1.24–1.41, indicated the existence of two long aliphatic chains and a methyl side chain. In addition, the ^1H NMR spectrum also presented a characteristic amide NH doublet at δ 8.58 (1H, *d*, *J* = 8.8 Hz; exchangeable with D_2O) and the ^{13}C NMR (DEPT) spectrum of **1** showed the presence of an amide functionality at δ 175.3 (C-1′) and 53.0 (C-2). This evidence led to the conclusion that compounds **4–6** were ceramides.

To determine the length of the FA and LCB of **4–6**, the mixture was treated as shown in Scheme 2 to afford long-chain methyl esters and peracetyl LCB. The long-chain methyl esters were identified as containing methyl 2-hydroxydocosanoate (**4a**), methyl 2-hydroxytricosanoate (**5a**), and methyl 2-hydroxytetracosanoate (**6a**) by the GC–MS analysis. The optical rotation ($[\alpha]_D^{20}$ –3.2°; *c* 0.09, C_5H_5N) of the mixture **4a–6a** is very close ($[\alpha]_D^{25}$ –3.0°; *c* 0.02, CHC_{13}) to that of the mixture of homologs reported earlier (25) with *R*-configuration at C-2, indicative that the stereochemistry of C-2 in **4a–6a** is *R*.

The sphingoid base was deduced to be 4-hydroxyl-6-methyl-sphinganine on the basis of ^1H-^1H COSY (see Table 2) and the HMBC spectrum, which showed correlation peaks between NH and C-1, NH and C-3, and the CH_3-18 group and C-6. EI–MS of peracetyl LCB displayed the molecular ion at *m/z* 485 $[M]^+$ and prominent fragment ions at *m/z* 425 $[M - CH_3COOH]^+$, 365 $[M - 2CH_3COOH]^+$, 305 $[M - 3CH_3COOH]^+$, 245 $[M - 4CH_3COOH]^+$, suggesting that the sphingosine part of **4–6** is 2-amino-1,3,4-trihydroxyl-6-methyl-heptadecane (**4b**).

In comparing the chemical shift of C-1, C-2, C-3, and C-4 with those of phytosphingosine-type LCB possessing (2*S*,3*S*,4*R*)-configurations (10,26,27), the relative configurations of C-2, C-3, and C-4 were predicted to be *S*, *R*, and *R*, respectively. The stereochemistry of position C-6 has not yet been determined. Based on the above facts, compounds **4–6** were established as (2*S*,3*S*,4*R*,2′*R*)-2-(2′-hydroxydocosanoylamino)heptadecane-6-methyl-1,3,4-triol, (2*S*,3*S*,4*R*,2′*R*)-2-(2′-hydroxytricosanoylamino)heptadecane-6-methyl-1,3,4-triol, and (2*S*,3*S*,4*R*,2′*R*)-(2′-hydroxytetracosanoylamino)-heptadecane-6-methyl-1,3,4-triol, respectively.

In nature, the most widely occurring sphingoid base is D-*erythro*-4*E*-sphingenine, whereas 6- and 9-branched methyls in the hydrocarbon chain are minor sphingoid bases. The present investigation demonstrated the presence in *C. umidicola* of a previously unrecognized sphingolipid and three ceramides, consisting of 9-methyl-4,8-sphingadienine in an amide linkage with a hydroxy FA; 9-methyl-4,8-sphingadienine in a β-glycosidic bond with glucose; and 6-methyl-4-hydroxysphingosine in amide linkage with different hydroxy FA, respectively. The natural occurrence of similar molecules has been found in a unique marine protist *Thraustochytrium globosum* (28), an imperfect fungus *Pachbasium* sp. (18), a



SCHEME 2

pathogenic fungus *Fusicoccum amygdali* (29), a sea anemone *Metridium senile* (30), and a basidiomycete *Polyporus ellisii* (8) and is presumed to be a characteristic component of lower organisms.

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Determination of the Phospholipase Activity of Patatin by a Continuous Spectrophotometric Assay

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ABSTRACT: Patatin is a family of glycoproteins that accounts for 30–40% of the total soluble protein in potato (*Solanum tuberosum* L.) tubers. This protein has been reported to serve as a storage protein and also to exhibit lipid phospholipase activity. This paper describes a simple continuous spectrophotometric method for assaying patatin phospholipase activity. The procedure is based on a coupled enzymatic assay using [1,2-dilinoleoyl]PC as the phospholipase substrate and lipoxygenase as the coupling enzyme. In the procedure developed in this work, lipoxygenase oxidizes the linoleic acid released by the phospholipase activity of patatin. This activity can then be followed spectrophotometrically by recording the increase in absorbance at 234 nm that results from the formation of the corresponding hydroperoxide from linoleic acid by the action of lipoxygenase. The optimal assay concentrations of patatin and lipoxygenase were established. Phospholipase activity varied with pH, reaching its optimal value at pH 9.5. Scans of the deoxycholate concentration pointed to an optimal detergent concentration of 3 mM. Phospholipid hydrolysis followed classical Michaelis–Menten kinetics (V_m = 9.8 × 10^{-3} μmol/min·μg protein, K_m = 7.8 μM, V_m/K_m = 1.3 min^{-1} · μg protein). This method proved to be specific since there was no activity in the absence of patatin. It also had the advantages of a short analysis time and the use of commercially nonradiolabeled and inexpensive substrates, which are, furthermore, natural substrates of phospholipase.

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Patatin is the trivial name given to a group of immunologically related glycoproteins with a M.W. of 40 kDa that have been found in practically all potato (*Solanum tuberosum* L.) cultivars thus far examined. Patatin accounts for up to 40% of the total soluble protein present in tubers (1,2), and the high levels accumulated suggest that it functions as a storage protein (3). As a major component of potato tuber protein, the study of patatin and its contribution to nutritional quality is important.

Several reports have shown that the storage proteins of plants have a function other than serving merely as a protein reserve. For example, several seed proteins act as proteinase inhibitors or have antifungal or antibacterial activity (4). Patatin itself has been reported to show esterase activity with a large number of lipid substrates. By using a baculovirus system to express protein from the patatin cDNA, it has been shown that the patatin coded by this DNA shows lipid acyl hydrolase activity (5). The enzyme is active with phospholipids, sulfolipids, MAG, and *p*-nitrophenyl esters; moderately active with galactolipids; but apparently inactive with DAG and TAG (6). The activity of patatin is therefore equivalent to a combination of many enzyme activities, such as those of phospholipase, glycolipase, sulfolipase, MAG lipase, and esterase (7).

Strickland *et al.* (7) examined the phospholipase activity of patatin. Product analysis revealed that patatin hydrolyzed both acyl groups from PC to produce FFA and glycerophosphorylcholine, a mechanism consistent with phospholipase B activity. This term was originally applied to an enzyme that removed both acyl groups from a diacyl phospholipid, indicating that this activity could be ascribed to a combination of phospholipase A_1 and A_2 (8,9). Other authors have recently investigated patatin phospholipase activity. Although the amino-terminal amino acid sequence showed homology to phospholipase A_2, the protein exhibited both activities (10–12).

The physiological function of patatin in the tuber is not clear, although it has been proposed that patatin may act to mobilize or degrade lipids during tuber development and sprouting. It also has been suggested to play a role in wound response (13), to afford defense by mediating phytoalexin production (14), or to be involved in signal transduction, the latter by virtue of its phospholipase A_2-like activity (15). Currently, no direct evidence to support any of these hypotheses is available.

The kinetic characteristics of the esterase activity of patatin have recently been studied by means of a mixed-micellar assay using the nonionic detergent octaethylene glycol monododecyl ether ($C_{12}E_8$) (15), which is a chemically pure detergent that has been well characterized physicochemically (16). The lipid acyl hydrolase activity of patatin also has been analyzed in reverse micelles made of Aerosol OT in isooctane, a system that allows study of the enzyme in conditions similar to that prevailing *in vivo* (17). However, in the literature, information on the kinetic characteristics of the phospholipase activity of patatin is limited, probably due to the poor solubility of its substrates in an aqueous medium and, furthermore, to the methods used to determine the activity.

In general, phospholipase A_2 is detected by determining the catalytic activity of the enzyme or from the concentration of the enzyme protein, as determined by immunoassay. The most routinely used methods for determining phospholipase catalytic activity are radiometric assays, with a substrate of synthetic phospholipids (e.g., PC or PE) containing a ^{14}C- or ^{3}H-labeled

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FA in the *sn*-2-position. The FA released are separated from the unreacted substrate and quantified by liquid scintillation counting (18–22). Continuous spectrophotometric assays for the determination of lipases, lysophospholipases, and phospholipases by using thioester substrate analogs also have been described (23,24). Other authors have described a fluorescence displacement assay, which involves the displacement of a fluorescent FA probe from albumin or rat liver FA-binding protein by the decanoic acid released as a result of the phospholipase A_2–catalyzed hydrolysis of didecanoyl-PC (25,26). Egg yolk emulsions containing phospholipids are also used as substrates for measuring phospholipase A_2 activity by the pHStat method (27,28), although egg yolk lipoproteins were found to be effective substrates for all the lipases tested. Consequently, the egg yolk assay cannot be considered a specific phospholipase A_2 assay (29). Phospholipase A_2 also can be detected in serum by determining the concentration of the enzyme protein using immunoassays, radioimmunoassays, ELISA, or a time-resolved fluoroimmunoassay, all of which are based on the use of polyclonal or monoclonal antibodies to purified phospholipase (30).

In particular, the phospholipase activity of patatin has usually been determined by radiometric methods (7,11). Other methods involve incubation of the substrate (PC) with patatin and further extraction of the reaction products with an organic solvent (5). The nonesterified FA released by the action of patatin are then determined by the rhodamine method (31).

Determination of the phospholipase activity of patatin therefore usually involves expensive radioactive or chromogenic substrates. However, in this paper we report a simple coupled spectrophotometric assay using [1,2-dilinoleoyl]PC as the patatin substrate and lipoxygenase as the coupling enzyme.

Lipoxygenase (linoleate/oxygen oxidoreductase, EC 1.13.11.12) catalyzes the addition of molecular oxygen to FA containing at least one (*Z,Z*)-pentadiene system to give the corresponding hydroperoxides (32,33). In the procedure developed in this work, lipoxygenase oxidizes the linoleic acid released by the phospholipase activity of patatin. This activity can then be followed spectrophotometrically by recording the increase in absorbance at 234 nm that results from the formation of the corresponding hydroperoxide from linoleic acid by the action of lipoxygenase (Fig. 1).

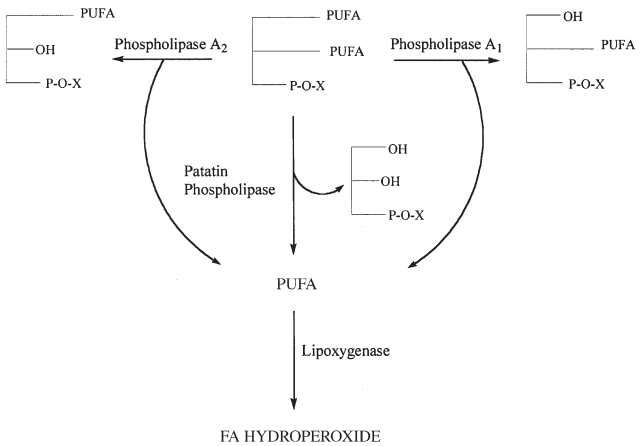

FIG. 1. Procedure developed to determine phospholipase activity. Lipoxygenase oxidizes the PUFA released by the action of phospholipase A_1, A_2, or the phospholipase activity of patatin ("phospholipase B," a combination of phospholipase A_1 and A_2). The formation of the conjugated diene hydroperoxide by lipoxygenase was followed spectrophotometrically at 234 nm. X, choline.

This method provides a continuous record of phospholipid hydrolysis and has proved to be specific, since no activity was recorded in the absence of patatin. In addition, this assay possesses the advantages of a short analysis time, straightforward measurement, and reproducibility. It also uses a substrate that is commercially available, nonradiolabelled, and inexpensive. Furthermore, this substrate is a natural substrate (i.e., it is not a phospholipid analog containing a thioester bond instead of the *sn*-2 ester) of the kind which, although not commercial products, have been used as substrates to measure phospholipase A_2 activity (34,35).

EXPERIMENTAL PROCEDURES

Plant materials. The potatoes used, *S. tuberosum* L. cv Spunta, were obtained from a local market.

Reagents. BSA and Bradford reagent were obtained from Bio-Rad (Madrid, Spain). [1,2-dilinoleoyl]PC and lipoxygenase from soybeans (Lipoxidase Type V, EC 1.13.11.12; 1,000,000 U/mg) were purchased from Sigma (Madrid, Spain). Phospholipase A_2 from hog pancreas (EC 3.1.1.4; 500 U/mg) was obtained from Fluka (Madrid, Spain). All other chemicals used were of analytical grade.

Enzyme extraction. Patatin was extracted from potatoes by using temperature-induced phase separation in Triton X-114 at pH 4.0 (36). The solution thus obtained was submitted to salt fractionation (30–60% ammonium sulfate). Following the salt fractionation treatment, the enzyme extract was further purified by consecutively applying anion exchange chromatography (Resource-Q column, connected to an Äkta purifier; Pharmacia Biotech, Barcelona, Spain) and affinity chromatography (HiTrap Concanavalin A column; Pharmacia Biotech). Purity of the isolated protein was checked by silver staining of SDS-PAGE (15)

Substrate preparation. Aqueous PC substrate was prepared by drying aliquots of a [1,2-dilinoleoyl]PC stock solution in chloroform (12.5 mg/mL) under a stream of N_2; the film obtained was rapidly dispersed in 10 mM deoxycholate dissolved in 50 mM Tris buffer (pH 8.5). The resulting substrate solution (1.3 mM) was allowed to equilibrate for 10 min at 25°C (37).

Enzymatic activity. Phospholipase activity was determined by means of a coupled assay using [1,2-dilinoleoyl]PC as substrate and lipoxygenase as the coupling enzyme. The linoleic acid released by phospholipase activity was oxidized by lipoxygenase, giving rise to the corresponding hydroperoxide derivative. The phospholipase activity was then followed spectrophotometrically by measuring the increase in absorbance at 234 nm that resulted from the formation of the conjugated diene hydroperoxide (ε_{234} = 25,000 M^{-1} cm^{-1}) (38). Spectrophotometric

measurements were carried out with a Uvikon 940 spectrophotometer. The standard reaction medium (1 mL) at 25°C contained the following: 65 μM [1,2-dilinoleoyl]PC, 0.2 μg/mL lipoxygenase, and 0.4 μg/mL patatin in 50 mM borate buffer (pH 9.5) containing 3 mM deoxycholate. The reaction was started by adding patatin to the assay medium. Controls either without phospholipase or without lipoxygenase were always carried out.

Optimal pH. pH studies were carried out using 50 mM Tris-HCl and sodium borate buffers from pH 7.4 to 10 in the presence of 3 mM deoxycholate. The concentrations of [1,2-dilinoleoyl]PC, lipoxygenase, and patatin were as in the standard assay. The pH of the assay solution was determined at room temperature using a Crison 2002 micro pH meter. After catalysis, the pH of the assay solution was again measured.

Protein determination. Protein concentration was determined according to Bradford's dye-binding method (39) using BSA as standard.

RESULTS AND DISCUSSION

Patatin phospholipase activity was monitored spectrophotometrically by using [1,2-dilinoleoyl]PC as substrate. The linoleic acid released by the action of patatin was used as substrate by the coupling enzyme lipoxygenase, which catalyzes the hydroperoxidation and double-bond arrangement of the FA. Figure 2 shows the UV spectral changes with time corresponding to the coupled enzymatic assay. Maximal spectral changes could best be detected by subtracting recording 2 from the rest of the recordings (inset in Fig. 1). This calculation allowed us to confirm the presence of a maximum at 234 nm, corresponding to the formation of the conjugated diene hydroperoxide of the linoleic acid.

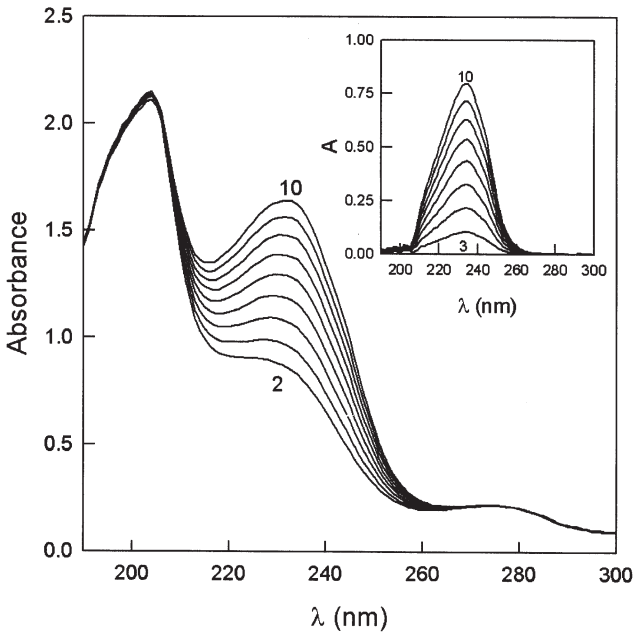

FIG. 2. Consecutive spectra obtained for the oxidation by lipoxygenase of the linoleic acid released by the action of patatin on [1,2-dilinoleoyl]PC, at 25°C. The assay medium (1.0 mL) contained 39 μM [1,2-dilinoleoyl]PC and 0.2 μg/mL lipoxygenase in 50 mM borate buffer (pH 9.5) containing 3 mM sodium deoxycholate. The reaction was started by the addition of patatin (0.46 μg/mL). The scan speed was 2000 nm/min, with scanning at 1-min intervals for 10 min. Inset: Spectra obtained by subtracting recording 2 from the rest of the recordings shown in the main figure.

Although it is generally acknowledged that free PUFA are the best lipoxygenase substrates, soybean lipoxygenase has reportedly been able to oxidize [1,2-dilinoleoyl]PC (34). Controls in the absence of patatin were routinely carried out. We can therefore state that under the experimental conditions used in our assays, there was no oxidation of [1,2-dilinoleoyl]PC by lipoxygenase.

The spectral changes depicted in Figure 2 were not observed in the absence of patatin and were, consequently, considered to be the result of the action of lipoxygenase on the product of patatin phospholipase activity (free linoleic acid). The nature of the spectral changes during the enzyme assay showed that the formation of the reaction product was proportional to time. This observation and the dependence of these spectral changes on the presence of the enzyme indicate that such changes are a reliable measure of patatin phospholipase activity.

From the results shown in Figure 2, we can conclude that the phospholipase activity of patatin can be monitored by recording the increase in absorbance at 234 nm, using [1,2-dilinoleoyl]PC as substrate and lipoxygenase as the coupled enzyme. In addition, the increase in activity was linear during the time of the assay.

To establish the optimal conditions for the coupled enzymatic assay, the lipoxygenase concentration was varied (Fig. 3). As can be seen, phospholipase activity increased as the lipoxygenase concentration was raised. However, there was a limit to this increase, since no more product was formed when the lipoxygenase concentration was higher than 0.2 μg/mL, as shown in Figure 3. In accordance with these results, this lipoxygenase concentration was chosen to assay phospholipase activity routinely because, at this concentration, the enzymatic assay became independent of the lipoxygenase concentration. In these conditions therefore the increase in absorbance at 234 nm that resulted exclusively from the formation of the hydroperoxide reflects the phospholipase activity of patatin.

Once the lipoxygenase concentration had been selected, the next parameter to be fixed to optimize the coupled assay was patatin concentration. The inset in Figure 3 shows that phospholipase activity was dependent on patatin concentration. This dependency was linear in an enzyme concentration range of 0–0.7 μg/mL. Over this range, enzyme activity started to deviate from linearity. Consequently, for a routine assay, an optimal concentration of 0.35 μg/mL was used for patatin.

To further characterize the phospholipase activity of patatin by this coupling assay, the effects of pH and detergent concentration were studied. These two factors had to be analyzed independently to establish the optimal experimental conditions for measurement.

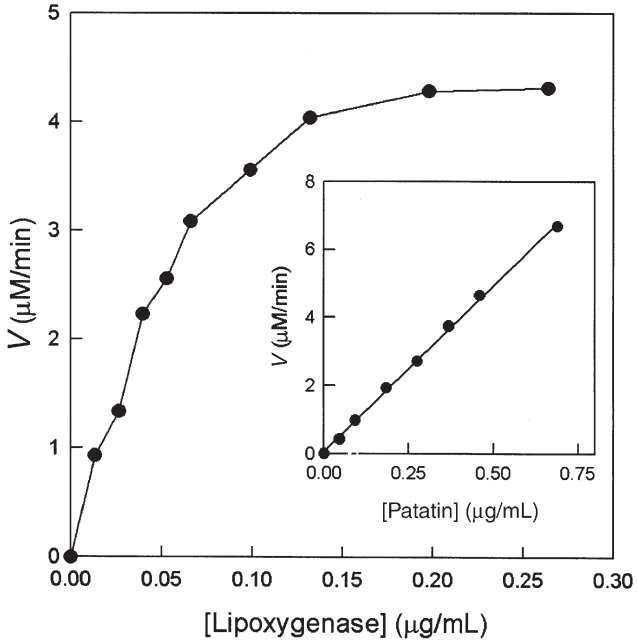

FIG. 3. Effect of variations in lipoxygenase concentration on the rate of the enzyme-coupled reaction. The reaction medium, at 25°C, contained 65 μM [1,2-dilinoleoyl]PC, lipoxygenase at the indicated concentration, and 0.4 μg/mL patatin in 50 mM borate buffer (pH 9.5) containing 3 mM deoxycholate. The reaction was started by adding patatin. Inset: Dependence of phospholipase activity on patatin concentration. The reaction medium contained 65 μM [1,2-dilinoleoyl]PC, 0.2 μg/mL lipoxygenase, and patatin, at the indicated concentration, in 50 mM borate buffer (pH 9.5) containing 3 mM deoxycholate.

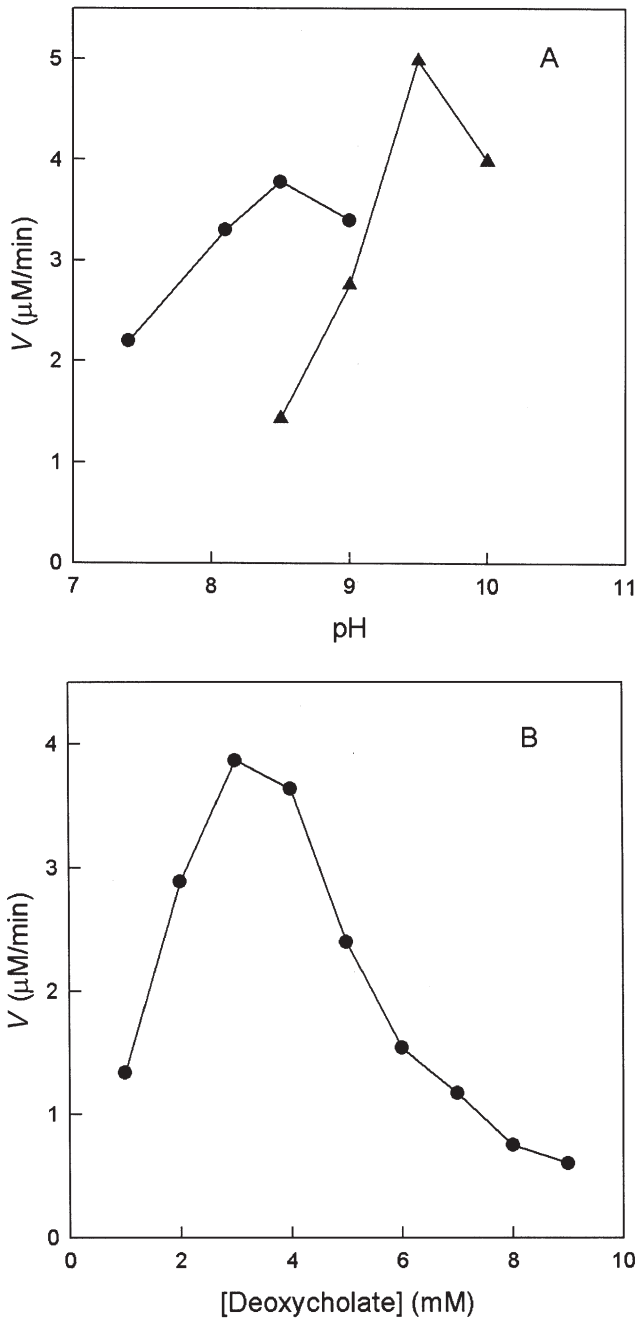

FIG. 4. (A) Effect of pH on the phospholipase activity of patatin. The assay medium contained 65 μM [1,2-dilinoleoyl]PC, 0.2 μg/mL lipoxygenase, and 0.4 μg/mL patatin in 50 mM buffer, containing 3 mM deoxycholate. (●) Tris-HCl buffer, (▲) sodium borate buffer. (B) Effect of deoxycholate concentration on phospholipase activity. The reaction medium was similar to that above but contained the indicated concentration of detergent.

Figure 4A shows that phospholipase activity varied with pH, being strongly dependent on the nature of the buffer. As shown, when the buffer in the assay medium was Tris-HCl, an optimal pH value appeared at pH 8.5. However, when the buffer was sodium borate, the optimal pH was 9.5. Since the activity was much higher at this pH value, the phospholipase activity of patatin was routinely measured in borate buffer pH 9.5.

Another factor influencing phospholipase activity was the concentration of detergent. The presence of detergent in the assay was necessary to solubilize the substrate and, as shown in Figure 4B, phospholipase activity also depended on deoxycholate concentration. Scans of the detergent concentration showed the optimal deoxycholate concentration to be 3 mM, since higher detergent concentrations decreased the enzymatic activity. Previous results obtained with different types of phospholipases also have shown that the enzymatic activity is stimulated at low concentrations of detergent but inhibited at higher values (20,40,41).

The dependence of phospholipase activity on substrate concentration was also examined. Enzyme activity followed the classical Michaelis–Menten kinetics previously reported for phospholipase A_2 activity (34,42,43). Figure 5 shows the results obtained when [1,2-dilinoleoyl]PC concentration was varied. Hanes–Woolf plots for the kinetic data resulted in linear relationships (inset in Fig. 5), from which the kinetic parameters were evaluated ($V_m = 9.8 \times 10^{-3}$ μmol/min·μg protein, K_m = 7.8 μM, $V_m/K_m = 1.3$ min^{-1} · μg protein). These values were of the same order as that reported for phospholipase A_2 in which other methods were used to determine activity (44).

Although a number of methods of determining phospholipase activity have been described, to our knowledge, the most

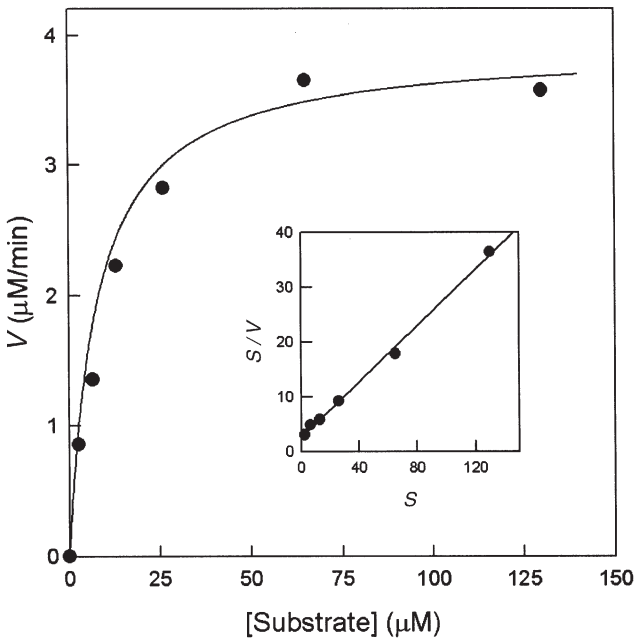

FIG. 5. Dependence of the phospholipase activity of patatin on [1,2-dilinoleoyl]PC concentration. The reaction medium contained [1,2-dilinoleoyl]-PC at the indicated concentrations, 0.2 μg/mL lipoxygenase, and 0.4 μg/mL patatin in 50 mM borate buffer (pH 9.5) containing 3 mM deoxycholate. Inset: Hanes–Woolf plot of the kinetic results.

routinely used assays for the phospholipase activity of patatin involve radioactive substrates or tedious chromatographic procedures. This paper, however, describes a continuous spectrophotometric method of measuring the phospholipase activity of patatin. The assay possesses the advantages of using commercially available, nonradiolabeled, inexpensive substrates. In addition, the substrate used in this procedure is a phospholipid, the natural substrate for determining phospholipase activity. This method also allows a simple measurement of enzymatic activity, involves a short analysis time, and provides reproducibility. Moreover, the assay allows the kinetic characteristics of the enzyme to be studied.

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COMMUNICATION

Impact of Dietary n-3 FA Deficiency on Rat Bone Tissue FA Composition

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ABSTRACT: The effect of dietary n-3 FA deficiency on bone tissue FA composition was evaluated in growing rats. Two mixtures combining hydrogenated coconut oil with safflower oil served as the n-3–deficient dietary treatments and provided two levels of linoleic acid (LA). The n-3 treatments were formulated with added α-linolenic acid (LNA) from flaxseed oil (diet LNA) or LNA plus DHA, and both were balanced for LA. This study showed that bone is sensitive to changes in dietary n-3 FA and that DHA is more effective than LNA in maintaining DHA levels in these tissues.

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Dietary n-6 and n-3 PUFA affect bone formation rate and are associated with altered FA composition of bone tissue compartments and changes in biochemical factors and functional properties during bone modeling in the young (1,2). We investigated how dietary ratios of n-6/n-3 PUFA modulate *ex vivo* production of prostaglandin E_2 (PGE_2) and localized growth factors in bone as well as protein expression in osteoblast cell cultures (2). We demonstrated that the ratio of 20:4n-6 arachidonic acid (AA)/20:5n-3 (EPA) in bone is positively correlated with *ex vivo* PGE_2 production and that by moderating its production, bone formation can be optimized in the rat by decreasing the dietary ratio of n-6/n-3 PUFA.

In our previous bone studies, menhaden oil, which contains a significant amount of EPA, was used to reduce the dietary ratio of n-6/n-3 PUFA. The n-3 diets in the present study were formulated using 18:3n-3 (α-linolenic acid; LNA)-rich flaxseed oil with or without an added source of 22:6n-3 (DHA). These dietary lipid treatments provide methods to examine the efficiency of LNA conversion to longer-chain n-3 PUFA and to measure the incorporation of DHA into rat bone tissues. Evaluating how dietary LNA or LNA + DHA, as well as high levels of LA, affect bone tissue FA profiles in growing animals will provide valuable insight into the incorporation and metabolism of n-3 and n-6 PUFA in bone. Therefore, the aim of this study was to investigate how dietary LNA and DHA influence the tissue levels of long-chain PUFA in bone compartments.

EXPERIMENTAL PROCEDURES

Animals and diets. Forty 21-d-old Long–Evans female rats (F1 generation) (Charles River, Portage, MI) were delivered to the animal facility of the National Institute on Alcohol Abuse and Alcoholism (NIAAA), randomized into four groups, and fed one of four experimental diets (n = 10 per dietary group). Animals were maintained on a 12-h light/dark cycle at a temperature of 20–21°C and fed *ad libitum*. At 8 wk of age, the F1 females were mated with chow-fed males of the same strain. Their litters were culled to 10 female pups (the F2 generation) per treatment group, and these pups were weaned to the same diet of the dam and maintained on these diets throughout the study until they were 12 wk old (3). The NIAAA Animal Care and Use Committee approved all animal procedures. The treatment diets were based on the AIN-93 formulation with modifications to the fat sources (4). The basal fat ingredients consisted of hydrogenated coconut and safflower oils for the n-3–deficient diet (Diet LA). The n-3–deficient high-LA diet (Diet High-LA) was formulated with added safflower oil at the expense of hydrogenated coconut oil. In the n-3–adequate diet (Diet +LNA), 0.48% of flaxseed oil was added to supply LNA. DHASCO oil (42 ± 1% of FA as DHA; Martek Biosciences, Columbia, MD) was added to Diet +LNA to form the n-3–adequate plus DHA diet (Diet +LNA/DHA). The n-3–adequate diets +LNA and +LNA/DHA had 3.12 and 3.83% of total n-3 PUFA, respectively, with the latter containing 1.28% of DHA. The total fat content of all diets was 100 g/kg.

Sample collections and analytical procedures. At the end of the study, the rats were euthanized by decapitation, and bone samples (right tibia and femur) were collected for FA analysis. Lipids in the diet and bone marrow were extracted with chloroform/methanol (2:1, vol/vol). Cortical bone samples were cooled in liquid nitrogen, pulverized, and sonicated for lipid extraction (1,5). The extracted lipids were converted to FAME and analyzed by GC as previously described .

Statistical analysis. Data were analyzed by one-way ANOVA and, where significant differences were found, a Student–Newman–Keuls multiple range test was performed at a probability of $P = 0.05$ (SAS software package for UNIX; SAS

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Abbreviations: AA, arachidonic acid; LA, linoleic acid; LNA, α-linolenic acid; $n\text{-}3_{LC}$, sum of 20:5n-3 + 22:6n-3 + 22:6n-3; $n\text{-}6_{LC}$, sum of 20:4n-6 + 22:4n-6 + 22:5n-6; NIAAA, National Institute on Alcohol Abuse and Alcoholism; PGE_2, prostaglandin E_2.

Institute Inc., Cary, NC). Variations between treatment groups were expressed as the pooled SD.

RESULTS AND DISCUSSION

The effect of dietary lipid treatments on rat bone tissues (cortical bone and marrow) was evaluated in both femur and tibia. In the femoral cortical bone (Table 1), the n-3–adequate diets, +LNA and +LNA/DHA, greatly enriched the tissue concentrations of 18:3n-3, 22:5n-3, 22:6n-3, and total n-3 but had no effect on EPA, which was barely detectable in the tissue. The two n-3–adequate diets resulted in increased concentrations of 14:1n-5, 16:1n-7, 18:1n-9, and total monounsaturated FA in the bone. The Diet High-LA elevated the level of 18:2n-6 in the tissue but failed to increase the concentrations of longer-chain n-6 FA (20:4n-6, 22:4n-6, and 22:5n-6) compared to the values in rats fed Diet LA. The dietary n-3 level did not affect the total amount of n-6 PUFA in femur cortical bone (treatments LA, +LNA, and +LNA/DHA). In n-3–deficient rats, long-chain n-6 PUFA contents were significantly higher compared to long-chain n-6 PUFA in rats fed n-3–sufficient diets. To further assess the effect of the diets on long-chain PUFA content, the ratio of these PUFA was calculated as follows: The n-6_{LC} numerator was the sum of (20:4n-6 + 22:4n-6 + 22:5n-6), and the n-3_{LC} denominator was the sum of (20:5n-3 + 22:5n-3 + 22:6n-3). The FA composition of rat tibial cortical bone was similarly affected by the dietary treatments. (Data for bone and diet not shown.)

The femur bone marrow FA profile was affected in a similar manner as the cortical bone but more closely reflected the dietary lipid treatments fed (Table 2). Rats fed the High-LA diet had the highest levels of 18:2n-6, 22:4n-6, and 22:5n-6, whereas those given diet +LNA/DHA showed the lowest levels for these FA. AA concentration was different only between

TABLE 1
FA Composition (wt%) of Femoral Cortical Bone from Rats Fed Different Dietary Lipid Treatments[a]

	Dietary treatment[b]					ANOVA *P*
FA	LA	High-LA	+LNA	+LNA/DHA	Pooled SD	value
12:0	3.2[b]	1.4[c]	4.3[a]	4.7[a]	1.0	0.0001
14:0	3.1[b]	1.9[c]	4.1[a]	4.5[a]	0.9	0.0001
15:0	0.4	0.2	0.3	0.3	0.3	NS
16:0	35.2[a]	28.4[b]	30.5[b]	31.6[a,b]	3.6	0.004
17:0	1.0[a]	0.7[a,b]	0.5[b]	0.7[a,b]	0.3	0.02
18:0	11.7[a]	8.7[b]	9.2[b]	8.7[b]	1.8	0.003
20:0	1.0[a]	0.6[b]	0.5[b]	0.7[b]	0.2	0.01
22:0	3.1	2.4	1.9	2.1	1.1	NS
24:0	1.5[a]	1.1[b]	0.7[b]	1.0[b]	0.4	0.007
Total saturates	60.3[a]	45.4[c]	52.1[b]	54.2[b]	5.0	0.0001
14:1n-5	0.1[b]	0.1[b]	0.3[a]	0.4[a]	0.2	0.006
16:1n-7	3.1[b]	2.7[b]	3.9[a,b]	5.4[a]	1.5	0.009
18:1n-9	10.0[b]	11.6[a,b]	14.1[a]	14.0[a]	2.7	0.01
18:1n-7	2.7[a]	2.1[b]	3.0[a]	2.8[a]	0.5	0.006
20:1n-9	Tr	Tr	0.1	Tr	0.1	NS
22:1n-9	0.1	ND	ND	ND	0.2	NS
Total monounsaturates	16.1[b]	16.5[b]	21.3[a]	22.5[a]	4.2	0.008
18:2n-6	5.6[c]	18.1[a]	8.1[b]	8.2[b]	1.8	0.0001
18:3n-6	ND	0.1	0.1	ND	0.1	NS
20:2n-6	Tr[b]	0.4[a]	0.1[b]	0.1[b]	0.1	0.0001
20:3n-6	0.2[b]	0.3[b]	0.5[a,b]	0.5[a]	0.2	0.005
20:4n-6	8.0[a]	6.8[a]	6.3[a]	4.9[b]	1.4	0.001
22:4n-6	3.3	2.7	1.4	0.8	2.3	NS
22:5n-6	1.5[a]	1.2[a]	Tr[b]	0.1[b]	0.8	0.002
Total n-6 FA	18.5[b]	29.5[a]	16.5[b]	14.6[b]	3.3	0.0001
18:3n-3	Tr[b]	Tr[b]	0.6[a]	0.6[a]	0.2	0.0001
20:5n-3	ND	Tr	0.1	ND	0.1	NS
22:5n-3	ND[b]	ND[b]	0.2[a]	0.2[a]	0.1	0.0001
22:6n-3	0.1[b]	0.1[b]	1.0[a]	1.3[a]	0.4	0.0001
Total n-3 FA	0.2[b]	0.1[b]	1.9[a]	2.1[a]	0.4	0.0001
Total PUFA	18.7[b]	29.6[a]	18.3[b]	16.7[b]	3.3	0.0001
n-6_{LC}/n-3_{LC}[c]	23.4[b]	86.6[a]	6.3[b]	4.7[b]	29.6	0.02

[a]Mean values for femur cortical bone FA composition ($n = 9$ for treatment LA and High-LA, $n = 7$ for the other two treatments) within a row having different roman superscripts are significantly different by one-way ANOVA and Student–Newman–Keuls multiple range test ($P < 0.05$). ND, not detected; NS, not significant; LA, linoleic acid; LNA, α-linolenic acid; Tr, trace amount.

[b]Dietary lipid treatments included four formulations that provided varied amounts of n-3 and n-6 FA: Diet LA, hydrogenated coconut (8.1%) and safflower oils (1.9%); Diet High-LA, increased amount of safflower oil (8.5%) at the expense of hydrogenated coconut oil (1.5%); Diet +LNA, a small amount of flaxseed oil (0.48%) was added to supply LNA; and Diet +LNA/DHA, a small amount of DHASCO oil (42 ± 1% of FA as DHA) was added to Diet +LNA.

[c]n-6_{LC}/n-3_{LC} = (20:4n-6 + 22:4n-6 + 22:5n-6)/(20:5n-3 + 22:5n-3 + 22:6n-3).

rats fed diets LA and +LNA/DHA. Both n-3–adequate diets led to increased concentrations of EPA in bone marrow independent of the presence of DHA. However, tissue 22:6n-3 level was greatly elevated by dietary DHA addition. In rats given Diet +LNA/DHA, tissue 22:6n-3 was almost doubled compared to tissue in rats given the Diet +LNA. However, dietary DHA supplementation suppressed femoral bone marrow 22:4n-6 and 22:5n-6. In rats given the +LNA/DHA diet, the concentration of EPA was five times higher than in those fed an n-3–adequate diet containing only 18:3n-3. In general, the total n-6 PUFA content in bone marrow (femur and tibia) was not affected in rats fed similar amounts of 18:2n-6 in the diet but was greatly increased in rats given the High-LA diet. Total n-3 PUFA content of bone marrow was increased by n-3 PUFA feeding and more so in rats given the +LNA/DHA diet. The ratio of $n\text{-}6_{LC}/n\text{-}3_{LC}$ was greatly reduced in marrow of rats fed n-3 PUFA.

FA analysis of bone tissue compartments demonstrated that DHA was preferentially accumulated in bone tissue compared to EPA. Li *et al.* (5) reported that feeding growing male rats n-3 diets (DHA and EPA each ~8% in the diets) greatly enriched DHA (2.5 to 3.3%) compared to EPA (1.4 to 1.8%) in femur bone marrow. Watkins *et al.* (1) reported similar findings, DHA was more efficiently incorporated in femur marrow polar lipids than EPA despite the two- to threefold higher concentrations of EPA compared to DHA in the diets. In the same study, the concentrations of DHA in the polar lipid fraction of femur bone periosteum were 10 to 14% of total FA, whereas those of EPA were only 0.4 to 3.3%. It seems that bone tissues selectively accumulate DHA over EPA. Hence, DHA may be more critical in supporting osteoblastic cell functions for normal bone development and growth. These data suggest that a sufficient dietary supply of n-3 PUFA will sustain DHA concentrations in bone tissue and that dietary DHA is able to maintain a higher level of bone DHA than dietary LNA. These results also indicate that DHA could be a candidate biomarker for n-3 status in bone.

During the conversion process of LNA to DHA, EPA acts as an intermediate, but it was practically absent in bone tissues in the current study, which suggests that it was entirely converted

TABLE 2
FA Composition (wt%) of Femoral Bone Marrow from Rats Fed Different Dietary Lipid Treatments[a]

	Dietary treatment[b]					ANOVA *P*
FA	LA	High-LA	+LNA	+LNA/DHA	Pooled SD	value
12:0	8.6[a]	1.8[c]	7.3[b]	7.4[b]	0.6	0.0001
14:0	7.2[a]	2.8[c]	6.5[b]	6.6[b]	0.5	0.0001
15:0	0.2	0.2	0.2	0.2	0.1	NS
16:0	24.4[b]	22.5[c]	24.2[b]	26.3[a]	1.5	0.0001
17:0	0.2	0.2	0.1	0.1	0.1	NS
18:0	6.4	6.8	6.5	6.7	1.0	NS
20:0	Tr[a,b]	Tr[a]	Tr[a,b]	ND[b]	<0.1	0.03
22:0	Tr	0.1	ND	0.1	0.1	NS
24:0	Tr	0.1	ND	ND	0.1	NS
Total saturates	47.1[a]	34.4[c]	44.7[b]	47.4[a]	1.3	0.0001
14:1n-5	0.6[a]	0.1[b]	0.6[a]	0.5[a]	0.2	0.0001
16:1n-7	6.6[a]	3.7[b]	6.8[a]	7.1[a]	1.3	0.0001
18:1n-9	20.9[a]	16.6[b]	20.4[a]	19.9[a]	1.5	0.0001
18:1n-7	5.8[a]	3.5[d]	5.1[b]	4.2[c]	0.4	0.0001
20:1n-9	0.1	0.3	0.2	0.2	0.1	NS
Total monounsaturates	34.0[a]	24.2[b]	33.0[a]	31.8[a]	2.5	0.0001
18:2n-6	11.1[b]	31.8[a]	11.6[b]	10.7[b]	1.6	0.0001
18:3n-6	Tr	0.2	0.3	0.1	0.2	NS
20:2n-6	0.2[b]	0.9[a]	0.3[b]	0.3[b]	0.1	0.0001
20:3n-6	0.1	0.2[a]	0.3[a]	0.3[a]	0.1	0.0002
20:4n-6	4.0	4.8	4.6	4.0	1.4	NS
22:4n-6	0.6[b]	1.0[a]	0.5[b]	0.3[c]	0.2	0.0001
22:5n-6	0.5[b]	0.7[a]	0.1[c]	Tr[c]	0.2	0.0001
Total n-6 FA	16.6[b]	39.7[a]	17.7[b]	15.8[b]	2.6	0.0001
18:3n-3	ND[b]	Tr[b]	0.9[a]	1.0[a]	0.1	0.0001
20:5n-3	ND[b]	ND[b]	Tr[b]	0.1[a]	<0.1	0.03
22:5n-3	ND[b]	ND[b]	0.2[a]	0.3[a]	0.1	0.0001
22:6n-3	ND[c]	ND[c]	0.6[b]	1.2[a]	0.3	0.0001
Total n-3 FA	ND	Tr[c]	1.8[b]	2.5[a]	0.4	0.0001
Total PUFA	16.6[b]	39.7[a]	19.5[b]	18.3[b]	2.8	0.0001
$n\text{-}6_{LC}/n\text{-}3_{LC}$[c]	NA	NA	6.2[a]	2.8[b]	0.5	0.0001

[a]Mean values for femural bone marrow FA composition ($n = 8$ for treatment LA and +LNA/DHA, $n = 10$ for treatment High-LA, and $n = 7$ for treatment +LNA) within a row having different roman superscripts are significantly different by one-way ANOVA and Student–Newman–Keuls multiple range test ($P < 0.05$). For abbreviations see Table 1.

[b,c]See Footnotes, Table 1.

to DHA. Moreover, the findings imply that, compared to DHA, EPA may be less critical for stem cell differentiation in marrow and for osteoblastic cell functions in modeling bone. Another important observation of this study is that the AA concentration was only suppressed by dietary DHA, which appears to be important for modulating PGE_2 production to support osteoblastic activity (1,2). In contrast, dietary LNA did not demonstrate a competitive effect in reducing the AA content of bone.

Since we have shown that dietary PUFA influence bone formation during growth, characterizing the changes in FA composition of femur bone compartments is emerging as an important indicator of skeletal health. How PUFA influence the FA composition of femur bone compartments may give insight into the potential role that lipids play in osteoporotic fractures of the femoral neck in high-risk populations, such as postmenopausal women. We believe that a marginal status of total n-3 PUFA and perhaps limited dietary DHA intake would have a detrimental outcome on skeletal modeling and bone architecture in the young that compromise bone health with aging. Future investigations should examine the effect of dietary n-3 PUFA on bone cell functions and bone modeling parameters (histomorphometry) early in life. These studies should address the metabolic conversion and tissue needs for LNA, EPA, and DHA in various bone compartments of young animals. The results of this study also provide a rationale to characterize the FA of lipid classes in bone tissues to further understand their actions in bone biology.

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A Novel Type Hypertriglyceridemia Observed in FLS Mice

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ABSTRACT: The unique inborn hypertriglyceridemia seen in FLS (fatty liver Shionogi) mice was relieved by the administration of purified apolipoprotein (apo) C-II. Lipoprotein lipase (LPL) and its cofactor, apoC-II, play a pivotal role in VLDL metabolism. Therefore, we investigated the genetic background involved in this hypertriglyceridemia. Plasma levels of TG and total cholesterol as well as LPL activity were measured in male FLS mice and C57/BL6J mice. Agarose gel electrophoresis and fast protein liquid chromatography were used to analyze the lipoprotein profile. A cross experiment was done to determine the genetic background of hypertriglyceridemia observed in FLS mice. cDNA sequences of apoC-II and apoC-III of FLS mice were determined. Preα-lipoprotein was the predominant lipoprotein class in FLS mouse plasma. LPL activity remained in the range observed in C57/BL6J mice, and purified apoC-II transiently relieved FLS mice from hypertriglyceridemia. Preα-lipoproteinemia was inherited in an autosomal recessive manner. ApoC-III appeared to be a causal factor for this unique hypertriglyceridemia. Microsatellite analysis, however, revealed that the responsible chromosome was not 7; rather, apoC-III mapped onto chromosome 9. Therefore, we suggest apoC-III as a candidate causative factor for the hypertriglyceridemia observed in FLS mice because an excessive amount of apoC-III attenuates LPL activity *in vivo* and *in vitro*.

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Lipoprotein lipase (LPL) is the major enzyme involved in the hydrolysis of plasma TG, and apolipoprotein (apo) C-II, a cofactor for LPL, plays a pivotal role in lipid metabolism (1–3). Human apoC-II undergoes posttranslational processing in the form of glycosylation, deglycosylation, and cotranslational cleavage to the major plasma isoforms of apoC-II (4,5). The majority of apoC-II in plasma is reversibly bound to HDL and TG-rich lipoproteins such as chylomicrons and VLDL (6). Its distribution on these plasma lipoproteins continually changes as a result of the secretion, metabolic conversion, and catabolism of the plasma lipoproteins. This occurs because apoC-II in HDL rapidly associates with the newly secreted TG-rich lipoproteins that are synthesized by the liver and intestine (6). TG-rich lipoproteins are then converted to remnants by lipolysis in which apoC-II dissociates from these particles and reassociates with HDL (7).

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Abbreviations: apo, apolipoprotein; EMBL, European Molecular Biology Laboratory; FLS, fatty liver Shionogi; FPLC, fast protein liquid chromatography; LPL, lipoprotein lipase.

ApoC-II deficiency is an autosomal recessive disease, which has been described in various independent forms. This disease is characterized by an increased incidence of pancreatitis with a marked elevation in the levels of plasma TG and/or chylomicrons due to impaired hydrolysis of TG (8–14). Classically, intravenous infusions of normal plasma containing apoC-II or isolated apoC-II fractions have been used to show a transient normalization of plasma levels of TG and lipoproteins for the diagnosis of apoC-II deficiency in humans (8,11,13,14). Various unusual isoforms of apoC-II were reported in apoC-II-deficient patients, but Southern blot analysis revealed no major rearrangements in the apoC-II gene (15,16). Amino acid substitutions, nonsense mutations, and posttranslational processing, such as glycosylation, deamidation, and proteolytic cleavage, accounted for the differences in charge and M.W. of the various apo isoforms in most cases (17–20). From these findings, it was further confirmed that functional impairment of apoC-II, an activator of LPL, may easily result in hypertriglyceridemia.

FLS (fatty liver Shionogi) mice were reported to have fatty livers with normal serum lipid conditions and lipoprotein profiles after overnight food deprivation (21). In this paper, however, we report that FLS mice consuming food *ad libitum* showed the characteristic hypertriglyceridemia, which was transiently relieved by apoC-II infusion.

MATERIALS AND METHODS

Animals. FLS mice, originating from ddN mice (21,22), were inbred at the Aburahi Laboratory of Shionogi & Co., Ltd. (Shiga, Japan). Normolipidemic mice, C57BL/6J, were purchased from SLC (Shizuoka, Japan). MSM, a well-characterized mouse strain originating from Japanese wild mice and kindly provided by the National Institute for Genetics (Mishima, Japan), were used for cross experiments. Mice were kept in our specific pathogen-free facilities. Commercially available laboratory chow diet (CE-2; Clea Japan, Tokyo, Japan), containing 4.4% crude fat and 25.4% protein, and tap water were consumed *ad libitum*. Blood samples were collected under fed conditions at 9–10 A.M. Animal care and experiments were carried out in accordance with institutional animal care regulations.

Plasma preparation and laboratory tests. Blood samples were collected under fed conditions at 9–10 A.M. from

18-wk-old mice using sodium EDTA as the anticoagulant. Plasma was immediately separated by centrifugation and used for transfer experiments or applied to laboratory tests including plasma levels of TG and total cholesterol, agarose gel electrophoresis, and fast protein liquid chromatography (FPLC) for analyses of the lipoprotein profile.

Agarose gel electrophoresis and FPLC for lipoprotein profile analysis. Aliquots of plasma (1 μL) were applied to precast agarose gels and were electrophoresed to fractionate lipoproteins using a commercially available kit (RET LIPO-30 plate; Helena Laboratories, Saitama, Japan). Cholesterol in the gels was stained for analysis (Titan gel S-cholesterol; Helena Laboratories).

Aliquots of plasma (50 μL) were also applied to the SMART System equipped with a Superose 6 PC 3.2/30 (Pharmacia Biotech, Uppsala, Sweden) at a flow rate 40 μL/min of 0.15 M NaCl supplemented with 0.05 M sodium phosphate (pH 7.0) at 4°C to fractionate VLDL, LDL, and HDL (80 μL/fraction). The cholesterol and TG contents in each fraction were quantified enzymatically using commercially available kits (Nissui, Tokyo, Japan).

LPL activity. Heparin (100 U/kg body weight) was administered to 4 FLS mice and 4 C57BL/6J mice. Plasma was collected 10 min later for analysis of LPL activity, which was measured in the presence of a sufficient amount of human apoC-II as described previously (23).

Transfusion of C57BL/6J plasma or purified human apoC-II to FLS mice. Male FLS mice were administered 0.1 mL of C57BL/6J plasma, saline, or saline with purified human apoC-II (25 μg) (Athens Research & Technology, Athens, GA) *via* the tail vein. Before transfusion and 4 h after transfusion, 50-μL plasma samples were collected from FLS mice and applied to the SMART System equipped with a Superose 6 PC 3.2/30 as described above for FPLC.

cDNA sequences of apoC-II and apoC-III. Total mRNA was prepared from the liver of FLS and C57BL/6J mice. After reverse transcription, the coding sequence was amplified using a sense primer GTGAGCCAGGATAGTCCTTC and an anti-sense primer AGCAAAGAGGCGCGGTGTCTGT for apoC-II and a sense primer 5′TTTTATCCCTAGAAGCAG3′ and an anti-sense primer 5′AGCAGGATGGAGGAACAG3′ for apoC-III. The product was sequenced directly using a commercially available kit (PerkinElmer Japan, Tokyo, Japan).

Cross experiment. Male FLS mice were mated with female C57BL/6J mice to obtain F1 mice. F1 mice were mated to obtain male F2 mice. Phenotypes of 50 male F2 mice were determined using a preα-lipoprotein class on agarose gel as the marker (Fig. 1).

Because FLS mice originating from ddN mice share microsatellites with B6 mice, we chose MSM, a well-characterized mouse strain originated from Japanese wild mice, for the cross experiments. Male FLS mice were mated with female MSM mice to obtain F1 mice. F1 mice were mated to obtain male F2 mice. Microsatellites (n = 92) on autosomal chromosomes were analyzed to define the chromosome responsible for the hypertriglyceridemia observed in FLS mice. Five microsatellites, D9MIT59, D9MIT2, D9MIT229, D9MIT11, and D9MIT18, were used to analyze chromosome 9.

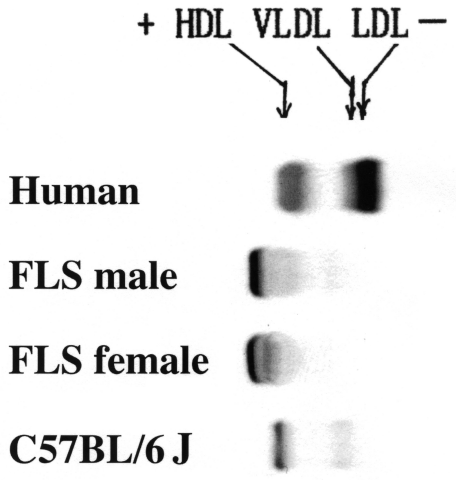

FIG. 1. Lipoprotein analysis of fatty liver Shionogi (FLS) mouse plasma using agarose gel electrophoresis. The cholesterol content in plasma lipoproteins was analyzed. α-Lipoprotein (HDL) and β-lipoprotein (LDL)/preβ-lipoprotein (VLDL) comprised two major lipoprotein classes in C57BL/6J mouse plasma, whereas a preα-lipoprotein class was the major lipoprotein class in FLS mouse plasma.

Statistical analysis. Data are shown as means ± 1 SD and were analyzed using Student's t-test. Microsatellite markers (n = 92) on 20 autosomal chromosomes, including D9MIT59, D9MIT2, D9MIT229, D9MIT11, and D9MIT18 on chromosome 9, were typed for genetic mapping of the preα-lipoprotein locus. The Friedman test and Fischer's exact test were used for linkage analysis using categorical data. All statistical analyses were done with StatView J-5.0 (Abacus Concepts, Cary, NC).

RESULTS

Agarose gel electrophoresis and FPLC for lipoprotein profile analysis. Male FLS mice were hypertriglyceridemic (168.3 ± 37.1 vs. 73.7 ± 32.9 mg/dL, $P < 0.001$) and mildly hypercholesterolemic (116.9 ± 26.3 vs. 78.9 ± 25.8 mg/dL, $P < 0.01$) compared with male C57BL/6J mice (n = 10/group).

Agarose gel electrophoresis revealed that α-lipoprotein (HDL) and β-lipoprotein (LDL)/preβ-lipoprotein (VLDL) were the two major lipoprotein classes in C57BL/6J plasma, whereas a preα-lipoprotein class was the predominant lipoprotein class in male and female FLS mouse plasma (Fig. 1, Table 1).

Concentrations of cholesterol and TG in the VLDL fraction of ultracentrifuged FLS plasma were 10- and 14-fold higher than those of C57BL/6J plasma (21 vs. 2 and 142 vs. 10 mg/dL, respectively) (n = 10/group). These findings were further confirmed by FPLC analysis showing increased concentrations of cholesterol and TG in the VLDL fraction of FLS mouse plasma (Fig. 2).

LPL activity. Postheparin LPL activity was 0.63 ± 0.12 μmol/(mL·min) in FLS mice and 0.68 ± 0.15 μmol/(mL·min) in C57BL/6J mice; hepatic TG lipase activity was 0.29 ± 0.08

TABLE 1
A Tight Correlation Between Preα-Lipoproteinemia and Chromosome 9 Genotype[a]

Genotype	FLS/FLS	FLS/C57BL	C57BL/C57BL
Phenotype			
Preα-lipoprotein (+)	23	0	0
Preα-lipoprotein (–)	0	19	11

[a]Fatty liver Shionogi (FLS) mice were mated with female MSM mice. Preα-lipoproteinemia in FLS mice was inherited in an autosomal recessive manner, and microsatellite analysis revealed that the gene responsible mapped onto chromosome 9 ($P < 0.0001$).

μmol/(mL·min) in FLS mice and 0.24 ± 0.05 μmol/(mL·min) in C57BL/6J mice. There was no significant difference in LPL and hepatic TG lipase activity.

Transfusion of C57BL/6J plasma or purified human apoC-II to FLS mice. Intravenous administration of either a small amount of C57BL/6J plasma or purified human apoC-II redistributed cholesterol from VLDL to HDL and LDL/VLDL within 4 h (Figs. 3A and 3C). The TG concentration in VLDL was also diminished (Figs. 3B and 3D).

Agarose gel electrophoresis revealed that the preα-lipoprotein class disappeared and lipoprotein classes of HDL and LDL/VLDL emerged within 4 h after intravenous administration of C57BL/6J plasma or purified human apoC-II (Fig. 4).

cDNA sequence of apoC-II and apoC-III. ApoC-II and apoC-III mRNA were detectable in the livers of FLS and C57BL/6J mice. The coding sequence of apoC-II of FLS mice was identical to that of BALB/c mice [Accession No. z15090 in the European Molecular Biology Laboratory (EMBL) nucleotide sequence database]. The coding sequence of the apoC-III gene carried three substitution sites at 122 (G→A; silent mutation), 196 (C→T; Ala→Val), and 232 (T→C; Phe→Ser) (Accession No. L04151 in EMBL).

A cross experiment for hereditary analysis. A preα-lipoprotein was detected in 23 of 100 male FLS × MSM F2 mice (23%). The average rank of chromosome 9 in the Friedman test was 4.20, whereas those of the other 19 chromosomes ranged between 7.41 and 14.26. This observation suggests that preα-lipoproteinemia in FLS mice was inherited in an autosomal recessive manner, and microsatellite analysis revealed that the responsible gene was mapped on chromosome 9 ($P < 0.0001$). Indeed, patterns of five microsatellites, D9MIT59, D9MIT2, D9MIT229, D9MIT11, and D9MIT18, in all 23 mice with preα-lipoprotein were identical to those of FLS mice (homozygote). Of the other 77 mice, 30 were randomly selected for microsatellite analysis; for all 30 mice without preα-lipoproteinemia, patterns of the five microsatellites, D9MIT59, D9MIT2, D9MIT229, D9MIT11, and D9MIT18, differed from those of FLS mice. Fischer's exact test confirmed the responsibility of chromosome 9 for the appearance of preα-lipoproteinemia ($P < 0.0001$, Table 1).

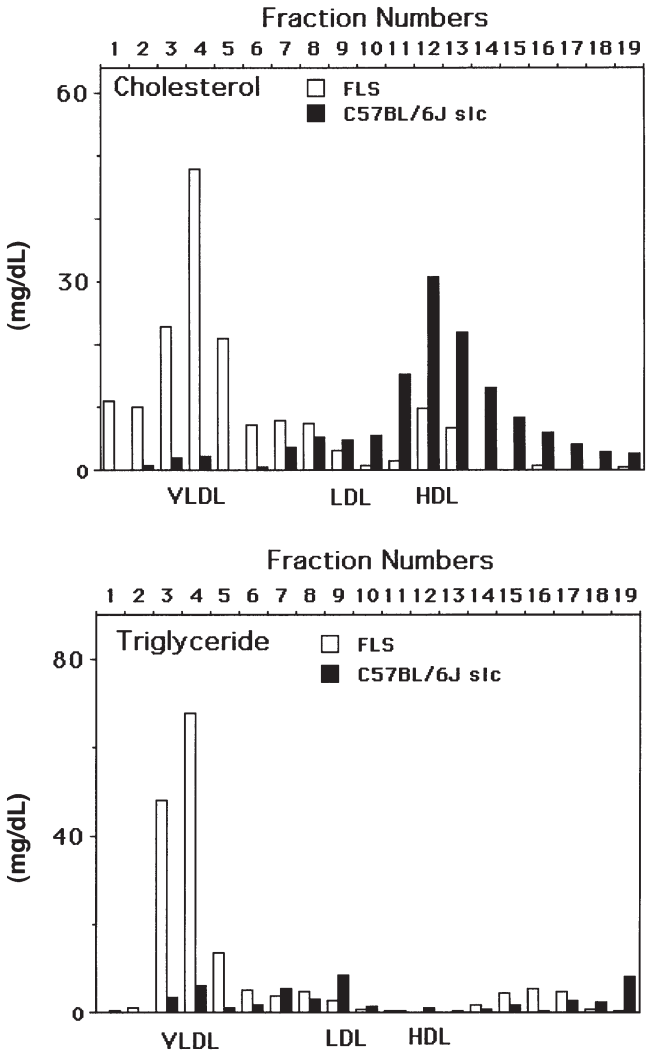

FIG. 2. Fast protein liquid chromatography (FPLC) analysis of FLS mouse plasma. FPLC analysis of C57BL/6J plasma revealed trace, moderate, and high amounts of VLDL, LDL, and HDL, respectively, whereas that of FLS mouse plasma revealed massive, trace, and trace amounts of VLDL, LDL, and HDL, respectively. The concentrations of cholesterol and TG in the VLDL fraction of FLS mouse plasma were much higher than those in the VLDL fraction of C57BL/6J mouse plasma. For other abbreviations see Figure 1.

DISCUSSION

Hypertriglyceridemia is common in the general population (24); it is frequently observed in association with a number of factors, including dietary habits, alcohol intake, physical activity, medication, and different diseases (25). Hypertriglyceridemia can also occur on a primarily genetic basis. In familial hyperchylomicronemia (Type I hyperlipidemia), for example, a functional defect exists in the LPL enzyme, which hydrolyzes TG in chylomicrons and VLDL. Defects in either the LPL gene or the gene coding for its cofactor, apoC-II, are responsible for the disease, but its occurrence in the general population is <1 in 1 million. In the vast majority of subjects with primary hypertriglyceridemia, the genetic defects responsible are not known.

Here we show FLS mice as a novel model of hypertriglyceridemia inherited in an autosomal recessive manner. FLS mice have a characteristic hypertriglyceridemia in which the

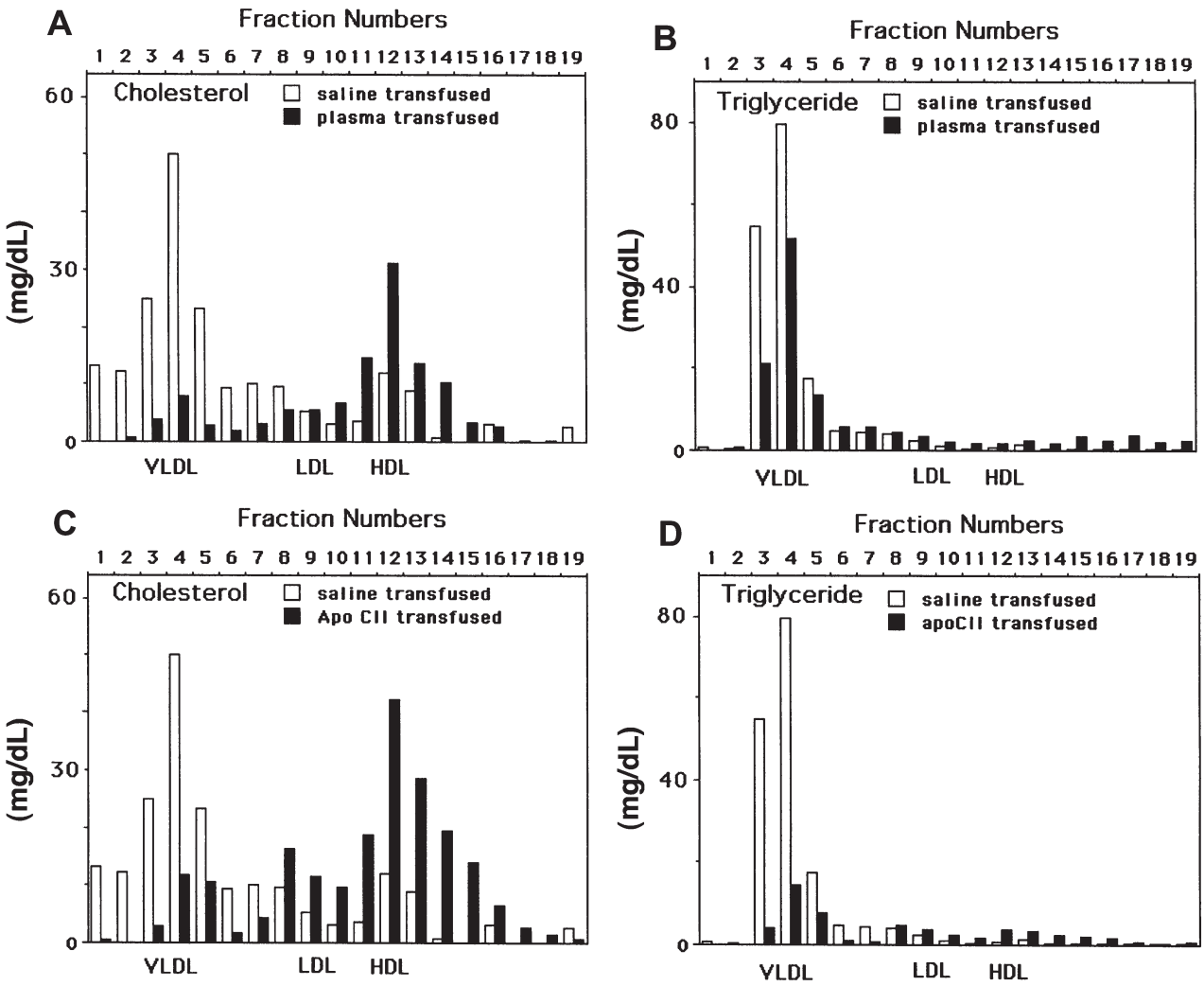

FIG. 3. Infusion of apolipoprotein (apo) C-II resulted in a redistribution of cholesterol from VLDL to HDL and LDL. The transfusion of either C57BL/6J plasma or purified human apoC-II to FLS mice dramatically normalized the pattern of FPLC analysis, i.e., transfusion resulted in a redistribution of cholesterol from the VLDL fraction to the HDL and LDL fractions within 4 h. For other abbreviations see Figures 1 and 2.

preα-lipoprotein class is the predominant lipoprotein according to agarose gel electrophoresis. Using FPLC, we showed the preα-lipoprotein of FLS mice to be VLDL, whereas α-lipoprotein (HDL) and β-lipoprotein (LDL)/preβ-lipoprotein (VLDL) are the two major lipoprotein classes in normal control mice (Fig. 1).

Because postheparin LPL activity remained normal in FLS mice, apoC-II could be responsible for the hypertriglyceridemia. To assess this possibility, a small amount of C57BL/6J plasma or purified human apoC-II was administered intravenously to FLS mice. Both of these procedures successfully eliminated the preα-lipoprotein class and facilitated transient emergence of α-lipoprotein (HDL) and β-lipoprotein (LDL)/preβ-lipoprotein (VLDL) (Figs. 3 and 4). TG and lipoproteins in the plasma of FLS mice returned transiently to normal levels. These results are in agreement with apoC-II deficiency in FLS mice (8,11,13,14).

VLDL should have been electrophoresed to the position of preβ-lipoprotein as observed in normal mice. Why are the VLDL of FLS mouse plasma electrophoresed to the position of preα-lipoprotein? This observation suggests an increased content of negatively charged proteins in VLDL of FLS mouse plasma. ApoC-III is the only protein known to be strongly negatively charged among apo, and its content was reported to be increased in hypertriglyceridemic patients (26). The plasma TG level is doubled when the apoC-III level is increased by as little as 40% because apoC-III can inhibit LPL-mediated lipolysis of TG-rich lipoproteins (26,27). Therefore, apoC-III could be regarded as another candidate factor responsible for hypertriglyceridemia in FLS mice.

Genes of these two candidate molecules are mapped onto two distinct chromosomes in mice: The apoC-II gene is mapped onto chromosome 7 and apoC-III gene onto chromosome 9. For this reason, microsatellite analysis was performed to define the chromosome responsible for the preα-lipoprotein phenotype in FLS mice. Chromosome 9 proved to be responsible for preα-lipoprotein phenotype of FLS mice. We tried to show concordant mapping of the preα-lipoprotein

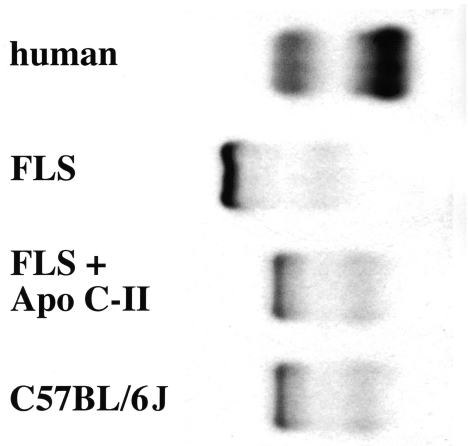

FIG. 4. Disappearance of the preα-lipoprotein class in FLS mice transfused with purified human apoC-II. The preα-lipoprotein class disappeared 4 h after intravenous administration of purified human apoC-II; lipoprotein classes of α-lipoprotein (HDL) and β-lipoprotein (LDL)/preβ-lipoprotein (VLDL) emerged in FLS mouse plasma. For other abbreviations see Figures 1 and 3.

phenotype gene and D9MIT229 because apoC-III and D9MIT229 are closely linked on chromosome 9; we were unable to achieve this because we could not detect a recombination on chromosome 9 in this experiment.

We sequenced the cDNA of the apoC-III gene of FLS mice and determined the three substitution sites at 122, 196, and 232. Although there is no evidence at this point to support the assumption that one of these substitutions is responsible for the preα-lipoprotein phenotype, polymorphism in the apoC-III gene was shown to be associated with hypertriglyceridemia in humans (27,28). Therefore, we assume that an excessive amount of apoC-III in plasma could be the primary difference in FLS mice. This may impair LPL activity, leading to the development of hypertriglyceridemia; intravenously administered apoC-II restores LPL activity. To test this hypothesis, further precise linkage analysis is required.

ACKNOWLEDGMENTS

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Chylomicron and VLDL TAG Structures and Postprandial Lipid Response Induced by Lard and Modified Lard

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ABSTRACT: Alterations in chylomicron and VLDL TAG and the magnitude of postprandial lipemia were studied in healthy volunteers after two meals of equal FA composition but different TAG FA positional distribution. Molecular level information of individual lipoprotein TAG regioisomers was obtained with a tandem MS method. The incremental area under the response curve of VLDL TAG was larger ($P = 0.021$) after modified lard than after lard. In plasma TAG, the difference did not quite reach statistical significance ($P = 0.086$). In general, there were less TAG with palmitic acid in the *sn*-2 position and more TAG with oleic acid in the *sn*-2 position in chylomicrons than in fat ingested. From 1.5 to 8 h postprandially, the proportion of individual chylomicron TAG was constant or influenced by TAG M.W. VLDL TAG regioisomerism was similar regardless of the positional distribution of fat ingested. Significant alterations were seen in VLDL TAG FA, in M.W. fractions, and in individual regioisomers with respect to time. The TAG *sn*-14:0-18:1-18:1 + *sn*-18:1-18:1-14:0, *sn*-16:0-16:1-18:1 + *sn*-18:1-16:1-16:0, and *sn*-16:1-18:1-18:1 + *sn*-18:1-18:1-16:1 decreased ($P < 0.05$); and *sn*-16:0-16:0-18:2 + *sn*-18:2-16:0-16:0, *sn*-16:0-16:0-18:1 + *sn*-18:1-16:0-16:0, *sn*-16:0-18:1-16:0, and *sn*-16:0-18:1-18:2 + *sn*-18:2-18:1-16:0 increased ($P < 0.05$) after both meals. In conclusion, positional distribution of TAG FA was found to affect postprandial lipid metabolism in healthy normolipidemic subjects.

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In addition to FA composition, the positional distribution of TAG FA influences postprandial lipid metabolism. Impaired absorption of palmitic and stearic acids has been observed from the *sn*-1 and *sn*-3 positions compared to the *sn*-2 position of TAG owing to loss of these FA in the feces of animals and infants, and saturated FA of the *sn*-2 position have delayed chylomicron clearance *in vitro* (see Refs. 1 and 2 for reviews). Although the positional distribution of saturated FA in dietary TAG has affected their absorption and metabolism *in vitro*, in animals, and in infants, effects on human adults have been unsubstantiated (3–5). Our previous work (6,7) suggested that, although the magnitude of postprandial lipemia in the human adult cannot be estimated from the positional distribution of palmitic acid, dietary TAG structure affects lipid metabolism.

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Abbreviations: ACN, acyl carbon number; apo, apolipoprotein; DB, double bond.

Chylomicron TAG composition is influenced by TAG hydrolysis in the gut, transfer of hydrolysis products to enterocytes, TAG reassembly, translocation of cellular lipid pools, assembly of lipid and apoprotein components into chylomicrons, TAG clearance from the blood, and remnant uptake by the liver. FA selectively released by digestive lipases from the primary positions of TAG regulate gastric secretion and motility (8–10). Many enzymes show FA specificity including FA transport proteins (11,12), enzymes of the 2-MAG pathway (13), and microsomal TAG transfer protein (14). The positional distribution of VLDL TAG depends on the FA available for TAG synthesis and the specificities of the enzymes of the glycerol-3-phosphate pathway for different FA. Availability of TAG FA as lipoprotein lipase substrate depends on the TAG structure (15).

Although many factors are known to possibly affect lipoprotein TAG structures, little is known of variation in TAG composition within the postprandial period or differences in the TAG structure-dependent clearance between chylomicron and VLDL TAG. Enzymatic methods for TAG structure analysis are laborious and require large sample amounts and are thus not suitable for large numbers of dilute physiological samples. Recently, the development of new analytical methods has led to first reports on selective postprandial behavior of TAG (7, 16,17).

Tandem MS is a powerful tool for TAG structure analysis of large numbers of dilute physiological samples. Several tandem MS methods potentially can be used for TAG structure determination. Information on TAG structure has been obtained with fragmentation of sodium (18) and lithium adducts of TAG (19), where the fragmentation of ammonium adducts produces only limited information on positional isomerism (20,21). Many of these methods have to date been applied to reference compounds only. The tandem MS analysis of TAG regioisomerism applied in our study is based on negative ionization with ammonia, collision-induced dissociation of selected TAG M.W. species, and calculation of results with a computer program (22,23). The method provides information on individual regioisomers and previously has been applied to various fats of vegetable and animal origin (24–26) as well as to chylomicron and VLDL TAG (6,7).

The aim of the current study was to quantify postprandial alterations in chylomicron and VLDL TAG structure and relate these alterations to the magnitude of lipemia. This is the first time, to our knowledge, that differences in the behavior of

individual TAG regioisomers between chylomicron and VLDL and postprandial changes in VLDL TAG regioisomer composition are reported. Understanding the factors influencing the magnitude of postprandial lipemia is important since increased postprandial lipemia is a risk factor for cardiovascular disease (27,28).

MATERIALS AND METHODS

Study design. This study had a randomized double-blind crossover design. Postprandial responses to two oral fat loads containing either lard or modified lard were measured over 8 h on two occasions 4 wk apart. The study plan was approved by the Ethics Committee of the University of Kuopio and Kuopio University Hospital.

Subjects. Seven female and two male volunteers were recruited from the student population of the University of Kuopio, Finland. Each subject gave written informed consent, and the subjects were free to discontinue their participation at any point without explanation. One volunteer discontinued the study during the second visit because of stomach discomfort 7 h after the lard meal, but her samples were included in the study.

The volunteers exhibited the following characteristics at the fasted state: age 24 ± 3 yr (mean ± SD), body mass index 21.5 ± 2.5 kg/m^2, total cholesterol 4.4 ± 0.7 mmol/L, LDL cholesterol 2.5 ± 0.7 mmol/L, HDL cholesterol 1.5 ± 0.3 mmol/L, TAG 0.9 ± 0.4 mmol/L, and plasma glucose 4.9 ± 0.4 mmol/L. There were no differences in the fasting values between the oral fat loads. Female volunteers were at the same stage of their menstrual cycle on both test days.

The subjects were asked to fast overnight (14 h) and advised not to consume alcohol or engage in strenuous exercise for 5 d before the fat load. The subjects were advised to eat as usual, but to avoid fatty foods the evening before the fat load. The subjects kept food records from Wednesday to Saturday the week prior to the test week and the day preceding the test day. According to the food diaries calculated with Diet 32 1.2 software (Aivo Finland Ltd., Turku, Finland), there were no statistical differences between the fat loads in the intake of energy nutrients (fat, protein, or carbohydrate), FA (saturated, monounsaturated, or polyunsaturated), or total energy.

Oral fat load. Refined lard was obtained from a meat processing company (Atria plc, Seinäjoki, Finland). Lard was further purified, and half of it was modified by an incomplete transesterification procedure at an oil processing company (Raisio Margarine, Raisio, Finland). The resulting two fats had different positional distributions of FA (Table 1) but similar FA compositions (Table 2), and they exhibited different melting characteristics. Measured by pulse NMR (Bruker Minispec NMS-100, Karlsruhe, Germany), 27.6% of lard and 18.3% of modified lard were solid at 30°C, 12.5% of lard and 11.0% of modified lard were solid at 35°C, and 8.3% of lard and 6.5% of modified lard were solid at 40°C.

The amount of fat in the test meal was 55 g per body square meter area according to the Dubois body surface chart. The fat was melted in a microwave oven and blended with hot ultra-high-temperature-treated skim milk low in lactose so that the fat percentage of the resulting mixture was 30. Chocolate aroma (0.1 g) (Chocolate flav-o-lok; Givaudan, Barneveld, The Netherlands) and 0.5 g of sweetener with 3% aspartame and 97% maltodextrin (G.D. Searle and Co., Paris, France) were added, and the blend was served. Five minutes prior to the ingestion of the test meal, a rice cake (8.2 g) topped with low-fat cheese [10 g cheese (12% fat, 7% saturated fat)/70 kg body weight; Valio Ltd., Helsinki, Finland] was served. Fat from the cheese accounted for 1–2% of the total fat. Water (1 dL) was provided with the meal. After 2 h subjects were provided water *ad libitum*.

Laboratory methods. A catheter was inserted into an antecubital vein for blood collection. Blood was drawn at the fasting

TABLE 1
Major TAG Positional Isomers of Lard and Modified Lard[a]

TAG	Lard	Modified lard
sn-18:1-16:0-18:0 + *sn*-18:0-16:0-18:1	19.9	12.7
sn-18:1-16:0-18:1	14.5	11.8
sn-18:2-16:0-18:1 + *sn*-18:1-16:0-18:2	7.6	7.0
sn-18:2-16:0-18:0 + *sn*-18:0-16:0-18:2	7.2	4.5
sn-18:0-16:0-18:0	6.5	1.3
sn-16:0-16:0-18:1 + *sn*-18:1-16:0-16:0	5.3	7.1
sn-16:0-16:0-18:0 + *sn*-18:0-16:0-16:0	4.2	4.1
sn-18:1-18:1-18:1	2.3	3.0
sn-18:1-18:1-18:0 + *sn*-18:0-18:1-18:1	2.3	2.9
sn-16:0-16:0-18:2 + *sn*-18:2-16:0-16:0	2.0	2.3
sn-16:0-18:1-18:0 + *sn*-18:0-18:1-16:0	1.9	1.0
sn-18:2-16:0-18:2	1.5	1.2
sn-16:0-18:0-18:1 + *sn*-18:1-18:0-16:0	1.3	2.2
sn-18:1-18:0-18:1	0.8	2.7
sn-18:1-18:0-18:0 + *sn*-18:0-18:0-18:1	0.8	1.8
sn-18:2-18:1-18:1 + *sn*-18:1-18:1-18:2	0.7	2.0
sn-16:0-18:1-18:1 + *sn*-18:1-18:1-16:0	0.1	1.8
Others	21.1	30.6

[a]Values are given as molar percentages of total fat.

TABLE 2
FA of Lard, Modified Lard, and Chylomicron TAG[a]

FA	Test fat	Chylomicrons						
		1.5 h	2 h	3 h	4 h	5 h	6 h	8 h
Lard treatment								
14:0[b]	1.4	1.87 ± 0.2	1.82 ± 0.2	1.69 ± 0.2	1.66 ± 0.2	1.61 ± 0.3	1.55 ± 0.1	1.40 ± 0.2
16:0	30.0	30.4 ± 1.7	30.2 ± 1.5	30.5 ± 1.9	30.1 ± 1.4[a]	30.7 ± 1.0[a]	30.4 ± 1.7	30.2 ± 1.1
16:1n-7	1.4	2.43 ± 1.0	2.32 ± 0.5	2.21 ± 0.7	2.32 ± 0.7	1.83 ± 0.5	1.89 ± 0.3	1.78 ± 0.2
18:0[b]	21.0	11.6 ± 2.3[a]	12.5 ± 1.6	11.8 ± 1.9[a]	12.6 ± 1.7[a]	13.0 ± 1.3[a]	13.1 ± 1.3[a]	13.8 ± 1.4[a]
18:1n-9	31.4	37.3 ± 0.7	36.2 ± 1.5	36.9 ± 0.6	36.5 ± 1.4	36.9 ± 1.0	36.5 ± 1.3	36.5 ± 0.9
18:1n-7	2.3	3.02 ± 0.6	2.97 ± 0.4	2.98 ± 0.8	2.89 ± 0.3	2.79 ± 0.2	2.68 ± 0.2	2.62 ± 0.4
18:2n-6	10.8	12.2 ± 0.9	11.7 ± 0.9	11.7 ± 1.3	11.9 ± 0.7	11.8 ± 0.7	11.6 ± 0.7	11.8 ± 1.0
18:3n-3	0.8	0.80 ± 0.5	0.85 ± 0.3	0.71 ± 0.5	0.95 ± 0.4	0.91 ± 0.1	0.88 ± 0.1	0.73 ± 0.3
Modified lard treatment								
14:0	1.5	1.66 ± 0.3	1.69 ± 0.2	1.69 ± 0.2	1.66 ± 0.2	1.60 ± 0.4	1.46 ± 0.2	1.43 ± 0.2
16:0	29.1	29.5 ± 1.1	29.6 ± 1.3	29.2 ± 1.5	29.1 ± 1.3[b]	29.2 ± 1.3[b]	29.4 ± 1.1	29.8 ± 0.9
16:1n-7[b]	1.5	2.25 ± 0.6	2.17 ± 0.5	2.18 ± 0.7	2.08 ± 0.4	1.90 ± 0.4	1.85 ± 0.3	1.80 ± 0.4
18:0	20.1	13.5 ± 2.0[b]	13.9 ± 2.0	13.8 ± 2.4[b]	14.0 ± 1.5[b]	14.2 ± 1.7[b]	14.8 ± 1.3[b]	14.4 ± 1.9[b]
18:1n-9	32.4	36.2 ± 1.7	36.6 ± 1.2	37.1 ± 1.1	37.0 ± 0.8	36.9 ± 0.8	36.5 ± 1.0	36.7 ± 0.7
18:1n-7	2.4	2.83 ± 0.6	2.88 ± 0.5	2.86 ± 0.6	2.96 ± 0.5	2.88 ± 0.6	2.73 ± 0.2	2.64 ± 0.4
18:2n-6	11.2	11.9 ± 1.2	11.7 ± 0.8	11.6 ± 1.0	11.6 ± 0.7	11.6 ± 0.6	11.6 ± 1.0	11.9 ± 0.9
18:3n-3	0.9	0.95 ± 0.4	0.92 ± 0.4	0.92 ± 0.4	0.98 ± 0.1	0.84 ± 0.3	0.92 ± 0.1	0.74 ± 0.4

[a]Values are relative proportions of TAG FA. Data are means of four determinations in lard and modified lard and mean ± SD in chylomicron TAG; $n = 9$ except for the 8-h time point after lard where $n = 8$. Values marked with different superscript roman letters (a,b) indicate a difference between treatments at the indicated time point ($P < 0.05$).
[b]Difference from the linear trend between the measured chylomicron TAG samples within treatment, $P < 0.05$.

state. After the oral fat load, blood was drawn at 20-min intervals during the first hour; at 30-min intervals during the second hour; and at 3, 4, 5, 6, and 8 h postprandially.

The isolation of chylomicron (Svedberg flotation > 400)- and VLDL (Svedberg flotation 20 to 400)-rich fractions and precipitation of LDL were performed as previously described (29). Plasma cholesterol, plasma TAG, and serum FFA concentrations were determined by enzymatic colorimetric methods (Monotest Cholesterol and Triglyceride GPO-PAP; Boehringer-Mannheim, Germany; NEFA C, ACS-ACOD method; Wako Chemicals GmbH, Neuss, Germany). Serum insulin was measured by an RIA method (Phadeseph Insulin RIA 100; Pharmacia Diagnostics, Uppsala, Sweden). Plasma glucose was analyzed with a glucose oxidase method (Glucose Auto&Stat, model GA-110; Daiichi, Kyoto, Japan). Incremental areas under the response curves for TAG in plasma, chylomicrons, and VLDL, and for cholesterol in chylomicrons and VLDL were calculated by the trapezoidal rule with Canvas™ software (Version 6; Deneba Software Inc., Miami, FL).

Lipids from the chylomicron and VLDL samples, collected at 90 min to 8 h postprandially, were extracted and TAG were separated from the extracted lipid mixture (30,31). FAME prepared from the TAG fraction for FA composition analysis (32) were analyzed by GC (PerkinElmer AutoSystem; Norwalk, CT) with HP INNOWax column (30 m × 0.32 mm i.d., 0.25 μm film thickness).

The M.W. distributions of the TAG of the test fats and the TAG extracted from the chylomicron and VLDL samples were determined with a triple quadrupole tandem mass spectrometer (TSQ-700; Finnigan MAT, San Jose, CA) in quadruplicate (24,33). TAG regioisomerism was determined with a tandem MS analysis based on negative ion CI and collision-induced dissociation with argon gas in quadruplicate and by using the same instrument as in the M.W. determination (22,24,34). The results were calculated with an MSPECTRA program (Nutrifen, Turku, Finland) (23). M.W. species corresponding to ACN:DB (acyl carbon number:number of double bonds) 50:1, 52:3, 52:2, and 54:3 were analyzed from chylomicron TAG and ACN:DB 50:2, 50:1, 52:3, and 52:2 from VLDL TAG.

Statistical methods. Normal distribution of the data was tested with the Shapiro–Wilk test. A paired-samples *t*-test or Wilcoxon matched-pairs signed rank test, depending on the normality of the distribution, were used when areas of total, chylomicron, or VLDL TAG; areas of plasma, chylomicron, or VLDL cholesterol; slopes of FFA curves from 1 to 3 h; individual time points of plasma, chylomicron, or VLDL TAG; chylomicron or VLDL FA and chylomicron or VLDL ACN:DB fractions were compared between the oral fat loads. ANOVA for repeated measures (general linear model test) was used when differences between chylomicron and VLDL attributes were compared within treatment. Student's *t*-test was used when chylomicron ACN:DB fractions were compared to those of fat ingested. SPSS for Windows (Version 10, SPSS Inc., Chicago, IL) was used.

RESULTS

Postprandial lipemia. The incremental area under the response curve of plasma TAG tended to be larger after modified lard than after lard ($P = 0.086$) (Fig. 1). Although significant differences were found in chylomicron TAG between the fat loads at the 60-min ($P = 0.039$) and 2-h ($P = 0.027$) time points, the incremental

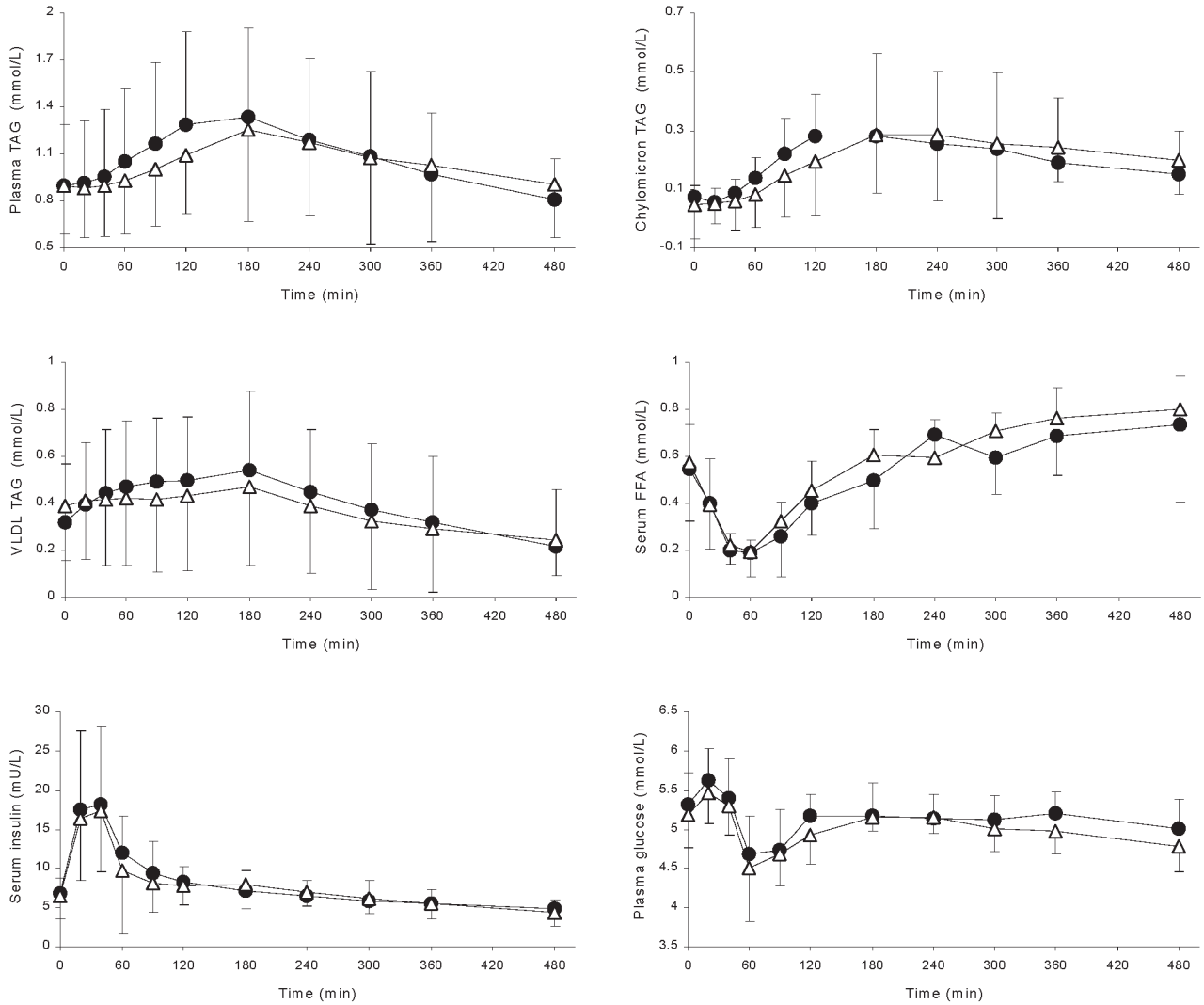

FIG. 1. Postprandial responses of plasma TAG, chylomicron TAG, VLDL TAG, FFA, insulin, and glucose after the meal containing lard (△) and modified lard (●).

areas did not differ. The incremental area under the response curve of VLDL TAG was larger after modified lard than after lard ($P = 0.021$) (Fig. 1).

Plasma FFA rose faster after lard than after modified lard 60 to 180 min postprandially ($P = 0.001$). Otherwise, there were no statistical differences in insulin, glucose, or FFA concentrations between the treatments (Fig. 1). There were no differences in the areas of plasma, chylomicron, or VLDL cholesterol (data not shown).

Chylomicron TAG FA composition, M.W. distribution, and structure. The proportion of stearic acid was larger in the fats ingested than in chylomicrons. Chylomicrons contained more stearic acid after consumption of modified lard than after lard ($P < 0.05$) at every measured time point except for the 2-h time point (Table 2).

Decreases of myristic acid ($P < 0.01$ after lard, $P = 0.118$ after modified lard) and palmitoleic acid ($P < 0.001$ after modified lard, $P = 0.099$ after lard), and an increase of stearic acid ($P = 0.002$ after lard, $P = 0.282$ after modified lard) were seen in chylomicrons from 1.5 to 8 h (Table 2).

The ACN:DB distribution of chylomicron TAG was different from that of fat ingested. The few exceptions are indicated in Figure 2. The drop of ACN:DB species 52:2 and 52:1, and in the case of lard also 52:0, from fat ingested to chylomicrons was large. TAG 48:0 (largely tripalmitin), 54:4, 54:3, 54:2, and 54:1 were present in higher quantities ($P < 0.05$ in five to seven time points) and TAG 52:4, 52:3, and 52:2 in lower quantities ($P < 0.05$ in four to seven time points) in chylomicrons after modified lard than after lard. M.W. fractions that behaved nonlinearly during the course of postprandial lipemia are shown in Figure 2.

Of the four ACN:DB species chosen for analysis of TAG structure (50:1, 52:3, 52:2, and 54:3), we detected 37 different regioisomers, of which 14 were abundant enough to be quantified. These isomers and their behavior during postprandial lipemia are presented in Figure 3. In general, TAG with

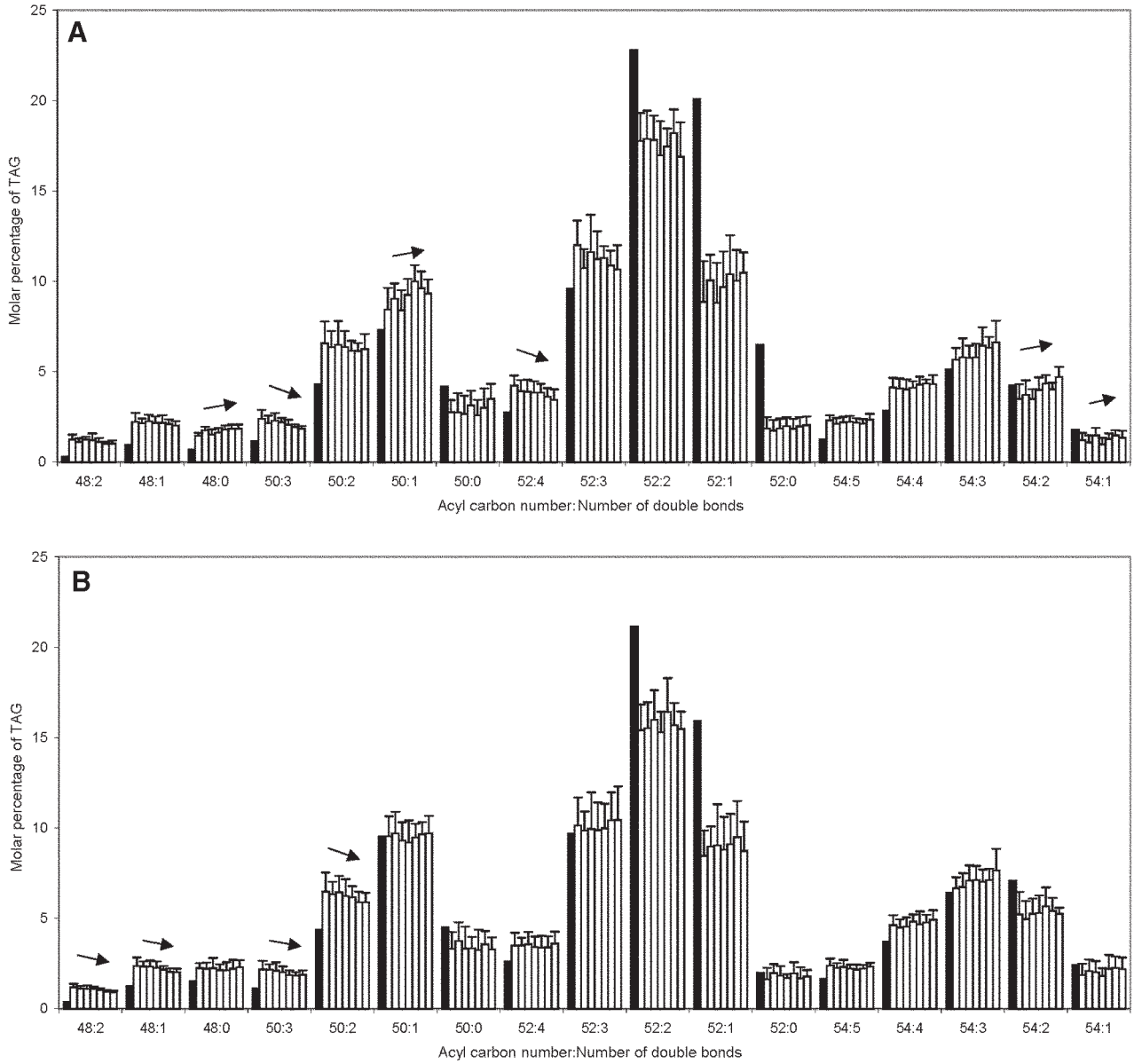

FIG. 2. Chylomicron TAG M.W. distribution during postprandial lipemia in subjects receiving an oral fat load containing lard (A) or modified lard (B). Values are means ± SD in chylomicrons; $n = 9$ except for the 8-h time point after lard where $n = 8$. Values of lard and modified lard are the mean of four determinations. Times of blood draw, 1.5, 2, 3, 4, 5, 6, and 8 h, from left to right, respectively, are represented by the open bars. Amount of each TAG in lard and modified lard is represented by the solid bars. An arrow indicates a difference from the linear trend in the general linear model test, $P < 0.05$, in the corresponding ACN:DB species between the measured time points in chylomicrons within treatment. All chylomicron TAG differ significantly ($P < 0.05$) from the same TAG in the corresponding fat load except the following: Lard: 50:0 8 h, 52:3 8 h, 54:3 2 h, 54:2 2 h, 4 h, 5 h, 6 h, 8 h, and 54:1 3 h. Modified lard: 50:1 all time points; 52:3 all time points; 52:0 1.5 h, 2 h, 3 h, 5 h, 6 h, 8 h; 54:3 1.5 h, 2 h, and 54:1 2 h, 3 h, 5 h, 6 h, 8 h. ACN, acyl carbon number; DB, double bond.

palmitic acid in the *sn*-2 position were underrepresented and TAG with oleic acid in the *sn*-2 position were overrepresented in chylomicrons when compared to fats ingested. Differences were greater after lard than after modified lard.

The between-subject variation was large, and no significant indications of selective chylomicron TAG formation or clearance were seen. The only significant differences during lipemia were an increase in *sn*-18:1-18:2-18:0 + *sn*-18:0-18:2-18:1 after the lard meal, and fluctuation of *sn*-18:2-18:0-18:1 + *sn*-18:1-18:0-18:2 after both meals ($P < 0.05$) (Fig. 3). The decrease of ACN:DB 52:2 from fat ingested to chylomicrons was seen in *sn*-18:2-16:0-18:0 + *sn*-18:0-16:0-18:2 and *sn*-18:1-16:0-18:1 but not in *sn*-16:0-18:1-18:1 + *sn*-18:1-18:1-16:0.

VLDL TAG FA composition, M.W. distribution, and structure. FA composition of VLDL TAG was similar regardless of the positional distribution of fat ingested. The proportion of stearic acid in VLDL TAG was one-sixth to one-fourth of that in fat ingested and one-third to one-half of that in chylomicrons (Table 3).

The behavior of all studied FA was nonlinear ($P < 0.05$) with respect to time except for the behavior of linoleic acid

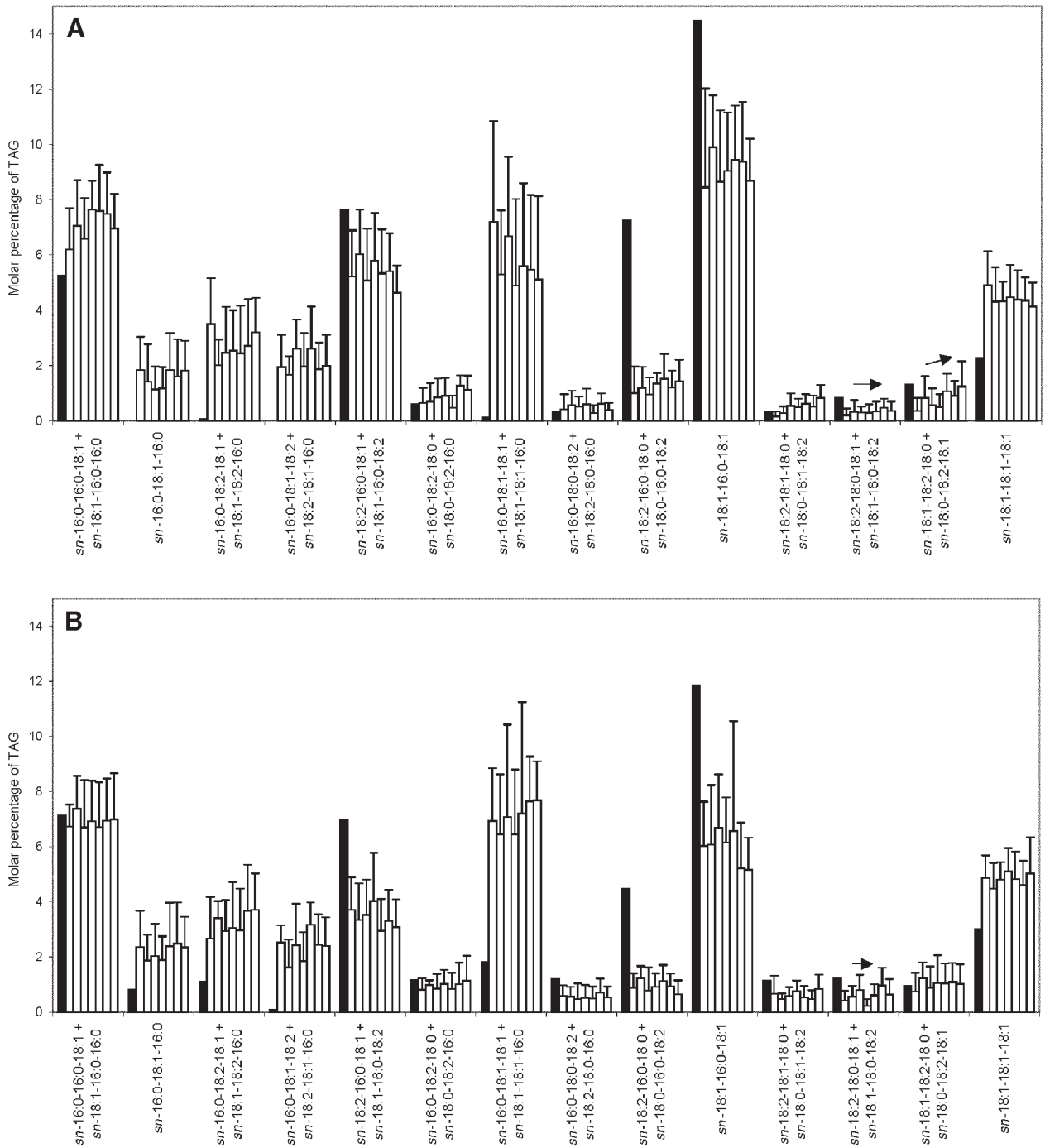

FIG. 3. Positional distribution of chylomicron TAG with ACN:DB 50:1, 52:3, 52:2, and 54:3 during postprandial lipemia in subjects receiving an oral fat load containing lard (A) or modified lard (B). Values are mean ± SD; $n = 9$ except for the 8-h time point after lard where $n = 8$. Values of lard and modified lard are the mean of four determinations. Times of blood draw, 1.5, 2, 3, 4, 5, 6, and 8 h, from left to right, respectively, are represented by the open bars. Amount of each TAG in lard and modified lard is represented by the solid bars. An arrow indicates a difference from the linear trend in the general linear model test, $P < 0.05$, in the corresponding isomer between the measured time points in chylomicrons within treatment. For abbreviations see Figure 2.

after the lard meal. The relative proportions of palmitic and stearic acids increased during the postprandial time period of 1.5 to 8 h, whereas the proportions of myristic and unsaturated FA decreased (Table 3).

Clear increasing and decreasing ($P < 0.05$) trends were seen in the VLDL TAG ACN:DB distribution during postprandial lipemia (Fig. 4). A total of 39 regioisomers were identified from VLDL ACN:DB fractions 50:2, 50:1, 52:3, and 52:2. Of these, 19 were abundant enough to be quantified (Fig. 5). Between-treatment differences were modest. Many VLDL regioisomers behaved nonlinearly during postprandial lipemia. The relative proportion of TAG with palmitic and linoleic acids in the *sn*-2

TABLE 3
FA of VLDL TAG[a]

	1.5 h	2 h	3 h	4 h	5 h	6 h	8 h
Lard treatment							
14:0[b]	1.86 ± 0.4	1.88 ± 0.4	1.88 ± 0.4	1.55 ± 0.2	1.34 ± 0.2	1.31 ± 0.5	0.94 ± 0.2
16:0[b]	28.0 ± 4.0	28.4 ± 4.1	29.6 ± 4.0	31.2 ± 4.8	32.0 ± 3.8	32.2 ± 3.6	33.2 ± 2.5
16:1n-7[b]	4.66 ± 1.0	4.52 ± 0.9	4.14 ± 0.8	3.63 ± 0.7	3.25 ± 0.6	2.86 ± 0.6	2.37 ± 0.5
18:0[b]	3.34 ± 0.9	3.80 ± 0.9	4.68 ± 1.1	4.90 ± 1.1	5.16 ± 1.3	5.57 ± 1.2	6.19 ± 2.1
18:1n-9[b]	41.6 ± 2.8	41.2 ± 2.8	40.0 ± 2.5	39.2 ± 2.9[a]	38.9 ± 2.7[a]	38.1 ± 2.1[a]	37.3 ± 1.9
18:1n-7[b]	3.83 ± 0.7	3.67 ± 0.7	3.47 ± 0.6	3.30 ± 0.6	3.26 ± 0.5	3.08 ± 0.4	3.17 ± 1.0
18:2n-6	15.0 ± 2.8	14.9 ± 2.7	14.5 ± 2.5	14.4 ± 2.6	14.6 ± 2.2	14.8 ± 1.8	15.0 ± 1.6
18:3n-3[b]	1.40 ± 0.3	1.39 ± 0.3	1.32 ± 0.2	1.20 ± 0.3	1.15 ± 0.2	1.07 ± 0.2	0.89 ± 0.2
Modified lard treatment							
14:0[b]	1.90 ± 0.6	1.97 ± 0.5	1.87 ± 0.4	1.68 ± 0.4	1.44 ± 0.3	1.27 ± 0.3	1.10 ± 0.2
16:0[b]	27.1 ± 3.6	27.8 ± 3.4	28.4 ± 3.4	29.7 ± 3.7	30.5 ± 3.4	30.6 ± 2.9	31.9 ± 3.0
16:1n-7[b]	4.63 ± 1.2	4.50 ± 1.1	3.61 ± 1.6	3.75 ± 0.7	3.37 ± 0.6	3.11 ± 0.5	2.72 ± 0.6
18:0[b]	3.78 ± 0.8	4.36 ± 1.1	4.97 ± 1.0	5.13 ± 1.1	5.35 ± 1.1	5.36 ± 0.8	5.75 ± 1.2
18:1n-9[b]	42.2 ± 2.7	41.4 ± 2.5	41.3 ± 2.3	40.6 ± 2.4[b]	40.2 ± 1.9[b]	39.6 ± 2.7[b]	38.6 ± 2.6
18:1n-7[b]	3.66 ± 0.8	3.57 ± 0.8	3.46 ± 0.8	3.32 ± 0.6	3.29 ± 0.6	3.15 ± 0.5	3.07 ± 0.5
18:2n-6[b]	15.0 ± 2.4	14.6 ± 2.4	14.5 ± 2.0	14.3 ± 2.1	14.4 ± 1.9	14.7 ± 2.0	15.1 ± 1.8
18:3n-3[b]	1.43 ± 0.3	1.41 ± 0.3	1.39 ± 0.3	1.27 ± 0.3	1.17 ± 0.3	1.13 ± 0.2	0.83 ± 0.5

[a]Values are relative proportions of VLDL TAG FA. Values are mean ± SD; $n = 9$ except for the 8-h time point after lard where $n = 8$. Values marked with different superscript roman letters (a,b) indicate a difference between treatments at the indicated time ($P < 0.05$).
[b]Difference from the linear trend between the measured time points within treatment, $P < 0.05$.

positions increased ($P < 0.05$) during the course of postprandial lipemia, and the relative proportion of TAG with palmitoleic acid in the *sn*-2 position decreased (combined data not shown). Of the individual TAG, *sn*-14:0-18:1-18:1 + *sn*-18:1-18:1-14:0, *sn*-16:0-16:1-18:1 + *sn*-18:1-16:1-16:0, and *sn*-16:1-18:1-18:1 + *sn*-18:1-18:1-16:1 decreased ($P < 0.05$); and *sn*-16:0-16:0-18:2 + *sn*-18:2-16:0-16:0, *sn*-16:0-16:0-18:1 + *sn*-18:1-16:0-16:0, *sn*-16:0-18:1-16:0, and *sn*-16:0-18:1-18:2 + *sn*-18:2-18:1-16:0 increased ($P < 0.05$) after both fats. In agreement with decreasing palmitoleic acid concentration, all TAG containing palmitoleic acid either decreased or remained constant. However, the behavior of TAG containing palmitoleic acid did not seem to depend on the position of palmitoleic acid in the molecule. All four TAG with two palmitic acid residues increased, although the increase of one of these four regioisomers was significant only after the lard meal. The behavior of TAG containing one palmitic acid residue seemed to be related more to the M.W. of TAG than to the location of palmitic acid in the TAG molecule. Palmitic acid was preferentially situated at the *sn*-1/3 position, but, interestingly, when TAG contained two palmitic acid residues, they were preferentially at the *sn*-1(3),2-positions instead of the *sn*-1 and *sn*-3 positions. The most abundant (15–17% of all VLDL TAG after both oils) TAG in VLDL was *sn*-16:0-18:1-18:1 + *sn*-18:1-18:1-16:0.

DISCUSSION

It is evident from plasma, chylomicron, and VLDL TAG responses that lard and modified lard were metabolized differently. Although modified lard caused larger incremental plasma and VLDL TAG areas, lard caused longer-lasting TAG responses.

Modified lard, containing less solid fat at body temperature, may have been more easily emulsified and therefore more rapidly absorbed than lard. Lard contains more stearic acid at the *sn*-1/3 positions of TAG, and thus more stearic acid could have been lost to feces as calcium soaps after lard than after modified lard as indicated by the chylomicron FA composition. Stearic acid was probably also burned to energy, metabolized to oleic acid, and blended with endogenous FA. Differences in FFA curves between treatments may be an indication of between-treatment differences in clearance rates. Although the proportion of stearic acid and the proportion of saturated FA in the *sn*-2 position of TAG have been linked to delayed chylomicron clearance rates in animal studies (35,36), the constantly larger *sn*-2 saturated FA or smaller stearic acid concentration after lard than after modified lard are unlikely causes for the differences observed in the chylomicron TAG curves.

Both constant (37) and changing (16) behaviors of chylomicron FA composition have been reported. After a meal containing olive oil, triolein and oleic acid decreased whereas polyunsaturated TAG, saturated TAG, and palmitic and stearic acids tended to increase (16). In our study, triolein, oleic acid, and palmitic acid remained constant, but the proportion of stearic acid increased after lard. Since we observed similar behavior after both fats, strong stereoselective chylomicron TAG FA uptake to adipose tissue or release to plasma is unlikely. It has been proposed that, *in vivo,* chylomicrons are subjected to the action of lipoprotein lipase long enough for the isomerization of 2-MAG to 1(3)-MAG and complete hydrolysis (5).

The differences between the ACN:DB distribution of fat ingested and chylomicron TAG were due to the rearrangement of FA of the *sn*-1 and *sn*-3 positions and the influence of FA of endogenous origin. The availability of both free palmitic acid and 2-monopalmitin for TAG synthesis explains the larger proportion of 48:0 after the modified lard meal.

We reported (7) that chylomicron TAG M.W. affected TAG composition. The chylomicron TAG that decreased significantly during the course of postprandial lipemia after the meals

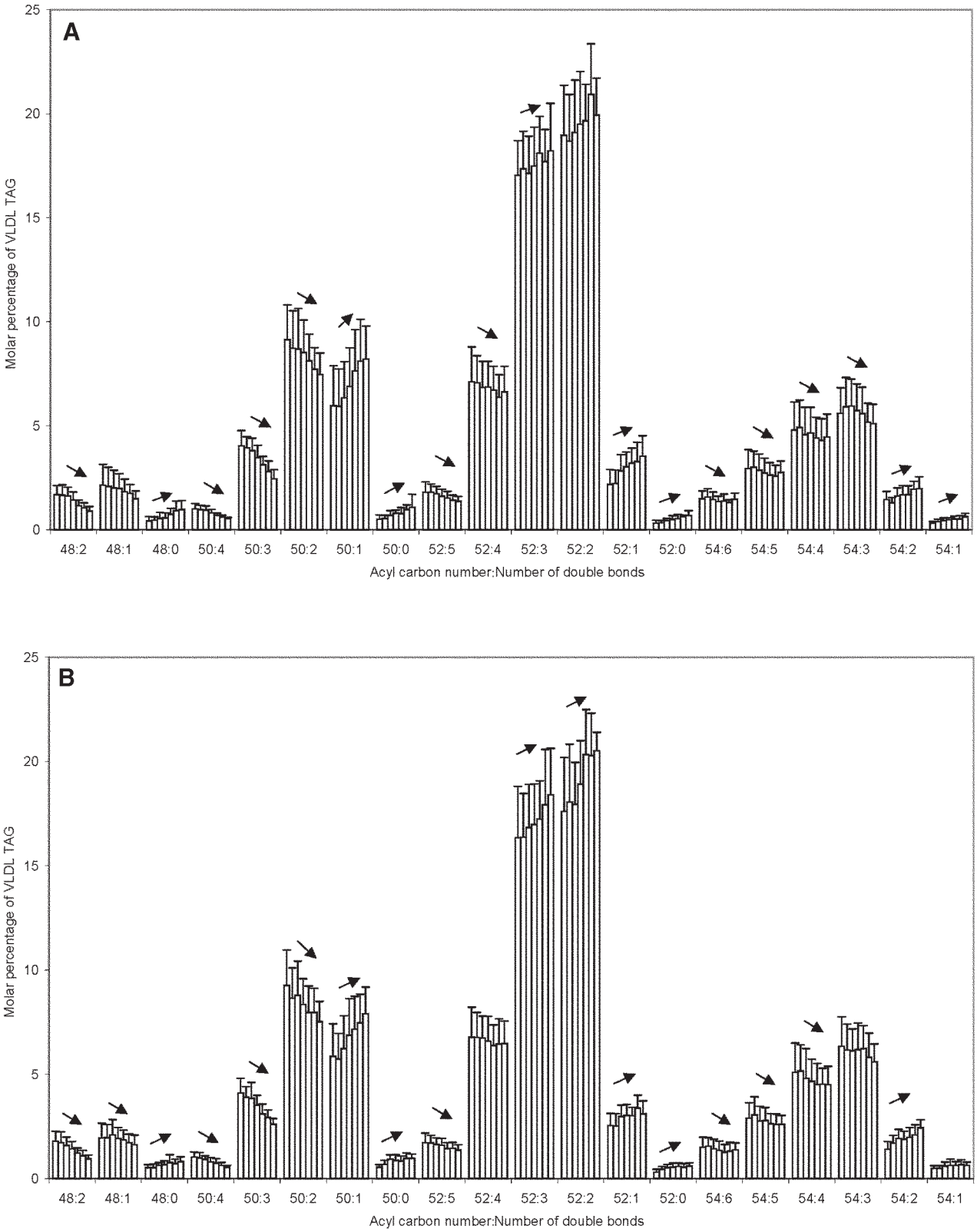

FIG. 4. VLDL TAG M.W. distribution during postprandial lipemia in subjects receiving an oral fat load containing lard (A) or modified lard (B). Values are means ± SD; $n = 9$ except for the 8-h time point after lard where $n = 8$. Times of blood draw, 1.5, 2, 3, 4, 5, 6, and 8 h, from left to right, respectively, are represented by the bars. An arrow indicates a difference from the linear trend in the general linear model test, $P < 0.05$, in the corresponding ACN:DB species between the measured time points. For abbreviations see Figure 2.

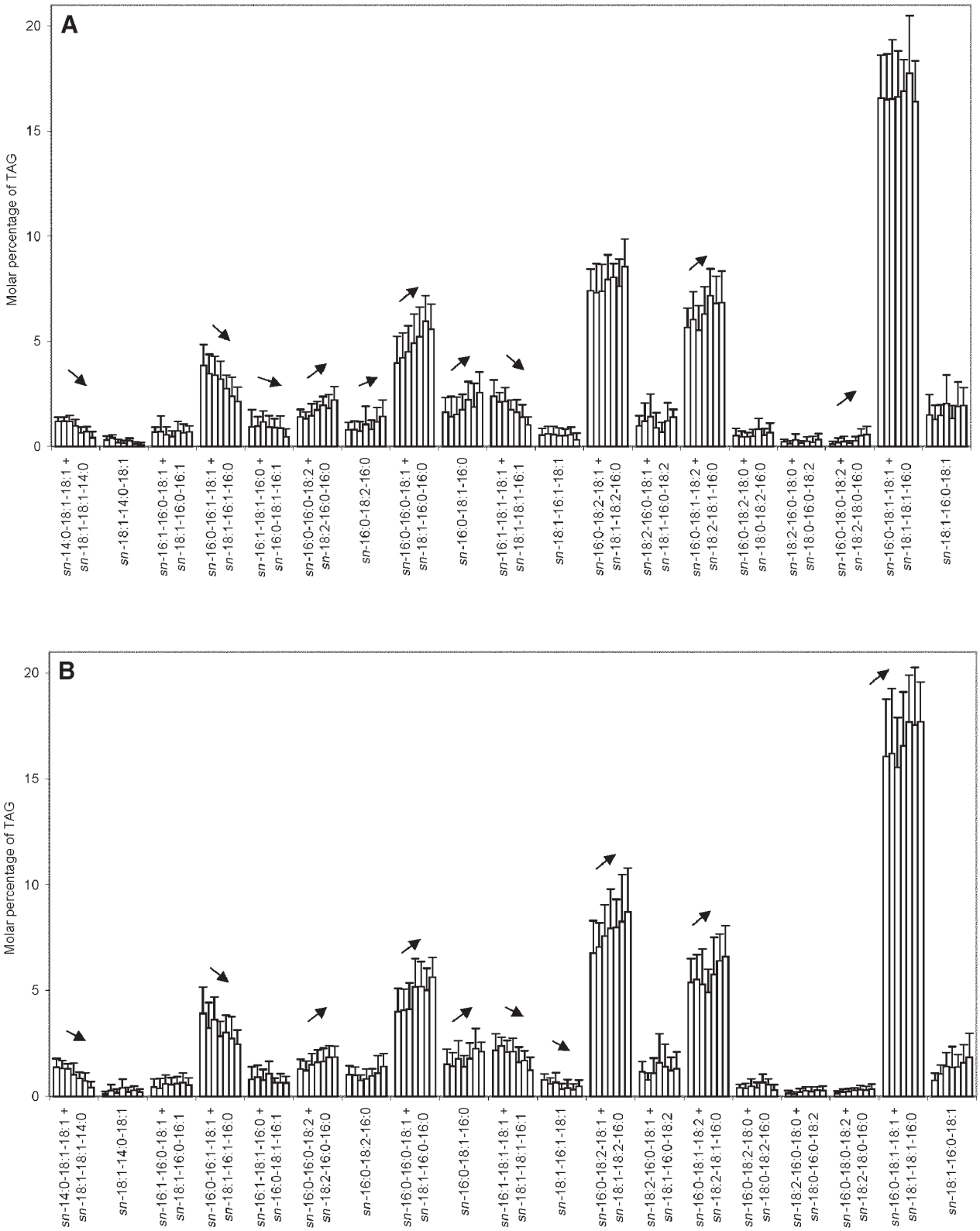

FIG. 5. Positional distribution of VLDL TAG with ACN:DB 50:2, 50:1, 52:3, and 52:2 during postprandial lipemia in subjects receiving an oral fat load containing lard (A) or modified lard (B). Values are mean ± SD; $n = 9$ except for the 8-h time point after lard where $n = 8$. Times of blood draw, 1.5, 2, 3, 4, 5, 6, and 8 h, from left to right, respectively, are represented by the bars. An arrow indicates a difference from the linear trend in the general linear model test, $P < 0.05$, in the corresponding isomer between the measured time points. For abbreviations see Figure 2.

containing palm oil and modified palm oil generally also tended to decrease in this study. TAG that remained constant in the palm oil trial remained constant in the current study with the exception of 48:0, which increased after lard. In the palm oil-based trial, TAG clearance was more rapid as the number of double bonds increased in each ACN fraction. This effect was less clearly seen in the current study. Despite differences of FA composition and positional distribution, M.W. distribution seems to be a relatively constant determinant affecting the clearance of TAG from chylomicrons.

We previously found that the proportion of *sn*-16:0-18:1-16:0 was less at 1.5 h postprandially than at later time points (7), possibly indicating initial loss of palmitic acid from the *sn*-1/3 positions. In the current study, the behavior of this TAG was linear throughout the observed time period, possibly due to lesser content of the isomer. Unlike in the palm oil-based trial, no significant differences were found in the behavior of *sn*-16:0-18:1-18:1 + *sn*-18:1-18:1-16:0 in chylomicron TAG during the postprandial period. The contents of the two TAG, *sn*-18:2-18:0-18:1 + *sn*-18:1-18:0-18:2 (after both oils) and *sn*-18:1-18:2-18:0 + *sn*-18:0-18:2-18:1 (after lard only), in which significantly nonlinear behavior with respect to time was found, were among the smallest quantified, and their fluctuation has little physiological significance. Our studies indicate that TAG regioisomers remain in chylomicrons in fairly constant proportions during the course of postprandial lipemia, but that the behavior of some TAG may depend on their content in plasma.

FA from chylomicron TAG are transferred to the liver both as albumin-bound FFA and as TAG in chylomicron remnants, and these FA are largely packed into the VLDL particles. It is therefore possible that the stronger initial chylomicron TAG response after modified lard triggered the larger response measured in VLDL TAG. It has been suggested that when more lipid is delivered to the liver in chylomicron remnants, the secretion of VLDL apolipoprotein (apo)B-100 increases (38). VLDL TAG FA, M.W., or regioisomers do not explain the difference in the plasma VLDL TAG concentrations.

Although dietary factors influence VLDL TAG composition (39), there is a correlation in the human VLDL structures between different studies. The main VLDL TAG structures of people on a high oleic acid diet were dioleoylpalmitoylglycerol and linoleoyloleoylpalmitoylglycerol (in Ref. 39, co-eluted with dioleoylpalmitoleoylglycerol), both comprising more than or nearly 20% of all TAG (39,40). Of the four M.W. we studied, dioleoylpalmitoylglycerol was the most abundant TAG, consisting largely of *sn*-16:0-18:1-18:1 + *sn*-18:1-18:1-16:0 and little *sn*-18:1-16:0-18:1. In our study linoleoyloleoylpalmitoylglycerol was divided into three regioisomers of which *sn*-16:0-18:2-18:1 + *sn*-18:1-18:2-16:0 was most abundant, followed by *sn*-16:0-18:1-18:2 + *sn*-18:2-18:1-16:0 in almost the same quantity, and a minor proportion of *sn*-18:2-16:0-18:1 + *sn*-18:1-16:0-18:2.

Whereas the chylomicon TAG structure remained constant, selective formation or clearance was seen in almost every VLDL TAG studied. Structural changes in VLDL composition have been observed previously: Ågren *et al.* (17) compared the composition of normal fasting VLDL TAG of rats to that obtained after blocking lipolysis by Triton WR1339. They suggested that the FA of the *sn*-1 position would be the most decisive factor in determining the sensitivity for hydrolysis, and that the nature of the FA, its position, and other FA in the TAG molecule affect the accessibility of TAG to lipase (17). The differences between chylomicron and VLDL TAG behavior were likely to be related to differences in apoB-48- and apoB-100-induced functions. Lipid composition varies between chylomicrons and VLDL also because, in the intestine, lipids are used mainly for chylomicron assembly, whereas in the liver lipids are used, in addition to VLDL assembly, for oxidation and bile acid synthesis. Chylomicrons compete with VLDL for removal of TAG by lipoprotein lipase (41). It can thus be speculated that VLDL TAG could be exposed to the stereoselective lipolysis of lipoprotein lipase longer than chylomicron TAG.

We have shown in this study that the magnitude of postprandial lipemia is altered by the positional distribution of saturated FA in the fat ingested. Unlike that of chylomicron TAG, the formation and/or clearance of VLDL TAG was selective 1.5 to 8 h postprandially. New molecular-level information on the postprandial alterations in chylomicron and VLDL TAG was obtained.

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Phospholipids Affect the Intestinal Absorption of Carotenoids in Mice

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ABSTRACT: Previously, we have shown that uptake of carotenoids solubilized with mixed micelles by human intestinal Caco-2 cells is enhanced by lysophosphatidylcholine (lysoPC) and suppressed by PC. This study determined the effect of PC and lysoPC in mixed micelles on the accumulation of β-carotene and lutein in mice in order to elucidate the roles of micellar phospholipid in the intestinal uptake of carotenoids *in vivo*. Mixed micelles were composed of 2.5 mM monooleoylglycerol, 7.5 mM oleic acid, 12 mM sodium taurocholate, 200 μM carotenoid, and 3 mM phospholipid in PBS. The mice were fed single doses of β-carotene or lutein solubilized in PC (PC group), lysoPC (LPC group), and no phospholipid (NoPL group) micelles. The β-carotene responses in the plasma and liver of the PC group were markedly lower than those of the other two groups, whereas no differences were noticed between the LPC and NoPL groups. The average level of lutein in the plasma of the PC group after administration was significantly ($P < 0.05$) lower than those of the other groups. Moreover, the average level of lutein in the liver was significantly ($P < 0.05$) different among the groups in the order of LPC > NoPL >PC. Thus, the results clearly indicate that PC suppressed the accumulation of β-carotene and lutein in plasma and liver and that lysoPC enhanced the accumulation of lutein in liver. These results suggest that the hydrolysis of PC to lysoPC plays an important role in the intestinal uptake of carotenoids solubilized in mixed micelles.

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Carotenoids are known to have beneficial functions in human health against major clinical diseases such as cancer, cardiovascular diseases, and age-related macular degeneration (1–4), although the mechanisms of their action at the molecular level still remain unknown (5,6). More information on the absorption and metabolic conversion of carotenoids is required to further understand their biological actions. The absorption of dietary carotenoids from foods involves several steps: the breakdown of the food matrix to release the carotenoids, dispersion in lipid emulsion particles, solubilization in mixed micelles, movement across the unstirred water layer adjacent to the microvilli, uptake by the cells of intestinal mucosa, and incorporation into chylomicron (7–9). Thus, the carotenoids must be solubilized in mixed micelles before cellular uptake. The mixed micelles are composed of bile acids, cholesterol, and PC, which are secreted as bile fluid. Dietary fat induces the secretion of bile, and its hydrolysates, such as MAG and FA, are also included in the mixed micelles. The micelles have a disklike structure, where phospholipids and FA form a bilayer with bile acids located at the edges of the disk (10,11). Thus, the processes up to the solubilization in the mixed micelles are dependent mostly on the physicochemical properties of food and carotenoids, and on bile secretion and dietary components such as fats, sterols, and fiber (12–18).

However, the detailed absorption processes of carotenoids after solubilization in mixed micelles have not been fully revealed. The early findings in perfused rat intestine and in hBRIE380 rat intestinal cells (19,20) support a simple diffusion mechanism for the cellular uptake of carotenoids. That mechanism was also supported by the linear relationship found in our recent study between the cellular uptake of micellar carotenoid and its hydrophobicity (21). Thus, the size and lipid composition of the mixed micelles would affect the movement of carotenoids from micelles to the intestinal cells by diffusion. Recent studies have indicated that phospholipids in the mixed micelles and phospholipase A_2 profoundly affect the cellular uptake of cholesterol and α-tocopherol (22–25). We also have found that PC suppresses the cellular uptake of carotenoids solubilized in mixed micelles by the human intestinal Caco-2 cells, whereas lysophosphatidylcholine (lysoPC) significantly enhances their uptake (21). Thus, phospholipids derived from bile and foods would affect the cellular uptake of carotenoids solubilized in mixed micelles formed in the intestinal tract. The present study was conducted to evaluate the accumulation of carotenoids in the plasma and liver of mice fed mixed micelles in order to clarify the *in vivo* effects of PC and lysoPC in mixed micelles upon intestinal uptake of carotenoids.

MATERIALS AND METHODS

Materials. All-*trans*-β-carotene (type IV), all-*trans*-retinol, retinyl palmitate, monooleoylglycerol, and sodium taurocholate were purchased from Sigma Chemical Co. (St. Louis, MO). Lutein was kindly donated by Kyowa Hakko Kogyo (Tokyo, Japan). *d*-α-Tocopherol was obtained from Eisai Co. (Tokyo, Japan). Egg-yolk PC and lysoPC were obtained from Q.P. Co. (Tokyo, Japan). HPLC grade acetonitrile and oleic acid (>99%) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Other chemicals and solvents were of reagent

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Abbreviations: AUC, area under the curve; LPC group, group fed mixed micelles containing lysoPC; lysoPC, lysophosphatidylcholine; NoPL group, group fed mixed micelles containing no phospholipid; PC group, group fed mixed micelles containing PC.

grade. All-*trans*-β-carotene (>98.8%) and lutein (>99%) were purified by passing them through a neutral alumina column (Brockman III; ICN Biomedicals, Eschwege, Germany) using hexane and methanol, respectively. Purified all-*trans*-β-carotene and lutein were dissolved in hexane and dichloromethane, respectively, and stored at −80°C until used. Retinyl palmitate was purified (>99.0%) by HPLC on a TSK gel ODS 80Ts column, 6.4 × 250 mm (Tosoh, Tokyo, Japan) with ethyl acetate/methanol (30:70, vol/vol) as the mobile phase. The purity of β-carotene, lutein, and retinyl palmitate was checked by HPLC, and the purity was estimated based on the peak area of components absorbing at specific wavelengths.

Animals. Male ICR mice (7 wk old) obtained from Clea, Japan Inc. (Tokyo, Japan) were housed at 25°C with a 12-h light/dark cycle. The animals had free access to tap water and a commercial diet (MF, Oriental Yeast Co., Osaka, Japan). After 7 d of feeding, mice weighing 32 ± 2 g were deprived of food for 14–15 h before carotenoid administration. The mice were handled according to the guidelines for experimental animals of the National Food Research Institute, Ibaraki, Japan.

Preparation of micelles and feeding. Mixed micelles in PBS containing 2.5 mM monooleoylglycerol, 7.5 mM oleic acid, 12 mM sodium taurocholate, and 200 μM β-carotene or lutein with either 3 mM PC, 3 mM lysoPC, or no phospholipid were prepared. Appropriate amounts of these chemicals dissolved in hexane or methanol were mixed to reach the final concentration. The solvent was evaporated using argon gas and the dried mixture was redissolved in PBS with vigorous mixing using a vortex mixer to obtain an optically clear solution. The micelle composition chosen was based on the composition of the clear layer obtained by ultracentrifugation of the duodenal content of healthy adult human subjects given a TG-rich meal (26). It would hypothetically produce a mixture of mixed micelles and small unilamellar vesicles, according to the phase diagram indicated by Staggers *et al.* (27). The vesicles can resolve spontaneously into the mixed micelles as the ratio of lipid to cholic acid decreases during the absorption process. Thus, the optically clear solutions obtained by the procedure described above were used as the mixed micelles in the present study.

The concentrations of β-carotene and lutein in the micelles were checked by HPLC before the mice were fed. The mice were randomly divided into seven groups. Six groups were fed with either β-carotene or lutein solubilized in the mixed micelles containing no phospholipid (NoPL group), PC (PC group), or lysoPC (LPC group). The other group was not fed mixed micelles (zero-time control). The mixed micelles (0.2 mL/mouse) were administered to the mice by direct intubation to the stomach. The volume size of intubation had no adverse effects on mice. Mice in the zero-time control (n = 5) and in each treatment group (n = 5/time point) at 1, 2, 3, 6, and 9 h after administration were anesthetized with diethyl ether and killed by exsanguination. Blood was collected from the caudal vena cava with a heparinized syringe. The livers were removed and washed with ice-cold isotonic saline. Blood was immediately separated into plasma by centrifugation at 1000 × g for 15 min at 4°C. The plasma and liver were immediately stored at −80°C until analyzed. The amount of carotenoid fed was calculated to be 0.671 mg/kg body weight and was comparable to the amounts reported in studies in which β-carotene was supplemented to human subjects. In a preliminary experiment in mice, one-tenth of the carotenoid level used in the present study was administered, but the accumulation of carotenoids in plasma was undetectable.

Extraction from plasma and liver. β-Carotene, retinol, retinyl palmitate, and lutein were extracted from the plasma according to the procedures described previously by Sugawara *et al.* (21) with slight modifications. Briefly, the plasma (0.4 mL) was diluted to 0.8 mL with ice-cold deionized water, and 3 mL of dichloromethane/methanol (1:2, vol/vol) containing 0.2 μmol α-tocopherol was vigorously mixed with the plasma for 1 min using a vortex mixer. Then 1.5 mL of hexane was mixed with the solution. The resulting upper layer of hexane/dichloromethane was withdrawn. The extraction procedure was repeated for the lower layer two more times using 1 mL of dichloromethane and 1.5 mL of hexane. The combined extract was evaporated to dryness under a stream of argon gas. The plasma extract used for the analyses of β-carotene, retinol, and retinyl palmitate was dissolved in dichloromethane/methanol (2:1, vol/vol), and that used for the analysis of lutein was dissolved in methanol.

Liver samples were homogenized with 9 parts ice-cold isotonic saline with a Potter–Elvehjem homogenizer, and 0.8 mL of the homogenate was used for extraction by the same procedure as described for the plasma. In the case of the groups fed lutein, the extract was further saponified by incubating in 2 mL of 10 M KOH at 60°C for 45 min. Subsequently, 2 mL of ice-cold deionized water was added, and lutein was extracted as described above. Samples were handled and homogenization and extraction were carried out on ice under dim yellow light to minimize isomerization and oxidation by light irradiation.

HPLC analyses. β-Carotene, retinol, retinyl palmitate, and lutein in the extracts of plasma and liver were quantified with an HPLC system consisting of an LC-10AD pump, an SPD-10A UV-vis absorbance detector (Shimadzu, Kyoto, Japan), an AS-8020 autosampler (Tosoh), and a personal computer equipped with EZChrome Chromatography Data System software (Scientific Software Inc., Pleasanton, CA). All the components were separated on a TSK gel ODS-80Ts column (Tosoh), 4.6 × 150 mm, attached to a precolumn (2 × 20 mm) of Pelliguard LC-18 (Supelco Inc., Bellefonte, PA). The column was kept in an oven at 20°C. Ethyl acetate/methanol (30:70, vol/vol) containing 0.1% ammonium acetate was used as a mobile phase for the analyses of β-carotene, retinol, and retinyl ester, and ethyl acetate/acetonitrile/methanol/water (21:23:53:3, by vol) containing 0.1% ammonium acetate was used for the analysis of lutein. An isocratic analysis was performed at a flow rate of 1 mL/min. β-Carotene and lutein were monitored at 450 nm with a UV-vis absorbance detector, and retinol and retinyl palmitate were monitored at 325 nm. They were quantified from their peak areas by use of the standard curves of reference compounds. The peak identity

of these components was further confirmed by their characteristic UV-vis spectra, recorded with a model 1100 HPLC system equipped with a photodiode array detector (Hewlett-Packard, Palo Alto, CA).

Statistical analysis. To quantify the postprandial β-carotene and lutein levels in the plasma and liver over 9 h, the area under the curve (AUC) was calculated by trapezoidal approximation. Data were tested for the homogeneity of variances by the Bartlett test. When homogenous variances were confirmed, the data were tested by ANOVA, and significant differences in means among groups and at different time intervals were evaluated by Tukey's test. The values underwent log transformation before the tests if necessary. The differences between β-carotene and lutein levels were analyzed nonparametrically by the Kruskal–Wallis test, and significant differences in means were evaluated by the Mann–Whitney U test. Differences were considered significant at a level of $P < 0.05$. All analyses were performed using StatView software version 5.0J (SAS Institute Inc., Cary, NC).

RESULTS

Neither β-carotene nor lutein was detected in the plasma and liver of mice before the administration of micellar carotenoids. After the administration of β-carotene solubilized in the micelles, the β-carotene level in the plasma reached a maximum at 2 h in the PC and LPC groups and at 1 h in the NoPL group (Fig. 1A). The maximal levels in the PC, LPC, and NoPL groups were 2.6 ± 0.2, 36.4 ± 11.3, and 26.3 ± 10.4 nM, respectively. The β-carotene in the plasma then decreased to levels significantly lower than the maximal levels observed at 6 h after administration in all of the groups ($P < 0.05$). No significant differences were found between the NoPL and LPC groups by two-way ANOVA, whereas the β-carotene level of the PC group was markedly lower than those of the NoPL and lysoPC groups. The AUC of plasma β-carotene was calculated from the curves shown in Figure 1A. The NoPL and LPC groups had similar AUC values, whereas the PC group had an extremely low AUC value (Table 1).

Lutein levels in the plasma after administration of micellar lutein are shown in Figure 1B. Levels in the NoPL and PC groups reached maxima of 4.75 and 4.30 nM, respectively, at 1 h after administration, whereas that in the LPC group reached a maximum of 9.99 nM at 2 h. The average levels of lutein at 1 to 6 h after administration were significantly different ($P < 0.05$) among the three groups by two-way ANOVA. The average level of plasma lutein in the PC group (2.72 pmol/mL) during that time was significantly lower ($P < 0.05$) than those in the NoPL and LPC groups (3.64 and 4.80 pmol/mL, respectively). The AUC value of lutein in the PC group was lower than those in the NoPL and LPC groups, consistent with the average levels. The average level of lutein in the LPC group was not significantly different from that in the NoPL group, and the AUC value of the LPC group was the same as that of the NoPL group. However, the lutein in the LPC group reached the maximum, which was significantly higher than those of the NoPL

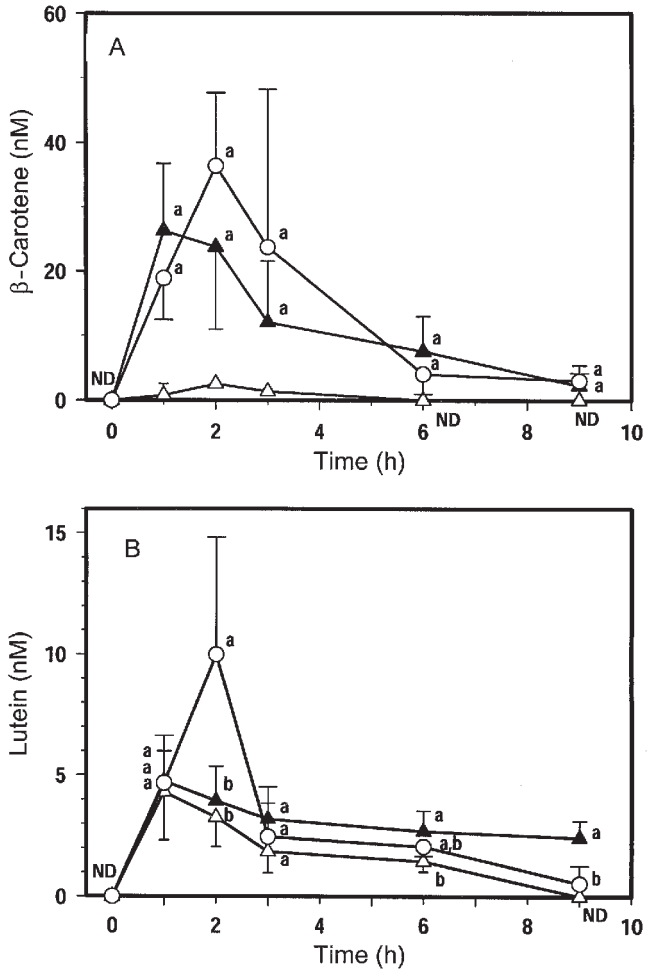

FIG. 1. β-Carotene and lutein levels in the plasma of mice after the administration of carotenoids solubilized in mixed micelles. Micelles were composed of 2.5 mM monooleoylglycerol, 7.5 mM oleic acid, 12 mM sodium taurocholate, and 200 μM β-carotene (A) or lutein (B) with 3 mM PC (△), lysophosphatidylcholine (lysoPC) (○), or no phospholipid (▲). Mice were fed a single dose of micelles (0.2 mL) and then sacrificed after various time intervals. β-Carotene and lutein in the plasma were analyzed by HPLC. Data represent the mean ± SD ($n = 5$). The values at each time point not sharing a common letter are significantly different ($P < 0.05$) between groups as determined by one-way ANOVA and Tukey's test after log transformation. β-Carotene and lutein at zero hour, β-carotene at 6 and 9 h, and lutein at 9 h in the PC group were not detected (ND). Data on β-carotene in the PC group were not included in statistical analyses.

TABLE 1
Area Under the Curve for β-Carotene and Lutein Levels in the Plasma and Liver of Mice over 9 h After Administration of Carotenoids Solubilized in Mixed Micelles[a]

	β-Carotene		Lutein	
Group	Plasma (pmol/mL·h)	Liver (pmol/g.·h)	Plasma (pmol/mL·h)	Liver (pmol/g·h)
NoPL	100.3	505.3	26.8	109.5
PC	6.2	ND	15.6	78.4
LPC	119.5	467.7	26.6	148.4

[a]NoPL, group fed mixed micelles containing no phospholipid; PC, group fed mixed micelles containing PC; LPC, group fed mixed micelles containing lysophosphatidylcholine; ND, not detected.

and PC groups at 2 h after administration ($P < 0.05$). The levels of β-carotene in the plasma at 1 and 2 h after administration were significantly higher than those of lutein in both the NoPL and LPC groups ($P < 0.05$). Consequently, the AUC value of β-carotene was approximately four times the value of lutein in the NoPL and LPC groups.

The response of plasma retinyl palmitate to β-carotene administration is shown in Figure 2. No significant difference was found among the three groups by two-way ANOVA. In both the PC and NoPL groups, no significant difference was observed between time points. On the other hand, the plasma retinyl palmitate in the LPC group was significantly ($P < 0.05$) increased from the baseline level (24.7 ± 10.7 nM) to 65.8 ± 27.7 nM at 3 h after administration. There was no significant difference in the levels of free retinol in the plasma among the treatment groups (data not shown).

β-Carotene levels in the liver after the administration of micellar β-carotene are shown in Figure 3A. No β-carotene was detected in the liver of the PC group at any time point (detection limit, 0.13 pmol/g liver). On the other hand, β-carotene levels in the liver of the LPC and NoPL groups reached maxima at 3 h after administration. The maximal levels in the NoPL and LPC groups were 79.4 ± 33.9 and 90.1 ± 44.6 pmol/g, respectively. The levels then decreased significantly at 9 h after administration ($P < 0.05$). No significant difference in the average levels of β-carotene was found between the LPC and NoPL groups by two-way ANOVA. The AUC value of liver β-carotene in the NoPL group was similar to that in the LPC group. Thus, the effects of micelles on the accumulation of β-carotene in the liver were similar to those observed in plasma.

Lutein levels in the liver after the administration of micellar lutein are shown in Figure 3B. They reached maxima at 2 h after administration. The maximal levels in the NoPL, PC, and LPC groups were 16.9 ± 2.3, 18.0 ± 4.7, and 21.1 ± 2.3 pmol/g, respectively, without any significant difference among the groups. After reaching the maximal level, the lutein level of the LPC group remained elevated, whereas those of the other two groups decreased significantly by 3 h after administration ($P < 0.05$). The average values of lutein after administration in the NoPL, PC, and LPC groups were 13.05, 9.88, and 17.16 pmol/g, respectively, and were significantly different between the groups ($P < 0.05$, by two-way ANOVA). Consistent with the average values, the AUC values of liver lutein could be ordered by group as follows: LPC > NoPL > PC. The β-carotene levels in the liver were significantly higher than those of lutein at the time points from 1 to 6 h after administration in the NoPL and LPC groups ($P <$

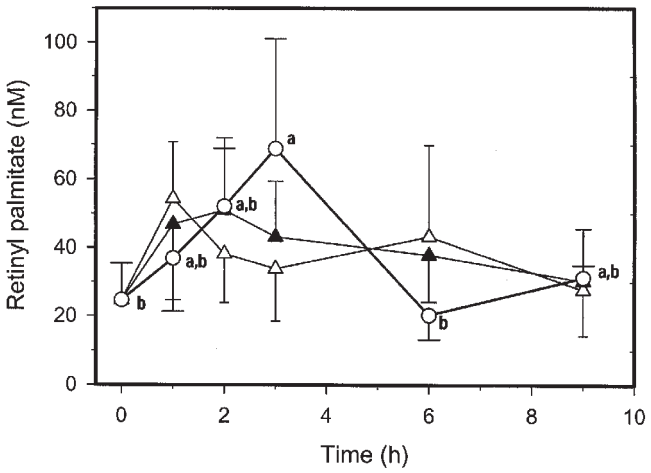

FIG. 2. Retinyl palmitate level in the plasma of mice after the administration of β-carotene solubilized in mixed micelles. Mice were fed micelles containing β-carotene and treated as described in Figure 1. Retinyl palmitate in the plasma was analyzed by HPLC. Data represent the mean ± SD ($n = 5$). Values not sharing a common letter in the lysoPC group are significantly different ($P < 0.05$) among time points as determined by one-way ANOVA and Tukey's test. PC, △; lysoPC, ○; no phospholipid, ▲. For abbreviation see Figure 1.

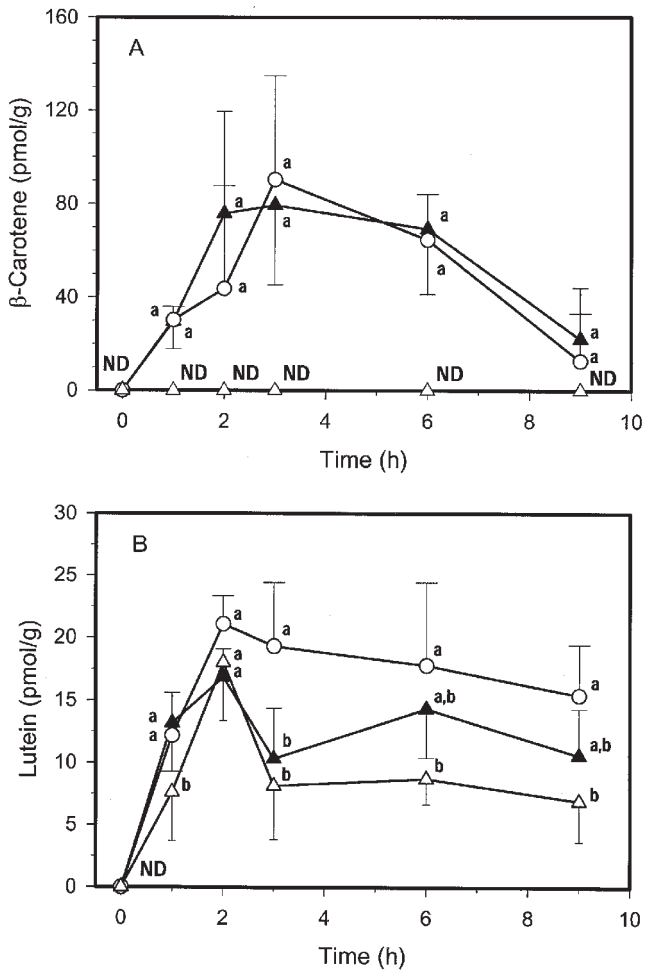

FIG. 3. β-Carotene and lutein levels in the liver of mice after administration of carotenoids solubilized in mixed micelles. Mice were fed micelles containing β-carotene (A) or lutein (B) and treated as described in Figure 1. β-Carotene and lutein in the liver were analyzed by HPLC. Data represent the mean ± SD ($n = 5$). The values at each time point not sharing a common letter are significantly different ($P < 0.05$) among groups as determined by one-way ANOVA and Tukey's test. β-Carotene and lutein at zero hour and β-carotene in the PC group at any of the time points were not detected (ND). PC, △; lysoPC, ○; no phospholipid, ▲. For abbreviation see Figure 1.

0.05), except for the 2-h point in the LPC group. As observed in plasma, the AUC value of β-carotene in the liver was about four times the value of lutein, except in the PC group.

DISCUSSION

The present study was conducted to elucidate the *in vivo* effects of phospholipids in mixed micelles on the intestinal uptake of carotenoids by monitoring the appearance of carotenoids in plasma and liver after feeding carotenoids and phospholipids to mice. To eliminate the uncertain solubilization state of carotenoids, the carotenoids were solubilized in the respective mixed micelles, and mice were then intubated with the micelles, instead of feeding diets mixed with carotenoids and phospholipids. β-Carotene levels in the plasma and liver after oral administration were not significantly different between the NoPL and LPC groups, but were markedly lower in the PC group. The AUC values and the average levels of lutein both in plasma and in liver were lower in the PC group than in the NoPL group. Thus, the results indicated clearly that PC in the mixed micelles suppressed the accumulation of lutein as well as β-carotene. The higher average level of lutein in the liver and its AUC value in the LPC group compared with those in the other two groups indicated that lysoPC enhanced accumulation of lutein in the liver. The lutein level in the plasma of the LPC group at 2 h after administration was higher than those of the other two groups, whereas the average level of plasma lutein was not significantly different between the NoPL and LPC groups. These results suggest that the lutein accumulated quickly in the liver, although its appearance in plasma was temporarily enhanced by lysoPC. The enhanced accumulation of β-carotene by lysoPC was not clearly observed from the data on β-carotene in the plasma and liver. However, plasma retinyl palmitate in the LPC group increased significantly to a level higher than the baseline level at 2 h after administration. The increase in retinyl palmitate might be due to the enhanced uptake of β-carotene in the intestinal cells, where β-carotene was converted to retinyl ester. The enhancement in the level of retinyl palmitate in the plasma was estimated to be 41.1 nM. This value corresponded to *ca.* 20 nM β-carotene, on the assumption that one molecule of β-carotene was converted to two molecules of retinal by the central cleavage enzyme in the intestinal cells (28). As the maximal β-carotene in the plasma of the LPC group was 36.4 nM, a considerable amount of β-carotene might have been converted to retinyl palmitate. However, it was not clear from the data on plasma retinyl palmitate whether lysoPC enhanced the uptake of β-carotene, because no significant differences in retinyl palmitate levels in the plasma were found between the groups. In particular, the levels of retinyl palmitate in the PC group were not significantly different from those of the other two groups, although the intestinal uptake of β-carotene was remarkably suppressed in the PC group. The large variance in the background level of retinyl palmitate made it difficult to evaluate the postprandial increase in retinyl palmitate. Taken together, these results suggest that PC suppresses the intestinal uptake of both β-carotene and lutein, whereas lysoPC enhances the uptake of lutein. However, the reason lysoPC had no effect on the level of β-carotene remains to be clarified.

It is not certain whether the mixed micelles fed by direct intubation to the stomach in the present study reached the intestinal tract or were reconstituted as micelles in the intestinal tract after disintegrating in the stomach. Nonetheless, the results of the present study were basically consistent with those of our previous study, in which the carotenoids solubilized in micelles were directly incubated with cultured human intestinal Caco-2 cells. Moreover, in the present study, the accumulation of micellar lutein was lower than that of β-carotene in both the plasma and liver except in the PC group, even though a significant amount of β-carotene might have been converted to vitamin A. This result was consistent with the previous finding in which a linear relationship between the uptake of carotenoids solubilized in lysoPC micelles and their hydrophobicity was observed in Caco-2 cells.

The ratio between the AUC values of β-carotene and lutein was approximately 4 in both the plasma and liver except in the PC group. This finding indicates that there was no discrimination between β-carotene and lutein in the incorporation from the plasma to the liver once the carotenoids were absorbed. As the incorporation of carotenoids into the liver is mediated through a chylomicron remnant, whole carotenoids present in chylomicron would be incorporated into the liver (29). In contrast, micellar carotenoids would be discriminated in the uptake by intestinal cells, and some part of the β-carotene might be converted to retinyl ester in the cells. The decline in carotenoid levels in the plasma and liver after reaching maxima would reflect the distribution of carotenoids to other tissues. As the liver has the second-highest activity of β-carotene dioxygenase among the tissues (30), conversion of β-carotene to vitamin A may be involved in the decline of β-carotene levels in the liver.

The results of the present study and the previous study with Caco-2 cells suggest that the hydrolysis of phospholipids in the intestinal tract by phospholipase A_2 is required for the efficient uptake of carotenoids into intestinal cells, although PC plays an important role in the solubilization of carotenoids in lipid emulsions (31). The mechanism underlying these effects is not yet fully understood. PC, with two long-chain acyl moieties, is more hydrophobic than lysoPC, with one acyl moiety and a free hydroxyl group. Therefore, PC has a greater affinity for hydrophobic carotenoid molecules than does lysoPC (32). The uptake of PC itself by intestinal cells is known to be much lower than that of lysoPC (23,24). Thus, PC can strongly retain the carotenoid in mixed micelles so that the uptake of carotenoid to intestinal cells is suppressed. LysoPC can be taken up by the cells of the jejunum across the unstirred water layer, whereas bile acids are taken up later in the ileum. LysoPC might associate with the carotenoid and facilitate its diffusion across the water layer from micelles to the brush border membrane of intestinal cells. Moreover, the lysoPC taken up into the intestinal cells is quickly converted

to PC and TAG, which then stimulates the synthesis of TAG and the secretion of chylomicron (33–35). Therefore, the increased cellular level of lipids and their secretion into the lymph may shift the equilibrium of the carotenoid partition from the micelles toward the cells and lymph.

The *in vivo* effects of phospholipids on carotenoid uptake observed in the present study were similar to those found on the uptake of cholesterol and α-tocopherol (23–25). The intestinal uptake of such highly hydrophobic substances may partly follow an identical mechanism. These properties of phospholipids would make it possible to modify the bioavailability of the hydrophobic substances with diets and supplements rich in phospholipids. Since carotenoids were fed to mice after direct solubilization in mixed micelles in the present study, it is uncertain whether phospholipids present in foods can influence the bioavailability of dietary carotenoids. However, the amount of phospholipids fed to mice in the present study was *ca.* 19 μmol/kg body weight, which was comparable to the daily ingestion of dietary PC (0.91–1.85 mmol) in the Western diet (36). Moreover, dietary supplementation with PC was reported to decrease cholesterol absorption in the human intestine (37). Therefore, dietary phospholipids have high potential to modify the bioavailability of carotenoids.

The present study is the first report to indicate that PC in mixed micelles suppresses the accumulation of carotenoids in mouse plasma and liver while lysoPC enhances the accumulation of lutein in liver. Thus, our earlier *in vitro* results and the present *in vivo* results suggest that the hydrolysis of PC to lysoPC plays an important role in the intestinal uptake of carotenoids solubilized in the mixed micelles and that dietary phospholipids modify the bioavailability of carotenoids. The mechanism of these effects of phospholipids in mixed micelles on the intestinal uptake of carotenoids and, in particular, its relationship to intestinal lipid metabolism, deserve further study.

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Effect of Structure and Form on the Ability of Plant Sterols to Inhibit Cholesterol Absorption in Hamsters

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ABSTRACT: We investigated the effect of three types of plant sterols (4-desmethylsterols, 4,4′-dimethylsterols, and pentacyclic triterpene alcohols) in three forms (free, esterified with FA, or with phenolic acids) on cholesterol absorption. Plant sterol fractions derived from soybean (99% 4-desmethylsterols), rice bran (70% 4,4′-dimethylsterols), or shea nut (89% pentacyclic triterpene alcohols) were fed to male hamsters (n = 20/group) as free sterols or esterified with FA or phenolic acids (cinnamic or ferulic). Cholesterol absorption was measured after 5–8.5 (mean, 7) wk by a dual-isotope technique. Soybean sterol intake significantly reduced cholesterol absorption efficiency (23%) and plasma total cholesterol (11%). Rice bran sterols tended to lower cholesterol absorption efficiency by 7% and plasma total cholesterol by 5%, whereas shea nut sterols had no effect. In hamsters, dietary 4-desmethylsterols were more effective than 4,4′-dimethylsterols in lowering cholesterol absorption and levels of cholesterol in blood. Pentacyclic triterpene alcohols had no effect on the absorption of cholesterol or on its level in blood. Esterification with FA did not impair the ability of 4-desmethylsterols and 4,4′-dimethylsterols to inhibit cholesterol absorption, whereas esterification with phenolic acids reduced this ability. This study supports the use of 4-desmethylsterols, esterified with FA to increase solubility, as the most effective cholesterol-lowering plant sterols in the diet.

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Blood cholesterol levels can be modified by dietary changes. A decreased intake of saturated FA and/or cholesterol and a higher consumption of unsaturated FA, especially PUFA, reduce blood total and LDL cholesterol levels (1,2). In addition, soluble or viscous fibers in the diet (3) or the substitution of soy protein for animal protein (4) can lower total and LDL cholesterol levels. Other dietary components that reduce blood total and LDL cholesterol levels are phytosterols or plant sterols, minor constituents of vegetable oils present in the unsaponifiable fraction. The majority of crude vegetable oils contain 0.1–0.5 g phytosterols/100 g of oil (5). Phytosterols are related structurally to cholesterol, but differ in their side-chain configuration and are not synthesized endogenously in mammals. There is a wide variety of phytosterol structures, which can be classified into three groups (Fig. 1): 4-desmethylsterols, 4-monomethylsterols, and 4,4′-dimethylsterols (6). Strictly speaking, pentacyclic triterpene alcohols are not plant sterols, but they are commonly categorized as a type of 4,4′-dimethylsterol. Phytosterols in the Western diet are almost exclusively 4-desmethylsterols (5).

Plant sterols inhibit the intestinal absorption of cholesterol in animals and humans (7), leading to lowered blood cholesterol levels. In general, plant sterols themselves are poorly absorbed (efficiency typically <5%) compared with cholesterol (>40%), but differences in the effect on cholesterol absorption efficacy have been suggested for different 4-desmethylsterols (8). Saturated and unsaturated 4-desmethylsterols esterified with FA do not differ in their capacity to lower cholesterol absorption efficiency (9,10) and their hypocholesterolemic effect (11).

Plant sterols from rice bran (largely 4,4′-dimethylsterols) have been reported to lower blood cholesterol effectively in one study with human subjects (12) but not in another study (11). In hamsters, 4,4′-dimethylsterols from rice bran oil lowered blood cholesterol to a lesser extent than 4-desmethylsterols and stanols (13). Shea nut sterols (largely pentacyclic

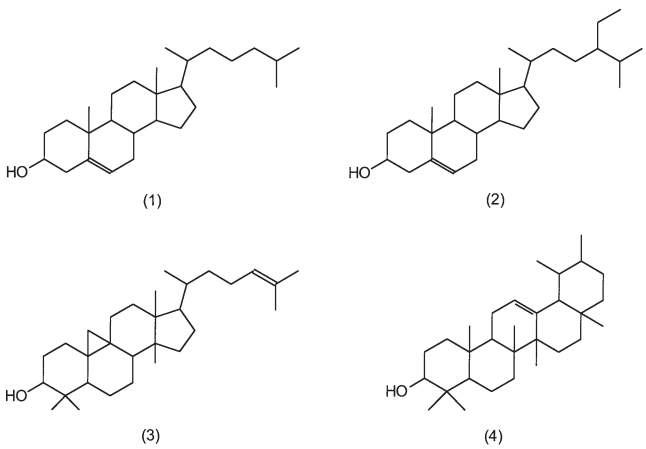

FIG. 1. Structures of plant sterols and pentacyclic triterpene alcohols in soybean oil, rice bran oil, and shea nut fat compared with the structure of cholesterol (1). Soybean oil contains mainly β-sitosterol (2) and other 4-desmethylsterols. Rice bran oil contains mainly the 4,4′-dimethylsterols cycloartenol (3) and 24-methylene cycloartanol. Shea nut fat contains mainly pentacyclic triterpene alcohols such as α-amyrine (4). In pentacyclic triterpene alcohols, the cyclopentane ring specific for sterols is replaced by two cyclohexane rings.

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Abbreviations: FG, free glycerol; IDL, intermediate-density lipoproteins; MCT, medium-chain triglycerides; TC, total cholesterol; TG, total glycerol.

triterpene alcohols) were reported to be ineffective in lowering levels of blood cholesterol in humans (11,12,14).

From the preceding, it appears that the structure of plant sterols affects their ability to inhibit cholesterol absorption and lower levels of cholesterol in blood. The effect of plant sterol esterification beyond increasing solubility is not clear. Esterification of 4-desmethylsterols to FA improves their solubility in dietary fat and may affect the interaction with cholesterol in the gut, thereby influencing the ability to inhibit cholesterol absorption. Rice bran and shea nut sterols occur as esters of phenolic acids (ferulic and cinnamic, respectively). Whether this affects the ability of these types of sterol structures to inhibit cholesterol absorption is unknown.

The objective of this study was to investigate the effects of sterol structure and form on cholesterol absorption and on the resulting blood and hepatic cholesterol levels. The hamster was chosen as the animal model because characteristics of cholesterol metabolism in hamsters resemble the human situation more closely than in other animal models such as rabbits or rats (15).

EXPERIMENTAL PROCEDURES

Animals and study design. The Animal Ethics Committee of Unilever Research, Vlaardingen, The Netherlands approved the study protocol. Male F1B hybrid Syrian golden hamsters (n = 240; Bio Breeder Inc., Fitchburg, MA), 8–9 wk old at arrival, were used. After arrival, the hamsters were allowed to adapt for 4 wk and were fed a regular SDS diet (Special Diet Services, Witham, England). The hamsters were housed individually in Makrolon type II cages, with a layer of sawdust as bedding under a 12-h light/dark cycle with the light cycle starting at 7:00 A.M. Hamsters were allocated to 12 experimental groups (n = 20/group), on the basis of similar mean and distribution of body weights. Owing to the large number of animals in this study, a staggered design was used. Each experimental group was divided in two subsets of 10 hamsters; the first subset started the experiment 1 wk earlier then the second subset. The 12 groups consumed 12 different semipurified diets (see below) *ad libitum* for 4 wk. Food intake was determined over four consecutive days in weeks 2 and 4 of the experiment. Body weight was determined weekly.

After 4 wk of the experiment on two consecutive days, <1 mL blood was obtained under inhalation anesthesia by retroorbital puncture. Only plasma total cholesterol was measured. Based on the differences in blood total cholesterol with the control group, it was decided to determine cholesterol absorption in all groups except for those fed cinnamic or ferulic acid. To measure cholesterol absorption, the dietary phase of the study was extended on average for three more weeks (range 1–4.5 wk). After determination of cholesterol absorption at the end of the experiment (see below), all hamsters that had not been food deprived (two of each group per day) were killed, after blood sampling by retroorbital puncture in the first 5 h of the light period, over 10 d in four consecutive weeks. Autopsy and macroscopical inspection were conducted on all hamsters.

Diets. Plant sterols were fed in their unesterified form, or esterified with different acids, i.e., FA or phenolic (ferulic or cinnamic) acids. Rice bran oil and shea nut fat contain 4,4′-dimethylsterols, in nature esterified mainly to ferulic acid (6) and cinnamic acid (16), respectively.

Diets, to which sodium salts of cinnamic acid or ferulic acid were added, were also included in the study to establish whether these phenolic acids had an independent effect on cholesterol metabolism. Thus, the following test substances were used: sodium salt of cinnamic acid; sodium salt of ferulic acid; unesterified shea nut sterols; shea nut sterols esterified with cinnamic acid; shea nut sterols esterified with sunflower FA; unesterified rice bran sterols; rice bran sterols esterified with ferulic acid; rice bran sterols esterified with sunflower FA; unesterified soybean sterols; soybean sterols esterified with cinnamic acid; and soybean sterols esterified with sunflower FA.

The levels of cinnamic and ferulic acid in the two phenolic acid diets were made equal to those in the diets containing plant sterols esterified with these phenolic acids. The sodium salt of either acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was prepared by dissolving each acid in a solution of sodium chloride. After freeze-drying for 1 d, the remaining salt powder of phenolic acids was mixed into the diets.

Soybean sterols occur predominantly in their unesterified form and are commercially isolated from oil deodorizer distillates (Cognis Corporation, LaGrange, IL). Rice bran sterols occur mainly as ferulic acid esters, which are called oryzanol, and are commercially isolated as such from soapstocks of the alkali refining of rice bran oil (Tsuno, Wakayama, Japan). Shea nut sterols occur mainly as cinnamic acid esters. A shea nut sterol concentrate was obtained from Loders Croklaan (Wormerveer, The Netherlands). The three sources of phytosterols were used for this hamster trial without further changes and were also used to prepare the other forms of sterols (esters) tested.

Free sterols from rice bran and shea nut were prepared in crude form by alkaline hydrolysis of the naturally occurring ester form. Sunflower FA esters of all three types of sterols and cinnamic acid ester of soybean sterols were prepared by a transesterification reaction of sterols with sunflower FA methyl esters or cinnamic acid methyl ester.

In Table 1, the composition of the control diet is shown. Cinnamic and ferulic acid (either unesterified or esterified) and sterols (expressed as unesterified sterol equivalents when sterol esters were used) were exchanged for dietary fiber (Solka-Floc; International Fiber Corp., North Tonawanda, NY). FA esterified with sterols were exchanged for FA of the TAG in the dietary fat blend. The unesterified sterols and sterol esters were dissolved in the fat blend. Cholesterol (0.01% w/w) was added to the diet to amplify differences in blood cholesterol-lowering efficacy of phytosterols, if any. Diets contained 30 energy percentage (en%) fat with a Western composition (PUFA/monousaturated FA/saturated FA = 1:1.65:1.5). The dietary fat blend was composed of 61.05% palm oil + 15.55% high-oleic acid sunflower oil (Trisun 80;

TABLE 1
Diet Composition

Diet component	(g/1000 kJ)	(g/100 g)	Energy (kJ)	Energy (en%)
Calcium caseinate	14.640	23.565	230.1	23.0
Fat blend[a]	8.100	13.038	299.7	30.0
Maize starch	34.420	55.403	469.8	47.0
Solka-Floc[b]	3.900	6.278		
Mineral mixture RMH.88[c]	0.860	1.384		
Vitamin mixture VIT'90[d]	0.200	0.322		
Cholesterol	0.006	0.010		
Total	62.126	100.000	999.6	100.0

[a]FA of the sterols esterified with sunflower oil FA were exchanged for TAG in the dietary fat blend.
[b]Cinnamic and ferulic acid (either free or esterified) and sterols (expressed as free sterol equivalents when esters were used) were exchanged for Solka-Floc.
[c]Composition in mg/MJ: primary potassium phosphate, K_2HPO_4, 112.89; potassium bicarbonate, $KHCO_3$, 170.88; potassium chloride, KCl, 83.18; calcium carbonate, $CaCO_3$, 70.11; secondary magnesium phosphate, $MgHPO_4 \cdot 3H_2O$, 227.21; trisodium citrate-dihydrate, $Na_3C_6H_5O_7 \cdot 2H_2O$, 168.98; manganese sulfate, $MnSO_4 \cdot H_2O$, 12.22; ferric citrate (19% Fe), $FeC_6H_5O_7 \cdot 5H_2O$, 10.43; copper citrate (35% Cu), $CuC_6H_6O_7 \cdot xH_2O$, 1.12; zinc citrate, $Zn_3(C_6H_5O_7)_2 \cdot 3H_2O$, 2.97; potassium iodate, KIO_3, 0.017.
[d]Composition in mg/MJ: thiamine mononitrate, 0.36; riboflavin, 0.36; nicotinamide, 1.20; calcium pantothenate, 1.20; choline chloride 50%, 119.5; myo-inositol, 5.98; folic acid, 0.06; biotin, 0.012; pyridoxine, 0.38; vitamin B_{12}, 3.10; vitamin A (325 IU/mg), 1.84; vitamin D_3 (1000 IU/mg), 0.60; vitamin K_3 (22.7%), 0.24; *dl*-α-tocopherol (dry powder 50%), 9.56; calcium silicate, 11.95; sucrose, 43.658.

SVO Specialty Products, Cleveland, OH) + 12.65% safflower oil + 10.75% soybean oil.

Two batches of each test diet were produced; the first batch was prepared 1 wk before the experiment started and the second batch was produced after 4 wk of the experiment. Total sterols (unesterified and esterified) were measured in the first batch of each test diet. To check homogeneity, dietary fat concentration was determined in both batches of each test diet.

Analyses of diets. Fat was extracted from diets (aliquots of 15–20 g) using dichloromethane. After evaporation of dichloromethane, the fat content in the extract (aliquot of 2.5–3.0 g) was determined gravimetrically. Extracted fat phases of the diets were saponified by methanolic NaOH and the FA were methylated with catalyst BF_3. Methylated FA were analyzed by GC using a polar capillary column (CP-Sil 88; length, 50 m; i.d., 0.25 mm; film thickness, 0.20 μm; Chrompack, Bergen op Zoom, The Netherlands) on the basis of chain length, degree of (un)saturation, geometry, and the position of the double bonds. Detection was with an FID (Hewlett-Packard Series II, HP 5890 with HP Prepstation and FID-detector; Amstelveen, The Netherlands) (17).

For quantification of plant sterols, an internal standard was added to part of the extracted fat of the diets. Fat was interesterified with NaOMe and nonpolar compounds were extracted with hexane (LiChrosolv; Merck, Darmstadt, Germany). By means of HPLC (515 HPLC pump; Spherisorb 5 Si; length, 150 mm; i.d., 4.6 mm; particle size, 5 μm; Waters, Etten-Leur, The Netherlands), the 4-desmethylsterols, 4-monomethylsterols, and 4,4′-dimethylsterols were separated into fractions. These three fractions were analyzed by GC using an nonpolar capillary column (CP-Sil 13 CB; length, 50 m; i.d., 0.32 mm; film thickness, 0.20 μm; Interscience, Breda, The Netherlands). Detection was with an FID (GC 8000 series, FID detection; Interscience).

A mixture of internal standards for every form of sterol (ester), i.e., β-cholestanol for unesterified sterols, cholesteryl acetate for steryl acetates, cholesteryl cinnamate for steryl cinnamates, cholesteryl palmitate for steryl FA, and cholesteryl ferulate for steryl ferulates, was added to a part of the extracted fat of the diets. By using HPLC, unesterified and esterified sterols were separated from the fat matrix and quantified.

Cholesterol absorption. The absorption efficiency of an oral dose of cholesterol was measured by the dual-isotope plasma ratio method originally described by Zilversmit and Hughes (18) and modified by Turley *et al*. (19), with minor modifications. Starting in week 6 of the experiment, hamsters were administered intravenously ~2.5 μCi [7(n)-^{3}H]cholesterol (Amersham, 's-Hertogenbosch, The Netherlands) dissolved in Intralipid (20%) (Pharmacia BV, Woerden, The Netherlands), followed by oral dosing of ~1.0 μCi [4-^{14}C]cholesterol (Amersham) dissolved in medium-chain triglyceride (MCT)-oil (Union Deutsche Lebensmittelwerke GmbH, Kleve, Germany) for rapid digestion. For each day on which 20 hamsters (2 hamsters of each group) were treated, the required total activity of [^{3}H]cholesterol was dried under nitrogen in a sterilized glass infusing bottle of ~30 mL and then redissolved in absolute ethanol (2 μL ethanol/μCi of ^{3}H). To this solution, undiluted Intralipid was added (0.16 mL Intralipid/μCi of ^{3}H). This mixture was vortexed vigorously for ~3 min. The required total activity of [^{14}C]cholesterol for 1 d, after being dried under nitrogen in a sterilized glass infusing bottle of ~30 mL, was redissolved in ethanol (10 μL/μCi of ^{14}C). To this solution, MCT oil was added (0.60 mL/μCi of ^{14}C).

Hamsters had free access to their diets up until the time of label administration, which was carried out approximately within the 5 h after onset of the light period. Hamsters were

anesthetized lightly with halothane/nitrous oxide (N_2O)/oxygen (O_2) and in the upper layer of the penis skin an incision of about 0.5 cm was made. The dose of [^{3}H]cholesterol in Intralipid (0.40 mL) was then injected into the penis vein using a plastic 1.0-mL tuberculin syringe (Becton, Dickinson, Drogheda, Ireland) with a 30G × 1/2 needle (Becton, Dickinson). This injection was done slowly over ~30 s to prevent any back bleeding after withdrawal of the needle. Each hamster was administered orally the required dose of [^{14}C]cholesterol in 0.60 mL MCT oil after recovering from anesthesia (1–2 min). This was done using a glass 1.0-mL tuberculin syringe fitted with a bending hypodermic needle (1G; stainless steel 18/8; record 1) with a blunt tin top. Intravenous and intragastric dosing of each hamster was routinely completed within an average of 6 min (range 4–12 min). The hamsters were then returned to their Makrolon cages where food was immediately available to them.

In a pilot experiment, under the same conditions as in this study, we measured cholesterol absorption in five hamsters (10–11 wk old), consuming the regular SDS diet, after 24, 48, 72, and 96 h. The plasma isotope ratio of oral and intravenously administered cholesterol was constant for 72 h after isotope dosage. An aliquot of 100 μL of plasma was found to contain enough ^{14}C and ^{3}H to measure their activities with sufficient accuracy. In addition, the reproducibility was tested in 14 hamsters (11–12 wk old) consuming the regular SDS diet after 72 h. Cholesterol absorption in these hamsters was (mean ± SEM) 44.6 ± 12.5%.

Consequently, in this study 72 h after dosing, hamsters were lightly anesthetized under inhalation anesthesia with halothane/N_2O/O_2 and killed after blood sampling by retroorbital puncture. To determine the proportion of the administered doses of [^{3}H]- and [^{14}C]cholesterol remaining in plasma after 72 h, 100-μL aliquots of plasma were added directly to 10 mL emulsifier scintillator plus (Packard Instrument BV-Chemical Operations, Groningen, The Netherlands). Emulsifier scintillator plus (10 mL) was added to the total original dosing mixtures between 2 h and 2 d after administration. Vials were counted in a liquid scintillation analyzer (1900 CA Tri-carb; Packard Instrument BV-Chemical Operations) in the presence of an external standard to determine the level of quenching. Data for the level of ^{3}H and ^{14}C activity in samples and standards were used to calculate the percentage of cholesterol absorption using the following expression (18):

$$\% \text{ cholesterol absorption} = \frac{(\text{dpm } [^{14}\text{C}]\text{cholesterol/mL plasma})/(\text{oral dose dpm } [^{14}\text{C}]\text{cholesterol})}{(\text{dpm } [^{3}\text{H}]\text{cholesterol/mL plasma})/(\text{IV dose dpm } [^{3}\text{H}]\text{cholesterol})} \times 100 = \frac{\%\text{ of oral dose } ([^{14}\text{C}]\text{cholesterol})/\text{mL plasma}}{\%\text{ of IV dose } ([^{3}\text{H}]\text{cholesterol})/\text{mL plasma}} \times 100 \quad [1]$$

Laboratory measurements. Blood was collected in K_3-EDTA (as anticoagulant, 1.8 mg/mL) tubes (Greiner Labortechnik, Alphen a/d Rijn, The Netherlands). Plasma was prepared by centrifuging the tubes for 10 min at 1500 × *g* and was divided into portions for different determinations. Plasma was stored at −20°C until analysis.

Plasma total cholesterol (TC) concentration was determined according to the CHOD-PAP (cholesterol oxidase-peroxidase amino phenazon) method (20) using a total cholesterol test kit (Boehringer, Mannheim, Germany).

Plasma total glycerol (TG) and free glycerol (FG) were analyzed according to the GPO (glycerol phosphate oxidase)-Trinder (PAP) method using a total glycerol test kit (Roche, Basel, Switzerland) and a free glycerol test kit (Sigma, St. Louis, MO). TAG were calculated as the difference between TG (mmol/L) and FG (mmol/L).

Three fractions of lipoproteins were separated (21,22): VLDL plus intermediate-density lipoproteins (IDL; $d < 1.006$ g/mL), LDL ($1.006 < d < 1.063$ g/mL), and HDL ($d > 1.063$ g/mL). TC and TG were measured in the three fractions of lipoproteins as described above. All determinations were done on a Cobas Mira S automated analyzer (Roche).

After the hamsters were killed, the liver was removed and weighed. The right median lobe was prepared for microscopical inspection. A sample from the left part of the median lobe was taken and stored at −20°C for lipid analysis. Lipids were extracted from liver tissue according to the method of Bligh and Dyer (23). After extraction, the dry vaporized extract was mixed with Triton X-100 in dichloromethane. This mixture was dried under nitrogen and dissolved in demineralized water (24). The liver extract was stored at −20°C. Liver TC and TG were measured as described above.

Statistical analysis. Values for general characteristics (body weight and food consumption) were analyzed using one-way ANOVA. Cholesterol absorption, plasma lipids, liver lipids, and liver weight were analyzed by two-way [type of phytosterol ($n = 3$) and type of esterification ($n = 3$)] ANOVA using the General Linear Model procedure of SAS version 6.12 (SAS Institute, Cary, NC). Tukey's multiple comparison test was used to assess differences among the nine sterol treatments. ANOVA was used to assess differences between the three unesterified phytosterol and the six esterified phytosterol treatments and between diets containing phenolic ($n = 3$) and FA ($n = 3$) phytosterol esters. Differences between separate diets that differed in only one compound (type of ester or type of acid) were assessed using the Student–Newman–Keuls (SNK) procedure. Only 27 of the 66 possible comparisons were used. A two-sided P-value <0.05 was considered to be significant.

RESULTS

Diet composition. The phytosterol composition of the different diets used in the study is shown in Tables 2 and 3. The amount of sterols in the control diet was 0.3 g/kg. Diets were prepared to contain 0.5% sterols (w/w; 5 g/kg diet; expressed as unesterified sterol equivalents). The diets with added soybean sterols contained on average 5.0 g sterols/kg diet,

whereas the diets with added rice bran sterols contained 4.4 g sterols/kg and the diets with added shea nut sterols, 4.1 g sterols/kg. The 4-desmethylsterols, especially β-sitosterol, campesterol, and stigmasterol, constituted 99% of soybean sterols (Table 2). The majority of shea nut (89%) and rice bran (70%) sterols consisted of 4,4′-dimethylsterols, especially α-amyrin, butyrospermol, and lupeol, and 24-methylenecycloartanol and cycloartenol, respectively. Esterification of the sterols did not affect sterol profiles. The degree of esterification of the sterols in the unesterified phytosterol diets was <10% (Table 3). Esterified phytosterols were esterified to >85%. For the diet containing the cinnamic esters of shea nut sterols, the degree of esterification was 66% with cinnamic acid and 27% of the sterols was esterified with acetate. The FA composition of all diets was largely equivalent (Table 4).

Animal characteristics. Mean body weight was 109 g at allocation to the different experimental groups, and mean terminal body weight was 123 g. Weight gain did not differ among the groups (data not shown). Feed intake was similar among the groups (data not shown). One hamster in the group fed shea nut sterols esterified with phenolic acid died during the experimental period. In this hamster only, pathological evaluation was performed.

Cholesterol absorption. Intravenous injection of ^{3}H-labeled mixture in 12 of 199 hamsters and the oral administration of ^{14}C-labeled mixture in one hamster were not successful. Consequently, in 186 hamsters (93%), cholesterol absorption could be measured after 72 h. Effects on cholesterol absorption did not differ between the two study subsets, and the actual length of the feeding period had no effect (data not shown). In hamsters fed phytosterols from soybeans, cholesterol absorption was decreased compared with all other groups (Table 5). There were no significant differences between the groups fed phytosterols from shea nut or rice bran and the control group. There were also no overall effects on cholesterol absorption from esterification of phytosterols or from the type of esterified acid. However, cholesterol absorption was lower in hamsters fed FA esters of soybean sterols than in their counterparts fed soybean sterols esterified with cinnamic acid.

Plasma lipids. Hamsters fed soybean sterols had lower plasma TC levels than hamsters fed sterols from shea nut or rice bran, or those fed the control diet (Table 5). Feeding unesterified phytosterols resulted in lower TC levels compared with feeding esterified phytosterols. Soybean sterols esterified with FA lowered TC more than the phenolic esters of soybean sterols and the FA esters of the other phytosterols. The addition of unesterified rice bran sterols to the diet lowered the TC level, compared with the control group, whereas addition of unesterified shea nut sterols did not. Ferulic acid and

TABLE 2
Sterol Composition (in mg/kg) of the Different Diets

		Type of diet								
		Shea nut			Rice bran			Soybean		
Sterol	Control	Free	Phenolic acid	FA	Free	Phenolic acid	FA	Free	Phenolic acid	FA
4-Desmethylsterols	259	310	310	300	1229	1284	1144	4853	5311	4734
Cholesterol	77	86	83	85	112	96	114	90	119	88
Brassicasterol	2	2	2	2	3	2	3	59	64	57
24-Methylcholesterol	—[a]	—	—	—	—	—	—	0	4	0
Campesterol	26	28	26	28	564	603	519	1238	1333	1208
Campestanol	2	2	3	2	25	33	19	83	80	72
Stigmasterol	18	19	17	20	63	60	59	943	1019	916
D7-Campesterol	4	4	5	4	13	15	12	16	17	15
Clerosterol	1	2	1	1	4	5	4	22	25	21
β-Sitosterol	87	96	91	95	357	360	338	2104	2275	2035
Sitostanol	8	12	22	11	21	36	19	99	109	98
D5-Avenasterol	7	6	8	6	10	12	10	48	52	48
D7-Stigmastenol	14	24	28	20	20	21	17	30	31	30
D7-Avenasterol	4	5	5	5	7	7	7	10	16	15
4-α-Methylsterols	25	115	109	122	99	120	105	21	22	16
Obtusifoliol	5	11	11	10	6	7	6	4	4	3
Citrostadienol	6	8	7	8	6	7	6	5	5	4
4,4′-Dimethylsterols	19	4003	3151	3599	3074	3341	2976	21	20	17
Cycloartanol	0	48	46	39	56	59	56	1	0	0
β-Amyrin	0	208	167	186	—	—	—	—	—	—
Butyrospermol	0	815	689	711	—	—	—	—	—	—
Cycloartenol	5	0	0	0	1300	1393	1254	5	5	4
α-Amyrin	0	1532	1182	1352	—	—	—	—	—	—
Lupeol	0	745	523	689	—	—	—	—	—	—
24-Methylcycloartanol	2	0	0	0	1653	1836	1608	3	3	2
Total sterols	303	4428	3570	4021	4402	4745	4225	4895	5353	4767

[a]—, not detectable; 0, <0.5 mg/kg.

TABLE 3
Sterol (ester) Concentration (in mg/kg) of the Different Diets

		Type of diet								
		Shea nut			Rice bran			Soybean		
Sterol (ester)	Control	Free	Phenolic acid	FA	Free	Phenolic acid	FA	Free	Phenolic acid	FA
Free	223	3876	134	218	4325	262	536	4793	257	378
Acetates	—[a]	—	1126	27	—	—	—	—	27	—
Cinnamates	68	3417	54	—	—	—	—	6021	—	
FA	197	150	228	7004	231	290	6646	203	135	8316
Ferulates	—	—	—	—	449	4816	174	—	—	—
Total sterol equivalent[b]	341	4026	3899	4542	4774	3823	4703	4914	4901	5427

[a]—, not detectable (<1 mg/kg).
[b]Total sterol equivalent = free sterols + 0.932 × acetate-esters + 0.754 × cinnamate-esters + 0.608 × FA-esters + 0.703 × ferulate-esters.

TABLE 4
FA Composition (in %) of the Fat Blends[a]

		Type of diet								
		Shea nut			Rice bran			Soybean		
Sterol	Control	Free	Phenolic acid	FA	Free	Phenolic acid	FA	Free	Phenolic acid	FA
12:0	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2
14:0	0.8	0.8	0.8	0.8	0.8	0.8	0.7	0.8	0.8	0.8
16:0	29.7	29.7	28.9	29.7	29.5	29.6	29.7	29.6	29.7	29.6
16:1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1
17:0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
18:0	4.0	4.1	4.8	4.1	4.1	4.1	4.1	4.0	4.1	4.1
18:1	40.0	40.0	39.6	40.0	40.1	40.1	40.0	40.0	40.0	40.0
18:2	23.0	23.1	23.4	23.1	23.1	23.1	23.0	23.0	22.8	23.1
18:3	0.8	0.8	0.6	0.8	0.8	0.8	0.8	0.9	0.9	0.9
20:0	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
20:1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
22:0	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
24:0	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1
Σ PUFA	23.8	23.8	24.0	23.9	23.9	23.9	23.8	23.9	23.7	24.0
Σ MUFA[b]	40.3	40.3	39.9	40.3	40.4	40.4	40.4	40.4	40.4	40.3
Σ SFA	35.7	35.7	35.7	35.6	35.5	35.6	35.6	35.6	35.6	35.5

[a]The sterol esters (and free sterols) were dissolved in the fat blend.
[b]MUFA, monounsaturated FA; SFA, saturated FA.

cinnamic acid increased plasma TC levels, with the strongest effect for cinnamic acid.

Effects on VLDL + IDL cholesterol and LDL cholesterol levels largely mirrored the effects on plasma TC (Table 5). Cinnamic acid intake increased plasma VLDL + IDL and LDL cholesterol levels. Rice bran and soybean sterols reduced plasma HDL cholesterol levels. Esterification of sterols with FA resulted in higher plasma LDL cholesterol to HDL cholesterol ratios compared with unesterified sterols (Table 5). Compared with shea nut sterols, soybean sterols lowered TAG levels in blood (Table 5). TAG levels of the groups fed ferulic and particularly cinnamic acid were higher than in the groups fed sterols esterified with phenolic acids. Differences in plasma total TAG levels were reflected in plasma VLDL + IDL-TAG levels.

Liver lipids. The addition of phytosterols to the diet, especially those from soybean, lowered liver weight (Table 5). No overall effect on liver weight was observed due to sterol esterification or the type of esterified acid. Feeding phytosterols reduced liver TC levels for all types of unesterified phytosterols compared with the control diet (Table 5). Ferulic acid also reduced liver total cholesterol level, in contrast to cinnamic acid, relative to the control diet. Total liver cholesterol pools were lower in hamsters fed soybean sterols than in those fed both other types of sterols. Hamsters fed ferulic acid and those fed nonesterified phytosterols also had reduced liver cholesterol pools compared with hamsters fed the control diet. Compared with shea nut sterols, rice bran sterols increased liver TAG levels, but to a lesser extent than soybean sterols (Table 5). Liver TAG levels in hamsters fed the shea nut sterols did not differ from those in the control group.

Pathology. At the macroscopic level, livers of hamsters in the control and phenolic acid groups showed a higher incidence of pallor (data not shown). A pronounced lobular pattern

TABLE 5
Cholesterol Absorption Efficiency, Total Cholesterol (TC), and TAG Concentrations in Plasma and Plasma Lipoproteins, Liver Weight, and Liver Lipid Levels in Hamsters Fed the Different Diets[a]

	Type of diet													
				Shea nut			Rice bran			Soybean				
Sterol (ester) Measure	Control	Cinnamic	Ferulic	Free	Phenolic acid[b]	FA	Free	Phenolic acid[c]	FA	Free	Phenolic acid[b]	FA	ANOVA[d]	
Cholesterol absorption (%)	57.6	—	—	59.7	56.4	57.9	51.3	55.3	53.8	43.3	50.0	40.4	P	(2.56)
Plasma TC (mM)														
TC	3.34	3.87	3.62	3.24	3.20	3.43	2.99	3.31	3.18	2.92	3.16	2.84	P,E,P × E	(0.082)
VLDL + IDL	0.46	0.76	0.54	0.35	0.33	0.32	0.27	0.36	0.34	0.25	0.29	0.24	P	(0.031)
LDL	0.69	0.83	0.80	0.60	0.68	0.72	0.60	0.69	0.69	0.59	0.60	0.58	P,E	(0.028)
HDL	1.88	1.88	1.90	1.88	1.84	1.93	1.75	1.81	1.78	1.69	1.88	1.69	P	(0.053)
LDL/HDL	0.38	0.45	0.42	0.32	0.38	0.38	0.35	0.38	0.39	0.35	0.32	0.34	P,E	(0.020)
Plasma TAG (mM)														
TG	2.14	3.34	2.78	1.93	1.96	1.80	1.72	1.91	2.02	1.74	1.67	1.71	P	(0.132)
TAG	1.85	3.05	2.49	1.69	1.71	1.56	1.50	1.69	1.75	1.51	1.46	1.48	P	(0.121)
VLDL + IDL	1.63	2.48	2.06	1.37	1.41	1.23	1.19	1.40	1.49	1.24	1.16	1.16	P	(0.106)
LDL	0.18	0.16	0.17	0.17	0.18	0.19	0.18	0.17	0.19	0.20	0.17	0.19	E	(0.007)
HDL	0.09	0.09	0.09	0.08	0.08	0.08	0.08	0.07	0.08	0.08	0.07	0.07	NS	(0.004)
LDL/HDL	2.04	2.04	2.30	2.36	2.46	2.52	2.51	2.42	2.38	2.75	2.64	2.72	NS	(0.140)
Liver														
Weight (g)	4.81	4.85	4.74	4.70	4.54	4.84	4.53	4.64	4.66	4.33	4.59	4.22	P	(0.12)
TC (pmol/g)	8.1	8.6	6.7	5.7	5.6	5.8	5.2	5.9	5.5	5.1	5.6	5.1	P	(0.28)
TC (μmol)[e]	39.4	41.4	31.9	27.0	25.6	27.8	23.5	27.6	25.8	22.1	25.6	21.6	P,P × E	(1.63)
TAG (pmol/g)	4.1	3.2	3.3	4.2	4.2	4.2	5.5	4.3	4.7	5.7	5.4	5.7	P	(0.28)
TAG (μmol[f]	19.7	15.5	15.5	19.7	19.3	20.6	24.9	20.2	22.5	25.0	24.9	24.2	P	(1.52)

[a]Values are means, $n = 20$, for all groups except for the group fed shea nut sterols esterified with phenolic acid ($n = 19$), as measured after feeding the diet for at least 5 wk.
[b]Phytosterols esterified with cinnamic acid.
[c]Phytosterols esterified with ferulic acid.
[d]Pooled SEM in parentheses; P, significant effect of type of sterol; E, significant effect of type of ester; P × E, significant interaction; NS, not significant.
[e]Total liver cholesterol pool, calculated by multiplying liver weight (g) by total cholesterol level (μmol/g).
[f]TAG liver pool, calculated by multiplying liver weight (g) by TAG level (μmol/g).

was more abundant in the two groups fed phenolic acids. The most frequently observed microscopic feature was the vacuolation in hepatocytes (data not shown), but the incidence was not treatment related. In the liver, cell infiltrates of an inflammatory nature (consisting mainly of mononuclear cells) with or without necrotic cells were found frequently. The incidence of these infiltrates was not treatment related.

DISCUSSION

The effect of different types of dietary plant sterols, in their unesterified form or esterified with different types of acids, on cholesterol absorption and plasma cholesterol levels was determined in hamsters. De Deckere and Korver (25) suggested a hypocholesterolemic potential for oryzanol, a mixture of ferulic acid esters of 4,4′-dimethyl- and 4-desmethylsterols present in the nonsaponifiable fraction of rice bran oil. Dietary oryzanol was shown previously to have a cholesterol-lowering effect in hamsters (26). Feeding oryzanol reduced cholesterol absorption efficiency by 25% and plasma total cholesterol levels by 28%, but the composition of the oryzanol was not reported. In our study in hamsters, rice bran sterols lowered cholesterol absorption efficiency by 7% and plasma cholesterol levels by 5%, but neither effect was significant.

In humans, rice bran sterols (65% 4,4′-dimethylsterols + 35% 4-desmethylsterols) at an intake level of 1.7 g/d did not lower blood total and LDL cholesterol (11). However, in another study with human subjects, rice bran sterols with a higher concentration of 4-desmethylsterols (48%) and consumed at a higher level (2.1 g/d) did lower blood cholesterol levels (12). In a study with hamsters, 4,4′-dimethylsterols from rice bran oil lowered blood cholesterol to a lesser extent than 4-desmethylsterols (13). In the present study, we fed hamsters rice bran sterols with a relatively high level of 4,4′-dimethylsterols (70%), and the efficiency of cholesterol absorption and blood cholesterol levels were reduced to a lesser extent than in hamsters fed the same level of 4-desmethylsterols from soybean.

At relatively high levels of intake (2.6–3.3 g/d) the pentacyclic triterpene alcohols from shea nut have repeatedly been shown not to affect blood cholesterol levels in humans (11,12,14). Also, in this study using hamsters, the addition of shea nut sterols to the diet did not lower the efficiency of cholesterol absorption and blood cholesterol levels.

The addition of phytosterols from soybean to the diet in this study decreased cholesterol absorption by 23% and plasma total cholesterol by 11%, compared with the control diet. The cholesterol-lowering effect of dietary soybean

sterols has also been demonstrated in humans (11,27), rats (28,29), and chickens (30). Taken together, the data suggest that 4-desmethylsterols are very effective hypocholesterolemic naturally occurring dietary plant sterols.

A lower efficiency of cholesterol absorption will lead to a lower flux of absorbed cholesterol into the liver, most likely leading to lower liver cholesterol pools. In our study, differences in liver cholesterol pools between the dietary treatment groups were more pronounced than differences in cholesterol absorption efficiency and blood cholesterol levels. However, we cannot explain the observation that hamsters fed the diets with shea nut sterols had lower hepatic cholesterol pools than hamsters in other groups.

Our results indicate that different structures have differing abilities to inhibit cholesterol absorption. It is to be expected that the structure of a plant sterol will influence its ability to compete with cholesterol in the luminal, cell-surface, and intracellular phases of the cholesterol absorption process. The structure of 4-desmethylsterols is more similar to cholesterol than the structure of 4,4′-dimethylsterols or pentacyclic triterpene alcohols (Fig. 1); thus, the 4-desmethylsterols may compete more efficiently with cholesterol for absorption. However, the 4,4′-dimethylsterols in rice bran oil may also inhibit cholesterol absorption, albeit to a lesser extent than the 4-desmethylsterols. The structure of the 4,4′-dimethylsterols in rice bran oil, mainly 24-methylenecycloartanol and cycloartenol, is more similar to cholesterol than that of the pentacyclic triterpene alcohols from shea nut, α-amyrin, butyro-spermol, lupeol, and β-amyrin. Our results suggest that structural differences at the 4-position and at the position of the side chain in cholesterol influence the ability of other sterols to interfere with cholesterol absorption.

The type of plant sterol may not be the only explanation for the differences observed in this study. Unesterified phytosterols lowered plasma cholesterol levels more than esterified phytosterols, particularly phytosterols esterified with phenolic acids. This may be because hydrolysis must occur before cholesterol absorption can be affected. Unesterified phytosterols do not have to be hydrolyzed and are immediately available for incorporation into intestinal micelles, whereas esterified phytosterols first have to be hydrolyzed by cholesterase or lipase. The unesterified plant sterols fed in this experiment were completely dissolved in the fat incorporated into the hamster diet, whereas the amount of plant sterols required for a significant hypocholesterolemic effect in humans cannot be dissolved in the amount of table spread that is typically consumed on a daily basis (11). The observations in our study suggest that plant sterols esterified with phenolic acids are hydrolyzed less efficiently in the intestinal tract. In addition, the observed hypercholesterolemic action of cinnamic acid and ferulic acid may have contributed to the reduced hypocholesterolemic potency of plant sterols esterified with phenolic acids.

In summary, dietary 4-desmethylsterols inhibit cholesterol absorption more effectively than 4,4′-dimethylsterols, and pentacyclic triterpene alcohols are not effective. Esterification of plant sterols to FA does not impair their ability to inhibit cholesterol absorption, whereas esterification with phenolic acids reduces this ability. Our results suggest that structural differences at the 4-position and at the position of the side chain in cholesterol influence the ability of other sterols to interfere with cholesterol absorption.

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Effects of Dietary Vegetable Oil on Atlantic Salmon Hepatocyte Fatty Acid Desaturation and Liver Fatty Acid Compositions

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ABSTRACT: Fatty acyl desaturase activities, involved in the conversion of the C_{18} EFA 18:2n-6 and 18:3n-3 to the highly unsaturated fatty acids (HUFA) 20:4n-6, 20:5n-3, and 22:6n-3, are known to be under nutritional regulation. Specifically, the activity of the desaturation/elongation pathway is depressed when animals, including fish, are fed fish oils rich in n-3 HUFA compared to animals fed vegetable oils rich in C_{18} EFA. The primary aims of the present study were (i) to establish the relative importance of product inhibition (n-3 HUFA) vs. increased substrate concentration (C_{18} EFA) and (ii) to determine whether 18:2n-6 and 18:3n-3 differ in their effects on the hepatic fatty acyl desaturation/elongation pathway in Atlantic salmon (*Salmo salar*). Smolts were fed 10 experimental diets containing blends of two vegetable oils, linseed (LO) and rapeseed oil (RO), and fish oil (FO) in a triangular mixture design for 50 wk. Fish were sampled after 32 and 50 wk, lipid and FA composition of liver determined, fatty acyl desaturation/elongation activity estimated in hepatocytes using [1-^{14}C]18:3n-3 as substrate, and the data subjected to regression analyses. Dietary 18:2n-6 was positively correlated, and n-3 HUFA negatively correlated, with lipid content of liver. Dietary 20:5n-3 and 22:6n-3 were positively correlated with liver FA with a slope greater than unity suggesting relative retention and deposition of these HUFA. In contrast, dietary 18:2n-6 and 18:3n-3 were positively correlated with liver FA with a slope of less than unity suggesting metabolism *via* β-oxidation and/or desaturation/elongation. Consistent with this, fatty acyl desaturation/elongation in hepatocytes was significantly increased by feeding diets containing vegetable oils. Dietary 20:5n-3 and 22:6n-3 levels were negatively correlated with hepatocyte fatty acyl desaturation. At 32 wk, 18:2n-6 but not 18:3n-3 was positively correlated with hepatocyte fatty acyl desaturation, whereas the reverse was true at 50 wk. The data indicate that both feedback inhibition through increased n-3 HUFA and decreased C_{18} fatty acyl substrate concentration are probably important in determining the level of hepatocyte fatty acyl desaturation and that 18:2n-6 and 18:3n-3 may differ in their effects on this pathway.

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Virtually all animals lack Δ12 and Δ15 fatty acyl desaturases and thus are unable to biosynthesize *de novo* the PUFA linoleate (18:2n-6) and linolenate (18:3n-3). Therefore, these PUFA are essential fatty acids (EFA) for animals, although the qualitative and quantitative requirements vary among species (1). Salmonid fish such as rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) require both 18:3n-3 and 18:2n-6 at a combined level of around 1% of the diet, although the C_{18} EFA have no direct physiological role in fish (2). Rather, their essentiality derives from their conversion to the functionally active highly unsaturated FA (HUFA) eicosapentaenoate (20:5n-3), docosahexaenoate (22:6n-3), and arachidonate (20:4n-6) that are formed by desaturation and elongation of the C_{18} EFA (3,4).

Fatty acyl desaturase enzyme activities are known to be under nutritional regulation in mammals (5), and this has also been demonstrated in fish. The activity of Δ9 desaturase (stearoyl CoA desaturase) in rainbow trout was low in starved fish and increased by feeding but was similar in fish fed a diet rich in palmitic acid (16:0) compared to fish fed a standard diet containing fish oil (FO) (6). However, the desaturation of 18:3n-3 and 18:2n-6 in isolated hepatocytes from Atlantic salmon was shown to be greater in fish fed a diet containing a vegetable oil [a 1:1 blend of linseed oil (LO) and rapeseed oil (RO)] rich in 18:2n-6 and 18:3n-3 compared to fish fed a diet containing FO and thus rich in 20:5n-3 and 22:6n-3 (7). Several further studies have confirmed that PUFA desaturation and elongation in hepatocytes from salmonid fish were increased in fish fed diets rich in C_{18} EFA compared to fish fed standard diets containing FO rich in C_{20} and C_{22} HUFA (8–13). The regulation of FA desaturation pathways in fish is currently of great interest as there is an urgent need to replace the $C_{20/22}$ HUFA-rich FO, derived from potentially nonsustainable wild marine fish resources, with vegetable oils, rich in C_{18} PUFA, in the diets of aquacultured fish species (14). Demand for FO is rapidly outstripping supply, and current estimates suggest aquaculture feeds will consume more than 85% of world FO supplies by 2010, and so, if aquaculture is to continue to expand and supply more of the global demand for fish, alternatives to FO must be found (15).

The biochemical mechanisms underpinning the nutritional regulation of the fatty acyl desaturation/elongation pathway are unclear. In broad terms, feeding vegetable oils could increase the activity of the PUFA desaturation/elongation pathway through two mechanisms. The pathway could simply be stimulated by increased substrate C_{18} PUFA concentrations, and/or the lack of C_{20} and C_{22} HUFA could increase activity of the

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Abbreviations: FAF-BSA, fatty acid free bovine serum albumin; FO, fish oil; HUFA, highly unsaturated FA (carbon chain length ≥C_{20} with ≥3 double bonds); LO, linseed oil; PPAR, peroxisome proliferator-activated receptor; RO, rapeseed oil.

pathway through decreased product inhibition. Thus, the primary aims of the present study are (i) to establish the relative importance of decreased product inhibition and increased substrate concentration and (ii) to determine whether 18:2n-6 and 18:3n-3 differ in their effects on the hepatic PUFA desaturation/elongation pathway in Atlantic salmon.

Salmon smolts were randomly stocked into 10 seawater pens and, after acclimatization for 2 wk, were fed for 50 wk on nine experimental diets containing various blends of two vegetable oils, LO and RO, and FO, and a control diet containing only FO. Fish were sampled twice, after 32 and 50 wk of feeding the experimental diets. At each sampling time, fatty acyl desaturation and elongation were estimated in isolated hepatocytes using [1-^{14}C]18:3n-3 as substrate, and samples of liver were collected for analysis of lipid and FA composition.

MATERIALS AND METHODS

Animals and diets. The experimental fish were Atlantic salmon post-smolts of initial weight 120 ± 10 g. In February 1999, FA desaturation in hepatocytes was measured in a sample of fish immediately before the fish were randomly assigned to 10 cages (5 × 5 m; 600 fish per cage). The smolts were fed one of 10 diets, consisting of a control diet containing FO alone and 9 diets containing different combinations of FO and/or vegetable oils (RO and LO) in a mixture design. Specifically, the 10 diets were 100% FO, 100% LO and 100% RO, FO/RO (2:1 and 1:2), FO/LO (2:1 and 1:2), RO/LO (2:1 and 1:2), and FO/RO/LO (1:1:1) forming a triangular design. The experimental diets were prepared by the Ewos Technology Centre, Livingston, Scotland. Initially, the diets contained 47.0% protein, 24.1% lipid, and 7.6% moisture (3 mm pellet) and later (6 mm pellet) 41.8% protein, 30.5% lipid, and 6.8% moisture. The formulation and FA compositions of the diets (6 mm pellet) are shown in Tables 1 and 2. All diets were formulated to satisfy the nutritional requirements of salmonid fish (16). Fish were sampled twice, after feeding the experimental diets for 32 wk (October 1999), with a final sampling performed 18 wk later in February 2000. There were no significant differences between the weights of the fish on the different dietary treatments sampled after 32 wk (ANOVA, $P > 0.05$). However, the range of weights was greater at 50 wk (1924 ± 564 to 2586 ± 841 g; n = 200), and there were some significant differences between treatments (ANOVA, $P = 0.0004$). However, regression analyses showed no relationship between final weight and any dietary FA (including 16:0, 18:1n-9, and total monoenes), indicating that dietary treatment was not responsible for the differences. Up to 32 wk, feed was distributed manually, but from 32 to 50 wk the method of feeding was changed from manual to automatic feeders controlled by Akvasmart pellet counters. However, logistical problems at the commercial farm meant that some treatments had to be fed by hand, which may have affected ration and final weight to some extent.

Lipid extraction and lipid class composition. Intact livers were dissected from three fish per dietary treatment at each sampling point and immediately frozen in liquid nitrogen. Total lipid content of livers and diet samples was determined gravimetrically after extraction by homogenization in chloroform/methanol (2:1, vol/vol) containing 0.01% BHT as antioxidant, basically according to Folch *et al*. (17). Separation of lipid classes was performed by one-dimensional, double development high-performance thin-layer chromatography (HPTLC) with classes quantified by charring followed by calibrated densitometry as described previously (18).

FA analysis. FAME were prepared from total lipid by acid-catalyzed transesterification using 2 mL of 1% H_2SO_4 in methanol plus 1 mL toluene as described by Christie (19), and FAME extracted and purified as described previously (20). FAME were separated and quantified by GLC (Fisons GC8600, Fisons Ltd.) using a 30 m × 0.32 mm capillary column (CP wax 52CB; Chrompak Ltd., London, United Kingdom). Hydrogen was used as carrier gas, and temperature programming was from 50 to 180°C at 40°C/min and then to 225°C at 2°C/min. Individual methyl esters were identified by comparison to known standards and by reference to published data (21).

Preparation of isolated hepatocytes. Isolated hepatocytes were prepared by collagenase digestion essentially as described previously (11–13) except that the sieved cells were washed

TABLE 1
Feed Composition (g/100 g)

Component	FO	FO/LO (2:1)	FO/LO (1:2)	LO	FO/RO (2:1)	FO:RO (1:2)	RO	LO/RO (2:1)	LO/RO (1:2)	FO/LO/RO (1:1:1)
Fish meal[a]	53.8	53.8	53.8	53.8	53.8	53.8	53.8	53.8	53.8	53.8
Soya (Hi Pro)[b]	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6
Wheat[c]	14	14	14	14	14	14	14	14	14	14
Fish oil[d]	23.6	15.7	7.9	0	15.7	7.9	0	0	0	7.9
Rapeseed oil[e]	0	0	0	0	7.9	15.7	23.6	7.9	15.7	7.9
Linseed oil[e]	0	7.9	15.7	23.6	0	0	0	15.7	7.9	7.9
Micronutrients[f]	1	1	1	1	1	1	1	1	1	1

[a]Norseameal, London, England.
[b]Grosvenor Grain, Perth, Scotland.
[c]Stewarts of Larbert, Larbert, Scotland.
[d]United Fish Products, Aberdeen, Scotland.
[e]Meade-King Robinson & Co., Liverpool, England.
[f]Vitamins, minerals, and carotenoid pigment (Roche Products, Heanor, England, to specification by Ewos Ltd., Bathgate, Scotland). FO, fish oil; LO, linseed oil; RO, rapeseed oil.

TABLE 2
FA Compositions[a] of the 10 Experimental Diets

FA/diet	FO	FO/LO (2:1)	FO/LO (1:2)	LO	FO/RO (2:1)	FO/RO (1:2)	RO	LO/RO (2:1)	LO/RO (1:2)	FO/LO/RO (1:1:1)
14:0	5.6	3.6	2.2	1.1	3.7	2.3	1.2	1.2	1.1	1.9
16:0	13.6	11.6	9.6	7.6	11.7	9.4	7.2	7.7	7.6	8.8
18:0	2.5	2.9	3.4	3.6	2.3	2.0	1.9	3.0	2.4	2.7
Total saturates[b]	2.4	20.2	16.7	13.2	19.9	15.5	11.4	13.0	10.9	14.9
16:1n-7	5.5	3.5	2.2	1.0	3.8	2.5	1.3	1.2	1.3	1.9
18:1n-9	15.1	16.1	16.4	16.6	26.7	37.5	48.3	25.9	36.9	28.8
20:1n-9	9.6	6.0	3.7	1.9	6.5	4.6	3.0	2.2	2.6	3.5
22:1n-11	13.2	8.2	4.8	2.3	8.3	5.4	2.7	2.6	2.7	4.3
Total monounsaturates[c]		44.3	34.6	27.6	22.1	46.2	50.6	55.6	32.3	45.5
39.0										
18:2n-6	4.5	7.9	11.4	13.6	9.5	14.0	17.9	14.6	16.3	13.7
20:4n-6	0.6	0.4	0.3	0.2	0.5	0.3	0.2	0.2	0.2	0.2
Total n-6 PUFA[d]	5.5	8.7	11.8	13.9	10.4	14.6	18.1	15.0	16.8	14.1
18:3n-3	1.7	18.6	33.0	45.4	4.7	6.6	8.9	32.9	20.2	22.0
18:4n-3	2.8	1.8	1.0	0.5	1.9	1.1	0.4	0.4	0.4	0.9
20:4n-3	0.8	0.6	0.3	0.1	0.6	0.4	0.1	0.2	0.1	0.2
20:5n-3	7.3	5.3	3.3	1.8	5.7	3.9	1.9	2.2	2.1	3.1
22:5n-3	1.2	1.0	0.6	0.3	1.1	0.6	0.3	0.4	0.4	0.5
22:6n-3	10.5	8.4	5.3	2.7	8.7	5.9	2.9	3.4	3.5	4.9
Total n-3 PUFA	24.5	35.8	43.6	50.8	22.8	18.6	14.5	39.6	26.7	31.5
n-3/n-6	4.4	4.1	3.7	3.7	2.2	1.3	0.8	2.6	1.6	2.2

[a]Values are weight percentages of total FA.
[b]Includes 10:0, 12:0, 17:0, 20:0, and 22:0.
[c]Includes 14:1, 17:1, 20:1n-7, 22:1n-9, and 24:1.
[d]Includes 18:3n-6, 20:2n-6, 20:3n-6, and 22:5n-6. For abbreviations see Table 1.

twice with 20 mL of calcium- and magnesium-free HBSS containing 10 mM HEPES and 1 mM EDTA, with the first wash also containing 1% wt/vol FA-free BSA (FAF-BSA). The hepatocytes were resuspended in 10 mL of Medium 199 containing 10 mM HEPES and 2 mM glutamine. Cell suspension (100 μL) was mixed with 400 μL of Trypan Blue, and hepatocytes were counted and their viability assessed using a hemocytometer. One hundred microliters of the cell suspension was retained for protein determination.

Assay of hepatocyte fatty acyl desaturation/elongation activities. Five milliters of each hepatocyte suspension was dispensed into a 25-cm^2 tissue culture flask. Hepatocytes were incubated with 0.25 μCi (~1 μM) [1-^{14}C]18:3n-3, added as a complex with FAF-BSA in PBS, prepared as described previously (22). After addition of isotope, the flasks were incubated at 20°C for 2 h. The reaction was stopped, cells were washed, and total lipid was extracted as described in detail previously (23). Total lipid was transmethylated, FAME were prepared as above, methyl esters were separated according to degree of unsaturation and chain length by argentation chromatography, and autoradiography was performed as described previously (11,24). Areas of silica containing individual PUFA were scraped into scintillation minivials containing 2.5 mL of scintillation fluid (Ecoscint A; National Diagnostics, Atlanta, GA), and radioactivity was determined in a TRI-CARB 2000CA scintillation counter (United Technologies Packard). Results were corrected for counting efficiency and quenching of ^{14}C under exactly these conditions.

Protein determination. Protein concentration in isolated hepatocyte suspensions was determined according to the method of Lowry *et al*. (25) after incubation with 0.4 mL of 0.25% (wt/vol) SDS/1 M NaOH for 45 min at 60°C.

Materials. [1-^{14}C]18:3n-3 (50–55 mCi/mmol) was obtained from NEN [DuPont (U.K.) Ltd., Stevenage, United Kingdom). HBSS, Medium 199, HEPES buffer, glutamine, collagenase (type IV), FAF-BSA, BHT, and silver nitrate were obtained from Sigma Chemical Co. (Poole, United Kingdom). TLC plates, precoated with silica gel 60 (without fluorescent indicator), were obtained from Merck (Darmstadt, Germany). All solvents were HPLC grade and were obtained from Fisher Scientific UK, (Loughborough, England).

Statistical analysis. All the data are presented as means ± SD (n = 3) unless otherwise stated. The relationships between dietary FA contents and growth, liver total lipid and neutral lipid contents and liver FA compositions, and between hepatocyte fatty acyl desaturation activity and both dietary and liver FA compositions were determined by regression analyses (Prism 3; Graphpad Software, Inc., San Diego, CA). In addition, the relationship between hepatocyte fatty acyl desaturation activity and the source of dietary fat was examined by using stepwise multiple linear regression using a mixture design (Modde 4.0; Umetri AB, Umeå, Sweden). Data from different individual fish were treated as independent samples. Some data were also analyzed by one-way ANOVA to determine whether the overall effects of dietary treatment were significant. Percentage data and data that were identified as nonhomogeneous (Bartlett's test) were subjected to either arcsine or log transformation before analysis. Differences were regarded as significant when $P < 0.05$ (26).

RESULTS

Dietary FA compositions. The graded and systematic substitution of the three dietary oils was clearly reflected in the FA compositions of the 10 resultant diets (Table 2). Thus, increasing inclusion of RO resulted in increased proportions of 18:2n-6 and 18:1n-9 irrespective of whether RO was replacing FO or LO (Table 2). Similarly, increasing inclusion of LO resulted in increased proportions of 18:3n-3 irrespective of which oil it was replacing, whereas 18:2n-6 levels in diets containing LO were dependent on which oil it was replacing, increasing if FO was being replaced and decreasing if RO was being replaced (Table 2). The dietary levels of 20:5n-3 and 22:6n-3, as well as 20:4n-6, 16:0, 20:1n-9, and 22:1n-11 were all decreased in a similarly graded manner irrespective of which vegetable oil was replacing FO. In general, the levels of FA were very similar in diets with the same proportion of the oil that predominantly supplied that FA. For example, the level of 18:3n-3 was similar in diets containing the same proportion of LO. An important exception to this relationship was 18:2n-6, whose level was more dependent on the inclusion level of all three dietary oils rather than one in particular.

Effects of diet on liver FA compositions. The graded and systematic variation in the FA compositions of the 10 experimental diets was reflected in the fatty acid compositions of liver total lipid at both 32 and 50 wk, but was quantitatively greater at the latter time point (Table 3). In particular, increasing inclusion of LO resulted in an increased proportion of 18:3n-3, and inclusion of RO resulted in increased proportions of 18:2n-6 and 18:1n-9, with the relative proportions of 20:5n-3, 22:6n-3, and, to a lesser extent, 20:4n-6, 16:0, 20:1n-9, and 22:1n-11 all decreased in liver lipid of fish fed diets in which the FO was replaced by vegetable oils.

Effects of diet on hepatocyte FA desaturation/elongation. A mixture design necessitates that all three main terms (level of FO, LO, and RO) be entered together or not at all. The simple linear model explained a significant amount of variance (*P*-value of regression equation with constant plus linear terms at mid- and end points were $7.35E^{-11}$ and $3.196E^{-6}$, respectively, with adjusted r^2 values being 0.81 and 0.58, respectively). Adding a square term of the inclusion of RO, thus forming a binomial regression equation, appreciably improved the adjusted r^2 of the regression for the end point to 0.81 and had rather little effect on the value for the midpoint

TABLE 3
FA Compositions[a] (% of total FA by weight) of Total Lipid of Liver from Atlantic Salmon (*Salmo salar* L.) Fed the Experimental Diets for 50 wk

	FO	FO/LO (2:1)	FO/LO (1:2)	LO	FO/RO) (2:1)	FO/RO (1:2)	RO	LO/RO (2:1)	LO/RO (1:2)	FO/LO/RO (1:1:1)
14:0	1.9 ± 0.2	1.3 ± 0.1	1.2 ± 0.2	0.7 ± 0.1	1.4 ± 0.2	1.0 ± 0.1	0.8 ± 0.2	0.7 ± 0.1	0.6 ± 0.0	1.0 ± 0.0
16:0	14.1 ± 2.0	12.9 ± 0.8	8.2 ± 0.8	9.8 ± 1.2	11.1 ± 1.2	8.8 ± 1.4	9.2 ± 2.0	6.6 ± 1.3	6.4 ± 1.5	11.7 ± 1.2
18:0	4.3 ± 0.3	4.6 ± 0.3	3.9 ± 1.1	6.3 ± 0.6	4.5 ± 0.3	3.7 ± 0.2	4.5 ± 0.4	4.3 ± 0.8	3.8 ± 0.3	5.0 ± 0.6
Total saturated[b]	20.5 ± 2.1	19.0 ± 0.7	13.4 ± 1.7	16.7 ± 1.8	17.1 ± 1.4	13.7 ± 1.5	14.5 ± 2.4	11.6 ± 2.1	10.8 ± 1.8	17.8 ± 1.7
16:1n-7	2.4 ± 0.2	2.0 ± 0.3	2.6 ± 1.6	1.6 ± 1.0	2.1 ± 0.1	1.7 ± 0.3	1.8 ± 0.7	1.9 ± 0.6	0.9 ± 0.1	1.1 ± 0.1
18:1n-9	14.9 ± 2.3	15.1 ± 1.3	21.8 ± 6.3	22.2 ± 5.3	22.7 ± 2.5	32.2 ± 6.4	40.1 ± 3.8	33.0 ± 2.6	32.8 ± 5.0	17.4 ± 3.0
18:1n-7	2.8 ± 0.1	2.0 ± 0.1	2.1 ± 0.4	1.3 ± 0.2	2.9 ± 0.1	3.1 ± 0.3	3.0 ± 0.3	1.5 ± 1.3	2.7 ± 0.2	1.9 ± 0.2
20:1n-9	6.2 ± 0.8	4.3 ± 1.0	4.2 ± 0.7	2.5 ± 0.2	6.2 ± 0.6	6.0 ± 0.5	5.4 ± 1.0	3.9 ± 0.2	4.1 ± 0.5	3.1 ± 0.5
22:1	2.2 ± 0.4	1.5 ± 0.4	1.4 ± 0.3	0.7 ± 0.2	1.6 ± 0.6	1.3 ± 0.2	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.9 ± 0.2
24:1n-9	1.2 ± 0.2	0.9 ± 0.1	0.5 ± 0.3	0.5 ± 0.1	0.8 ± 0.0	0.6 ± 0.3	0.6 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	1.2 ± 0.2
Total monoenes[c]	30.1 ± 3.3	26.2 ± 2.4	33.1 ± 8.3	29.0 ± 6.8	36.7 ± 3.4	45.4 ± 7.5	52.3 ± 5.0	41.8 ± 2.1	42.3 ± 5.5	25.8 ± 3.9
18:2n-6	2.1 ± 0.1	3.7 ± 0.6	6.7 ± 0.8	7.3 ± 1.0	4.4 ± 0.6	7.6 ± 1.0	9.8 ± 0.8	9.0 ± 1.2	11.3 ± 1.0	6.8 ± 1.0
20:2n-6	0.7 ± 0.1	1.0 ± 0.1	1.4 ± 0.3	1.4 ± 0.3	1.4 ± 0.1	2.1 ± 0.1	2.2 ± 0.3	1.7 ± 0.1	1.9 ± 0.1	1.2 ± 0.1
20:3n-6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.4 ± 0.1	0.8 ± 0.2	0.4 ± 0.1	0.6 ± 0.0	0.4 ± 0.1
20:4n-6	2.2 ± 0.2	1.9 ± 0.1	0.9 ± 0.7	0.7 ± 0.2	1.7 ± 0.2	1.1 ± 0.4	0.7 ± 0.2	0.4 ± 0.0	0.8 ± 0.2	2.1 ± 0.5
22:5n-6	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0
Total n-6 PUFA[d]	5.7 ± 0.1	7.1 ± 0.5	9.4 ± 0.6	10.0 ± 1.4	8.2 ± 0.5	11.5 ± 0.4	13.7 ± 1.2	11.7 ± 1.2	14.8 ± 0.8	10.7 ± 0.6
18:3n-3	0.6 ± 0.0	6.3 ± 1.6	16.4 ± 2.1	18.5 ± 3.1	1.5 ± 0.3	2.9 ± 0.6	3.4 ± 0.2	16.4 ± 3.0	11.3 ± 0.6	7.7 ± 0.7
20:3n-3	0.2 ± 0.0	1.5 ± 0.3	3.9 ± 0.8	3.7 ± 0.8	0.5 ± 0.0	0.8 ± 0.1	0.7 ± 0.1	3.7 ± 0.8	2.1 ± 0.2	1.3 ± 0.1
20:4n-3	1.4 ± 0.2	1.5 ± 0.1	1.9 ± 0.3	2.2 ± 0.4	1.3 ± 0.2	0.9 ± 0.1	0.7 ± 0.2	2.1 ± 0.3	2.0 ± 0.3	1.5 ± 0.4
20:5n-3	10.3 ± 0.3	9.0 ± 0.7	4.6 ± 1.8	5.2 ± 0.7	7.6 ± 0.4	4.8 ± 2.0	3.2 ± 0.8	3.1 ± 0.5	4.1 ± 1.2	8.0 ± 1.2
22:5n-3	3.3 ± 0.5	2.9 ± 0.4	1.9 ± 0.2	1.2 ± 0.1	2.9 ± 0.1	2.0 ± 0.5	0.8 ± 0.2	1.0 ± 0.2	1.0 ± 0.3	1.8 ± 0.2
22:6n-3	27.6 ± 1.4	26.0 ± 1.9	14.9 ± 6.5	13.0 ± 3.6	23.8 ± 2.8	17.8 ± 4.7	10.6 ± 3.1	8.1 ± 1.4	11.2 ± 3.9	25.0 ± 2.7
Total n-3 PUFA[e]	43.7 ± 1.2	47.6 ± 1.7	44.1 ± 6.3	44.3 ± 6.7	37.9 ± 2.4	29.4 ± 6.5	19.5 ± 4.2	35.0 ± 3.1	32.1 ± 4.5	45.6 ± 2.7
n-3/n-6	7.7 ± 0.3	6.7 ± 0.4	4.7 ± 0.6	4.4 ± 0.1	4.6 ± 0.6	2.6 ± 0.7	1.4 ± 0.3	3.0 ± 0.1	2.2 ± 0.4	4.3 ± 0.5

[a]All data are presented as means ± SD (n = 3).
[b]Includes 15:0 present at up to 0.5%.
[c]Includes 16:1n-9 and 20:1n-7, each present at up to 0.5%.
[d]Includes 18:3n-6 and 22:4n-6, each present at up to 0.5%.
[e]Includes 18:4n-3 and 22:4n-3, each present at up to 0.5%. For abbreviations see Table 1.

TABLE 4
Coefficients of the Model Shown in Figure 1 and Related Statistical Evaluation[a]

	Midpoint			End point		
Term	Value	SE	*P*-value	Value	SE	*P*-value
Coefficients of the model						
Constant	8.49	1.321	$8.28E^{-7}$	2.894	1.17	0.02
Proportion of FO	–5.178	1.433	$1.27E^{-3}$	–0.278	1.269	0.828
Proportion of LO	–1.725	1.433	0.239	6.248	1.269	$4.11E^{-5}$
Proportion of RO	6.903	1.797	$7.08E^{-4}$	–5.97	1.592	$8.95E^{-4}$
(Proportion of RO)^2	–3.779	2.169	0.093	11.429	1.922	$2.82E^{-6}$

Statistical evaluation	Midpoint	End point
Number of cases	30	30
Degrees of freedom, regression	3	3
Degrees of freedom, residual	26	26
F value	45.8	43.5
Adjusted r^2	0.823	0.815
P-value regression	$1.61E^{-10}$	$2.78E^{-10}$
Lack-of-fit *P*-value	0.075	$6.03E^{-8}$
Residual SD	1.21	1.07

[a]For abbreviations see Table 1.

sampling, which changed to 0.82. The use of other square terms made no appreciable change to the adjusted r^2 or *P*-values. Hence, the use of linear terms and a square term for the inclusion of RO was chosen as the most appropriate regression model. Table 4 gives full details of the model, and Figure 1 represents it graphically. The lack-of-fit term for the end point data was noteworthy, as the fact that the *P*-value was less than 0.05 suggested that, although the model chosen gives a good fit explaining over 80% of the variance, there are still significant effects that are not explained by the model. In contrast, the fact that the midpoint data showed a lack-of-fit *P*-value of greater than 0.05 suggested that much of the error for these data is replicate error.

Flux through the FA desaturation/elongation pathway was greater in hepatocytes from fish fed diets containing vegetable oils compared to the fish fed the standard diet containing only FO (Fig. 1). The activities reflected the level of vegetable oil substitution, increasing linearly as the level of substitution increased. Hepatocyte FA desaturation/elongation was approximately 50% higher in fish fed RO-substituted diets than in fish fed LO-substituted diets. However, after 50 wk and using the modeled levels, the lowest level of hepatocyte FA desaturation occurred in a wide range of RO substitution from no substitution to the 50% level (Fig. 1). LO-fed fish had generally higher levels than RO-fed fish when the same levels of substitution were compared. The changes with substitution were not linear.

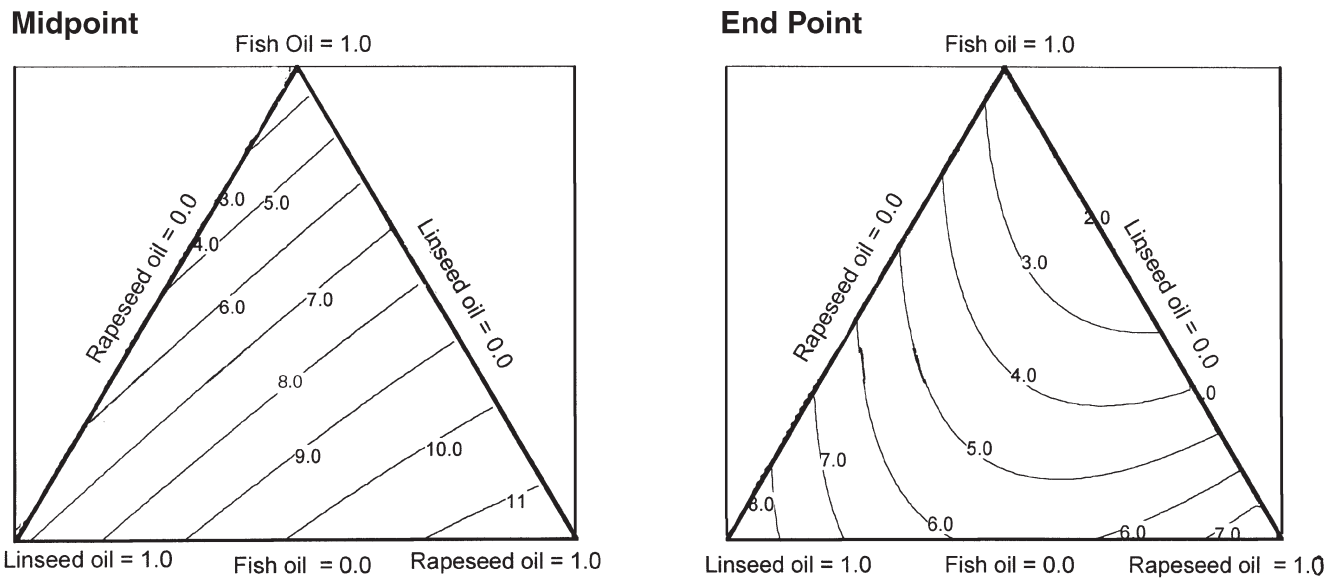

FIG. 1. Mixture contour plot of levels of hepatocyte FA desaturation at mid- and end point sampling. Vertices of the triangles represent 100% of the added oil from fish oil (upper vertex), linseed oil (lower left vertex), or rapeseed oil (lower right vertex). Contour lines represent the modeled levels of FA desaturation using the model shown in Table 4.

TABLE 5
Correlation[a] (regression) Analyses (r^2 slope values and significance) for Dietary FA and Liver Total Lipid Content, Dietary and Liver FA Compositions, and Hepatocyte Fatty Acyl Desaturation Activity, and Dietary and Liver FA Compositions

	32 wk			50 wk		
FA	r^2	Slope	Significance	r^2	Slope	Significance
(A) Diet vs. liver total lipid						
18:2n-6	0.04	0.02 ± 0.04	0.5714	0.45	0.41 ± 0.16	0.0326
18:3n-3	0.05	0.01 ± 0.01	0.5203	0.09	0.05 ± 0.06	0.3984
Total C_{18} PUFA	0.02	0.00 ± 0.01	0.6690	0.19	0.07 ± 0.05	0.2048
20:5n-3	0.01	–0.02 ± 0.08	0.8435	0.44	–0.87 ± 0.35	0.0371
22:6n-3	0.01	–0.01 ± 0.06	0.7575	0.46	–0.61 ± 0.23	0.0300
Total n-3 HUFA	0.01	–0.01 ± 0.03	0.8033	0.44	–0.31 ± 0.12	0.0359
(B) Diet vs. liver FA						
18:2n-6	0.89	0.49 ± 0.06	<0.0001	0.91	0.67 ± 0.07	<0.0001
18:3n-3	0.93	0.28 ± 0.03	<0.0001	0.94	0.46 ± 0.04	<0.0001
20:5n-3	0.19	0.35 ± 0.26	0.2052	0.73	1.15 ± 0.25	0.0017
22:6n-3	0.51	1.11 ± 0.38	0.0204	0.75	2.30 ± 0.47	0.0012
(C) Diet vs. desaturation						
18:2n-6	0.80	0.63 ± 0.11	0.0005	0.34	0.37 ± 0.18	0.0785
18:3n-3	0.00	–0.003 ± 0.07	0.9645	0.43	0.12 ± 0.05	0.0398
Total C_{18} PUFA	0.05	0.04 ± 0.06	0.5588	0.54	0.12 ± 0.04	0.0162
20:5n-3	0.50	–1.07 ± 0.38	0.0223	0.58	–1.04 ± 0.31	0.0106
22:6n-3	0.50	–0.73 ± 0.26	0.0230	0.59	–0.72 ± 0.21	0.0097
Total n-3 HUFA	0.50	–0.39 ± 0.14	0.0219	0.58	–0.38 ± 0.11	0.0102
(D) Liver vs. desaturation						
18:2n-6	0.83	1.23 ± 0.2	0.0002	0.29	0.48 ± 0.27	0.1106
18:3n-3	0.002	–0.03 ± 0.24	0.8935	0.34	0.22 ± 0.11	0.0751
Total C_{18} PUFA	0.13	0.21 ± 0.19	0.3025	0.43	0.20 ± 0.08	0.0408
20:5n-3	0.26	–0.96 ± 0.57	0.1328	0.26	–0.52 ± 0.31	0.1311
22:6n-3	0.42	–0.43 ± 0.18	0.0436	0.38	–0.22 ± 0.10	0.0586
Total n-3 HUFA	0.47	–0.32 ± 0.12	0.0285	0.28	–0.16 ± 0.09	0.1136

[a]Correlation (regression) analyses of A: Dietary FA contents and liver total lipid contents. B: Dietary and liver FA composions. C: Dietary FA composition and hepatocyte fatty acyl desaturation. D: Liver FA composition and hepatocyte fatty acyl desaturation.

Regression analyses of lipid and desaturation data. Regression analyses were performed to determine correlations between specific individual dietary FA (18:2n-6, 18:3n-3, 20:5n-3, and 22:6n-3) or dietary FA groups (C_{18} PUFA and n-3 HUFA) and observed liver lipid data and levels of hepatocyte FA desaturation/elongation. At 32 wk, there was no correlation between dietary FA and liver total lipid contents (Table 5). However, after 50 wk liver total lipid levels were positively correlated with dietary 18:2n-6 and negatively correlated with dietary 20:5n-3, 22:6n-3, and total n-3 HUFA. There were positive correlations between dietary 18:2n-6 and 18:3n-3 levels and their level in liver total lipids both at 32 and 50 wk, with slopes all less than 1 (Table 5). The level of 20:5n-3 in liver total lipid was not correlated with dietary 20:5n-3 at 32 wk, although by 50 wk there was a positive correlation with a slope around 1 or perhaps higher (Table 5). In contrast, the relationship between dietary and liver lipid levels of 22:6n-3 was greater than with 20:5n-3, and the slope much greater than unity (Table 5, Fig. 2A).

Hepatocyte FA desaturation/elongation was negatively correlated with dietary 20:5n-3, 22:6n-3, and total n-3 HUFA at both 32 wk and 50 wk, although the r^2 values were higher at 50 wk (Table 5). At 32 wk, there was a strong positive correlation between dietary 18:2n-6 and hepatocyte FA desaturation, whereas there was no relationship between desaturation and dietary 18:3n-3. However, at 50 wk this situation was reversed, with only 18:3n-3, and not 18:2n-6, being positively correlated with hepatocyte FA desaturation/elongation (Table 5). Liver FA compositions were less strongly related to fatty acyl desaturation/elongation. There was a strong positive correlation between fatty acyl desaturation/elongation and liver 18:2n-6 levels and weak negative correlations between 22:6n-3 and total n-3 HUFA at 32 wk but little of significance at 50 wk (Table 5). The closer relationship between hepatocyte fatty acyl desaturation/elongation and dietary FA composition compared to liver FA composition is evident from Figures 2B and 2C.

DISCUSSION

The present study has confirmed results from earlier studies that showed increased flux through the FA desaturation/elongation pathway in fish fed diets containing vegetable oils compared

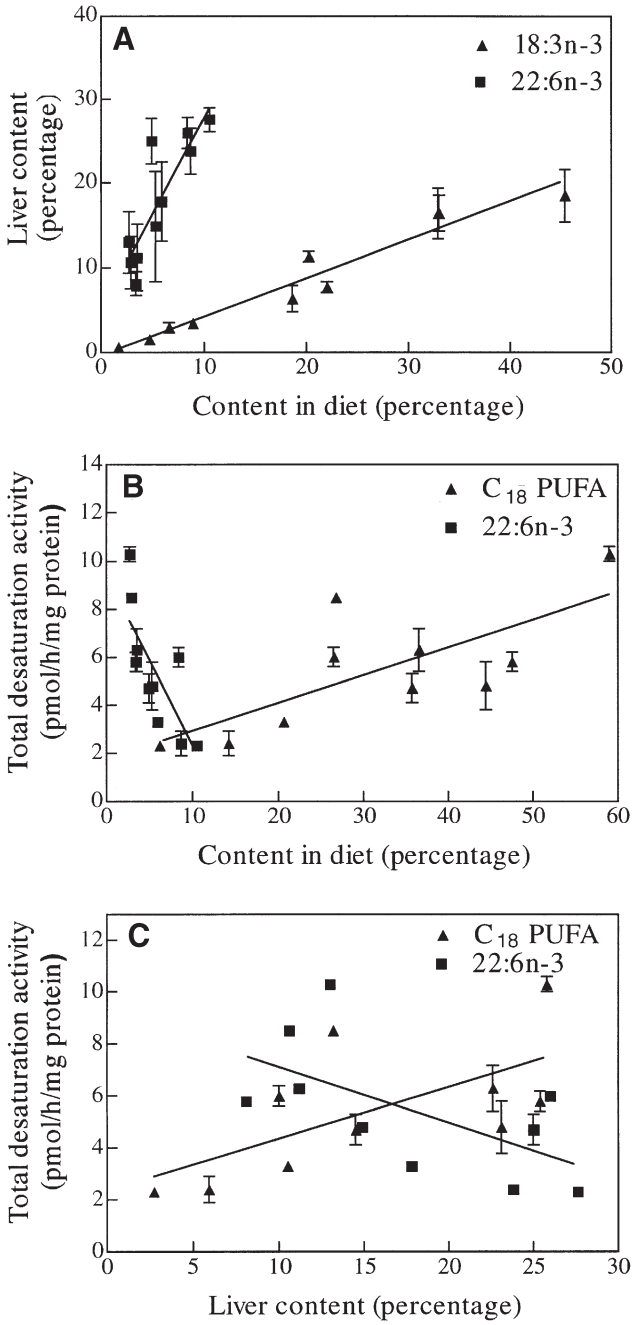

FIG. 2. Regression analyses showing relationships between FA compositions and hepatocyte fatty acyl desaturation/elongation after 50 wk. (A) Relationships between dietary and liver total lipid levels of 18:3n-3 and 22:6n-3; (B) relationships between dietary C_{18} PUFA and 22:6n-3 levels and hepatocyte FA desaturation/elongation; (C) relationships between levels of C_{18} PUFA and 22:6n-3 in liver total lipid and hepatocyte FA desaturation. Values represent mean ± SD (n = 3).

to fish fed diets containing FO. The levels of desaturation obtained with fish fed vegetable oil diets in the present study were up to fourfold higher than the levels in fish fed FO. In previous studies on salmonids in freshwater, activities were up to 2.5-fold (7), 2.4-fold (12), and 2.8-fold (13) greater in fish fed vegetable oil compared to fish fed FO. In salmon in seawater, 100% replacement of FO with RO resulted in a 2.7-fold increase in desaturation activity (8), whereas 100% replacement with palm oil increased the activity over 10-fold (9). Similar results have been obtained in mammals. Christiansen *et al.* (27) fed rats diets containing either sunflower oil (18:2n-6), LO (18:3n-3), a combination of these vegetable oils, or FO (n-3 HUFA) and investigated the effects on liver microsomal desaturase activities. Both Δ6 and Δ5 activities, as determined using 18:3n-3 and 20:3n-6 as respective substrates, were significantly stimulated by feeding the diets containing vegetable oils compared to the control laboratory chow diet or the FO diet. Microsomal Δ6 activity was significantly lower in rats fed the diet containing FO compared to rats fed the diets containing vegetable oils and the control chow diet when 18:2n-6 was used as the substrate (27).

The design of the present study lends itself to statistical treatment of the resultant data by regression analyses to determine potential correlations between specific dietary factors and specific outcomes. This is far more illuminating than ANOVA analyses, which simply indicate whether there is a significant difference between different diets. This was demonstrated very clearly when we sought to interpret and clarify dietary effects on two potentially related parameters, growth and lipid content. Both growth and liver lipid contents showed basically similar patterns in that there were no significant differences between treatments at 32 wk, but there were significant effects after 50 wk. There was no obvious pattern to these effects, but it is possible that the two were related, as lipid content is known to vary with fish weight in trout and salmon (28–30). The regression analyses showed that there was absolutely no relationship between dietary FA and growth. Furthermore, lipid levels in the liver were not significantly related to final fish weights ($P = 0.0561$, $r^2 = 0.3839$). Thus, the primary variation in final weights was almost certainly due to the change in feeding regime (from automatic to hand feeding) in the latter part of the experiment that probably affected the ration (30). In contrast, the variation in liver lipid content was related to dietary FA, with 18:2n-6 being positively correlated, indicating that it was associated with increased lipid deposition in the liver, whereas n-3 HUFA were negatively correlated and therefore associated with decreased liver lipid deposition. Thus, regression analyses proved to be a useful tool in interpreting the effects observed in this experiment.

The strongest correlations (highly significant, and with r^2 values near 1) were observed between dietary FA content and liver lipid FA composition. Similar strong correlations were observed between dietary FA content and muscle and liver FA compositions in salmon fed diets containing graded amounts of RO (8) and palm oil (9). In the present study, the slope for 22:6n-3 was greater than unity, which would indicate selective deposition and retention of this dietary FA in liver lipid (8,9). This may also be the case for 20:5n-3, although the slope was actually 1.15 ± 0.25. In contrast, the slopes for 18:2n-6 and 18:3n-3 were both less than one, which suggests that further metabolism of these FA had occurred. This could be the result of several processes, including catabolism for energy through β-oxidation and/or desaturation and elongation to HUFA (8,9).

The latter is certainly a factor, as evidenced by the increased desaturation and elongation observed with increasing dietary content of C_{18} PUFA-rich vegetable oils. A similar result was observed in a previous trial in salmon (8) in which FO was replaced incrementally with RO; in that work the correlations between dietary FA and liver FA compositions gave slopes of 0.52 and 0.31 for 18:2n-6 and 18:3n-3, respectively, and 0.75 and 1.36 for 20:5n-3 and 22:6n-3, respectively.

However, our primary aims in the present study were to determine the effects of specific dietary FA, namely, 18:2n-6, 18:3n-3, 20:5n-3, and 22:6n-3, both individually and in combination (C_{18} PUFA and n-3 HUFA), on the hepatocyte fatty acyl desaturation/elongation pathway. Strong negative correlations were obtained between dietary 20:5n-3, 22:6n-3, and total n-3 HUFA and the total flux through the FA desaturation/elongation pathway at both 32 and 50 wk. However, the most significant influence on hepatocyte FA desaturation was 18:2n-6, which was very strongly and positively correlated at 32 wk. In contrast, 18:3n-3 and total C_{18} PUFA were not correlated with FA desaturation at 32 wk but, conversely, the reverse was true at 50 wk, i.e., only 18:2n-6 was not correlated with desaturation. These data do not give unequivocal answers to either of our hypotheses/questions. Regarding the relative importance of substrate stimulation through C_{18} PUFA vs. product inhibition through n-3 HUFA, the rank order of importance changed from 18:2n-6 > 20:5n-3 = 22:6n-3 > 18:3n-3 at 32 wk to 22:6n-3 = 20:5n-3 > 18:3n-3 > 18:2n-6 at 50 wk using r^2 values as a measure of strength of the relationship. However, it could be argued that slope may be a better determinant of strength of effect and, as clearly shown in Figure 2B, a relatively small change in 22:6n-6 has a large effect on desaturase activity, whereas a much larger change in dietary C_{18} PUFA is required to produce the same magnitude of effect. This hypothesis is acknowledged as a particularly challenging one to test experimentally with practical diets, as the diets with high n-3 HUFA are necessarily the ones with low C_{18} PUFA and vice versa. The mixture model (Fig. 1, Table 4) used in the present study to analyze the desaturation data can be revealing, but we do not have a ready explanation for the change in pattern between the midpoint and end points. At the midpoint, the relationship was linear and, although there is a square term, it was not statistically significant and was only shown to maintain consistency with terms used with the end point. In contrast, the end point data were clearly nonlinear, and the square term was certainly significant. This would have been very difficult to predict, as would the minimum level of FA desaturation at the end point, which was no substitution to 50% RO substitution.

However, regarding our second hypothesis, it was evident that 18:2n-6 and 18:3n-3 indeed had different effects, with 18:2n-6 initially being more important, but 18:3n-3 having a more significant role later. Therefore, the mechanism of the observed effects on fatty acyl desaturation is not clear. Interestingly, though, the general lack of correlation between liver FA compositions and fatty acyl desaturation/elongation may be relevant. This could be due to the fact that the liver FA composition was that of total lipids, of which the majority was generally neutral lipid rather than membrane lipid (data not shown). However, in a study comparing the effects of dietary coconut and salmon oils in rats, the authors found no correlation between microsomal phospholipid FA profiles and microsomal desaturation rates (31).

In mammals, the situation regarding nutritional regulation of desaturase activities by dietary FA is unclear, and studies have been inconclusive. In rats, Δ6 desaturase is reported to be depressed by fasting and stimulated by EFA deficiency (5). Dietary FA were also reported to affect rat Δ6 desaturase activity, with PUFA, both 20:4n-6 and 18:2n-6, being depressors, although only in comparison to an EFA-deficient diet (5). More recently, desaturation of 18:2n-6 in small intestine was increased by feeding rats a diet containing 20% safflower oil, i.e., rich in 18:2n-6, in comparison to rats fed a diet containing 18% hydrogenated tallow and 2% safflower oil (32). Feeding the rats a diet containing 10% FO with 8% hydrogenated tallow and 2% safflower oil had no significant effect on intestinal Δ6 desaturase activity. Therefore, this study suggested that increased dietary substrate FA increased Δ6 activity whereas its activity was not apparently depressed by increased levels of 20:5n-3 and 22:6n-3, at least with respect to desaturation of 18:2n-6. However, the activity of rat liver Δ6 desaturase was higher in rats fed a diet rich in coconut oil compared to diets containing salmon oil (31). As neither of these oils provided much C_{18} precursor FA (no 18:3n-3 and only very low 18:2n-6), this study suggested that the presence of long-chain n-3 HUFA from the salmon oil was responsible for the lower Δ6 desaturase activity rather than an effect of dietary substrate FA concentration. Similarly, in mice previously fed an EFA-deficient diet, feeding a diet supplemented with corn oil did not alter tissue desaturase activities, whereas feeding a diet supplemented with FO inhibited both Δ6 and Δ5 desaturase activities (33). Thus, studies in mammals are consistent with the data obtained in the present study with salmon in suggesting that both substrate C_{18} PUFA content and product n-3 HUFA contents can be important in determining the level of FA desaturation. However, it is noteworthy that the greatest stimulation of hepatocyte desaturation that we have obtained in dietary trials with fish was with salmon fed a diet containing 100% palm oil, a diet that, as well as being low in n-3 HUFA, was also relatively low in C_{18} PUFA (9). Although this diet formulation was not EFA-deficient, with sufficient n-3 HUFA to satisfy basal EFA requirements being present in the fish meal, and there were no deficiency symptoms, the fish on the 100% palm oil diet may have been bordering on EFA deficiency (9).

It is important to note, however, that, despite increased hepatic FA desaturation/elongation, the liver fatty acyl compositions are considerably changed by the diets in the present study. Other studies also have demonstrated that replacement of large amounts of n-3 HUFA-rich FO by C_{18} PUFA-rich vegetable oils in salmon diets significantly changes the FA compositions of muscle (flesh) despite increased hepatic fatty acyl desaturation (7–9,11–13). This has been apparent even in studies, including this one, using diets rich in LO providing large amounts of 18:3n-3 (7,12). The conclusion must be that hepatic

and, presumably, total body fatty acyl desaturation capacity is not sufficient to convert the large amounts of dietary 18:3n-3 to 20:5n-3 and 22:6n-3 at the rates that would be required to maintain tissue n-3 HUFA at levels similar to those in fish fed FO. Substrate concentration is obviously not limiting, as 18:3n-3 is increased in both liver and muscle lipids and therefore 18:3n-3 is available for desaturation (7,12).

The precise molecular mechanisms underpinning the nutritional regulation of fatty acyl desaturation are unclear. In mammals, both Δ6 and Δ5 activities are depressed by fasting, and nutrients such as glucose, fructose, and glycerol are reportedly depressors, whereas protein is an activator (5). Regulation by nutritional state and specific nutrients is likely mediated by hormones. Certainly, insulin is known to activate both Δ6 and Δ5 desaturases (34). Cyclic AMP and glucagon (probably *via* cAMP) block the increase in Δ6 activity at refeeding, and epinephrine suppresses both Δ6 and Δ5 *via* activation of β-receptors (34). However, the involvement of protein kinases and phosphorylation in the regulation of fatty acyl desaturases has not been elucidated. Glucocorticoids and other steroids are also depressors of Δ6 and Δ5 desaturases, suggesting another mechanism, transcriptional control, in the regulation of desaturase activity. Recently, the cloning of PUFA desaturase enzymes from both mammals (35–37) and fish (38,39) has enabled gene expression to be studied. In mice fed 10% corn oil rich in 18:2n-6, the mRNA abundance and hepatic Δ6 activity were, respectively, 50 and 70% lower than those in mice fed 10% triolein, an EFA-deficient diet (35). The levels of hepatic mRNA for both Δ6 and Δ5 desaturases in rats fed either 10% safflower oil (18:2n-6) or menhaden oil (n-3 HUFA) were only 25% of those in rats fed a fat-free diet or a diet containing triolein (36). The liver mRNA level of a putative Δ6 desaturase cloned from rainbow trout was significantly higher in trout fed LO compared to trout fed FO (39). These results imply transcriptional regulation of desaturases in response to dietary FA.

The regulation of hepatic gene transcription by FA, particularly PUFA, is being increasingly studied (40). PUFA can potentially affect gene transcription by a number of direct and indirect mechanisms including changes in membrane composition, eicosanoid production, oxidant stress, nuclear receptor activation, or covalent modification of specific transcription factors (41). PUFA are now known to bind and directly influence the activities of a variety of transcription factors such as peroxisome proliferator-activated receptors (PPAR), which have in turn been shown to be critical regulators of a growing list of genes involved in lipid homeostatic processes (42). In rodents, peroxisomal proliferators, which also activate PPAR, are known to upregulate fatty acyl Δ6, Δ5, and Δ9 desaturases (43–45). PPAR genes have been identified in Atlantic salmon and plaice (46,47), and the peroxisomal proliferator clofibrate increased the desaturation of 20:5n-3 in rainbow trout (48). However, these data do not exclude the possibility that FA may also influence desaturase more directly at a membrane level through alterations in fluidity or membrane microenvironments.

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Desaturase Activities in Rat Model of Insulin Resistance Induced by a Sucrose-Rich Diet

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ABSTRACT: A sucrose-rich diet, as compared with a similar starch diet, induces a time-dependent typical noninsulin-dependent diabetes syndrome characterized by insulin resistance in rats. Within the first 3 wk, there was glucose intolerance associated with hyperinsulinemia, hypertriglyceridemia, and high plasma FFA. In this study, we examined the effect of the sucrose-rich diet vs. the starch diet during short- (3 wk) and long-term treatment (6 mon) on hepatic Δ9, Δ6, and Δ5 desaturases. These enzymes modulate monounsaturated FA and PUFA biosynthesis, respectively. Sucrose feeding (3 wk) caused an initial hyperinsulinemia that was normalized within 6 mon. In the early period (3 wk), stearoyl-CoA desaturase-1 (SCD-1) mRNA and activity were decreased, whereas Δ6 desaturase mRNA abundance and Δ6 and Δ5 desaturase activities remained unchanged. After 6 mon of sucrose feeding, activities of the Δ9, Δ6, and Δ5 desaturases were each increased. The SCD-1 and Δ6 desaturase mRNA were also correspondingly higher. These increases were consistent with an increase in oleic acid, the 20:4/18:2 ratio, and 22:4n-6 and 22:5n-6 acids in liver and muscle lipids. On the other hand, the percentage of 22:6n-3 acid was decreased. In conclusion, a sucrose-rich diet after 6 mon induces an increase in rat liver SCD-1 and Δ6 desaturase mRNA and enzymatic activities that are opposite to the changes reported in insulin-dependent diabetes mellitus. It appears that neither blood insulin levels nor insulin resistance is a factor affecting the Δ9, Δ6, and Δ5 desaturase changes in mRNA and activity found with the sucrose-rich diet.

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The two major types of diabetes mellitus are type 1, also called insulin-dependent diabetes mellitus (IDDM), which is associated with insulin deficiency, and type 2, also called noninsulin-dependent diabetes mellitus (NIDDM), which is strongly associated with insulin resistance. The latter is the most common form of the disease and has been investigated for many years (1,2). Studies have shown that alterations in carbohydrate and lipid metabolism contribute to the disease (3). Although it is generally considered that there are genetic or polygenetic explanations for type 2 diabetes (4), environmental factors also contribute to the disease process. However, the precise mechanism for these factors is yet to be determined.

Several animal models of type 2 diabetes are generally based on genetic alterations (5). However, we chose to study one based on the induction by fructose, or more specifically by a sucrose-rich diet. Similar to the genetic models, a sucrose-rich diet evokes glucose intolerance associated with hyperinsulinemia, increased plasma FFA, and hypertriglyceridemia (6,7). A three-step metabolic syndrome has been identified (8) depending on the time of administration of the sucrose diet. Step 1: Induction Period (3–5 wk) characterized by hypertriglyceridemia, a moderate increase in plasma FFA, hyperinsulinemia, and impaired glucose tolerance. Step 2: Adaptation Period (5–8 wk) featuring a spontaneous normalization of the aforementioned variables. Step 3: Recurrence Period (after 8 wk) involving moderate hyperglycemia with normoinsulinemia, hypertriglyceridemia, high plasma FFA, and severe glucose intolerance (insulin insensitivity).

A fructose diet also alters Δ9 desaturation activity. Early work (9) in our laboratories showed that after substituting fructose for dextrin in the diet for only 3 d, liver Δ9 desaturation of stearic acid was enhanced in both normal and streptozotocin-diabetic rats. In addition, Waters and Ntambi (10) recently showed that fructose, but not glucose, administration for 24 h to food-deprived diabetic mice increased the hepatic stearoyl-CoA desaturase-1 (SCD-1) mRNA 23-fold in a way similar to insulin administration. They also deduced that normal circulating insulin levels are not required for the fructose induction of the SCD-1 mRNA, and the simultaneous administration of fructose and insulin more than doubles the effect of each factor.

Given this information, we considered it important to extend our knowledge of the effect of a sucrose-rich diet and the resulting insulin resistance on the biosynthesis of PUFA modulated by Δ6 and Δ5 desaturase activities, and to compare these effects with the synthesis of the monounsaturated FA regulated by Δ9 desaturase activity. For this reason, we determined the effect of a sucrose diet on the hepatic activity of Δ6 and Δ5 desaturases, and Δ6 desaturase mRNA transcription. Moreover, the FA composition of different liver and muscle lipids was determined and correlated with changes in

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Abbreviations: IDDM insulin-dependent diabetes mellitus; NIDDM noninsulin-dependent diabetes mellitus; PPAR peroxisome proliferator-activating receptor; SCD-1 stearoyl-CoA desaturase-1.

mRNA and enzymatic activity of hepatic Δ6 desaturase and SCD-1 during the induction (3 wk) and recurrence (6 mon) periods. The results showed that a sucrose-rich diet resulted in an enhancement of Δ9, Δ6, and Δ5 desaturations after 6 mon, which was the reverse of what would be expected of an insulin resistance effect.

MATERIALS AND METHODS

Materials. [1-^{14}C]Stearic acid (56 mCi/mmol, 98% radiochemically pure) and [1-^{14}C]linoleic acid (55 mCi/mmol, 99% radiochemically pure) were purchased from Amersham Life Science (Amersham, Bucks, United Kingdom). [1-^{14}C]-Eicosa-8,11,14-trienoic acid (52 mCi/mmol, 98% radiochemically pure) was provided by New England Nuclear (Boston, MA). Unlabeled FA were provided by Nu-Chek-Prep (Elysian, MN). Cofactors used for enzymatic reactions were obtained from Sigma Chemical (St. Louis, MO). Solvents for HPLC were purchased from Carlo Erba (Milan, Italy).

Rat SCD-1 (Δ9 desaturase) cDNA was a kind gift from Dr. Juris Ozols (Department of Biochemistry, University of Connecticut, Central Health, Farmington, CT), and rat Δ6 desaturase cDNA was a kind gift from Dr. Tsunehiro Aki (Department of Molecular Biotechnology, Hiroshima University, Higashi-Hiroshima, Japan). Restriction enzymes and other DNA nuclei-modifying enzymes were obtained from Promega (Madison, WI), and were used for Northern blot determinations of mRNA (11).

Animal model and diets. Male Wistar rats, weighing 180–200 g, were purchased from the National Institute of Pharmacology (Buenos Aires, Argentina), and maintained in an animal room under controlled temperature (23°C) with a fixed 12-h light/dark cycle. Animal care followed international rules for experimentation with animals. They were initially fed a standard laboratory-rat diet (Ralston Purina, St. Louis, MO). After 1 wk, the rats were randomly distributed into two groups. The experimental group received a semisynthetic sucrose-rich diet containing by weight 63% sucrose, 17% vitamin-free casein, 5% corn oil, 10% cellulose, 3.5% salt mixture (AIN-93M-MX; 7), 1% vitamin mixture (AIN-93-VX; 12), 0.2% choline chloride, and 0.3% methionine. The control group received the same semisynthetic diet but with sucrose replaced by starch. The FA composition of both diets (% by weight) was as follows: 12.54% 16:0, 0.20% 16:1, 2.77% 18:0, 32.30% 18:1n-9, 51.52% 18:2n-6, and 0.67% 18:3n-3. The rats had free access to food and water. Diets were isoenergetic, providing ~15.28 kJ/g of food. The weight of each rat was recorded twice each week, and the individual energy intake of five rats in each group was also assessed twice each week. Energy intake in kJ/d, after 3 wk of diet, was as follows: control, 361.21 ± 30.55; sucrose-rich, 372.97 ± 13.62 ($P > 0.05$). Energy intake (kJ/d) after 30 wk of diet was control, 285.4 ± 16.75; sucrose-rich, 353.23 ± 14.8, $P < 0.01$). Rats fed sucrose for 6 mon gained ~15% more weight than controls. Control and experimental rat groups were processed and analyzed after 3 wk or 6 mon.

Analytical methods. Blood samples obtained at the times and conditions specified in the text were rapidly centrifuged at 4°C and 200 × *g* and analyzed immediately or stored at −20°C, and examined within the next 3 d. Plasma TG (13), FFA (14), and glucose (15) were determined by spectrophotometric methods. Immunoreactive insulin levels were measured by RIA using the method of Herbert *et al*. (16).

Liver organelle fractionation. After the specified times, lots of five rats from each group were killed by decapitation without anesthesia and exsanguinated. The liver and the gastrocnemius muscle of each rat were excised rapidly. The liver was placed in an ice-cold homogenizing solution (1:3 wt/vol) composed of 0.25 M sucrose, 0.15 M KCl, 0.1 mM EDTA, 1.41 mM *N*-acetyl cysteine, 5 mM $MgCl_2$, and 62 mM phosphate buffer (pH 7.4). Microsomes were obtained by differential ultracentrifugation at 100,000 × *g* (Beckman Ultracentrifuge) as described elsewhere (17). Samples were stored at −80°C. Protein concentration was measured according to the procedure of Lowry *et al*. (18).

Lipid fractionation and analyses. Lipids were extracted from total liver homogenate, microsomes, and gastrocnemius muscle according to the procedure of Folch *et al*. (19). Total phosphorus was determined by the method of Chen *et al*. (20). Free and esterified cholesterol were separated by TLC on Whatman high-performance TLC plates (Alltech, Deerfield, IL), 20 × 20 cm (Linear-K preabsorbed strip) using hexane/ethyl ether/acetic acid (80:20:1, by vol). Free and esterified cholesterol spots were visualized by the ferric chloride method of Lowry (21). They were quantified by comparison to curves constructed using commercial standards (1–5 μg). After staining, the plates were scanned and densitometric quantitation was performed using ID Image Analyses Software (Kodak, Rochester, NY).

Total phospholipids and TAG were separated by TLC using hexane/ethyl ether/acetic acid (80:20:1 by vol). The FA composition of the lipids was determined by GLC of their methyl esters in a Hewlett-Packard HP 6890 apparatus. They were injected into an Omega Wax 250 (Supelco, Bellefonte, PA) capillary column of 30 m, 0.25 mm i.d. and 0.25 μm film. The temperature was programmed to obtain a linear increase of 3°C/min from 175 to 230°C . The chromatographic peaks were identified by comparison of their retention times with those of authentic standards.

Δ9, Δ6, and Δ5 desaturation activity determination. The Δ9, Δ6, and Δ5 desaturations were estimated in hepatic microsomes, using as substrates 50 μM [1-^{14}C]stearic acid, 33 μM [1-^{14}C]linoleic acid, and 50 μM [1-^{14}C]eicosa-8,11,14-trienoic acid, respectively. The acids were incubated with 2.5, 2.0, and 1.95 mg of microsomal protein, respectively, in a final volume of 1.5 mL at 36°C. The reaction mixture consisted of 0.25 M sucrose, 0.15 M KCl, 1.41 mM *N*-acetyl-L-cysteine, 40 mM NaF, 60 μM CoA (sodium salt), 1.3 mM ATP, 0.87 mM NADH, 5 mM $MgCl_2$, and 40 mM potassium phosphate buffer (pH 7.4). After a 1-min preincubation at 36°C, the reaction was started by the addition of microsomal protein, and the mixture was incubated in open tubes for 15 min in a thermoregulated shaking water bath. The desatura-

tion reaction was stopped with 10% (wt/vol) KOH in ethanol, followed by saponification. The extracted FFA were dissolved in methanol/water/acetic acid (85:15:0.2, by vol) and fractionated by RP-HPLC. Separations were performed on an Econosil C18, 10-μm particle size, reversed-phase column (250 × 4.6 mm) (Alltech), coupled to a guard column (10 × 4 mm) filled with pellicular C18. The mobile phase consisted of methanol/water/acetic acid (90:10:0.2, by vol) at a flow rate of 1 mL/min; a Merck-Hitachi L-6200 solvent delivery system (Darmstadt, Germany) was used. The column eluate was monitored by a UV spectrometer at 205 nm for FA identification on the basis of their retention times. The effluent was mixed with Ultima Flo-M scintillation cocktail (Packard Instruments, Downers Grove, IL) at a 1:3 ratio, and the radioactivity was measured by passing the mixture through an on-line Radiomatic Instruments Flo One-β radioactivity detector (Tampa, FL), fitted with a 0.5-mL cell, at a rate of 3 mL/min.

Measurements of desaturase mRNA. Total liver RNA of the different rats tested was isolated with a Wizard RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions. Total RNA (20 μg) was size fractionated on a 1% formaldehyde gel and then transferred to a Zeta-Probe nylon membrane (Bio-Rad, Richmond, CA). The Δ9 and Δ6 desaturase and β-actin probes were prepared by incorporating [^{32}P]dCTP by random prime labeling. Northern blot hybridization analyses were performed as described by Sambrook *et al.* (11). The autoradiographic signals for Δ9 and Δ6 desaturase mRNA were quantified using 1D Image Analysis Software (Kodak) from multiple exposures. They were normalized to mRNA for β-actin, with all mRNA probed on the same gel.

Statistical analyses. The results are expressed as means ± SEM or ± SD. Significance was determined by Student's *t*-test (unpaired) or, when appropriate, data were subjected to ANOVA (Instat v. 2.0 Graph Pad Software, San Diego, CA) with diet as the main factor. The Tukey–Kramer multiple comparison test was used. Differences were considered significant at $P < 0.05$.

RESULTS

Blood variables. As reported in other publications (22) and confirmed in this study, a sucrose-rich diet produced significant changes in blood metabolic variables in rats, which evolved with the duration of feeding (Table 1). In samples of blood taken from rats that had been food deprived overnight, it was determined that after 3 wk of feeding a sucrose-rich diet (Induction Period) the glycemia did not change, but the FFA and TG levels increased (Table 1). The insulinemia did not increase. After 6 mon of feeding a sucrose-rich diet (Recurrence Period), the blood samples under food-deprived conditions showed increased glycemia, FFA, and TG levels but normal insulin levels. The blood samples taken from fed rats showed similar increases in FFA and TG variables for rats fed sucrose-rich diets both short term and long term (Table 1). The glycemia was normal for the first 3 wk but increased after 6 mon. In contrast, in rats that were fed, there was a significant increase in the blood insulin level of sucrose-fed rats compared with the controls after 3 wk of treatment, which disappeared after 6 mon (Table 1).

Effect of a sucrose-rich diet on liver Δ9 desaturation activity and the abundance of SCD-1 mRNA. The liver microsomal Δ9 desaturation activity for the conversion of labeled stearic acid to oleic acid in control rats and rats fed a sucrose-rich diet for 3 wk and for 6 mon clearly differed (Table 2). After 3 wk of feeding the sucrose-rich diet, the liver Δ9 desaturation activity declined sharply compared with that of the control rats fed starch (Table 2). This response occurred despite the observation that the insulinemia was higher in the sucrose-fed rats. When these results were compared with the relative abundance of SCD-1 mRNA in the livers of rats fed a sucrose-rich diet and control rats, we also found a decrease in the mRNA induced by the sucrose diet (Fig. 1) that correlated rather well with the enzymatic measurements.

However, after 6 mon of sucrose feeding, when the rats were in the Recurrence Period, their Δ9 desaturation activity was more than double the control values (Table 2), despite the similarity of

TABLE 1
Plasma Parameters in Rats Fed a Control or a Sucrose-Rich Diet at Different Times of the Diet[a,b]

	3 wk		6 mon	
	Control	Sucrose-rich	Control	Sucrose-rich
Food-deprived rats				
FFA (μequiv/L)	440.0 ± 28.0	578.4 ± 20.0**	526.0 ± 19.6	930.0 ± 29.4***
TG (mM)	0.33 ± 0.04	0.67 ± 0.01***	0.54 ± 0.05	1.20 ± 0.09***
Glucose (mM)	5.67 ± 0.2	5.54 ± 0.07	6.1 ± 0.19	6.98 ± 0.16**
Insulin (μU/mL)	8.50 ± 0.44	10.34 ± 2.03	8.36 ± 1.48	7.45 ± 1.34
Fed rats				
FFA (μequiv/L)	257 ± 22	487 ± 25***	280 ± 20	700 ± 40***
TG (mM)	0.56 ± 0.04	1.62 ± 0.07***	0.73 ± 0.03	2.28 ± 0.14***
Glucose (mM)	6.20 ± 0.04	6.45 ± 0.08	6.50 ± 0.06	8.20 ± 0.12***
Insulin (μU/mL)	51.2 ± 4.5	80.2 ± 6.1**	48.1 ± 3.6	50.2 ± 4.1

[a]Values are means ± SEM, $n = 5$. Asterisks indicate values are different from control ***$P < 0.001$; **$P < 0.01$ evaluated by Student's *t*-test.
[b]Rats were food deprived overnight.

TABLE 2
Δ9, Δ6, and Δ5 Desaturation Activities of Liver Microsomes at 3 wk and 6 mon of Sucrose Feeding[a]

	3 wk		6 mon	
Desaturase	Control (a)	Sucrose-rich (b)	Control (c)	Sucrose-rich (d)
	[nmol product/(min·mg protein)]			
Δ9	0.285 ± 0.040	0.072 ± 0.010^{a***}	0.141 ± 0.039^{a***}	0.391 ± 0.070^{b***,c***}
Δ6	0.185 ± 0.030	0.177 ± 0.037	0.150 ± 0.029	0.276 ± 0.056^{b***,c***}
Δ5	0.111 ± 0.025	0.162 ± 0.027	0.137 ± 0.018	0.213 ± 0.033^{c**}

[a]Results are means ± SD, $n = 5$. Differences were evaluated using ANOVA. Different roman superscript letters indicate differences between this value and that in the group headed by that letter. ***$P < 0.001$ and **$P < 0.01$.

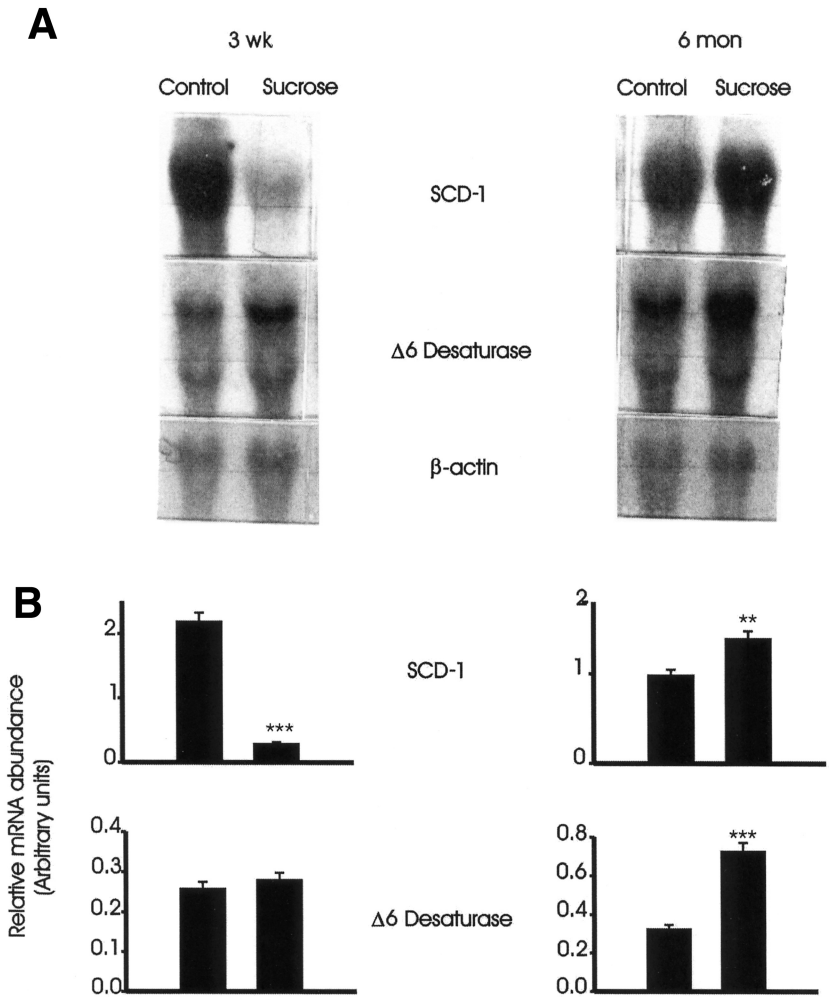

FIG. 1. Effect of a sucrose-rich diet on the levels of liver stearoyl-CoA desaturase-1 (SCD-1) and Δ6 desaturase mRNA. (A) Representative autoradiographs of Northern blot analysis of three rats. Total liver mRNA from control and sucrose-fed rats, at the periods indicated, were electrophoresed on a 1% agarose-formaldehyde gel, blotted to nylon membrane, and probed with ^{32}P random primed cDNA. mRNA levels were compared with β-actin signals. (B) The signals were quantified by ID Image System Software of Northern blots representing the ratio of the intensities of desaturases mRNA to β-actin mRNA. The results are means ± SD, $n = 3$. Asterisks indicate that values are different from control, ***$P < 0.001$; **$P < 0.05$; NS, not significant as evaluated by Student's *t*-test.

blood insulin levels in control and sucrose-fed rats (Table 1). Correspondingly, liver SCD-1 mRNA abundance was also much higher in the sucrose-fed rats than in the controls (Fig. 1).

Effect of a sucrose-rich diet on liver Δ6 and Δ5 desaturation activity and the abundance of Δ6 desaturase mRNA. The extent of liver microsomal Δ6 and Δ5 desaturations of labeled

linoleic and eicosatrienoic n-6 acids, respectively, in rats fed a control (starch-rich diet) and a sucrose-rich diet for 3 wk or 6 mon are shown in Table 2. After 3 wk, the Δ6 and Δ5 desaturation activities did not differ between diets. After 6 mon, both the Δ6 and Δ5 desaturation activities were significantly enhanced by the sucrose-rich diet compared with the control diet. The Δ6 and Δ5 desaturation activities of control rats did not change from 3 wk to 6 mon (Table 2).

The measurement of Δ6 desaturase mRNA abundance in rat liver (Fig. 1) showed similar changes to the activities of the enzyme in sucrose-fed rats after 3 wk and 6 mon. Δ6 Desaturase mRNA and enzymatic activity increased in the rats only after 6 mon of sucrose feeding compared with rats fed the control diet for the same time period.

Effect of diets and time on liver and muscle FA composition of lipids. Compared with the control starch-rich diet, the sucrose-rich diet did not affect free cholesterol or esterified cholesterol of liver and liver microsomes after 3 wk or 6 mon (Table 3). In addition, the diets did not affect the proportion of phospholipid in the lipids of liver and the corresponding microsomes.

The FA composition of the lipids of rats fed for 3 wk or 6 mon is shown in Table 4 for liver, Table 5 for liver microsomes, Table 6 for liver microsomal phospholipids and TAG, and Table 7 for muscle phospholipids and TAG. In all of the lipids studied, linoleic acid was a very important component due to its high concentration in the diet, whereas oleic acid was a minor component in all of the lipids except for TAG. Arachidonic acid was a major component in all of the lipids except the TAG.

The sucrose-rich diet generally produced some changes in the FA composition in all of the samples. Changes in the level of oleic acid correlated with the Δ9 desaturase activities, showing a significant decrease in the liver microsomal phospholipids of rats fed for 3 wk (Table 6) and a significant increase in the muscle and microsomal liver phospholipids and TAG (Tables 6 and 7) of rats fed the sucrose-rich diet for 6 mon. An enhancement of the 20:4/18:2 ratio and the n-6 PUFA, 22:4n-6, and 22:5n-6 in total liver lipids (Table 4) correlated with the increase in Δ6 and Δ5 desaturase activity in rats after 6 mon of sucrose feeding. Similar results were observed in liver microsomes (Table 5), and in phospholipids of liver microsomes (Table 6) and muscle (Table 7). The sucrose-rich diet increased the n-6 PUFA/18:2n-6 ratio after 6 mon, whereas the same diet simultaneously and antagonistically decreased the proportion of DHA (22:6n-3) of the n-3 family (Tables 4–7) at both 3 wk and 6 mon.

TABLE 3
Effect of the Diets on the Free and Esterified Cholesterol and Phospholipids in Total Liver and Liver Microsomes[a]

	3 wk		6 mon	
	Control	Sucrose-rich	Control	Sucrose-rich
	(% of total lipid weight)			
Free cholesterol	4.01 ± 0.20	3.71 ± 0.18	2.98 ± 0.43	3.31 ± 0.46
Cholesterol esters	2.23 ± 0.53	2.08 ± 0.38	2.34 ± 0.60	1.41 ± 0.53
Phospholipids	59.31 ± 2.22	58.79 ± 1.11	60.86 ± 4.52	66.49 ± 8.46
Liver microsomes				
Free cholesterol	2.93 ± 0.35	3.48 ± 1.08	3.06 ± 0.21	2.92 ± 0.22
Cholesterol esters	1.43 ± 0.51	0.91 ± 0.12	1.06 ± 0.30	0.86 ± 0.25
Phospholipids	84.59 ± 5.08	73.96 ± 7.66	74.78 ± 5.48	65.77 ± 2.91

[a]Results are means ± SD, $n = 4$. Groups did not differ when analyzed by Student's *t*-test.

TABLE 4
FA Composition of Total Liver[a,b]

	3 wk		6 mon	
FA	Control (a)	Sucrose-rich (b)	Control (c)	Sucrose-rich (d)
	(g/100 g)			
16:0	22.95 ± 3.08	21.75 ± 2.08	22.32 ± 1.14	21.04 ± 0.67
16:1	0.96 ± 0.20	1.41 ± 0.31	1.89 ± 0.30	1.69 ± 0.19
18:0	21.00 ± 2.88	20.36 ± 0.96	14.10 ± 1.39	15.44 ± 0.41
18:1n-9	6.31 ± 0.58	8.08 ± 1.00	10.74 ± 2.07	12.03 ± 0.55
18:2n-6	18.87 ± 2.14	14.65 ± 0.36	22.65 ± 1.35	16.68 ± 0.94^{c***}
20:4n-6	20.77 ± 2.50	22.87 ± 1.95	18.29 ± 1.41	20.52 ± 1.00
22:4n-6	0.42 ± 0.24	0.97 ± 0.16^{a**}	0.65 ± 0.08	1.10 ± 0.19^{c*}
22:5n-6	0.37 ± 0.14	2.55 ± 0.52^{a***}	0.94 ± 0.17	3.26 ± 0.72^{c***}
22:6n-3	6.10 ± 0.77	3.69 ± 0.16^{a***}	4.10 ± 0.64	2.96 ± 0.44
20:4/18:2	1.23	1.56	0.81	1.23

[a]Results are means ± SD, $n = 4$. Differences were analyzed by ANOVA. Different roman superscript letters indicate differences between this value and that in the group headed by that letter. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.
[b]Only the main FA are tabulated. Other FA make 100%.

TABLE 5
FA Composition of Liver Microsomes[a,b]

	3 wk		6 mon	
FA	Control (a)	Sucrose-rich (b)	Control (c)	Sucrose-rich (d)
		(g/100 g)		
16:0	19.95 ± 0.35	19.32 ± 0.76	21.37 ± 1.21	20.83 ± 2.04
16:1	0.90 ± 0.44	0.70 ± 0.13	0.80 ± 0.55	0.98 ± 0.22
18:0	21.39 ± 1.23	23.50 ± 0.47	18.18 ± 0.82	19.18 ± 1.77
18:1n-9	4.77 ± 0.35	4.73 ± 0.16	6.20 ± 1.46	7.13 ± 0.23
18:2n-6	13.84 ± 0.73	10.39 ± 0.48^{a**}	14.57 ± 1.64	11.41 ± 0.93^{c**}
20:4n-6	26.44 ± 0.45	29.47 ± 0.40	26.83 ± 1.97	27.49 ± 2.65
22:4n-6	0.82 ± 0.27	1.16 ± 0.22	0.59 ± 0.09	1.10 ± 0.22^{c*}
22:5n-6	0.52 ± 0.49	2.86 ± 0.60^{a**}	0.79 ± 0.16	3.71 ± 0.98^{c***}
22:6n-3	7.11 ± 0.69	4.80 ± 0.34^{a**}	6.21 ± 0.97	3.38 ± 0.32^{c***}
20:4/18:2	1.91	2.84	1.84	2.41

[a]Results are means ± SD, $n = 4$. Differences were analyzed by ANOVA. Different roman superscript letters indicate differences between this value and that in the group headed by that letter. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.
[b]Only the main FA are tabulated. Other FA make 100%.

TABLE 6
FA Composition of Hepatic Microsomal Phospholipids and TAG[a,b]

	3 wk		6 mon	
FA	Control (a)	Sucrose-rich (b)	Control (c)	Sucrose-rich (d)
		Phospholipids (g/100 g)		
16:0	22.95 ± 3.08	21.75 ± 2.08	22.32 ± 1.14	21.04 ± 0.67
16:0	20:17 ± 0.70	21.83 ± 3.93	22.36 ± 0.41	20.39 ± 1.19
16:1	0.56 ± 0.06	0.54 ± 0.05	0.64 ± 0.05	0.66 ± 0.15
18:0	23.42 ± 0.81	25.74 ± 1.06	21.67 ± 1.39	20.79 ± 0.40
18:1n-9	3.81 ± 0.28	2.65 ± 0.06^{a***}	3.24 ± 0.16	4.42 ± 0.27^{c***}
18:2n-6	12.60 ± 0.58	7.74 ± 0.53^{a***}	11.33 ± 0.64	9.25 ± 0.80^{c*}
20:4n-6	27.66 ± 0.69	29.90 ± 1.91	20.14 ± 1.19	30.79 ± 0.59^{c***}
22:4n-6	0.25 ± 0.03	0.95 ± 0.11^{a***}	0.50 ± 0.06	1.13 ± 0.08^{c***}
22:5n-6	0.17 ± 0.02	2.88 ± 0.78^{a*}	0.83 ± 0.28	4.11 ± 1.37^{c**}
22:6n-3	7.05 ± 0.53	4.79 ± 0.43^{a*}	6.16 ± 0.92	3.44 ± 0.55^{c**}
20:4/18:2	2.19	3.88	2.57	3.33
		TAG (g/100 g)		
16:0	31.34 ± 2.44	31.89 ± 4.49	23.88 ± 1.56	25.42 ± 0.61
16:1	2.41 ± 0.49	2.90 ± 0.19	2.75 ± 0.32	2.96 ± 0.22
18:0	10.41 ± 2.25	7.54 ± 0.99	5.16 ± 0.72	5.80 ± 0.81
18:1n-9	16.88 ± 1.55	18.47 ± 0.05	20.18 ± 1.73	26.61 ± 0.82^{c***}
18:2n-6	27.86 ± 1.45	27.46 ± 4.53	38.90 ± 0.18	30.41 ± 0.58^{c*}
20:4n-6	5.55 ± 0.95	7.35 ± 0.73^{a*}	6.86 ± 0.07	5.43 ± 0.32
22:6n-3	2.82 ± 0.24	0.55 ± 0.09^{a**}	Trace	Trace
20:4/18:2	0.19	0.27	0.17	0.18

[a]Results are means ± SD, $n = 4$. Differences were analyzed by ANOVA. Different roman superscript letters indicate differences between this value and that in the group headed by that letter. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.
[b]Only the main FA are tabulated. Other FA make 100%.

DISCUSSION

Blood parameters and insulin resistance. The data clearly show that during the Induction Period (3 wk), a sucrose-rich diet produces the typical plasmatic changes previously described (22), including increases in FFA and TG as well as hyperinsulinemia with normoglycemia. It is interesting to remark that this increase in plasma TG was shown (6) to be produced together with correlative increases in liver and heart TG. In addition, the group led by Lombardo (22) also demonstrated that during this period, the sucrose-rich diet moderately increased basal lipolysis and decreased the antilipolytic action of insulin in the adipocytes. Moreover, Lombardo *et al*. (7) showed that pancreas samples obtained from rats fed the sucrose-rich diet for 3 wk released three- to sixfold more insulin than those of the controls in the absence or presence of physiologic concentrations of glucose. Despite the changes in insulin secretion, a drop in glucose tolerance was shown, suggesting that a state of insulin resistance had developed. After 6 mon in the Recurrence Period, rats fed the sucrose-

TABLE 7
FA Composition of Muscle Phospholipids and TAG in Rats Fed a Sucrose-Rich Diet for 6 mon[a,b]

	Phospholipids		TAG	
FA	Control	Sucrose-rich	Control	Sucrose-rich
	(g/100 g)			
16:0	23.82 ± 0.85	23.63 ± 1.08	24.84 ± 1.01	23.49 ± 2.62
16:1	0.91 ± 0.14	0.74 ± 0.22	5.34 ± 0.91	4.49 ± 0.40
18:0	13.72 ± 0.16	13.65 ± 0.72	9.14 ± 3.49	5.12 ± 1.44
18:1n-9	4.65 ± 0.27	5.84 ± 0.54**	26.02 ± 0.68	33.19 ± 2.14**
18:2n-6	17.55 ± 0.91	15.26 ± 1.24**	30.39 ± 1.54	28.50 ± 2.64
20:4n-6	20.22 ± 0.71	22.44 ± 1.04**	0.99 ± 0.10	1.10 ± 0.19
22:4n-6	0.74 ± 0.05	1.28 ± 0.14***	ND	ND
22:5n-6	0.87 ± 0.13	4.47 ± 1.15***	ND	ND
22:6n-3	10.84 ± 1.26	6.90 ± 0.53***	ND	ND
20:4/18:2	1.15	1.47	0.03	0.04

[a]Results are means ± SD, $n = 5$. Asterisks indicate difference from controls, ***$P < 0.001$, **$P < 0.01$ (Student's *t*-test).
[b]Only the main FA are tabulated. Other FA make 100%; ND, not detected.

rich diet progressed to normoinsulinemia compared with controls; however, increased glycemia was now present, and plasma TG and FFA remained elevated.

Similar alterations in plasma variables were shown by Soria et *al*. (22) to be evident as early as 15 wk after treatment. These data suggest a minor response of the pancreas to the glycemic stimulus. To support this suggestion, Lombardo *et al*. (23) found that a prolonged period (30 wk) of sucrose-rich diet consumption significantly increased both islet number and β-cell area in the pancreas. However, this was not accompanied by an increase in immunoreactive insulin. Therefore, it was postulated that the newly emerged β-cells had some sort of derangement due to the increased insulin demand resulting from insulin resistance induced by the long-term feeding of the sucrose-rich diet. Tobey *et al*. (6) proposed in an early work that the insulin resistance resulting from chronic fructose feeding was due to the diminished ability of insulin to suppress hepatic glucose output, and not to a decrease in insulin-stimulated glucose uptake in muscle.

However, it is important to compare this insulin-resistance syndrome evoked by the sucrose-rich diet in rats with modern results and interpretations of the human insulin resistance found in NIDDM patients. In this respect, Shulman (2) found that under steady-state plasma concentrations of insulin and glucose, muscle glycogen synthesis was ~50% lower in diabetic subjects. Because muscle glycogen synthesis accounted for most of the whole-body glucose uptake, he considered it to play a major role in causing insulin resistance in NIDDM patients. Considering the possible rate-controlling steps in this process, including glycogen synthase, hexokinase II, and glucose transport, the Shulman laboratory (2) proved that glucose transport was the most effective. Moreover, FFA are associated with insulin resistance, and their increase causes a reduction of ~50% in the insulin-stimulated rates of muscle glycogen synthesis and whole-body glucose oxidation, consequently challenging some aspects of the old model of Randle *et al.* (24). Therefore, these analyses could help to explain the relevance and importance of lipid metabolism in muscle, liver, and adipose tissue.

In addition, peroxisome proliferator-activating receptors (PPAR) are involved in insulin resistance. Thiazolidinediones, which are high-affinity ligands of the nuclear receptor PPAR-γ, enhance target tissue sensitivity to insulin *in vivo* (25) and induce other antidiabetic effects. In fructose-fed rats, it was also shown that oral administration of troglitazone, a thiazolidinedione derivative, normalizes plasma TG and FFA concentration and glucose homeostasis, and completely prevents insulin resistance in either the 3-wk (26) or 6-mon long (8) experiments, without detectable changes in plasma insulin levels. PPAR-γ plays a specific role in fat cells and in other tissues, but not in liver. It has been suggested that PPAR-γ downregulates resistin, an adipocyte-secreted signaling molecule that induces insulin resistance, which has been suggested to increase in NIDDM (27). Although this conclusion has been refuted by some investigators (28–30), Stumvoll and Häring (31) indicated that the Pro12 Ala polymorphism in PPAR-γ2 represents the first genetic variant with a notable effect on the risk of common type 2 diabetes, modulating the production and release of adipose-derived factors, i.e., insulin-desensitizing FFA, tumor necrosis factor-α, resistin, and the insulin-sensitizing hormone adiponectin. Therefore, these aspects of human type 2 diabetes mellitus and the dietary fructose effect are very similar, consequently suggesting similar mechanisms of development.

Effect on Δ9 desaturase and monounsaturated FA biosynthesis. The presence of high levels of fructose in a sucrose-rich diet compared with a similar diet containing only starch altered liver Δ9 desaturase (SCD-1) activity and its mRNA abundance. However, the changes were quite different depending on the duration of the sucrose-rich diet feeding.

After 3 wk, comparative decreases were found in both the Δ9 desaturation activity and mRNA in spite of the significant increase found in the blood insulin level (Table 1). It has been known for a long time (32) that insulin injection activates the

depressed Δ9 desaturation activity found in livers of experimental insulin-dependent diabetic rats by a specific protein synthesis enhancement. However, from the present results, it may be deduced that the increased blood insulin levels resulting from administration of a sucrose-rich diet for 3 wk had no activating effect on the transcription of SCD-1 mRNA and its enzymatic activity, and that insulin was incapable of modulating this enzyme.

In addition, the FA compositions of the liver and muscle lipids show that the sucrose-rich diet evoked after 3 wk a significant, correlative decrease of oleic acid in liver microsomal phospholipids, but no differences in oleic or palmitoleic acids were found in other lipids. Therefore, the changes in these FA in this short period were limited.

A different scenario emerges in the biosynthesis of monounsaturated FA after 6 mon of sucrose feeding. Compared with control rats, a significant increase in the liver SCD-1 mRNA that correlated with a corresponding increase in the enzymatic activity was observed. In this case, the increased activity of Δ9 desaturation of stearic to oleic acid was also correlated with significant increases in oleic acid in the phospholipids and TAG of muscle and in total liver microsomes, and their phospholipid and TAG fractions. In these rats, the blood insulin level had no direct effect on the increased transcription of the SCD-1 mRNA and enzymatic activity because blood insulin levels were normal (Table 1). Moreover, an insulin resistance mechanism may also be rejected because it would have exactly the opposite effect to what was observed.

The SCD-1 isoform was tested in our experiments and this isoform is the only one present in rat liver. It is also present in adipocytes together with the SCD-2 isoform, and is the one depressed there by thiazolidinedione-specific ligands of PPAR-γ (33), demonstrating its importance in enzyme modulation. Consequently, the change in SCD-1 activity in liver of sucrose-fed rats cannot be ascribed to a direct effect of PPAR-γ; however, an indirect effect may be possible. This would be in accordance with the beneficial effects shown in the normalization of plasma TG, FFA, glucose, and insulin levels, and the elimination of insulin resistance by treatment of rats fed the sucrose-rich diet with troglitazone (8). However, a recently published work (34) showed that of all the thiazolidinediones tested, only troglitazone is able to induce PPAR-γ expression in rat liver, opening questions about PPAR-γ organ specificity.

Effect on Δ6 and Δ5 desaturases and PUFA biosynthesis. The effect of the sucrose-rich diet vs. a starch-rich diet on the Δ6 and Δ5 desaturation activities of liver microsomes in comparison with the blood insulin level showed that the 3-wk period of sucrose administration did not change their activity in spite of the hyperinsulinemia. After 6 mon, both enzymes were significantly activated in spite of invariance in insulinemia. Therefore, an insulin resistance mechanism affecting this enhancing activation must also be rejected.

We could measure the effect of sucrose on the mRNA transcription of only the Δ6 desaturase; correlative to the enzymatic activity, the abundance of Δ6 desaturase mRNA was not altered during the first 3 wk of sucrose treatment. After 6 mon, the increased activity of the Δ6 desaturation could be ascribed to a relevant enhancement of its mRNA transcription (Fig. 1).

The sucrose activation of the Δ6 and Δ5 desaturases after 6 mon was expressed in the FA composition of liver and muscle lipids in which the 20:4n-6/18:2n-6 ratio increased in each tissue examined. However, as indicated, the proportion of DHA (22:6n-3) was decreased at all times in all of the lipids in spite of the increase in the Δ6 and Δ5 desaturation activities.

The activity of these enzymes is a very important modulating factor in the biosynthesis of 20-carbon polyunsaturated EFA of both the n-6 and n-3 families, but this modulation does not always work in controlling the level of the n-3 acid DHA (22:6n-3) (35). This difference may be ascribed to the additional microsomal steps of chain elongations from 20:5n-3 to 22:5n-3 and 24:5n-3 and further Δ6 desaturation to 24:6n-3 acid, and a peroxisomal step of β-oxidation of the 24:6n-3 to 22:6n-3 acid, which are necessary after the sequence of desaturations and elongations of 18:3n-3 to the 20-carbon PUFA controlled by the Δ6 and Δ5 desaturases.

However, the specific depression of n-3 DHA is difficult to attribute to the above-mentioned peroxisomal steps because the same steps are required for the biosynthesis of docosapentaenoic acid (22:5n-6) of the n-6 family, which is significantly increased in all of the lipids. Therefore, we hypothesize that the effect is produced at a later step of oxidation, which would selectively differentiate the n-3 from the n-6 22-carbon PUFA. Nevertheless, both the starch-rich and sucrose-rich diets were n-3 FA deficient (0.03 wt% 18:3n-3 in total food). Consequently, the data indicate that a sucrose-rich diet exacerbates this deficiency. The present results suggest that the antagonistic behavior of the NIDDM syndrome is induced by the sucrose-rich diet compared with the experimental streptozotocin diabetes (IDDM) syndrome in which the diabetes status, while decreasing the 20:4n-6/18:2n-6 ratio through a depression of Δ6 (36) and Δ5 desaturases, increases the proportion of DHA (22:6n-3) in liver lipids as shown in our laboratory (35) and confirmed by others (37). The decrease in DHA may be important from many points of view because it has been shown (38) that replacement of corn oil by cod liver oil (rich in 22:6n-3) in the sucrose-rich diet provokes a reversal of the abnormal hypertriglyceridemia, high plasmatic FFA levels, and diminished peripheral insulin sensitivity. Moreover, Storlien *et al.* (39) suggested that in muscles, n-3 PUFA counteract insulin resistance, whereas saturated acids esterified in the muscle membrane phospholipids would enhance it, and a high n-6/n-3 ratio would be deleterious.

However, in spite of the similarity of the Δ9, Δ6, and Δ5 desaturase modulation of the activity and independence from insulin and insulin resistance found in sucrose-fed rats, different mechanisms modulate the Δ9 desaturases and the Δ6 and Δ5 desaturases, and although hormones such as glucocorticoids, mineralocorticoids, testosterone, and estradiol activate Δ9 desaturase, they deactivate Δ6 and Δ5 (37–40).

In the case of the increased expression of Δ6 and Δ5 desaturase, the contribution of PPAR-α, a nuclear receptor very important in liver and specifically activated by fibrates, and not by thiazolidinediones, has been proved (41). Therefore, it is possible that PPAR-α might be involved in some way in the sucrose modulation of these enzymes. This hypothesis gains special importance when we consider the latest findings of Nagai *et al*. (42), showing that a fructose-rich diet modifies the hepatic PPAR-α mRNA content and activity. In addition, the fructose-rich diet also increases the gene expression of sterol regulatory element binding protein-1 which, as recently shown (41), raises Δ6 and Δ5 desaturase mRNA expression. Therefore, those effects are coincident with the effects found in the present experiment with sucrose, and suggest another possible mechanism of enzyme activation.

It is important to remark that the basic underlying mechanism that brings about the effects shown in our experiments resides in the metabolic differences and interactions existing between fructose (sucrose) and glucose (starch). Moreover, because the metabolic effects induced by the sucrose-rich diet are very similar to those found in human NIDDM, it is an excellent and appropriate tool for studying and disclosing the developmental steps of the disease.

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Sterol-Mediated Regulation of Hormone-Sensitive Lipase in 3T3-L1 Adipocytes

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ABSTRACT: We previously reported that intracellular free cholesterol at physiological concentrations regulates the activity of neutral cholesterol esterase (N-CEase) in macrophages. The objective of the present study is to investigate whether the regulation of N-CEase by cholesterol is generally observed in other types of cells such as adipocytes with high activity of hormone-sensitive lipase (HSL), the same gene product as N-CEase. 3T3-L1 adipocytes were cultured with and without cholesterol (1–30 μg/mL) or 25-hydroxycholesterol (0.1–10 μg/mL), and changes in the N-CEase activity, expression of HSL mRNA, and protein were examined. Incubation (24 h) of cells with cholesterol did not change N-CEase activity, but incubation with 25-hydroxycholesterol decreased the activity in a concentration-dependent manner by 24 (24 h) and 54% (36 h). Quantitative reverse transcription-PCR indicated that 25-hydroxycholesterol (10 μg/mL) did not influence expression of HSL mRNA. However, Western blot analysis showed that this sterol reduced HSL protein by 72 (24 h) and by 93% (36 h), respectively. It was concluded that sterol-mediated regulation of HSL/N-CEase occurs not only in macrophages but also in adipocytes, and regulation appears to occur not at a transcriptional level but by a post-transcriptional process. Sterol-mediated proteolysis may be involved in the loss of HSL protein.

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The enzyme activity or immunoreactivity of hormone-sensitive lipase (HSL) was found originally in adipose tissue and subsequently in the heart, adrenal gland, ovary, placenta, testis, and skeletal muscle (1). It has been proposed that HSL in adipose tissue, heart, and skeletal muscle serves as a TG lipase in supplying FFA to these tissues (2). In contrast, the role of HSL in the adrenal gland and ovary may be related to its cholesteryl ester (CE) hydrolytic activity, for it mobilizes cholesterol from stored CE for steroid biosynthesis (2). HSL is also present in several mouse macrophage cell lines (3,4). Investigations have attributed the CE cycle hydrolase, neutral cholesterol esterase (N-CEase), and the activity in mouse macrophage cell lines to HSL (3,4). HSL mRNA is reportedly absent in human monocyte-derived macrophages, suggesting that a species difference may exist (5). However, Reue *et al.* (6) reported that HSL mRNA is detectable in human macrophages and that the level of HSL mRNA in monocyte-derived macrophages is approximately one-fortieth of the level in human adipose tissue.

The functional significance for macrophage expression of HSL has been speculated to be the hydrolysis of CE, liberating free cholesterol for efflux from the cells (3,4). Based on these observations, several investigators have postulated the importance of HSL in macrophage foam cell formation and in atherogenesis (3,4). For example, Escary *et al.* (7) demonstrated that essentially all N-CEase activity in RAW 264.7 cells, a murine macrophage cell line, and mouse peritoneal macrophages is accounted for by HSL. An increased expression of HSL in macrophage foam cells produced by transfection of RAW 264.7 cells with an HSL cDNA resulted in increased N-CEase activity and net hydrolysis leading to faster depletion of CE in foam cells. Recently, Okazaki *et al.* (8) reported that adenovirus-mediated overexpression of HSL eliminated CE from THP-1 foam cells at a faster rate, although a paradoxical effect of HSL on atherosclerosis was reported. The overexpression of macrophage-specific HSL did not improve susceptibility to diet-induced atherosclerosis in mice (9).

Intracellular free cholesterol concentration is tightly regulated by acyl-CoA:cholesterol acyltransferase (ACAT) and N-CEase in addition to regulation of cholesterol uptake through LDL receptors and cholesterol synthesis by HMG-CoA reductase (10). In the course of our study on the metabolism of CE in lipid droplets of J774 A.1 macrophages incubated with either LDL or HDL, we found that intracellular free cholesterol regulates N-CEase activity within a range of physiological concentrations; cholesterol at lower concentrations up-regulates the activity, and the activity is lowered at higher concentrations (11). Independently, Jepson *et al.* (12) reported that cholesterol accumulation in J774 A.2 macrophages leads to a decrease of both HSL activity and protein levels. These results suggest that HSL is regulated not only by cAMP-dependent mechanisms but also by a cholesterol-dependent mechanism. If the regulation of N-CEase by intracellular free cholesterol would occur universally in cells carrying this

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; CE, cholesteryl ester; CS, calf serum; ECL, enhanced chemiluminescence; HSL, hormone-sensitive lipase; LPDS, lipoprotein-deficient serum; N-CEase, neutral cholesterol esterase; RT, reverse transcription; SCAP, SREBP cleavage-activating protein; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein; SSD, sterol-sensing domain.

enzyme, such as steroidogenic cells (adrenal cortical cells, follicular granulosa cells, Leydig cells) and adipocytes, alterations in cellular cholesterol concentration may have an impact on reproductive physiology as well as atherogenesis.

To examine whether cholesterol-mediated regulation is a universal phenomenon in other cells, we studied the regulatory effect of cholesterol on 3T3-L1 adipocytes, which are the major site of lipolysis by HSL. We report that 25-hydroxycholesterol downregulates N-CEase activity by reducing the amount of HSL protein in adipocytes, not by affecting the mRNA level.

MATERIALS AND METHODS

Cell culture and differentiation. Mouse fibroblast 3T3-L1 cells (ATCC No. CL-173) were grown and maintained in 75-cm^2 flasks with DMEM containing 10% (vol/vol) calf serum (CS), penicillin G (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (0.3 mg/mL) (10% CS-DMEM) in 5% CO_2 and humidified air at 37°C. The cells were grown to confluence (Day 0) and treated with the first differentiation medium: DMEM containing 10% (vol/vol) FCS (10% FCS-DMEM) supplemented with 0.5 mM isobutylmethylxanthine, 0.25 μM dexamethasone, and 200 μM L-ascorbic acid for 48 h. The cells were then maintained in the second differentiation medium: 10% FCS-DMEM supplemented with 5 μg/mL insulin and 200 μM L-ascorbic acid. The second differentiation medium was changed every 3 d up to 5–7 d.

Measurement of N-CEase activity. Assay of N-CEase activity using CE as a substrate was carried out essentially as described in our previous papers (11,13,14).

Preparation of lipoprotein deficient serum (LPDS). LPDS ($d \geq 1.225$ g/mL) was prepared as described previously (11,15).

Incubation of 3T3-L1 adipocytes with cholesterol and 25-hydroxycholesterol. 3T3-L1 adipocytes (Day 7 differentiation) were washed with PBS (–) and cultured for the indicated periods in DMEM containing 10% (vol/vol) LPDS (10% LPDS-DMEM) supplemented with 200 μM L-ascorbic acid in the presence of cholesterol (0–30 μg/mL) or 25-hydroxycholesterol (0–10 μg/mL). Each medium was adjusted to contain the same concentration of vehicles: 0.3% ethanol in cholesterol experiment, and 0.1% ethanol in the 25-hydroxycholesterol experiment. Cholesterol and 25-hydroxycholesterol were dissolved in ethanol.

Reverse transcription-PCR (RT-PCR). RT-PCR was carried out as described in RNA PCR core kit protocols (Perkin Elmer Cetus Inc., Branchburg, NJ) with minor modifications. Total RNA from 3T3-L1 adipocytes was isolated as described previously (11,16). Total cellular RNA (0.25 μg) was subjected to reverse transcription with Oligo d $(T)_{16}$ nucleotide used as a primer in 5 μL of reaction mixture using murine leukemia virus RT for 15 min at 42°C. The reaction mixture was heated at 99°C for 5 min to inactivate RT. PCR was then carried out in a Gene Amp PCR System 2400 (Perkin-Elmer Cetus Inc.) for 18–40 cycles with 5 μL of the reverse-transcribed mixture, 0.625 units of AmpliTaq Polymerase and reaction kits in a final volume of 25 μL. Each cycle of PCR induced 1 min of denaturation at 95°C, 1 min of primer annealing at 55°C, and 2 min of polymerization at 72°C. The primers used were as follows: 5′-GCTGGTGCAGAGAGACAC-3′ (nucleotides 1515-1532 of mouse HSL coding sequence) as a forward primer and 5′-GAAAGCAGCGCACGCG-3′ (nucleotides 1906–1923 of mouse HSL coding sequence) as a reverse primer. PCR using these primers yields a 408-bp product. Each primer was added at 250 nM in the reaction mixture. For quantification, PCR products were electrophoresed in 1 × Tris-borate EDTA buffer on a 5% polyacrylamide gel for electrophoresis and stained with SYBR® Green I (Takara, Kyoto, Japan). The PCR products were evaluated by measuring the intensity of fluorescence of the bands using a FluorImager (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis. Cultured 3T3-L1 adipocytes were rinsed twice with 3 mL of PBS and scraped with a rubber spatula into 4 mL of PBS. The cell suspension was centrifuged at 100 × *g* for 5 min at 10°C. Cells in the pellets were homogenized in 0.2 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, 1 mM EDTA, 0.1% (wt/vol) *N*-lauroylsarcosine, 3% (vol/vol) Triton X-100, and 2 μg/mL leupeptin by freezing and thawing and centrifuging for 10 min at 13,000 × *g* at 4°C. The soluble extract was used immediately or stored at –80°C. The extracts of adipocytes were electrophoresed on 10% SDS-PAGE. Immunoblotting was performed using rabbit polyclonal anti-HSL IgG (1:10,000 dilution) as published previously (1). Immunoblots were visualized using enhanced chemiluminesence (ECL) detection reagent (Amersham Life Science, Little Chalfont, Buckinghamshire, England), and the relative amounts of immunodetectable HSL contained in each lane were determined by scanning with an LKB Ultra Scan XL enhanced laser densitometer and Gel Scan XL software (Pharmacia LKB Biotechnology, Uppsala, Sweden) on a NEC computer (Boxborough, MA).

Whole cell treatment with lipolytic drugs and glycerol assays. 3T3-L1 adipocytes (Day 7 differentiation) were cultured for 36 h in 10% LPDS-DMEM with and without 10 μg/mL 25-hydroxycholesterol. Cells on 3.6 cm^2 dishes were washed with PBS (–). Krebs–Henseleit original Ringer bicarbonate solution containing 2.5% FA-free BSA (Miles, Elkhart, IN) was then added to each dish. The cells were incubated for 1 h in the presence of 0–1 μM isoproterenol at 37°C in an atmosphere of 95% O_2–5% CO_2. Aliquots of samples withdrawn from lipolysis experiments were assayed for glycerol content using F-kit glycerol (Boehringer-Mannheim, Mannheim, Germany).

RESULTS

Effects of sterols on N-CEase activity. Figure 1 shows the change of N-CEase activity in 3T3-L1 adipocytes treated with sterols. 3T3-L1 adipocytes were cultured for 24 h in 10% LPDS-DMEM in the presence of cholesterol (0–30 μg/mL) or 25-hydroxycholesterol (0–10 μg/mL). Increasing concentrations of 25-hydroxycholesterol decreased N-CEase activity. There was a 15% reduction at 1 μg of 25-hydroxycholesterol/mL and a 25% reduction at 10 μg 25-hydroxycholes-

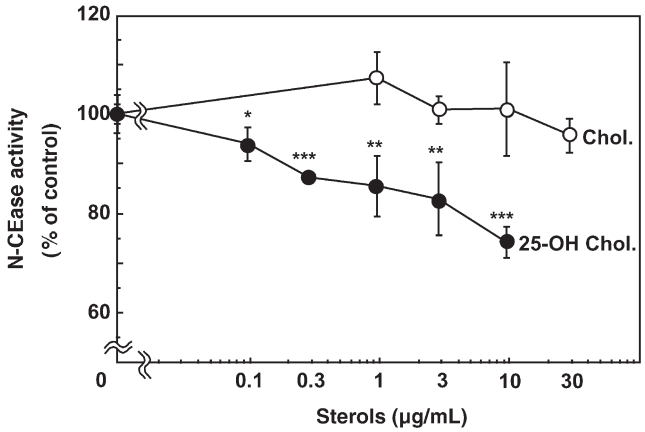

FIG. 1. Neutral cholesterol esterase (N-CEase) activity as a function of sterol concentrations in 3T3-L1 adipocytes. 3T3-L1 adipocytes (Day 8 differentiation) were cultured for 24 h in DMEM containing 10% lipoprotein-deficient serum (LPDS) in the presence of cholesterol (Chol.: ○, 0–30 μg/mL) or 25-hydroxycholesterol (25-OH Chol.: ●, 0–10 μg/mL). N-CEase activity in the supernatant (43,000 × *g*, 30 min) of cell homogenate was assayed as described in the Materials and Methods section. N-CEase activity in the absence of sterols was 156 ± 3.20 nmol cholesteryl ester (CE) hydrolyzed/h/mg protein ($n = 3$). Data represent mean ± SD of triplicate determinations. Significance: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. without sterols.

terol/mL. Cholesterol, however, did not affect N-CEase activity. 3T3-L1 adipocytes were cultured for various periods in 10% LPDS-DMEM with or without the addition of 25-hydroxycholesterol (10 μg/mL) (Fig. 2). N-CEase activity of cells cultured without 25-hydroxycholesterol was approximately twofold higher at 12 h and approximately 2.5-fold higher at 36 h compared with that of time 0, while N-CEase activity with the supplement of 25-hydroxycholesterol increased similarly up to 6 h when it reached a plateau and remained at the same level until 36 h.

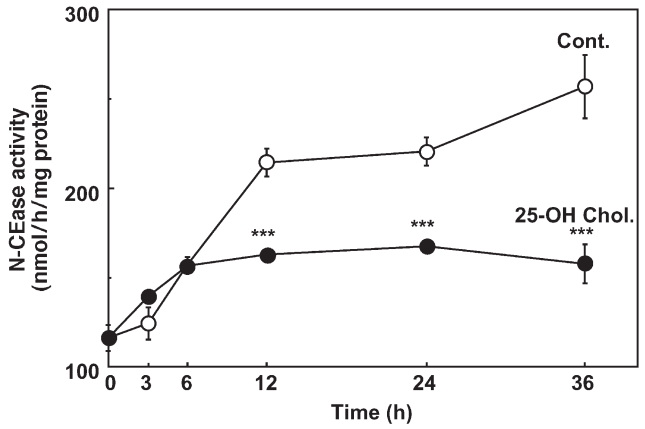

FIG. 2. Inhibition of N-CEase activity in 3T3-L1 adipocytes by 25-hydroxycholesterol (10 μg/mL). 3T3-L1 adipocytes (Day 8 differentiation) were cultured for indicated times in DMEM containing 10% LPDS in the presence (25-OH Chol.: ●) or the absence (Cont.: ○) of 25-hydroxycholesterol (10 μg/mL). N-CEase activity in the supernatants of cell homogenate was assayed as described in the Materials and Methods sectgion. Data represent mean ± SD of triplicate determinations. Significance: $^{***}P < 0.001$ vs. with 25-hydroxycholesterol. For abbreviations see Figure 1.

To determine whether 25-hydroxycholesterol exerts a direct effect on N-CEase, 25-hydroxycholesterol was added to the enzyme solution and the activity was assayed. Table 1 shows that addition of 100 ng/tube of 25-hydroxycholesterol to the enzyme solution did not show significant inhibition. These data suggest that 25-hydroxycholesterol does not inhibit N-CEase activity directly.

Effects of 25-hydroxycholesterol on expression of HSL mRNA. Quantitative RT-PCR was carried out to determine whether the 25-hydroxycholesterol-mediated changes in N-CEase activity are associated with the expression of HSL mRNA. When the PCR reaction was carried out with primers specific for HSL cDNA for 12–40 cycles, PCR products for 18–24 cycles reflected the first template cDNA that reverse-transcribed total RNA in adipocytes (Fig. 3). Therefore, a PCR reaction was performed for 20 cycles to determine the expression of HSL mRNA in adipocytes. When cells were incubated for 3, 6, 12, 24, and 36 h with 25-hydroxycholesterol (10 μg/mL) in 10% LPDS-DMEM, expression of HSL mRNA was not affected by the treatment with the sterol (Fig. 4).

Effects of 25-hydroxycholesterol on the amount of HSL protein. To examine whether the decrease in N-CEase activity associated with increasing concentrations of 25-hydroxycholesterol results from a corresponding reduction in the level of the HSL protein, Western blotting studies using polyclonal antibody raised against rat HSL were carried out on extracts of cells incubated in the presence and absence of 25-hydroxycholesterol (Fig. 5). When cells were incubated for 24 or 36 h in the presence of cholesterol, the amount of HSL protein was slightly decreased. In cells incubated with 25-hydroxycholesterol, the amount of enzyme protein was greatly reduced. Although addition of cholesterol to the medium decreased the amount of HSL protein by approximately 20%, 25-hydroxycholesterol markedly reduced it by 72 and 93% in the cells incubated for 24 and 36 h, respectively.

These results indicate that the loss of HSL protein is mainly responsible for the observed decrease in N-CEase activity in cells incubated with 25-hydroxycholesterol.

Effects of 25-hydroxycholesterol on lipolytic responses in cells. To see how the loss of HSL protein in the presence of

TABLE 1
Lack of Direct Effects of 25-Hydroxycholesterol on N-CEase Activity[a]

25-hydroxycholesterol (ng/tube)	N-CEase activity (% of control)	
	10% FCS	10% LPDS
0	100.0 ± 1.99	100.0 ± 0.93
1	99.9 ± 1.91	99.9 ± 1.13
10	99.6 ± 0.78	99.8 ± 0.84
100	98.4 ± 0.87	98.9 ± 2.09

[a]25-Hydroxycholesterol dissolved in ethanol was added to the assay buffer. The final concentration of ethanol in the assay mixture was less than 0.05%. 3T3-L1 adipocytes (Day 7 differentiation) were incubated for 24 h in DMEM containing 10% FCS or 10% lipoprotein-deficient serum (LPDS), and neutral cholesterol esterase (N-CEase) activity was assayed as described in the Materials and Methods section of the text. N-CEase activities in the absence of 25-hydroxycholesterol were 268 ± 2.49 (FCS) and 355 ± 7.06 (LPDS) nmol cholesteryl ester hydrolyzed/h/mg protein. Data represent mean ± SD of triplicate determinations.

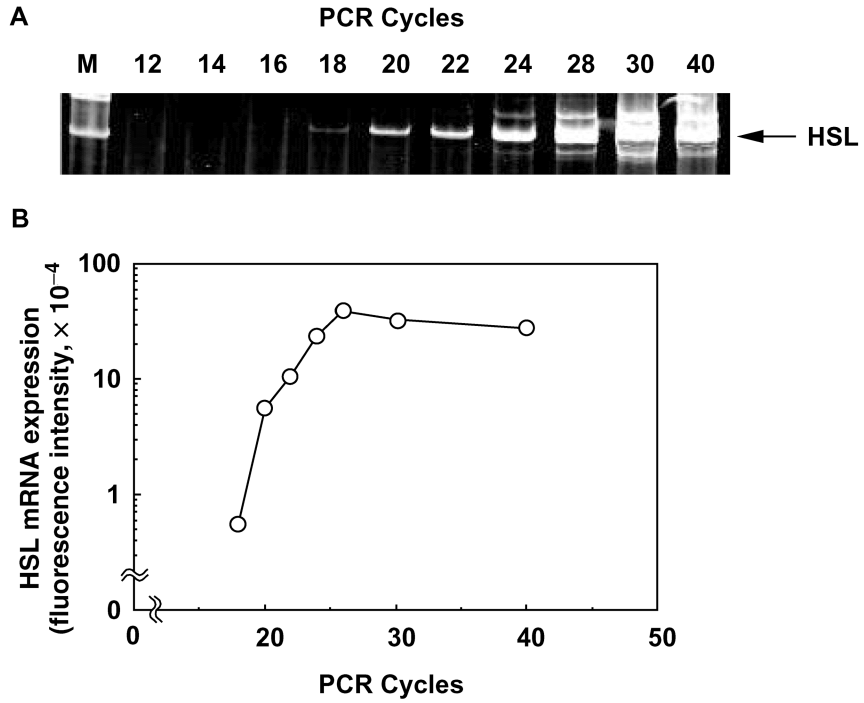

FIG. 3. Hormone-sensitive lipase (HSL) mRNA expression as a function of PCR cycles. Total RNA from 3T3-L1 adipocytes (Day 7 differentiation) was converted to cDNA by reverse transcription (RT). (A) PCR products were separated by 5.0% PAGE (100 V, 2 h) and detected by FluorImager (Molecular Dynamics). (B) HSL mRNA expression as a function of PCR cycles. M: size marker (pUC119 digested with *hinf* I). For other abbreviations see Figure 1.

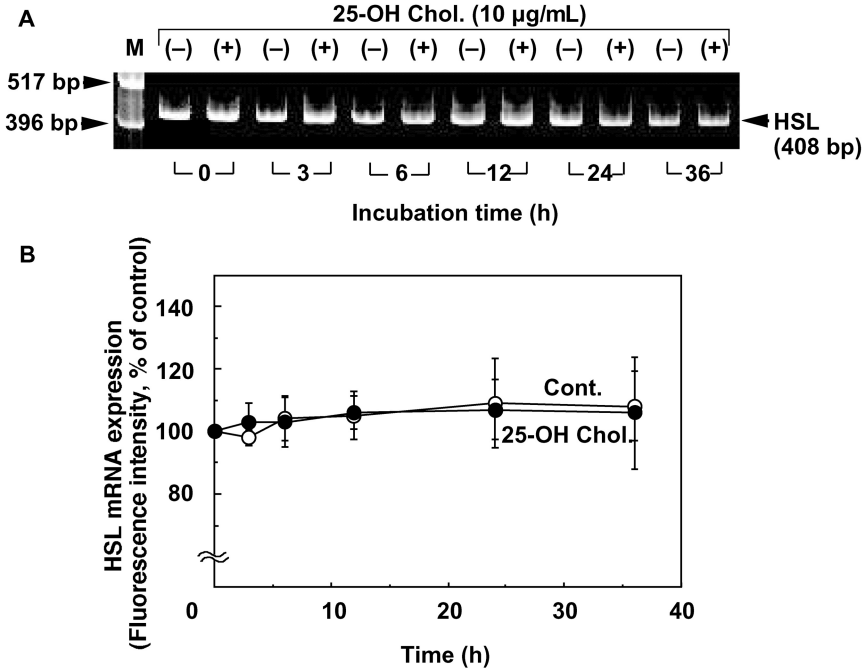

FIG. 4. Lack of transcriptional effects of 25-hydroxycholesterol on HSL mRNA expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes (Day 7 differentiation) were cultured for 0–36 h in DMEM containing 10% LPDS with (25-OH Chol.: ●) or without (Cont.: ○) 10 μg/mL 25-hydroxycholesterol. HSL mRNA was semiquantified by reverse transcription (RT)-PCR (20 cycles). (A) Time course of HSL mRNA expression. PCR products were separated on 5.0% PAGE (150 V, 2 h). (B) Densitometric analysis of RT-PCR. Data represent mean ± SD of quadruplicate determinations. M: size marker (pUC119 digested with *hinf* I). For abbreviations see Figures 1 and 3.

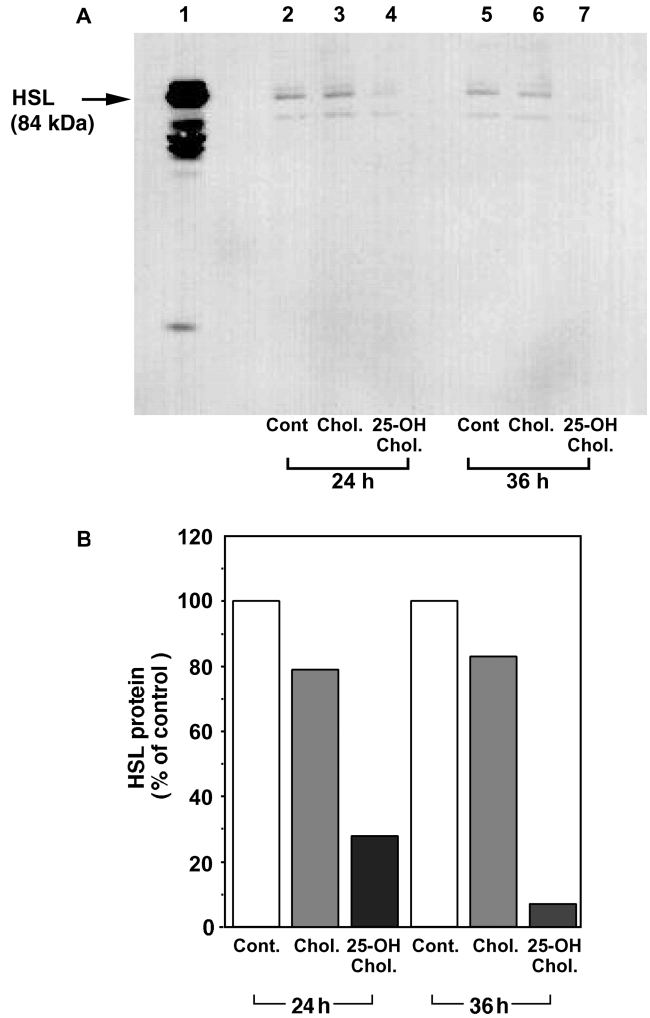

FIG. 5. Western blot analysis of HSL in 3T3-L1 adipocytes. 3T3-L1 adipocytes (Day 7 differentiation) were incubated for 24 or 36 h in the presence of 30 μg/mL cholesterol or 10 μg/mL 25-hydroxycholesterol. Cells were homogenated in lysis buffer by freezing and thawing (five times), and the mixtures were centrifuged for 10 min at 13,000 × *g*, 4°C. (A) The supernatant of rat epididymal adipose tissue homogenate (20 μg protein, lane 1) or cell homogenate (75 μg protein, lanes 2–7) was electrophoresed on SDS-PAGE (10%, 10 × 15 × 0.15 cm gel), transferred to nitrocellulose membrane, and then incubated with anti-HSL IgG, and subsequently with horseradish peroxidase-linked donkey anti-rabbit IgG. After washing, the membranes were then incubated with enhanced chemiluminescence detection reagents for 1 min prior to exposure to Kodak XAR film for 40 min at room temperature. (B) Densitometric scans of the ECL-stained Western blots were expressed a percentage of control. Data represent the mean of two independent experiments. Cont., control; for other abbreviations see Figure 3.

25-hydroxycholesterol influences cellular lipolytic response, 3T3-L1 adipocytes were preincubated for 36 h with and without 25-hydroxycholesterol and then stimulated with various concentrations of isoproterenol. Released glycerol concentrations in the medium were assayed (Fig. 6). Although basal lipolysis did not differ between adipocytes preincubated with and without 25-hydroxycholesterol, there were significant differences in lipolytic responses to 0.03, 0.1, and 1 μM isoproterenol. Treatment with 25-hydroxycholesterol significantly inhibited the lipolytic response by 18, 28, and 23% when cells were stimulated with 0.03, 0.1, and 1 μM isoproterenol, respectively, compared to that in cells without treatment. These results show that the treatment with 25-hydroxycholesterol reduces the lipolytic response to β-agonist and causes the loss of HSL protein.

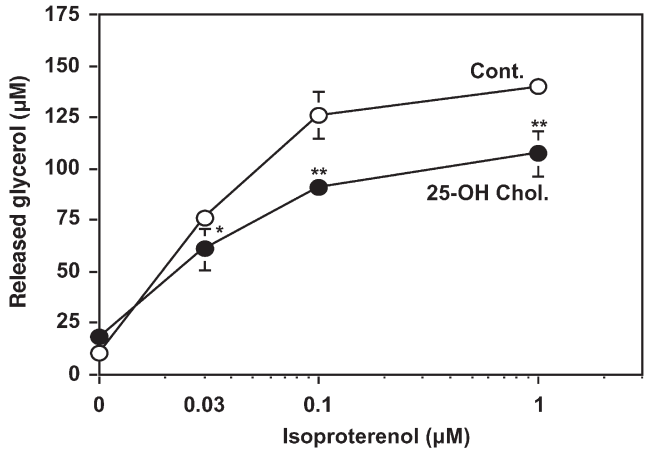

FIG. 6. 25-Hydroxycholesterol-mediated change of lipolysis in 3T3-L1 adipocytes. 3T3-L1 adipocytes (Day 7 differentiation) were cultured for 36 h in DMEM/10% LPDS with (25-OH Chol.: ●) and without (Cont.: ○) 10 μg/mL 25-hydroxycholesterol. Cells on 3.6-cm^2 dishes were washed with PBS (–). Krebs–Henseleit original Ringer bicarbonate solution containing 2.5% FA-free BSA was then added to each dish. The cells were incubated for 1 h in the presence of 0–1 μM isoproterenol at 37°C in an atmosphere of 95% O_2/5% CO_2. Aliquots of samples withdrawn from lipolysis experiments were assayed for glycerol content using F-kit glycerol. Results are mean ± SD of triplicate determinations. Significance: $^*P < 0.05$; $^{**}P < 0.01$ vs. with 25-hydroxycholesterol. For abbreviation see Figure 1.

DISCUSSION

HSL, a rate-limiting enzyme of the lipolytic cascade, hydrolyzes not only TG and DG but also CE, steroid FA esters, retinyl esters, and *p*-nitrophenyl esters (17). HSL/N-CEase activity is regulated by phosphorylation by cAMP-dependent protein kinase (18–21), and by dephoshorylation by protein phosphatase-1, -2A, and -2C (22,23). Anthonsen *et al*. (24) reported two novel phosphorylation sites affected by cAMP-dependent protein kinase, Ser-659 and Ser-660, responsible for *in vitro* activation of HSL. On the other hand, Olsson and Belfrage (22) and Wood *et al. (*23) reported that phosphatase PP2A and PP2C are involved in dephosphorylation of HSL/N-CEase, which are phosphorylated by cAMP-dependent protein kinase. Extracellular signal-regulated kinase was recently reported to regulate adipocyte lipolysis by phosphorylating HSL and increasing its activity (25).

Based on our findings that incubation of macrophages with HDL reduced CE content in lipid droplets while removing free cholesterol from cells, we hypothesized that cellular free cholesterol concentration might modulate N-CEase activity. This hypothesis was proven by the experiment in which intracellular cholesterol concentration was manipulated with an

HMG-CoA reductase inhibitor and an ACAT inhibitor (11). In the present study, this concept was extended to HSL in adipocytes, and we demonstrated that sterol reduces N-CEase activity through posttranslational mechanisms but not through transcriptional mechanisms. Our result is strongly supported by an observation in patients with familial combined hyperlipidemia (high serum cholesterol level), whose fat cells show decreased lipolytic activity compared with those of controls (26,27). HSL activity in fat cells from patients was reduced by 30%, which is attributed to a 70% reduction in HSL protein mass without any changes in mRNA level (28).

The reduction of N-CEase activity by 25-hydroxycholesterol treatment did not parallel that of protein mass in our experiment. The apparent discrepancy between enzyme activity and protein is often observed, as seen in Reference 28, which showed 70% loss of protein vs. 30% loss of activity. A somewhat similar discrepancy was observed in the 3T3-L1 adipocytes cultured with 10 μg of 25-hydroxycholesterol/mL (72% decrease in protein mass vs. 25% decrease in activity). In addition, the activity of CE hydrolysis increased only 5-fold in HSL overexpressing RAW264.7 cells that express 10- to 20-fold HSL protein compared to wild type (7). One possible reason for the discrepancy is that another TG lipase in 3T3-L1 adipocytes may compensate HSL activity that has been reduced by the protein mass. White adipose tissue from HSL–/– mice still retained 40% of TG lipase activities of the wild type. Release of FFA, but not of glycerol, increased by stimulation with isoproterenol in the HSL–/– adipocytes (29). Another possibility is that only phosphorylated HSL can be translocated to the surface of lipid droplets, access its substrate, and express activity (30). These possibilities may also explain the discrepancy between lipolytic reaction and protein reductions.

Cholesterol content of cells is controlled by a feedback system that governs the transcription of genes encoding enzymes of cholesterol synthesis and cell surface receptors for LDL (31). This system maintains a constant level of cholesterol in cells by governing the rate of cholesterol uptake from LDL and of cholesterol synthesis. Studies employing cloned genes for these proteins show that these negative feedback regulations are mediated at the transcriptional level and are determined by sequences (sterol regulatory elements, SRE) in the 5′ flanking regions of the genes (31). Li *et al.* (32) demonstrated that the mouse HSL gene has regulatory elements including SRE-1 in the 5′ flanking region. In our study with macrophages (11) and in 3T3-L1 adipocytes, the expression of HSL mRNA was regulated neither by cholesterol nor by 25-hydroxycholesterol. By contrast, Western blot analysis of HSL disclosed that the reduction of N-CEase activity in adipocytes treated with 25-hydroxycholesterol resulted from a decrease in the amount of the protein.

There are two possibilities for the decrease in HSL protein by the sterol: reduced translational efficiency of the mRNA and posttranslational mechanisms. Several factors decrease the expression of HSL mRNA (33–35), but changes in translation efficiency by sterols have not been reported so far. HSL protein degradation might occur by sterol-mediated proteases. The sterol-sensing domain (SSD) in HMG-CoA reductase (36,37) and in the NPC1 gene, which is involved in Nieman–Pick type C disease (38,39), targets degradation of the enzyme in response to intracellular sterol levels. Transcription of the gene encoding HMG-CoA reductase is also regulated by transcription factors, sterol regulatory element-binding protein (SREBP) 1 and SREBP2, whose cleavage and release from the membrane are in turn regulated by the sterol-responsive protein, SREBP cleavage-activating protein (SCAP) (40). The region of SCAP is also assumed to contain an SSD. Because an SSD is not found within the HSL amino acid sequence, HSL would presumably be degraded through some other pathway. In the future, experiments using inhibitors of translation and degradation and radiolabeling studies will be needed to clarify the mechanism(s).

Likewise, we speculated that FFA could be a modulator of HSL/N-CEase activity in macrophages and adipocytes. Treatment of cells with oleic acid did not change the activity (Miura, S., Nagura, H., and Tomita, T., unpublished data). Therefore, intracellular cholesterol concentration may play a specific role in maintaining cell homeostasis. We demonstrated in the present study that sterol-mediated downregulation of HSL/N-CEase also occurs in adipocytes. Although reproductive cells such as Leydig cells, follicular granulosa cells, and adrenal cortical cells were not examined in this study, it is possible that sterol-mediated regulation of HSL/N-CEase may influence these cell systems. This implies that the reproductive system could be under the control of the nutritional state. It is notable that targeted disruption of HSL in mice results in male sterility and adipocyte hypertrophy, but not in obesity (29).

In conclusion, sterol-mediated regulation of HSL/N-CEase occurs universally in cells carrying this enzyme, and the regulation site does not appear to be transcriptional but post-transcriptional. Sterol-mediated proteolysis might be involved in this regulation.

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Isoprenoid Alcohols Restore Protein Isoprenylation in a Time-Dependent Manner Independent of Protein Synthesis

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ABSTRACT: Mevalonic acid derivatives are required for the isoprenylation of a variety of growth-regulating proteins. Treatment of NIH3T3 cells with lovastatin (LOV), an HMG-CoA reductase inhibitor, depletes cells of these derivatives and impairs isoprenylation of RAS and RAS-related proteins. In LOV-treated cells, farnesol (FOH) and geranylgeraniol (GGOH) restore RAS and Rap1 isoprenylation, respectively. In this study, we further characterize the manner in which these isoprenoid alcohols are utilized for protein isoprenylation. Over a 48-h time span, FOH is unable to maintain RAS isoprenylation in the continuing presence of LOV, whereas GGOH is able to maintain Rap1 isoprenylation in the presence of LOV at all times tested. When cells are pretreated with LOV, the ability of both FOH and GGOH to restore protein isoprenylation is time dependent; as the LOV pretreatment time increases, the time required for FOH and GGOH to restore isoprenylation also increases. Despite this time dependence, the ability of FOH and GGOH to restore protein isoprenylation is not dependent on new protein synthesis and does not require alcohol dehydrogenase. These data support the existence of and further characterize the isoprenoid shunt, a novel metabolic pathway that utilizes FOH and GGOH for protein isoprenylation. The enzymes of the isoprenoid shunt are constitutively expressed, their activity may be modulated by isoprenoid depletion, and they are differentially regulated.

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Isoprenoid pyrophosphate intermediates are substrates for posttranslational modification of growth-regulating proteins. In particular, the farnesyl and geranylgeranyl moieties from farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are covalently linked to the C-terminus of RAS and RAS-related proteins (1). The corresponding alcohols for these pyrophosphates, farnesol (FOH) and geranylgeraniol (GGOH), restore cellular functions that have been altered by mevalonic acid depletion. For example, radiolabeled FOH is incorporated into cholesterol and proteins (2,3). Subsequent experiments have demonstrated that FOH is utilized for RAS farnesylation (4–6). Mevalonic acid depletion results in both FPP and GGPP deficiencies. Some characteristics of mevalonic acid depletion, such as cell cycle arrest, apoptosis, and morphology changes, are reversed by GGOH but not FOH (4,5,7–9), suggesting that the geranylgeranyl moiety is important in these cellular functions. Radiolabeled GGOH can be incorporated into proteins, and GGOH has been utilized for Rap1 isoprenylation (4,5,10). The common explanation for the utilization of FOH and GGOH for protein isoprenylation is their conversion to their pyrophosphorylated forms, although the precise metabolic steps catalyzing these reactions are largely unidentified. The activities of FOH and farnesyl phosphate kinases have been demonstrated in rat liver microsomes and peroxisomes, but the corresponding reactions for GGOH have yet to be described (11,12).

We previously demonstrated differences between the isoprenoid alcohols with regard to their abilities to reverse the changes induced by mevalonic acid depletion (6). GGOH both maintains and restores normal morphology in mevalonic acid-depleted fibroblasts (6), undoubtedly owing to the ability of GGOH or its pyrophosphate derivative to maintain and restore protein geranylgeranylation (4,5,13). In contrast, FOH does not uniformly alter the effects of mevalonic acid depletion, particularly in the case of RAS isoprenylation (6). In cells depleted of isoprenoid intermediates by treatment with the HMG-CoA reductase inhibitor lovastatin (LOV), concurrent treatment with FOH does not maintain RAS isoprenylation (6). However, in cells pretreated with LOV for 24 h, the addition of FOH for an additional 24 h restores RAS isoprenylation (6). This suggests that depletion of mevalonic acid or isoprenoid intermediates is necessary prior to utilization of FOH for protein isoprenylation. Because others have reported that FOH maintains isoprenylation when combined with LOV for shorter times (16–24 h) (4,5), the results observed with our longer incubations (6) may be a consequence of temporal differences in the activities of the enzymes necessary for the utilization of FOH. To explore this possibility further and to clarify the differing results, we characterized, in detail, the time course of maintenance and restoration of protein isoprenylation by both FOH and GGOH. Additionally, we assessed the requirement for *de novo* protein translation and alcohol dehydrogenase (ADH) on the ability of NIH3T3 cells to utilize FOH and GGOH for protein isoprenylation. FOH and GGOH are increasingly utilized experimentally as reagents to selectively replenish pharmacologically induced isoprenoid deficiencies. The data described herein are critical to interpreting the results of these experiments because, although the enzymes utilizing FOH and GGOH are

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Abbreviations: 4MP, 4-methylpyrazole; ADH, alcohol dehydrogenase; CHX, cycloheximide; FOH, farnesol; FPP, farnesyl pyrophosphate; FXR, farnesoid X receptor; GGOH, geranylgeraniol; GGPP, geranylgeranyl pyrophosphate; LOV, lovastatin; LXR, liver X receptor.

constitutively present, their activities appear to be modulated by isoprenoid deficiencies.

METHODS

Materials. DMEM, bovine calf serum, and trypsin-EDTA were from Gibco (Grand Island, NY). LOV was from Sigma (St. Louis, MO) and was converted to the free acid prior to use. Mevalonic acid, *trans-trans*-FOH, cycloheximide (CHX), and 4-methylpyrazole (4MP) were from Sigma. All-*trans*-GGOH was generously supplied by Dr. David Wiemer (University of Iowa, Iowa City, IA). Stock solutions of FOH, GGOH, and 4MP were prepared by diluting compounds in DMSO. Anti-Rap1 (sc-65) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). NCC-004 anti-RAS IgG was kindly provided by Dr. Setsuo Hirohashi (National Cancer Center, Tokyo, Japan), and rabbit antimouse IgG and Vectastain ABC kits were from Vector Laboratories (Burlingame, CA). ECL Plus™ kit was from Amersham Pharmacia Biotech (Buckinghamshire, England). All other chemicals were readily obtained from commercial sources.

Cell culture. NIH3T3 cells (American Type Culture Collection, Rockville, MD) were grown in DMEM supplemented with 10% heat-inactivated bovine calf serum and maintained at 37°C in humidified 5% CO_2 atmosphere.

Measurement of RAS and Rap1. Semiconfluent NIH3T3 cells were incubated in DMEM supplemented with 10% bovine calf serum containing 100 μM LOV or 200 μM zoledronic acid, 50 μM FOH, 50 μM GGOH, and/or 5 μg/mL CHX or 50 μM 4MP for the indicated time periods. As a control for each experiment, cells were incubated in 0.01% DMSO, as this served as the vehicle for FOH, GGOH, and 4MP. The cells were then harvested by trypsinizing, pelleted by centrifugation at 800 × *g* for 10 min, and washed twice with cold PBS (140 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). Cells were resuspended in 0.1 mL of PO_4-lysis buffer (10 mM Tris, 5 mM disodium EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 0.1 mM sodium orthovanadate, 0.1 mM PMSF) and vortexed intermittently for 30 min. The cell pellets were centrifuged at 10,000 × *g* for 1 h. The total protein concentration of the supernatant was determined using the method of Lowry *et al.* (14).

Cell lysate containing 25 μg of protein for RAS or 50 μg of protein for Rap1 was subjected to electrophoretic fractionation on denaturing 15% polyacrylamide gels (SDS-PAGE). After fractionation, the gel slab was transblotted to Immobilin-Polyvinylidene difluoride (Millipore, Bedford, MA) and the membrane blocked for 1 h at 37°C with 5% skim milk, 1% BSA, and 0.01% Antifoam A (Sigma) in PBS/0.1% Tween-20.

To detect RAS protein, as previously described (15), the membrane was sequentially incubated with NCC-004 anti-RAS antibody in 2 mL of 1:500 hybridoma supernatant for 1 h at 37°C, biotinylated rabbit antimouse IgG for 1 h at 37°C, and avidin-linked horseradish peroxidase (Vectastain ABC, Vector Laboratories) for 30 min. The membrane was washed five times with PBS/0.1% Tween-20 prior to each incubation. The RAS bands were visualized by incubation of the membrane with 0.7 mM 3,3-diaminobenzidine and 0.006% H_2O_2 in 10 mM Tris, pH 7.5. To detect Rap1 protein after blocking, the membrane was sequentially incubated with anti-Rap1 antibody, which will detect both modified and unmodified Rap1 (5) for 2 h at room temperature and horseradish peroxidase-conjugated antirabbit IgG for 1 h at 37°C. Rap1 protein was detected by enhanced chemiluminescence (ECL Plus, Amersham Pharmacia Biotech) and visualized by autoradiography.

Measurement of ADH. Lysate from untreated NIH3T3 cells or NIH3T3 cells that were incubated with 50 μM 4MP for 48 h or purified equine ADH (Sigma) plus or minus 50 μM 4MP was assayed for ADH activity. The conversion of ethanol to acetaldehyde at 30°C was measured by following the formation of reduced NADH from oxidized NAD^+ at 340 nm by use of a spectrophotometer (16). The final reaction volume was 1.5 mL, and reactions were carried out in 0.5 M Tris-HCl, pH 7.5, containing 2.8 mM NAD^+, 1 mg protein from cell lysate or 25 μg purified equine ADH, and/or 50 μM 4MP. Reactions were initiated by the addition of 5 mM ethanol. To determine whether 50 μM 4MP is sufficient to inhibit ADH activity, purified equine ADH was incubated with 50 μM 4MP for 5 m at 30°C prior to the addition of ethanol (16) or lysate from cells that were incubated with 50 μM 4MP for 48 h. ADH activity was calculated using the extinction coefficient for NADH at 340 nm, 6220 $M^{-1}cm^{-1}$. Activity was calculated as mol of NADH formed per min per mg protein.

Data analysis. Unmodified and modified RAS and Rap1 protein levels were quantified using the Scion Image Analysis program and normalized to the amount of modified RAS or Rap1 protein present in DMSO-treated NIH3T3 cells. The results from individual experiments were averaged and displayed beneath representative Western blots. Error bars on the graphs represent one SD from the mean. When appropriate, a two-tailed paired Student's *t*-test was performed to compare the levels of unmodified protein in cells treated with LOV alone to the levels of unmodified protein in cells treated under the described experimental conditions. Significance was defined as $P < 0.05$.

RESULTS

The effects of LOV concurrently with FOH or GGOH on protein isoprenylation. To determine the effects of FOH and GGOH on protein isoprenylation, we used LOV first to inhibit isoprenylation. RAS and Rap1 proteins were chosen because RAS is preferentially farnesylated, and Rap1 is geranylgeranylated. Before determining the time course of FOH and GGOH effects on RAS and Rap1 isoprenylation, respectively, NIH3T3 cells were incubated with LOV alone for times ranging from 12 to 48 h. Figure 1A depicts a Western blot of RAS protein from LOV-treated cells. At all times tested between 12 and 48 h, LOV inhibited isoprenylation of RAS. This can be seen by the appearance of the more slowly

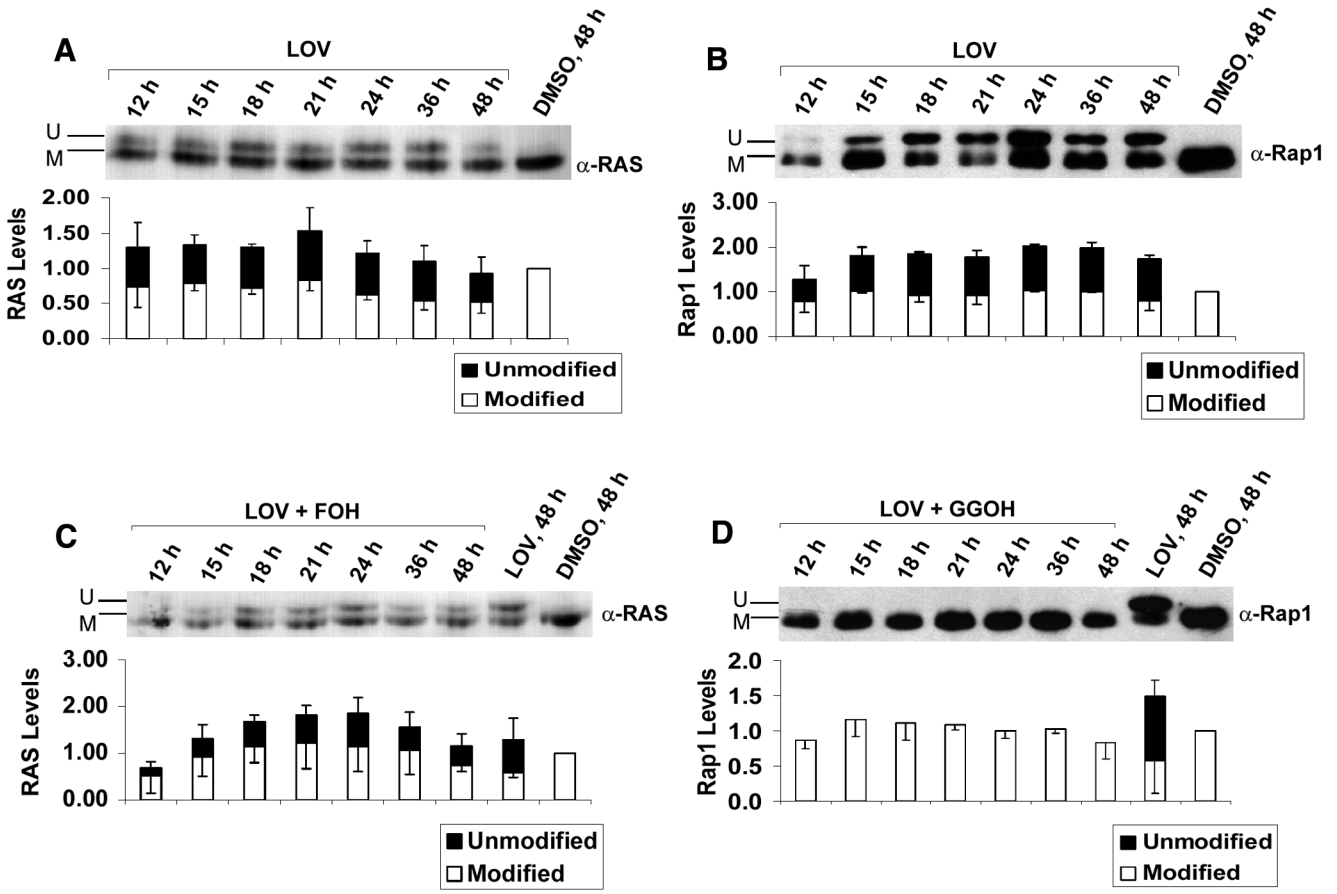

FIG. 1. The effects of lovastatin (LOV) concurrently with farnesol (FOH) or geranylgeraniol (GGOH) on protein isoprenylation. Western blot analysis of (A) RAS protein from NIH3T3 cells treated with 100 μM LOV for 12–48 h, (B) Rap1 protein from NIH3T3 cells treated with 100 μM LOV for 12–48 h, (C) RAS protein from NIH3T3 cells treated with 100 μM LOV plus 50 μM FOH for 12–48 h, and (D) Rap1 protein from NIH3T3 cells treated with 100 μM LOV plus 50 μM GGOH for 12–48 h. In this figure and all following figures, RAS or Rap1 protein from DMSO-treated cells served as controls. The relative levels of unmodified (U) and modified (M) protein from three individual experiments were quantified, normalized to the amount of modified protein present in DMSO-treated cells, averaged as described in the Materials and Methods section, and displayed beneath a representative Western blot. Error bars represent one SD from the mean.

migrating RAS band corresponding to the higher-M.W., unmodified RAS protein. Similarly, Figure 1B depicts a Western blot of Rap1 under the same conditions. LOV inhibits the isoprenylation of Rap1 at all times tested between 12 and 48 h, again reflected by the appearance of the more slowly migrating, higher-M.W., unmodified Rap1 protein band. We previously showed that FOH is unable to maintain RAS isoprenylation when added to cells concurrently with LOV for 48 h (6). We wanted to determine whether FOH, as well as GGOH, could maintain protein isoprenylation in the continuing presence of LOV prior to 48 h. Figure 1C is a Western blot of RAS protein from NIH3T3 cells treated with LOV plus FOH from 12 to 48 h. In spite of the continued presence of FOH, there is still an accumulation of unmodified RAS evident beyond 12 h. Figure 1D is a Western blot of Rap1 protein from cells treated concurrently with LOV and GGOH from 12 to 48 h. This figure demonstrates that, unlike FOH, GGOH is able to maintain Rap1 isoprenylation in the presence of LOV at all time points tested.

The effects of LOV pretreatment followed by FOH or GGOH on protein isoprenylation. To expand our prior studies demonstrating that treatment of cells with FOH for 24 h restores RAS isoprenylation after an initial 24-h LOV pretreatment (6), we determined the actual amount of time necessary for FOH or GGOH to restore protein isoprenylation after LOV pretreatment. NIH3T3 cells were incubated for 24 h with LOV, and then FOH or GGOH was added in the continuing presence of LOV for an additional 12 to 24 h. Figure 2A demonstrates that after a 24-h LOV pretreatment, exposure to FOH for longer than 18 h is required for partial restoration of RAS isoprenylation. Exposure of cells to FOH for 21 h or less is not sufficient for FOH to completely restore RAS isoprenylation. Figure 2B demonstrates that after a 24-h LOV pretreatment, exposure to GGOH must be longer than 21 h for restoration of Rap1 isoprenylation. Similar to FOH, 21 h or less is not sufficient for GGOH to restore Rap1 isoprenylation after a 24-h LOV pretreatment.

After determining the amount of time required for FOH- or GGOH-induced restoration of protein isoprenylation after a 24-h LOV pretreatment, cells were exposed to LOV for

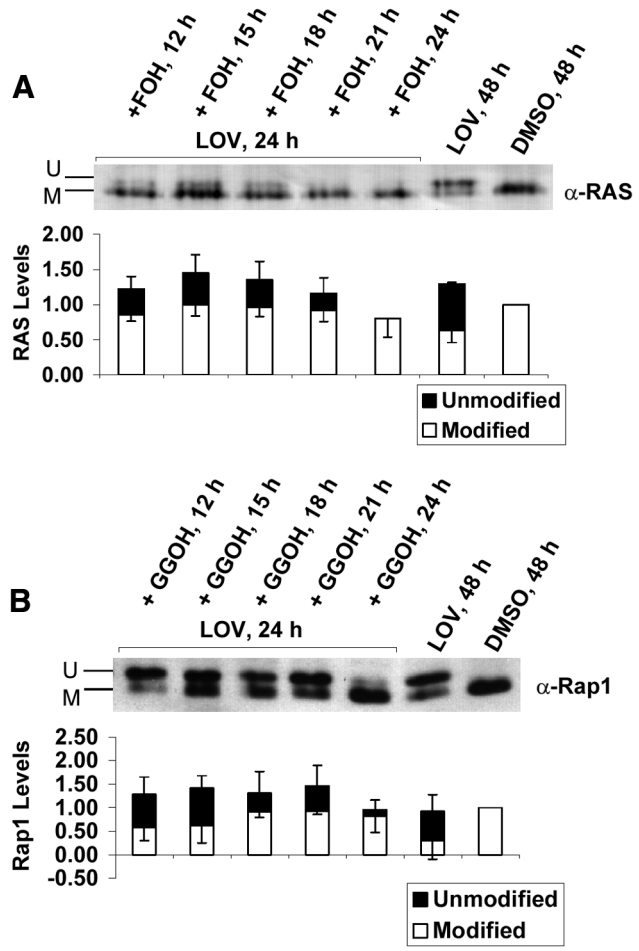

FIG. 2. The effects of LOV pretreatment followed by varying times of FOH or GGOH treatment on protein isoprenylation. Western blot analysis of (A) RAS protein from NIH3T3 cells pretreated with 100 μM LOV for 24 h and then 50 μM FOH added for an additional 12–24 h, and (B) Rap1 protein from NIH3T3 cells pretreated with 100 μM LOV for 24 h and then 50 μM GGOH added for an additional 12–24 h. The relative levels of U and M protein from three individual experiments were quantified, normalized to the amount of M protein present in DMSO-treated cells, averaged as described in the Materials and Methods section, and displayed beneath a representative Western blot. Error bars represent one SD from the mean. See Figure 1 for abbreviations.

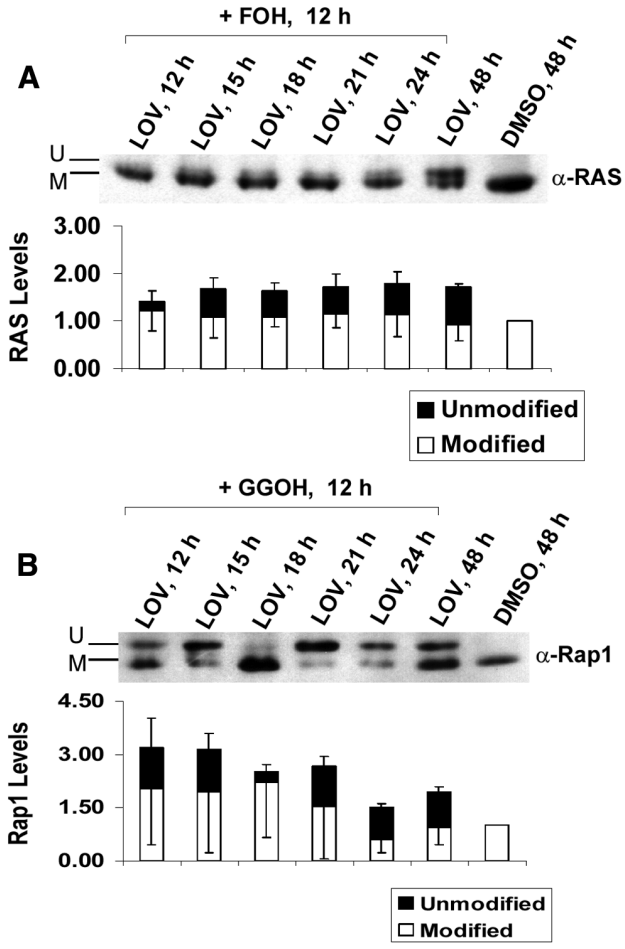

FIG. 3. The effects of varying LOV pretreatment times followed by FOH or GGOH on protein isoprenylation. Western blot analysis of (A) RAS protein from NIH3T3 cells pretreated with 100 μM LOV for 12–24 h, after which 50 μM FOH was added for an additional 12 h, and (B) Rap1 protein from NIH3T3 cells pretreated with 100 μM LOV for 12–24 h, after which 50 μM GGOH was added for an additional 12 h. The relative levels of U and M protein from three individual experiments were quantified, normalized to the amount of modified protein present in DMSO-treated cells, averaged as described in the Materials and Methods section, and displayed beneath a representative Western blot. Error bars represent one SD from the mean. See Figure 1 for abbreviations.

varying pretreatment times, and then FOH or GGOH was added for an additional 12 h in the continuing presence of LOV. This was done to determine the effect of LOV pretreatment time on the ability of FOH or GGOH to restore protein isoprenylation. Figure 3A is a Western blot of RAS protein from NIH3T3 cells that were pretreated with LOV for times varying from 12 to 24 h, after which FOH was added for an additional 12 h. Figure 3A demonstrates that, as LOV pretreatment time increased beyond 12 h, an additional 12 h exposure to FOH did not restore RAS isoprenylation. Figure 3B is a Western blot of Rap1 from NIH3T3 cells pretreated with LOV for 12 to 24 h, after which GGOH was added for an additional 12 h. For most of the LOV pretreatment times, an additional 12-h exposure to GGOH did not restore Rap1 isoprenylation. Interestingly, GGOH partially restored Rap1 isoprenylation after 18 h of LOV pretreatment. In fact, there was significantly less unmodified Rap1 present under this condition compared to the amount of unmodified Rap1 present when cells were treated with LOV alone ($P = 0.014$).

The effect of inhibiting protein translation on the ability of FOH or GGOH to restore protein isoprenylation after LOV pretreatment. The time-course studies described previously led us to hypothesize that transcription or translation of enzymes involved in the isoprenoid shunt may need to take place before FOH can be utilized for protein isoprenylation. NIH3T3 cells were pretreated with LOV for 12 h. At the end of the pretreatment, CHX, a potent protein synthesis inhibitor, was added concurrently with FOH for an additional 12 h in the continued presence of LOV. Alternatively, 6 h into the LOV pretreatment time, CHX was added to inhibit protein

synthesis before the addition of FOH. At the end of the 12-h pretreatment time, FOH was added for an additional 12 h in the continued presence of LOV and CHX. Figure 4A is a representative Western blot of RAS protein from cells treated in this manner. The ability of FOH to restore RAS isoprenylation was not inhibited by the addition of CHX. Unmodified RAS was undetectable in cells treated concurrently with CHX and FOH or in cells treated with CHX prior to the addition of FOH (lanes 4 and 6). To determine whether protein translation is required for the use of GGOH by enzymes of the isoprenoid shunt, NIH3T3 cells were pretreated with LOV for 18 h. This time course was chosen based on the results of Figure 3B, which shows that 12 additional hours of GGOH significantly restored Rap1 isoprenylation after an 18-h LOV pretreatment. During the final 3 h of the LOV pretreatment, CHX was added. At the end of the pretreatment time, GGOH was added for an additional 12 h. Figure 4B demonstrates that addition of CHX does not alter the ability of GGOH to restore Rap1 isoprenylation. Figure 4C represents the control to ensure that CHX is inhibiting protein translation at the concentration and times for which cells are exposed. Cells were pretreated with CHX for 3 or 6 h, and then LOV was added for an additional 12 h. Because LOV only inhibits the isoprenylation of newly synthesized RAS, the disappearance of the unmodified RAS protein band after CHX pretreatment indicates that CHX is inhibiting protein translation under the conditions used in the preceding experiments (Figs. 4A and 4B).

The effect of inhibiting ADH on the ability of FOH or GGOH to restore or maintain protein isoprenylation after LOV pretreatment. Because of previous reports that FOH is a

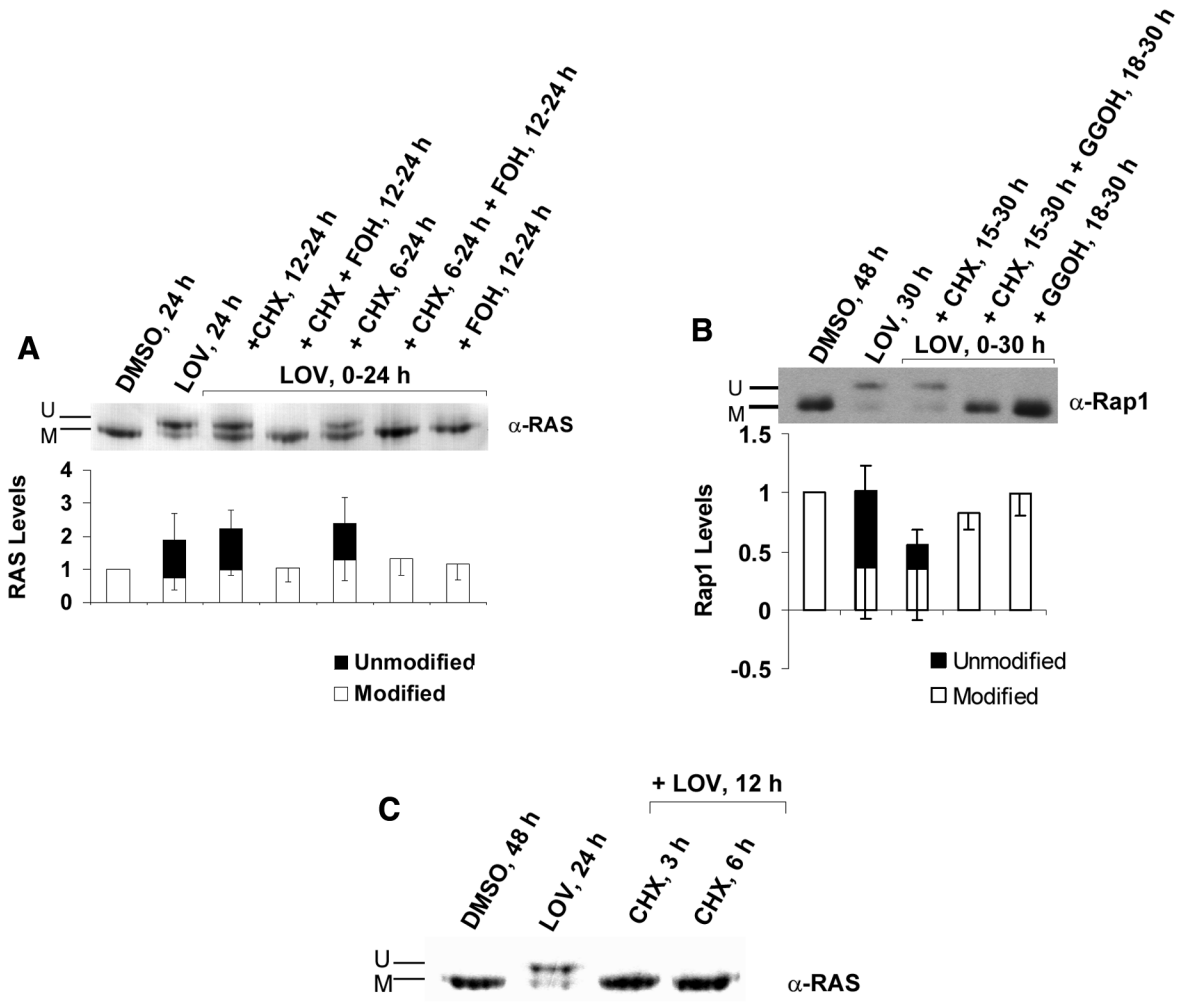

FIG. 4. The effect of inhibiting protein translation on the ability of FOH or GGOH to restore protein isoprenylation after LOV pretreatment. Western blot analysis of (A) RAS protein from NIH3T3 cells pretreated with 100 μM LOV for 12 h, with 5 μg/mL cycloheximide (CHX) added either with FOH or added for the last 3 h of pretreatment, after which 50 μM FOH was added for an additional 12 h, and (B) Rap1 protein from NIH3T3 cells pretreated with 100 μM lovastatin for 18 h, with 5 μg/mL CHX added for the last 3 h of pretreatment, after which 50 μM GGOH was added for an additional 12 h. As a control (C), Western blot analysis is shown of RAS protein from NIH3T3 cells pretreated with 5 μg/mL CHX for 3 or 6 h then 100 μM LOV added for an additional 12 h. The relative levels of U and M protein from three individual experiments were quantified, normalized to the amount of modified protein present in DMSO-treated cells, averaged as described in the Materials and Methods section, and displayed beneath a representative Western blot. Error bars represent one SD from the mean. See Figure 1 for other abbreviations.

substrate for ADH (17), we hypothesized that FOH may be converted into its aldehyde form prior to use by the isoprenoid shunt. An ADH inhibitor, 4MP, was used to determine whether ADH activity is required for use of FOH by the isoprenoid shunt. NIH3T3 cells were pretreated with both LOV and 4MP for 24 h, and then FOH was added for an additional 24 h. Alternatively, 4MP was added to cells with FOH after a 24-h LOV pretreatment. Figure 5A demonstrates that 4MP alone does not alter RAS isoprenylation (lane 3) and also does not alter the ability of LOV to inhibit RAS isoprenylation (lanes 4 and 5). Importantly, 4MP does not alter the ability of FOH to restore RAS isoprenylation, either when added with LOV (lane 6) or when added with FOH (lane 7). To determine whether inhibition of ADH would alter the ability of GGOH to maintain Rap1 isoprenylation in the presence of LOV, NIH3T3 cells were incubated concurrently with LOV, 4MP, and GGOH for 24 h. Figure 5B is a representative Western blot, demonstrating that inhibition of ADH does not inhibit the ability of GGOH to maintain Rap1 isoprenylation in the presence of LOV. An *in vitro* assay was used to confirm that 50 μM 4MP is sufficient to inhibit ADH activity in NIH3T3 cells as described in the methods (16). The activity of ADH in lysate from untreated NIH3T3 cells was 547.8 pmol/min/mg total protein, which was decreased to 16.1 pmol/min/mg protein in lysate from NIH3T3 cells that were incubated with 50 μM 4MP prior to lysing. As a control for this assay, the activity of purified equine ADH was determined to be 435 nmol/min/mg, which was decreased to 7.2 nmol/min/mg after incubation in 50 μM 4MP.

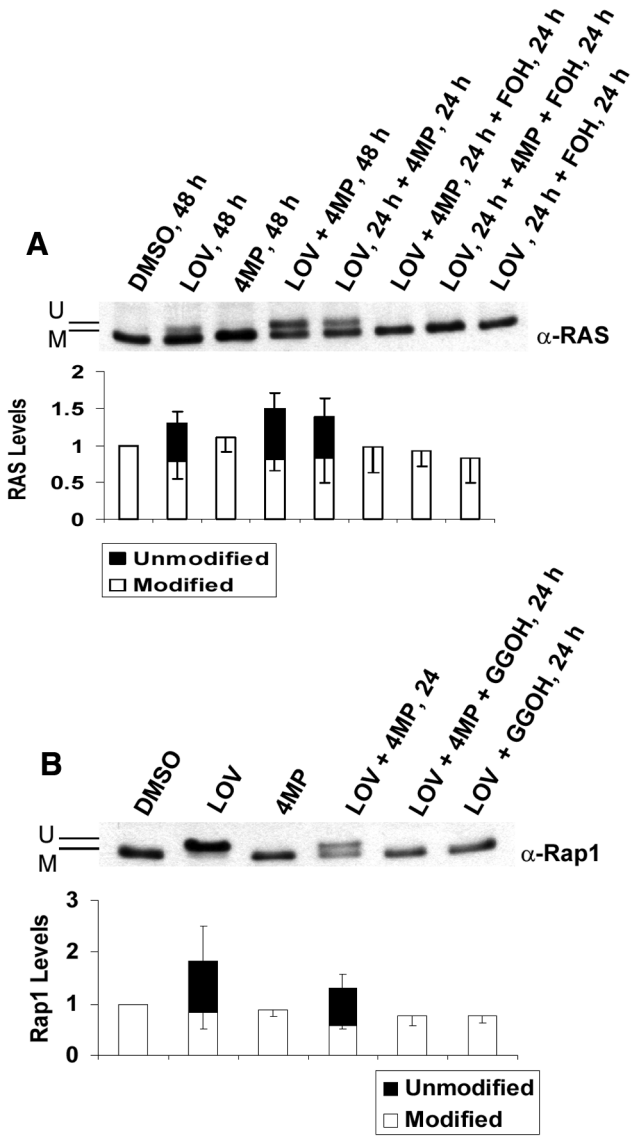

FIG. 5. The effect of inhibiting alcohol dehydrogenase on the ability of FOH or GGOH to restore or maintain protein isoprenylation after LOV pretreatment. Western blot analysis of (A) RAS protein from NIH3T3 cells pretreated with 100 μM LOV for 24 h, after which 50 μM FOH was added for an additional 24 h and 50 μM 4-methylpyrazole (4MP) added either at the same time as LOV or at the same time as FOH, and (B) Rap1 protein from NIH3T3 cells treated concurrently with 100 μM LOV, 50 μM GGOH, and 50 μM 4MP. The relative levels of U and M protein from three individual experiments were quantified, normalized to the amount of M protein present in DMSO-treated cells, averaged as described in the Materials and Methods section, and displayed beneath a representative Western blot. Error bars represent one SD from the mean. See Figure 1 for abbreviations.

DISCUSSION

FOH and GGOH are increasingly known to display distinct biological functions. In fact, FOH has been shown to modulate protein expression, calcium channel function, and cell differentiation, as well as to induce apoptosis in tumor-derived cell lines. For example, FOH regulates HMG-CoA reductase expression by increasing its rate of degradation (18–21). FOH also has been shown to modulate current through both N- and L-type voltage-gated calcium channels in neuronal, vascular smooth muscle, and retinal glial cells (22–25). Also, FOH induces the differentiation of epidermal keratinocytes *via* the peroxisome proliferator-activated receptor-α (26). FOH is also known to be a ligand for the farnesoid X receptor (FXR), a member of the nuclear hormone receptor superfamily (27). Another biological function ascribed to FOH is the induction of apoptosis (28,29). Interestingly, FOH selectively induces apoptosis in cell lines derived from neoplastic, but not nonneoplastic, sources (30,31). Although the potential biological functions of GGOH are less well characterized than those of FOH, GGOH has been shown to induce apoptosis in a variety of tumor cell lines (32–34). The mechanism of GGOH-induced apoptosis involves caspase cleavage and activation of c-Jun (33,34). In addition to inducing apoptosis in tumor cells, GGOH inhibits the activity of liver X receptor (LXR)α, another member of the nuclear hormone receptor superfamily (35).

In spite of the knowledge of these multiple functions, the metabolic dispositions of FOH and GGOH are, as yet, minimally understood. For some of these functions, such as calcium channel regulation and FXR binding, the alcohol, not the corresponding pyrophosphate, is the active species. For other functions, however, it is not clear whether the isoprenoid alcohol or the pyrophosphate is active. Our prior studies indicated

that FOH was utilized for protein isoprenylation in a manner highly dependent on prior depletion of mevalonic acid and its derivatives (6). When added to cells for an additional 24 h, FOH supported RAS isoprenylation after cells were pretreated with LOV for 24 h but not when added to cells concurrently with LOV (6). In view of the multiple studies by others of both FOH and GGOH, a detailed understanding of this time dependence is essential for correctly interpreting the results of these growing numbers of experiments.

The time-dependent utilization of FOH for protein isoprenylation may be a consequence of the requirement for induction of enzymes essential for FOH activity. To evaluate this hypothesis, we more completely defined the timing of FOH's ability either to maintain RAS isoprenylation during LOV treatment or to restore RAS isoprenylation after LOV pretreatment. As demonstrated by Figures 1C and 2A, maintenance and restoration of RAS isoprenylation by FOH is time dependent. Additionally, decreasing the amount of time that cells are pretreated with LOV decreases the amount of time necessary for FOH to restore RAS isoprenylation (Fig. 3A). If the postulated FOH kinase is constitutively expressed in cells, one might expect FOH to restore RAS isoprenylation quickly. The long time (>18 h) required before FOH is able to restore RAS isoprenylation suggests transcriptional or translational induction rather than simply activation of an enzyme(s) in the isoprenoid shunt. Although this process has yet to be described for isoprenoids, this positive feedback has been demonstrated for oxysterols. The oxysterols 24(*S*),25-epoxycholesterol and 24(*S*)hydroxycholesterol have been shown to activate the LXR, which then activates the transcription of the cholesterol 7α-hydroxylase gene (36). The protein synthesis inhibitor CHX was used to test the hypothesis that enzymes of the isoprenoid shunt must be synthesized before they can utilize FOH. Unexpectedly, treatment of cells with CHX did not inhibit the ability of FOH to restore RAS isoprenylation after LOV pretreatment (Fig. 4A). Thus, the enzymes responsible for utilizing FOH must be constitutively present, although not necessarily active.

Although FOH is presumed to be present in cells at low levels, the levels of mevalonic acid and its isoprenoid derivatives are not known due to experimental limitations. Our findings suggest that the requisite time needed for FOH to restore RAS isoprenylation may be that required for depletion of isoprenoids by LOV. This depletion may then lead to activation or disinhibition of the enzymes that utilize FOH. Furthermore, longer exposures to LOV and greater mevalonic acid depletion require longer periods of time for FOH to restore RAS isoprenylation (Fig. 3A). This may be because FOH is not merely being converted to FPP but is also being utilized for other cellular functions as described previously. Additionally, if FOH is being converted to FPP, the enzyme farnesyl protein transferase must then transfer the farnesyl group to newly synthesized RAS protein [reviewed in Rowinsky *et al.* (1)]. After farnesylation, the RAS protein then undergoes further modifications before it can localize to the cell membrane (1). These steps would occur subsequent to the conversion of FOH to FPP. Therefore, the lag time before FOH restores RAS isoprenylation after LOV pretreatment may be due to the time required for depletion of isoprenoids, the time required for accumulation of FPP from FOH, and the time required for FPP to modify RAS.

Because the data described earlier suggest that the enzyme(s) utilizing FOH are constitutively present, one possible candidate is ADH, which has been shown to utilize FOH as a substrate (17). Dependence on the reaction by which ADH converts FOH to an aldehyde also could contribute to the length of time required for FOH to restore RAS isoprenylation. Figure 5A demonstrates, however, that inhibition of ADH has no effect on the ability of FOH to restore RAS isoprenylation after LOV pretreatment. Therefore, although FOH is a substrate for ADH, this enzyme is not likely to be involved in the isoprenoid shunt.

Although GGOH restores protein geranylgeranylation (4,5,8–10), the mechanism(s) by which cells utilize this isoprenoid alcohol are not yet identified. Thus, we undertook experiments parallel to those just described for FOH to delineate the time required for GGOH to maintain or restore Rap1 geranylgeranylation in the presence of LOV. Unlike FOH, GGOH is able to maintain protein isoprenylation in the presence of LOV at all times tested (Fig. 1D). Similar to FOH, restoration of isoprenylation by GGOH after LOV pretreatment is time dependent (Fig. 2B), although GGOH is less effective at restoring Rap1 isoprenylation than FOH at restoring RAS isoprenylation. As the LOV pretreatment time increases, the amount of time required for GGOH to restore Rap1 isoprenylation increases (Figs. 2B and 3B). These results may be explained by an effect of LOV on the GGOH-utilizing enzymes of the isoprenoid shunt, as described for FOH. The differences seen in Figure 3B with LOV pretreatment for 15, 18, and 21 h plus GGOH for an additional 12 h may be due to biphasic activities of the enzymes utilizing GGOH. At the condition of LOV for 18 h then GGOH for 12 h, there may be optimal depletion of mevalonic acid and exposure to GGOH. At earlier pretreatment times, there is insufficient mevalonic acid depletion, whereas at later pretreatment times, there is insufficient exposure to GGOH. Because GGOH maintains Rap1 isoprenylation when added to cells concurrently with LOV, it seems likely that the enzyme(s) that utilize GGOH in the isoprenoid shunt are constitutively expressed and active. If these isoprenoid shunt enzyme(s) are constitutively expressed, addition of CHX before the addition of GGOH would not be expected to alter the ability of GGOH to restore Rap1 isoprenylation after LOV pretreatment. The Western blot of Rap1 protein in Figure 4B supports this hypothesis. As demonstrated for FOH, it is possible that ADH converts GGOH to its corresponding aldehyde before use by the isoprenoid shunt. This is not the case, however, given the data shown in Figure 5B.

Of note is that LOV increases total RAS (Fig. 1A) and Rap1 (Fig. 1B) protein levels, as described previously (37). Preservation and restoration of isoprenylation by FOH (Figs. 1C, 2A, and 3A) and GGOH (Figs. 1D, 2B, and 3B) parallel

the reduction of total RAS and Rap1 levels to control conditions. These findings are consistent with those previously reported (37), assuming FOH and GGOH are converted to their respective pyrophosphates.

In this current report, we advance the understanding of how the isoprenoid alcohols FOH and GGOH maintain and restore protein isoprenylation. The enzymes for FOH utilization require (i) prior depletion of mevalonic acid and/or its derivatives and (ii) exposure to FOH for a period proportional to the duration of mevalonic acid depletion. GGOH utilization differs in that it is, overall, less effective for restoration or maintenance of isoprenylation than utilization of FOH, and prior depletion of mevalonic acid and/or its derivatives is not required. However, prior mevalonic acid depletion requires proportional exposure to GGOH for this alcohol to restore protein isoprenylation. These results further support the existence of distinct and differentially regulated enzymes for FOH and GGOH utilization.

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Enterotoxigenic *Escherichia coli* Strains Bind Bovine Milk Gangliosides in a Ceramide-Dependent Process

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ABSTRACT: Diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC) is the main infectious disease of newborn calves. The first step of infection involves bacterial attachment to the intestinal mucosa. This adhesion is mediated by fimbriae that recognize some glycoconjugates on the host cell surface, in particular, several gangliosides. Because milk also contains gangliosides, these have been suggested to serve as ligands for bacterial fimbriae and thus prevent the bacterial attachment to mucosa. The most relevant ETEC strains in calves, including those with K99 and F41 fimbriae, were assayed to determine whether they are able to bind gangliosides isolated from several stages of bovine lactation. Both GM3 and GD3, the main gangliosides of milk, were recognized by ETEC strains, although the different fimbriae showed diverse levels of affinity. Unexpectedly, the adhesion to colostral gangliosides was considerably weaker than that to gangliosides from the other stages of lactation. Because the carbohydrate moiety did not change and because differences in the percentages of unsaturated FA and sphingosine between colostrum and other stages were observed, we conclude that the differences in adhesion could be due to a different composition of the ganglioside ceramide.

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Escherichia coli causes several types of infection in both human and animals. In developing countries, enterotoxigenic *E. coli* (ETEC) is a major cause of children's diarrhea as well as the well-known traveler's diarrhea (1). Although these colibacilloses are rare in human populations from developed countries, ETEC strains are often associated with severe diarrhea and septicemia in the youngest piglets, lambs, and calves in these countries.

ETEC have two virulence factors: fimbriae and toxins. Fimbriae, also called pili, are proteinaceous filaments that decorate the bacterial surface. They mediate the adherence to intestinal mucosa because they are constituted by adhesive proteins, called adhesins, which specifically recognize ligands on the host cell surface (2). Once fixed to the mucosa, bacteria can secrete the enterotoxin that enters the cell, altering its normal metabolism and promoting the symptoms of diarrhea.

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Abbreviations: cfu, colony-forming units; Cer, ceramide; ETEC, enterotoxigenic *Escherichia coli*; Gal, galactose; Glc, glucose; HPTLC, high-performance TLC; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid.

Both toxins and fimbriae can recognize and bind carbohydrate chains from the cell membrane glycoconjugates in a very specific way. Cholera toxin and *E. coli* LT bind GM1. Shiga toxin recognizes the Gal α1,4Galβ1,4 Glc sequence (where Gal = galactose and Glc = glucose) on human glycolipids. The P-fimbriated uropathogenic *E. coli* and the *Streptococcus suis* adhesins also recognize the Galα1,4Gal epitope (3). *Helicobacter pylori* bind several glycolipids, including lactosyl-gangliotriosyl- and gangliotetraosyl-ceramide (4). Although many of these sugar sequences are also present in glycoproteins, bacteria bind more strongly to glycolipids because their shorter carbohydrate moiety allows a closer attachment of the pathogen to the host cell membrane (5). Because ceramide and sphingosine act as second messengers, other authors have suggested that as bacteria attach to glycosphingolipids, they initiate certain signaling cascades within the host cell that would facilitate infection (4).

K99 is the most common fimbria expressed in ETEC strains isolated from calves (6). This type of fimbriae also appears in porcine and ovine strains (7). Other ETEC strains isolated from calves produce F41 fimbriae, both isolated and coexpressed with K99, and F17 (FY=Att25) fimbriae (8).

Ganglioside *N*-glycolylneuraminic acid (NeuGc)-GM3 and other NeuGc-containing gangliosides have been reported to be the main receptors for K99 (9). The susceptibility of newborn calves depends on the levels of this ganglioside in the intestinal epithelium. In an outstanding study conducted with piglets, NeuGcGM3 proved to be the most abundant ganglioside in the intestine of K99-susceptible pigs and GM2 predominated in the intestine of the nonsusceptible pigs (10). In the same way, the absence of NeuGcGM3 in the adult pig intestine has been associated with adult porcine resistance to K99 infections (11).

Colostrum and milk have been reported to be routes for the transference of immunity from the mother to the newborn because they contain several antimicrobial components, not only immunoglobulins but also a plethora of nonspecific factors such as lactoferrin or lactoperoxidase (12). Furthermore, bacterial fimbriae and toxins may recognize milk glycoconjugates because they contain the same carbohydrate sequences as those expressed on the mucosal surface (13). Thus, these soluble ligands would act as a decoy, blocking the bacterial adhesins and preventing them from infecting the newborn.

The aim of this study was to evaluate the ability of gangliosides from bovine colostrum and milk to bind several

ETEC strains isolated from diarrheic calves, including K99, F41, and F17. Both overlay and quantitative methods were used to study the adhesion.

EXPERIMENTAL PROCEDURES

Bacterial strains and media. Seven ETEC strains isolated from diarrheic calves were provided by Dr. Jorge Blanco from the Laboratorio de Referencia de *Escherichia coli* (Lugo, Spain). The adhesins synthesized by each strain are listed in Table 1 (7). Bacteria were cultured in Mueller–Hinton broth (Difco, Detroit, MI) for 3–5 d at 37°C. For fimbria expression, bacteria were grown on Minca-Agar containing IsovitaleX (Becton, Dickinson and Company, Cockeysville, MD) at 37°C for 16 h, as reported previously (14).

Milk samples. Milk was obtained from six Spanish-Brown cows from December to August. Colostrum, transitional milk, mature milk, and late lactation milk corresponded to the morning milking from days 2, 7, 90, and 270, respectively. All samples were immediately frozen at −20°C and then lyophilized. Lyophilized milk at each sampling time was pooled (six cows) and homogenized to ensure an accurate distribution of the components. Cows were fed ~50% hay and 50% standard concentrate. This diet is representative of what would normally be utilized for milk cattle production.

Gangliosides. NeuGc-GM3 was purified from horse erythrocytes as follows: erythrocytes were obtained by centrifuging fresh blood at 675 × *g*. Total lipids were extracted from erythrocytes with 7 vol of isopropanol. Chloroform (5 vol) was added after stirring for 1 h (15). The mixture was filtered under vacuum, and the filtrate was evaporated to dryness. Gangliosides were isolated from this total lipidic extract by a modification of the procedure of Folch. Briefly, total gangliosides were partitioned with 20 vol of chloroform/methanol (2:1, vol/vol) and 7 vol of 15 mM PBS. The upper phases, containing the gangliosides, were evaporated, dialyzed against distilled water for 48 h, and then lyophilized. Gangliosides were taken up in 1 mL chloroform/methanol (2:1). NeuGc-GM3, the most abundant ganglioside in horse erythrocyte membranes, was purified by preparative TLC (Merck, Darmstadt, Germany), using chloroform/methanol/water (50:45:10, by vol) containing 0.02% $CaCl_2$ as the solvent system.

Gangliosides from bovine milk were purified as reported previously (16). Briefly, lyophilized milk was homogenized twice with 10 vol of cold acetone (−20°C) (10 mL acetone/g lyophilized milk) to remove neutral lipids and filtered. The solid residue was successively extracted with 10 vol of chloroform/methanol (2:1, 1:2, and 1:1, vol/vol) and the extracts were evaporated to dryness, taken up in 10 vol of chloroform/methanol (2:1), and subjected to a Folch partition as modified by Vanier *et al.* (17). Briefly, crude extract was partitioned by the addition of 0.2 vol of 0.2% cold aqueous KCl. After centrifugation and withdrawal of the upper phase, 0.2 vol of cold methanol was added to the lower phase. After careful mixing, 0.2 vol of the saline solution was added. The sample was again shaken and centrifuged. The supernatant was pipetted off, and a third partition was performed similarly but with the saline solution replaced by distilled water. The upper phases, containing crude gangliosides, were combined, dialyzed against distilled water for 48 h, and lyophilized. Total gangliosides were also taken up in 1 mL chloroform/methanol (2:1). The main gangliosides from bovine milk, GM3 and GD3, were purified by preparative TLC as described above.

TABLE 1
Enterotoxigenic *Escherichia coli* Strains

Strain	Serogroup	Adhesin
K99–12	O8	K99
F41–15	O20	F41
K99–4	O101	K99+F41
CCB1	O101	F17 (Att25)
CCB22	O139	B16 (Vir)
CCB33	O21	B64
CCB37	O123	B23

Identification of FA by GC. The FA content of each ganglioside was determined as FAME by GC. Gangliosides were methanolyzed with 0.5 M HCl in anhydrous methanol (Supelco, Bellefonte, PA) for 20 h at 80°C. A KNK3000 GC equipped with an FID (Konik Instruments, Barcelona, Spain) was used to separate the FAME. A SUPELCOWAX 10 semicapillary column (30 m × 0.53 mm, with 1.0 µm film thickness; Supelco) was programmed for an initial time at 115°C of 5 min, 10°C/min from 115 to 190°C, 2°C/min from 190 to 230°C, and a final time at 230°C of 20 min with helium as carrier gas. FAME were identified by comparing their retention times with those of authentic standards.

Adhesion of bacteria to gangliosides on TLC. ETEC binding to gangliosides on TLC was done as described previously (18). Total milk gangliosides (2.4 µg of lipid-bound sialic acids in each lane) were separated on high-performance TLC (HPTLC) sheets (Merck) using chloroform/methanol/water (50:45:10, by vol) containing 0.02% $CaCl_2$ as the solvent system. Gangliosides from bovine brain were included as a control in a separate lane and developed with orcinol-H_2SO_4. For adhesion, the plate was dipped in 0.1% polyisobutyl methacrylate (Aldrich Chemical, Milwaukee, WI) in hexane for 75 s and allowed to dry. Once dried, it was soaked in PBS containing 2% BSA (Fluka, Buchs, Switzerland; blocking buffer) for 1 h. Bacteria grown on a Petri dish (~8×10^{10} cfu) were collected in 1 mL of sterile PBS containing 1% mannose. The plate was covered with the bacterial suspension and incubated for 2 h at 37°C in a humid chamber. The plate was then thoroughly washed (four times, 5 min each) with PBS and incubated with the anti-*E. coli* antibody (DAKO, Copenhagen, Denmark) at 1:25 in the blocking buffer for 1 h at room temperature. Biotinylated anti-rabbit IgG (Sigma, St Louis, MO) and streptavidin-alkaline phosphatase conjugate (Sigma) were added successively (1:1000 in the blocking buffer) for 1 h at room temperature before developing with the substrate.

Quantification of bacterial binding to gangliosides. To quantify the binding of each strain to total and individual ganglio-

sides from milk, a very simple method previously described by Mack and Blain-Nelson (19) was adapted. A microtiter plate (PolySorp™ Surface, Nalge Nunc International, Roskilde, Denmark) was coated with increasing amounts of gangliosides (from 30 to 2400 ng of lipid-bound sialic acids) diluted in methanol. After drying, each well was blocked with 300 μL of the same blocking buffer used for TLC-binding. Bacteria, collected as described above, were added (~3×10^{10} cfu/well) and kept for 2 h at 37°C. After being washed with PBS, the bound bacteria were stained with 0.01% (wt/vol) aqueous crystal violet dye for 5 min. The excess dye was removed by washing twice with PBS. A 2% (wt/vol) sodium deoxycholate aqueous solution was used to lyse the bound cells and release the dye. Plates were read at 504 nm using a 340 ATTC microplate reader (SLT Labinstruments, Salzburg, Austria).

RESULTS

Binding of the different strains to milk gangliosides. The composition and purity of total gangliosides and isolated GM3 and GD3 were assessed by TLC or HPTLC in several solvent systems and by immunostaining on HPTLC plates as previously described (20). The gangliosides identified in colostrum and milk are listed in Table 2. Their relative proportions in the samples are also found in a previous publication (20). The ability of ETEC fimbriae to bind gangliosides from the four stages of lactation was verified in both microtiter plates and overlay assays. Nonfimbriated bacteria (grown at 18°C) were also examined; they failed to bind gangliosides.

Increasing amounts of total gangliosides from each stage of lactation were incubated with the fimbriated bacteria in microtiter plates. NeuGc-GM3 from horse erythrocytes was included in each assay as a positive control. Figure 1 shows the binding curves for each strain.

As expected, K99-containing bacteria (both single-expressed and coexpressed with F41) strongly bound NeuGc-GM3 from horse erythrocytes in microtiter plates. Gangliosides from colostrum exhibited a lower ability to bind K99 than gangliosides from the other stages. The higher amounts of gangliosides seemed to be inhibitory. The explanation may be that overcoating of a well with glycolipids may produce unstable multilayers, which are washed away; alternatively, as concentrations increase, the amount of glycolipids bound to the bottom of the well should reach a maximum (saturation) with the excess simply washing off as micelles, paradoxically resulting in less binding with more glycolipids. Coexpression of F41 and K99 on the same bacterial cell proved to be less efficient than the K99-single fimbriated bacteria in binding milk gangliosides. The F41-single fimbriated strain (data not shown) failed to bind both milk gangliosides and NeuGc-GM3.

In microtiter plates, the B16 strain had a greater affinity for milk gangliosides than for horse erythrocyte NeuGc-GM3. Adhesion to late lactation milk gangliosides was again better than to those from the rest of the stages. The transitional gangliosides showed the second-best results in each case, and adhesion to colostral gangliosides was very poor. The strains B23 and B64 did not exhibit any binding, either to NeuGc-GM3 or to milk gangliosides, in microtiter plates.

Adhesion of bacteria to gangliosides separated on TLC. Gangliosides from colostrum, transitional, mature, and late lactation milks were separated using TLC and incubated with each strain. The anti-*E. coli* antibody was assayed in the absence of bacterial cells as a negative control but did not bind any gangliosides. The most abundant gangliosides in bovine milk were GD3 (50–70% of total) and GM3 (3–11%). Bacterial adhesins showed different affinities for these two gangliosides. Table 3 presents the results of the TLC overlay for all the strains tested. Data were a résumé of the binding tests regardless of the stage of lactation.

The $K99^+$ strain strongly bound GM3 and GD3. Binding of the double fimbriated $K99^+F41^+$ strain to GM3 was weaker than those of the single fimbriated strains $K99^+$ and $F41^+$. The F17, B16, and B23 adhesins exhibited strong adhesion to GD3. B16 also recognized and bound GM3, as expected after the quantitative assays. However, these data required further explanation. Bacterial adhesion to each ganglioside differed depending on the stage of lactation. In each case, adhesion to colostral GM3 or GD3 was weaker than adhesion to GM3 and GD3 from the other stages (Fig. 2).

The different level of K99 adhesion cannot have been due to a lower amount of NeuGc-GM3 in colostrum because colostrum was richer in GM3 than mid-lactation milk (11.5 vs. 2.5% of total gangliosides), and the GM3-bound NeuGc levels remained constant during lactation. Although the other strains have not been reported to require *N*-glycolylated gangliosides, the differences in binding exhibited the same pattern as K99. B23, which failed to bind gangliosides on the microtiter plates, exhibited a good adhesion to gangliosides on the TLC overlay. This could be due to a different conformation or arrangement of the gangliosides on these surfaces.

Because the carbohydrate moiety could not be invoked

TABLE 2
Gangliosides from Bovine Milk[a]

GM3	NeuAc α2-3Galβ1-4Glc-Cer
	NeuGc α2-3Galβ1-4Glc-Cer
9-*O*-Acetyl-GD3	Neu5,9Ac_2α2-8NeuAc α2-3Galβ1-4Glc-Cer
GD3	(NeuAc/NeuGc)α2-8(NeuAc/NeuGc)α2-3Galβ1-4Glc-Cer
GT3	NeuAcα2-8 NeuAcα2-8NeuAc α2-3Galβ1-4Glc-Cer

[a]The ganglioside nomenclature of Svennerholm (33) and IUPAC-IUB recommendations (34) were followed. Cer, ceramide; Gal, galactose; Glc, glucose, NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid.

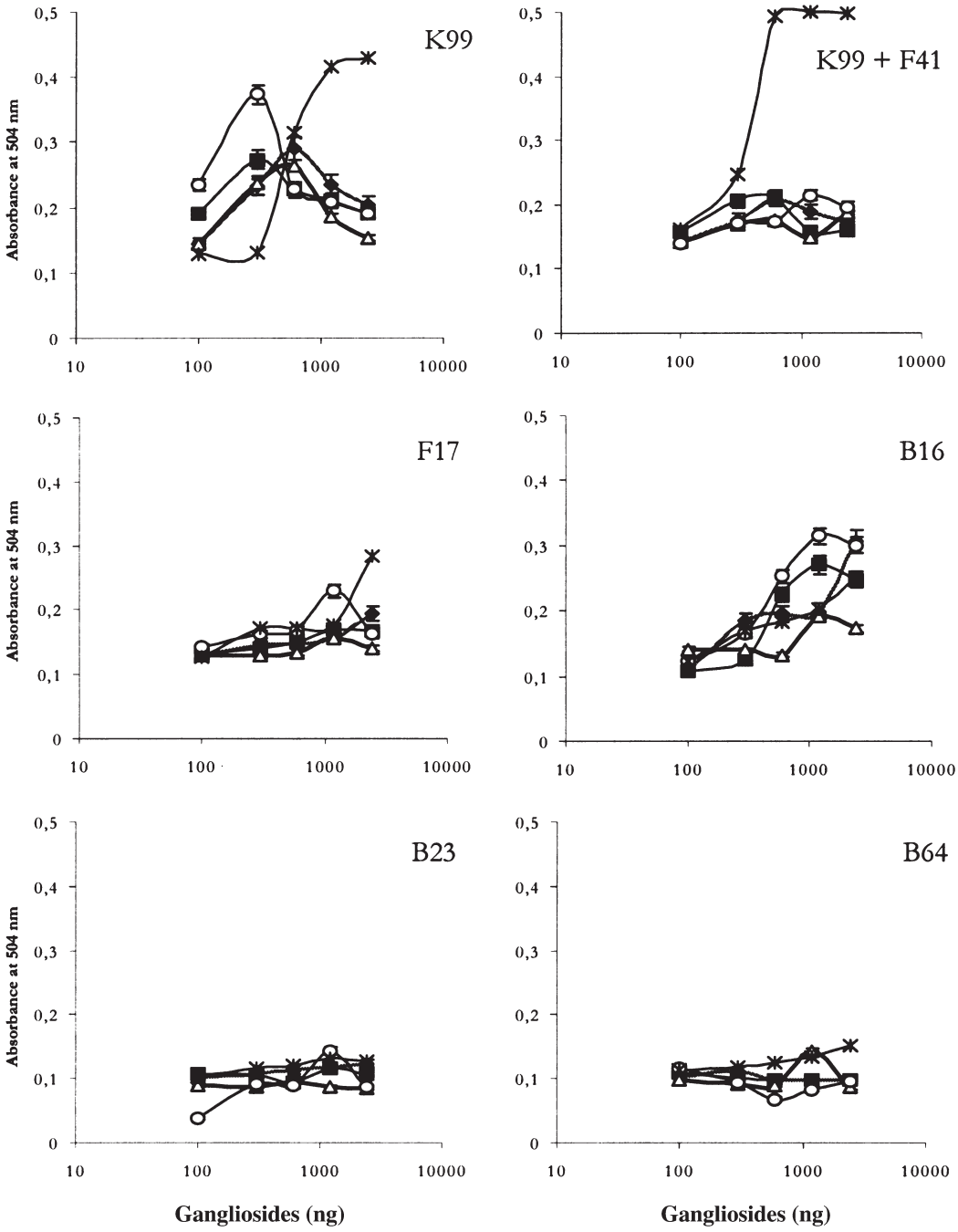

FIG. 1. Binding of enterotoxigenic *Escherichia coli* (ETEC) strains to milk gangliosides. ◆ Colostrum, ■ transitional milk, △ mature milk, ○ late lactation milk, ✳ NeuGc-GM3. Error bars represent ± SD. Each point was obtained in triplicate.

to account for the differences found, we considered the ceramide. Changes occurred over the course of lactation in the ceramide from milk GM3 and GD3, i.e., an increase in the unsaturated FA content just after colostrum (Table 4). As shown, both GM3 and GD3 from colostrum had almost exclusively saturated FA. The following stages of lactation showed a lower content of saturated FA. These changes were accompanied by a reduction in the length of the hydrocarbon chain. This means that colostral ceramide would be more rigid than the others. The results of the binding assays carried out in this work using solid-phase systems seem to indicate that the more rigid the ceramide, the worse the bacterial adhesion.

Binding of several bacterial strains to GM3 and GD3. Taking into account the TLC overlay results, a quantitative assay on binding to individual gangliosides was performed for K99, F41, F17, and B16. The crystal violet method was reported by the authors to be as accurate in quantifying bacte-

TABLE 3
Bacterial Binding to Individual Gangliosides Separated on TLC[a]

	Enterotoxigenic *Escherichia coli* (ETEC) strains[b,c]						
	K99	F41 + K99	F41	F17	B16	B23	B64
GM3	++	+	+	±	+	−	−
GD3	+	±	−	+	+	+	±

[a]2.4 μg of each ganglioside was spotted and incubated with 8×10^{10} colony-forming units/mL of PBS.
[b]Strains are identified by the name of the fimbriae expressed.
[c]++, very strong binding; +, strong binding; ±, weak binding; −, no binding.

rial adhesion as the use of ^{3}H-thymidine to radiolabel bacteria. All strains except K99 showed a weaker adhesion to individual gangliosides than to total gangliosides (Fig. 3).

As shown in the TLC overlays, binding to colostral GM3 was weaker than binding to mature or late lactation milk GM3. Nevertheless, GD3 from all stages showed similar levels of adhesion. The main differences in FA content between transitional and later-stage ceramides were found in 16:0 and 18:2. The former was significantly more abundant in mature and late-lactation GM3 (40 and 31%, respectively, of total FA vs. 21%), whereas transitional GM3 was richer in 18:2 (13 vs. 2.3 and 4.5%).

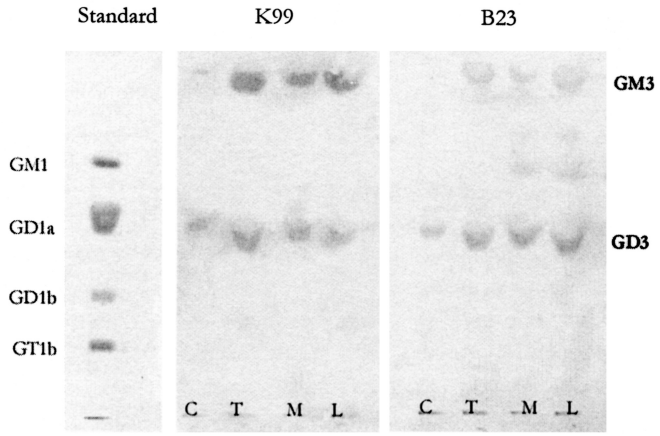

FIG. 2. Adhesion of ETEC strains to milk gangliosides on a TLC overlay. Gangliosides from bovine brain developed chemically were used as standards. Total gangliosides (2.4 μg) from each stage were spotted onto the TLC plate. C, colostrum; T, transitional milk; M, mature milk; L, late lactation milk; for other abbreviation see Figure 1.

DISCUSSION

In milk, gangliosides are partially localized in the fat globule membrane. They reach the small intestine without being hydrolyzed; for this reason, they were proposed to play a protective role in the newborn intestine (21). Human milk gangliosides were reported to bind several bacterial toxins, e.g., GM1 bound cholera toxin (22). Because gangliosides also act as ligands for bacterial fimbriae, here we assayed the ability of bovine milk gangliosides to bind a set of common ETEC strains isolated from diarrheic calves.

K99 are the most common fimbriae expressed in ETEC strains isolated from calves. The best-known receptor for K99 in the piglet and calf intestine is the ganglioside NeuGc-GM3. In a previous paper, we reported the presence of NeuGc-GM3 using the monoclonal antibody 14F7 (20). As expected, K99 bound total milk gangliosides as well as GM3 and GD3, presumably to the NeuGc-containing isoforms. Although the sequence *N*-acetylneuraminic acid NeuAcα2-8NeuAcα2-

TABLE 4
FA Content of Gangliosides GM3 and GD3 from the Different Stages of Lactation[a]

	GM3				GD3			
FA	C	T	M	L	C	T	M	L
12:0	1.2	3.3	0.1	1.6	0.7	1.5	1.8	3.6
13:0	—	—	—	—	—	0.5	0.2	—
14:0	5.3	5.9	5.1	5.3	8.3	8.3	8.3	5.4
14:1	—	0.1	—	—	—	0.4	—	—
15:0	1.8	6.6	2.2	1.9	3.6	4.8	4.0	3.6
16:0	37.9	20.5	40.0	31.2	38.7	29.8	30.0	25.1
16:1	—	7.6	—	5.3	—	11.2	6.0	4.5
17:0	0.2	2.4	1.8	5.7	2.4	6.7	2.9	4.1
17:1	—	2.4	—	—	—	—	—	—
18:0	38.1	17.1	25.4	24.9	29.8	19.0	22.3	18.2
18:1	—	12.8	15.7	10.6	—	10.2	14.2	21.6
18:2	—	13.1	2.3	4.5	—	4.7	0.7	4.9
19:0	—	—	—	0.2	—	0.4	0.1	0.2
20:0	—	2.3	—	0.6	—	1.1	2.6	0.4
22:0	9.8	4.4	5.0	6.7	16.5	2.7	5.0	7.2
23:0	—	0.4	0.9	0.1	—	—	0.1	0.1
24:0	5.7	0.8	1.5	1.1	—	1.1	1.3	0.8
25:0	—	0.2	—	0.2	—	0.3	0.4	0.4
Σ Saturated FA	99.5	63.9	82.0	79.6	99.7	73.4	79.0	69.0
Σ VLCFA	15.5	8.1	7.3	9.0	16.5	5.2	9.4	8.8

[a]Data are expressed as the means of three different experiments carried out in duplicate. FA were analyzed as FAME. C, colostrum; T, transitional milk; M, mature milk; L, late lactation milk; VLCFA, very long chain FA (≥20 carbon atoms).

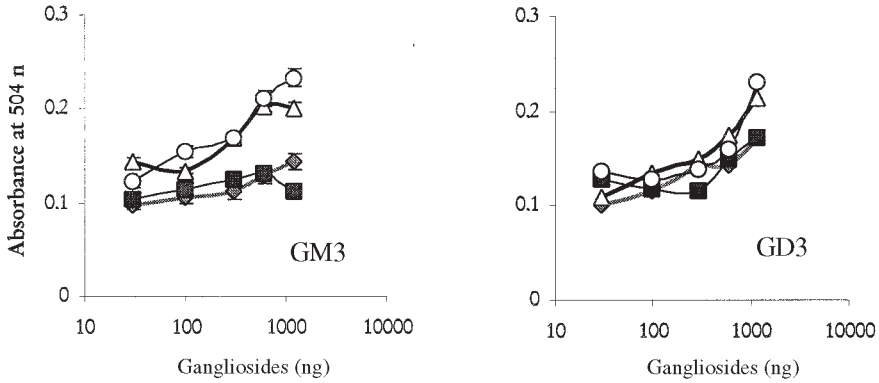

FIG. 3. Binding of K99-fimbriated ETEC to GM3 and GD3. ◆ Colostrum, ■ transitional milk, △ mature milk, ○ late lactation milk. Error bars represent ± SD. Each point was obtained in triplicate. For abbreviation see Figure 1.

3Galβ1-4Glc has been reported to be a very effective inhibitor of K99-mediated hemagglutination of human erythrocytes (23), some authors did not find binding of K99 to any of the possible conformations of GD3, not even the NeuGc-containing isoforms (24,25). However, here we found positive binding of K99 to milk GD3.

Expression of K99 and F41 fimbriae alone or in combination by *E. coli* has been studied (26). Coexpression of K99 and F41 fimbriae on the same bacterial cell seemed to reduce the efficiency of binding to milk gangliosides. It is possible that F41 could interfere with the correct recognition and adhesion of K99 fimbriae to their receptors. This phenomenon affected only gangliosides from milk because the binding of $K99^+F41^+$ bacteria to horse erythrocyte NeuGc-GM3 was even better than that of $K99^+$. Because the binding of gangliosides from horse erythrocytes and milk is affected in opposite directions, F41 pili expression probably modifies the interaction of K99 with its receptor by modifying the presentation of the receptor. The different NeuGc content of GM3 and GD3 from milk and GM3 from horse erythrocytes cannot be discarded as responsible for differences. When F41 was expressed alone, ETEC failed to recognize all gangliosides assayed.

Regarding the other strains, very little is known about their preferences in binding. F17 belongs to an adhesin family whose members exhibit a certain affinity for GlcNAc and highly sialylated proteins (27). Here, F17 fimbriae scarcely bound total milk gangliosides, but they did adhere to GD3 on the TLC overlay assays. B16 showed strong adherence levels to GM3 and GD3, in both the quantitative and the overlay assays. The fimbriae B23 and B64 did not seem to recognize any milk gangliosides in microtiter plate assays, although B23 bound quite well to GD3 on TLC overlays.

Bacterial adhesins recognize the carbohydrate moiety on glycoconjugates. However, the ceramide composition seems to affect the sugar conformation and hence modifies receptor recognition. In particular, the presence of free hydroxy groups has been shown to be essential for binding (5). Thus, *H. pylori* seems to bind only those species of lactosyl-ceramide containing phytosphingosine and 2-hydroxy FA (28). *Campylobacter jejuni* has been reported to bind several phospholipids, preferentially those containing unsaturated FA (i.e., 18:1) (29). Several reports have demonstrated that K99 binding to NeuGc-GM3 with phytosphingosine and 2-hydroxy 16:0 FA is stronger than to other NeuGc-GM3 species (24, 30).

Here we show that not only K99 but also other bovine ETEC strains are able to discriminate among several ganglioside species differing only in their ceramide composition and to bind weakly to those containing saturated FA. Thus, colostral NeuGc-GM3, which is very rich in saturated FA, was scarcely bound by the strains tested. In addition to the extremely high percentage of saturated FA, colostral ceramides have been found to contain mainly 3-*O*-ethoxy-C15 sphinganine as a long-chain base (63%), whereas ceramides from other stages are richer in C18 sphingosine than colostrum (20). The former long-chain base does not contain any free hydroxy group because C3 OH is substituted by an ethoxy group (see Fig. 4).

Some differences were found in the two binding methods used. A differential binding to different glycolipids depending on the characteristics of the matrix of the solid phase (TLC overlay or microtiter plates) was found. This phenome-

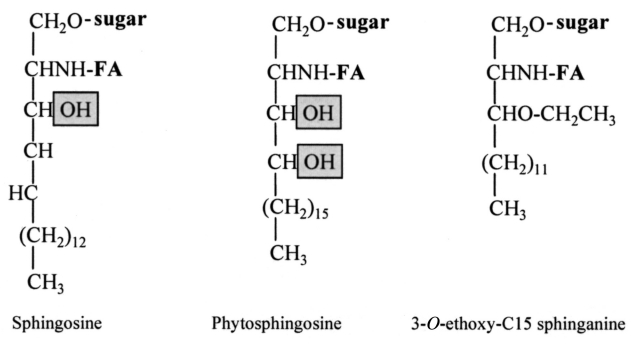

FIG. 4. Structures of long-chain bases. Free hydroxyl groups are marked.

non was also reported previously by others (31). TLC overlay is reported (5,18) to be a good model with which to test bacterial binding to lipids because lipids seem to adopt a conformation similar to the one they adopt *in vivo*; indeed, they could even form bilayers. Thus, the nature of the ceramide could affect the packing and presentation of the sugar ligand. Other authors have pointed to an interaction between silica gel and polar head groups (in gangliosides, the sugar part of the molecule); therefore, lipid tails may be more accessible to bacterial adhesins (29).

The calf small intestine contains sialoparagloboside, NeuAc-GM3, NeuGc-GM3, GM2, GM1, and GD1 (24). The ceramides of the intestinal glycolipids are very rich in hydroxylated FA and phytosphingosine (5); thus, ETEC binding to the epithelial membrane may be quite strong. Milk gangliosides would block bacterial adhesins only if the affinity of these molecules for them was similar to or greater than the affinity for epithelial cell gangliosides. As mentioned, colostral gangliosides lack free hydroxy groups; consequently, they would be weak competitors for preventing bacterial adhesion to the mucosa. The high level of saturated FA also seems to contribute to reducing fimbrial adhesion.

Nevertheless, because the milk lipid content is easily modifiable by changing the cow's diet, it could be of interest for cattle managers to increase the content of unsaturated FA in milk gangliosides during the first days of lactation. Just after calving, the mammary gland takes up plasma FA to synthesize the milk lipids. Although the ceramide FA content of milk gangliosides is likely under a stronger metabolic control than a simple reflection of adipose content, the adipose/lipoprotein FA content could also influence the FA composition of gangliosides by a higher availability of selected FA. To achieve higher levels of unsaturated FA, some methods for protecting dietary lipids against ruminal hydrogenation have been proposed (32).

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Fatty Acids Bound to *Fasciola hepatica* 12 kDa Fatty Acid-Binding Protein, a Candidate Vaccine, Differ from Fatty Acids in Extracts of Adult Flukes

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ABSTRACT: The FA composition of *Fasciola hepatica* 12 kDA purified native FA-binding protein (*n*Fh12), a candidate vaccine against fascioliasis, is described. The FA chain lengths ranged between 12 and 24 carbons. The principal FA were 16:0, 18:1n-9, 18:0, 20:4n-6, and 20:1n-9. The acids 16:0, 18:1n-9, and 18:0 comprised over half the FA that were bound to the whole FA-binding protein. Small amounts (1.0–2.8%) of *iso-anteiso* methyl-branched FA also were characterized. Forty-one different FA were identified in extracts of the adult flukes, with the three most abundant FA also being 16:0, 18:1n-9, and 18:0. A similar proportion of saturated vs. unsaturated FA was observed between the whole extract from *F. hepatica* and the *n*Fh12 protein. However, the n-3/n-6 ratio of PUFA was significantly different, being 1.2 in the whole extract vs. 9.6 in the *n*Fh12 protein complex. The *n*Fh12 protein binds more n-5, n-6, and n-7 PUFA and less n-3 and n-9 PUFA than the whole extract. In addition, cholesterol (56%), sitosterol (36%), and fucosterol (8%) also were bound to the *n*Fh12 protein complex.

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Fascioliasis, caused by the parasitic trematode *Fasciola hepatica* or *F. gigantica,* causes important economic losses in livestock worldwide. More recently, it has been recognized as an important parasite in humans, particularly the poor. Infections can be treated by chemotherapy although there is increasing evidence of resistance. Moreover, in the case of sheep and cattle, repeated chemotherapy is required to treat reinfections, as many as several times each year, making this a costly approach. There is increasing interest in developing another approach to control the disease *via* vaccination. Three major defined purified antigens are currently being evaluated as candidate vaccines against fascioliasis: the FA-binding proteins (FABP) (1–3), the glutathione S-transferases (4), and cathepsin L proteinase (5). Coincidentally, all have cross-reacting antigens with other candidate vaccine antigens against schistosomiasis, a major parasitic trematode in humans. The purposes of the current study were to define in detail the composition of FA that bind to a *F. hepatica* 12-kDa protein (*n*Fh12), which is involved in the intracellular transport of long-chain FA and their acyl-CoA esters, and to compare these FA with the total FA of the fluke homogenate. One or more of these FA could be involved in increasing the protective immune response or, alternatively, in increasing the allergic response *via* eosinophilia (the condition of having an absolute increase in eosinophils in the circulating blood), a hallmark of fascioliasis in humans. Therefore, a thorough knowledge of the FA composition of *n*Fh12 is important if we are to understand fully its role in fascioliasis. A preliminary report on the FA bound to the *n*FH12 protein complex revealed six main FA, namely 14:0, 16:0, 16:1, 18:0, 18:1, and 18:2, but many minor components remained unidentified (6).

Earlier studies with *F. hepatica* isolated from rat liver established the presence of eight FA in its phospholipids (mainly in PC and PE), and these were identified as 16:0, 18:0, 18:1, 18:2, 20:1, 20:2, 20:4, and 22:6 (7). The main FA identified were 16:0 and 18:0 (38–39% of the total), followed by 20:4 (14–22%). When a comparison was made between the FA of the rat liver with *F. hepatica,* it was found that the FA 20:1 and 20:2 occurred only in the parasite but not in the liver (7). However, the double bond positions in these mono- and diunsaturated FA were not determined.

EXPERIMENTAL PROCEDURES

Sampling and protein purification. Fasciola hepatica adult flukes were obtained by dissection from the bile ducts of infected bovine livers at a local abattoir. They were rinsed in ice cold 0.01 M PBS, pH 7.2, with a cocktail of protease inhibitors including EDTA, PMSF, iodoacetamide, and leupeptin. *Fasciola hepatica* whole worm extract (FhWWE) was obtained by grinding the above with a Ten Broeck tissue homogenizer in ice followed by centrifugation at 30,000 × *g* for 30 min. The supernatant was shell-frozen, lyophilized, and stored dry at –20°C until used (8,9). *Fasciola hepatica* excretion–secretion (FhES) antigens were obtained by incubating *F. hepatica* flukes obtained above in PBS for 1 h at 37°C, centrifuging the liquid and processing as above. A native 12 kDa protein (*n*Fh12) was isolated from FhWWE in a three-step process: (i) by partial purification by molecular sieve chromatography using Sephadex G-50; (ii) by preparative isoelectric focusing using a BioRad Rotofor Cell and an ampholyte

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Abbreviations: FABP, fatty acid-binding protein; FhES, *Fasciola hepatica* excretion–secretion; FhWWE, *F. hepatica* whole worm extract; *n*Fh12, native 12-kDa protein.

mixture of pH 3–10; and (iii) a second isoelectric focusing run with an ampholyte mixture of pH 4–7 (3,8,9). The fractions obtained were analyzed by SDS-PAGE, and those fractions with a single band in the 12-kDa region were lyophilized and stored as above.

Lipid analyses. For lipid analysis either 12 mg of adult *F. hepatica*, 3 mg of FhES, or 50 µg of *n*Fh12 protein complex was dissolved in 5–10 mL of 1.5 M HCl/methanol and refluxed for 24 h. The reaction mixture was then evaporated to dryness under vacuum. The FAME thus obtained (1 mg–10 µg) were then purified by silica gel column chromatography using a Pasteur pipette and eluting with hexane/ether (8:2 vol/vol). The solvent was evaporated to dryness, and the methyl esters were then dissolved in hexane and analyzed by GC–MS. The GC–MS analyses were recorded at 70 eV using a Hewlett-Packard 5972 A MS ChemStation equipped with a 30 m × 0.25 mm special performance capillary column (HP-5 MS) of polymethylsiloxane and He as the carrier gas. Analyses were performed using the following conditions: initial temperature, 130°C; rate of increase 3°C/min; final temperature, 270°C. The double-bond positions in the monounsaturated FA were determined by dimethyl disulfide derivatization as described by Dunkelblum *et al.* (10). Sterols were found in the FAME mixture from the *n*FH12 protein complex and characterized by GC–MS by comparison with authentic samples using the same GC conditions as outlined above.

RESULTS AND DISCUSSION

The total FA from FhWWE, the FhES, and the *n*Fh12 protein complex are presented in Table 1. Figure 1 shows a total ion gas chromatogram comparing the total FAME from FhWWE with the purified FABP *n*Fh12. All major peaks are represented in both parasite preparations. Thirty-nine FA were identified in FhWWE, 35 FA in FhES, and 29 FA in the *n*Fh12 FABP. The difference between the total FA from the whole worm extract and the *n*Fh12 protein complex is meaningful. The FA chain lengths ranged between 12 and 30 carbons. In each case the principal FA were 16:0, 18:1n-9, 18:0, 20:4n-6, and 20:1n-9, which correlates well with the FA identified before in *F. hepatica* from infected rats (7). In addition, small amounts (1.5–4.6%) of *iso-anteiso* methyl-branched FA were characterized by their GC retention times. A similar proportion of saturated vs. unsaturated FA was observed between the FhWWE (43% saturated vs. 54% unsaturated) and the *n*Fh12 protein complex (48% saturated vs. 52% unsaturated). However, the n-3/n-6 ratio of PUFA changed significantly between the FhWWE and the *n*Fh12 protein complex inasmuch as it went from 1.2 in the FhWWE to 9.6 in the *n*Fh12 protein complex. In fact, it seems that the protein contains more n-5, n-6, and n-7 PUFA and less n-3 and n-9 PUFA as compared to the FhWWE. The molar ratio of FA to the *n*Fh12 binding protein was estimated to be between 6 and 8.

The *n*Fh12 protein complex also contained at least three sterols. These were characterized as cholesterol (56%), sitosterol (36%), and fucosterol (8%) and identified by GC–MS and comparison with authentic samples. This finding implies that the *n*Fh12 protein is able to bind, besides FA, sterols. This fact has not been documented before for the *n*Fh12 protein. The molar ratio of sterols to the *n*Fh12 binding protein was estimated to be between 0.5 and 1.0.

FABP belong to a large family of small M.W. (*ca.* 14–15 kDa) cytosolic proteins that are found in abundance in a number of tissues of mammals, birds, fish, and insects (11) as well as in the parasitic trematodes of the genera *Schistosoma*, *Fasciola*, and *Echinococcus* (12–14). FABP are involved in the binding and transportation of a variety of hydrophobic lig-

TABLE 1
FA Composition (relative abundance in wt%) of *Fasciola hepatica* Whole Worm Extract, Excretion–Secretion Products (FhEs), and the *n*Fh12 Protein Complex[a]

FA	*Fasciola hepatica*[b]	FhES	*n*Fh12 protein complex
12:0	0.1	0.3	0.6
14:1n-9	0.01	—	—
i-14:0	0.08	0.1	—
14:0	0.5	0.9	1.2
i-15:0	0.3	0.6	1.0
ai-15:0	0.2	0.5	1.0
15:0	1.3	1.2	1.5
i-16:0	0.2	0.3	0.8
16:1n-7	0.2	0.3	0.1
16:1n-5	0.4	0.9	2.3
16:1n-4	0.2	0.3	1.0
16:0	19.0	28.1	18.2
i-17:0	0.3	0.6	1.8
ai-17:0	0.4	0.5	—
17:1n-8	0.3	0.3	0.8
17:1	—	0.2	—
17:0	1.3	1.1	1.6
18:2n-6	0.4	1.0	5.2
18:1n-9	16.7	14.3	16.0
18:1n-7	2.1	1.5	3.4
18:1n-5	1.0	1.1	2.1
18:0	18.0	25.8	17.8
19:0	0.2	0.2	0.4
20:4n-6	8.2	3.1	9.5
20:3n-3	4.4	0.6	—
20:3n-6	1.5	0.4	2.5
20:2	2.3	3.1	0.8
20:1n-9	7.9	7.0	4.7
20:1n-7	0.3	0.3	1.0
20:0	1.0	1.1	1.0
22:6n-3	3.0	—	—
22:4n-6	1.5	0.6	1.1
22:5n-3	3.4	0.9	1.9
22:3n-6	1.0	—	—
22:1n-9	1.6	0.8	—
22:0	0.2	0.5	0.4
23:0	0.1	—	—
24:2	0.2	—	—
24:1	—	0.3	—
24:0	0.1	0.5	0.3
30:0	0.05	—	—

[a]In % relative abundance greater than 1.0, the error was ±0.4%.
[b]In addition to the FA a total of 12 aldehydes, characterized as dimethyl acetals by GC–MS, also were identified in the whole worm extract. These aldehydes were 12:0, 13:0, *i*-14:0, 14:0, *i*-15:0, *ai*-15:0, 15:0, *i*-16:0, 16:0, *i*-17:0, *ai*-17:0, 17:0, and 18:0.

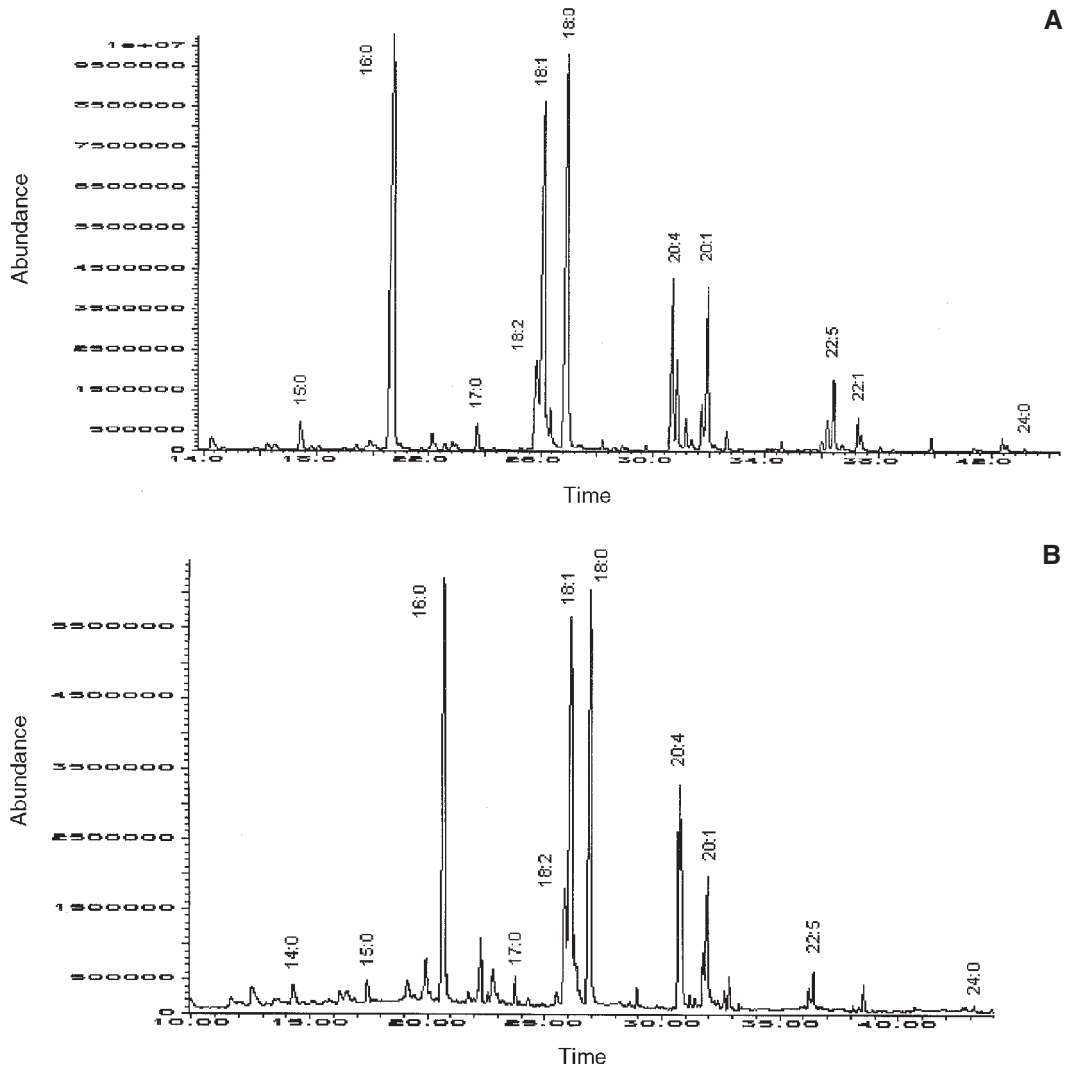

FIG. 1. Total ion gas chromatogram of the total FAME from *Fasciola hepatica* adult worm extracts (A) and the purified *n*Fh12 protein complex from *F. hepatica* (B).

ands such as oleate, palmitate, and a variety of bile acids. For example, rat intestinal FABP is a small monomeric protein composed of 131 residues that binds a single long-chain FA in a noncovalent fashion. It has no known co- or posttranslational modifications and can be efficiently produced in bacteria with full preservation of function (15). Intracellular FA are critical molecules for energy delivery and for the synthesis of membrane lipids and lipid mediators. Thus, FABP may have regulatory functions in energy production as well as in the synthesis of membrane lipids and lipid mediators including prostaglandins, leukotrienes, and thromboxanes (11).

FABP, and in particular *n*Fh12, were the first defined and purified antigen fractions to be tested as a vaccine against fascioliasis. The recognition of this antigen as protective came from a series of experiments begun in the 1970s in which a subset of *Fasciola* antigens could be purified by virtue of their cross-reactivity to antibodies against *S. mansoni* (16). A more purified subset of these cross-reactive *Fasciola* antigens was able to protect mice (1) and calves (2) against challenge infection with *F. hepatica*. This cross-protection also applied in mice challenged with *S. mansoni,* thereby confirming the conservation of immunoprotective epitopes between *Fasciola* and *Schistosoma* antigens (16–19).

Our present report presents a complete characterization of the FA in *F. hepatica* *n*Fh12 FABP as well as in *F. hepatica* adult worms. It shows a complex mixture of FA with differences in proportions between the *n*Fh12 protein and the total whole worm extracts. As already stated, 39 FA were identified in FhWWE, in contrast to the original analysis where 8 FA were detected (7). In addition, 29 FA were identified in the *n*Fh12 FABP complex, as the protein failed to bind 20:3n-6, 22:6n-3, 22:3n-6, and 22:1n-9. This means that there is some FA selectivity for the *n*Fh12 protein. In fact, the protein binds less n-3 and n-9 PUFA than the FhWWE.

Lipids, such as phospholipids, TAG, and cholesterol, are not synthesized *de novo* by *F. hepatica* but are obtained directly from the host (cholesterol or FA) or are synthesized from building blocks obtained from the host including certain

FA and, in the case of phospholipids, the FA and the head groups. Moreover, FA are not degraded by *F. hepatica* and thus are not used for energy generation. Because *de novo* FA synthesis does not occur in *F. hepatica*, the FA must be obtained directly from its mammalian host (20).

Some FA that are present in the host in only very low amounts are abundant in trematodes such as *Schistosoma* and *Fasciola* (21). This is possible because the flukes are able to modify FA obtained from the host (21). They are unable to desaturate FA, but they can elongate them, and the elongation of oleic acid (18:1) to eicosenoic acid (20:1) is the most important modification (21). The function of this extensive modification of lipids obtained from the host is unclear, but it is probably essential for the fitness of the flukes; otherwise, this remainder of the biosynthetic machinery for lipids would also have been lost during evolution (21). The possible role of FA as immunoadjuvants linked to *n*Fh12 or alone remains to be elucidated.

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Seed Oil Composition of *Paullinia cupana* var. *sorbilis* (Mart.) Ducke

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ABSTRACT: The chemical composition of the oil extracted from the seeds of *Paullinia cupana* var. *sorbilis* (Mart.) Ducke (syn. *P. sorbilis*) was investigated. Cyanolipids constituted 3% of the total oil from guaraná seeds, whereas acylglycerols accounted for 28%. ^{1}H and ^{13}C NMR analyses indicated that type I cyanolipids (1-cyano-2-hydroxymethylprop-2-ene-1-ol diesters) are present in the oil from *P. cupana*. GC and GC–MS analysis showed that *cis*-11-octadecenoic (*cis*-vaccenic acid) and *cis*-11-eicosenoic acids were the main FA (30.4 and 38.7%) esterified to the nitrile group. Paullinic acid (7.0%) was also an abundant component. Oleic acid (37.4%) was the dominant fatty acyl chain in the acylglycerols.

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The seeds of many species of Sapindaceae are rich in oils that contain acylglycerols (AG) and an unusual class of plant lipids, the cyanolipids (CL), derived from the amino acid leucine (1–3). Four types of CL structures (Fig. 1; I–IV), with FA esterified to a mono- or a dihydroxynitrile moiety, have been reported as occurring in this plant family (4,5); types I and IV CL are cyanogenic. Moreover, compositional studies on the overall lipids isolated up to now from Sapindaceae plants have indicated that they are characterized by a particularly high content of eicosanoic (20:0) and eicosenoic (20:1) FA (6,7).

The occurrence of CL in the plant kingdom is restricted to only a few families such as the Sapindaceae, the Hippocastanacaeae, and the Boraginaceae (1). The physiologic role of CL in the plant is still not fully understood. It has been suggested that these compounds may serve *in vivo* as storage compounds for reduced nitrogen (8). On the other hand, some structural types (CL I and IV) are involved in cyanogenesis, possibly providing a protective function for the plant (8).

Paullinia cupana var. *sorbilis* (Mart.) Ducke (syn. *P. sorbilis*), commonly known as guaraná, is a plant native to the Amazonas jungle, belonging to the Sapindaceae family (9). The plant is one of the most popular medicinal plants in Brazil, used mainly as a stimulant to relieve fatigue or exhaustion from hot weather. Seeds from this plant contain high amounts of caffeine and are also used to prepare a very popular carbonated soft drink in Brazil. Within the genus *Paullinia*, the species *P. elegans* (10), *P. meliaefolia* (5–7), *P. tomentosa*, and *P. carpopodea* (11,12) have been studied previously for the lipid content of their seed oils. Investigations on the oil composition of *P. cupana* have been limited and controversial (11,12). It was reported, for example, that the presence of CL and the cyanogenic features of the plant may depend on the different origins of the seeds (12).

The presence of cyanogenic phytochemicals, compounds that are able to release cyanide, has long been recognized in the vegetable kingdom, and their insufficient removal from food or forage plants may constitute a health hazard. Therefore, it appears of interest to better define the presence of those metabolites in seed extracts of *P. cupana*. Furthermore, to the best of our knowledge, no detailed compositional analysis of the FA occurring in the CL and AG seed oil fractions from this plant has been performed previously.

In the present work we report the results from the isolation and structural identification of the AG and CL components of the seed oil from *P. cupana* var. *sorbilis*. The chemical analysis was conducted on dried plant seeds collected in Brazil as well as on a commercial guaraná preparation from the "Regiao Amazonica." Our data add to and improve earlier findings.

EXPERIMENTAL PROCEDURES

Plant material. Seeds of *P. cupana* var. *sorbilis* (Mart.) Ducke (syn. *P. sorbilis*) originated from the city of Maurés, in the Amazonas State; they were collected from October to December. Voucher specimens are deposited at the Universidade Federal do Paraná, Laboratório de Farmacognosia, Jardim Botânico, Curitiba, Paraná, Brazil, as well as at the Dipartimento Farmaco-Chimico, Università di Bari, Italy.

Oil extraction and purification. Seeds of *P. cupana* (215 g) were stripped of their external coat and mechanically pulverized to obtain a fine powder, which was submitted to Soxhlet extraction by refluxing petroleum ether (b.p. 35–60°C) for 2.30 h (~10 cycles). Evaporation of the solvent under reduced pressure gave a reddish oil product, which was examined further. The total amount of material recovered was 4.34 g (2.2%). The same Soxhlet apparatus was used for the extraction

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Abbreviations: AG, acylglycerol; AMP, 2-amino-2-methyl-1-propanol; CC, column chromatography; CL, cyanolipids; DMOX, 4,4-dimethyloxazoline.

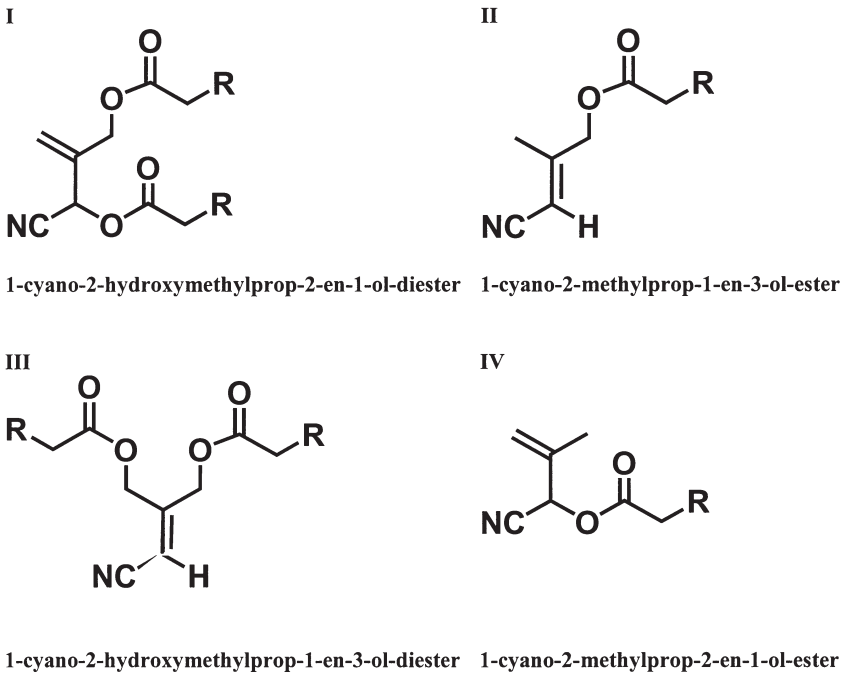


FIG. 1. Chemical structures of cyanolipids I–IV.

of a commercial guaraná powder (Guaraná em pó, Regiao Amazonica), with a total yield of 2.47 g (2.5%).

Guaraná extracts were first investigated by analytical precoated silica gel 60F254 TLC aluminum plates (10 × 20 and 5 × 7.5 cm; Merck, Milan, Italy) with benzene as the eluent. Visualization of the oil components was obtained by spraying the TLC plates with phosphomolybdic acid reagent (10% EtOH; Sigma, Milan, Italy) followed by heating at 110°C (TLC Plate Heater III; CAMAG, Milan, Italy). Individual components were separated by column chromatography (CC) (silica gel 60H; Merck), using benzene as the eluent to recover the pure lipid constituents.

Saponification and methylation. AG and CL (10 mg) dissolved in MeOH (1 mL) were both treated with 5% NaOH in MeOH (2 mL) for 40 min at 80°C using a Multiblock Module Heater (Lab-Line Instruments, Melrose Park, IL). Hexane and H_2O (1:1, vol/vol; 1 mL) were added to stop the reaction. The organic layer was then collected and dried overnight over Na_2SO_4. After filtration, the solvent was removed under vacuum and the residue reacted with CH_2N_2 according to conventional procedures (13). The resulting methyl derivatives were then analyzed by GC and GC–MS.

Standard methyl esters of commercial FA (oleic, palmitic, palmitoleic, stearic, linoleic, linolenic, and eicosanoic acids) were also prepared by reacting them with CH_2N_2 as above; the methyl esters were used as reference compounds for GC and GC–MS analyses together with commercial methyl esters of paullinic (*cis*-13-eicosenoic), *cis*- and *trans*-vaccenic, and *cis*-11-eicosenoic acids (Sigma).

Preparation of FA butyl esters. AG and CL (5 mg) extracted from the guaraná seeds were reacted with 1 mL of 0.1 M of Na *tert*-butoxide in *n*-butanol (Fluka, Milan, Italy). The reaction was performed at ambient temperature for 1 h; 5% $NaHSO_4$ in water (1 mL) was added to stop the reaction (14). The organic layer was separated and dried overnight over Na_2SO_4 before GC and GC–MS analyses. Reference *tert*-butyl esters of oleic, *cis*- and *trans*-vaccenic, palmitic, stearic, palmitoleic, eicosanoic, linoleic, linolenic, and *cis*-11- and *cis*-13-eicosenoic acids (Sigma) were also prepared and submitted to GC and GC–MS investigations.

4,4-Dimethyloxazoline (DMOX) derivatives. Oil fractions containing AG and CL (5 mg) were both treated with 1 mL of 2-amino-2-methyl-1-propanol by heating for 90 min at 170–180°C (15). After cooling, a solution of $CHCl_3$ and H_2O (1:1, vol/vol) was added to the reaction mixture, which was then vigorously shaken and allowed to separate into two phases. The organic phase was collected and dried overnight over Na_2SO_4 before GC and GC–MS analyses. DMOX derivatives of saturated (stearic, palmitic, and eicosanoic) and unsaturated (oleic, palmitoleic, linoleic, linolenic, and methyl *cis*-11- and methyl *cis*-13-eicosenoic) FA were also prepared according to the above procedure.

GC analysis. A Carlo Erba HRGC 5160 gas chromatograph with FID and on-column injection was used. Hydrogen was the carrier gas; air and H_2 were adjusted to yield the optimal separation. Data were processed by a Spectra Physics SP 4290 computing integrator.

Analysis of FAME and FA *tert*-butyl esters of AG and CL from *P. cupana*, as well as their DMOX derivatives, was carried out on a DB-23 (Superchrom, Milan, Italy) fused-silica capillary column, 30 m × 0.32 mm i.d.; 0.25 μm film thickness. FAME were analyzed under the following conditions: detector temperature 300°C; column temperature was programmed from 70°C, 10°C/min to 180°C and then up to

230°C at 3°C/min. Chromatographic conditions for the analyses of FA *tert*-butyl esters and DMOX derivatives were as follows: 70°C, 15°C/min to 110°C and then up to 240°C at 3°C/min. The detector port was maintained at 300°C. Alternatively, FAME and FA *tert*-butyl esters were analyzed with a DB-5 (Superchrom) fused-silica capillary column (30 m × 0.32 mm i.d.; 0.25 μm film thickness) under the following conditions: 70°C, 15°C/min to 180°C and then up to 260°C at 5°C/min; detector temperature 300°C.

GC–MS analysis. FAME, FA *tert*-butyl esters, and DMOX derivatives were analyzed by GC–MS with a Hewlett-Packard 6890–5973 mass spectrometer interfaced with an HP Chemstation. Analytical conditions were as follows: column oven program from 70°C (4 min isothermal) to 280°C (20 min isothermal) at 20°C/min; injector, 250°C. Helium was the carrier gas (flow rate, 1 mL/min). An HP-5-MS capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness) was used. MS operating parameters were as follows: ion source, 70 eV; ion source temperature, 230°C; electron current, 34.6 μA; vacuum, 10^{-5} torr. Mass spectra were acquired over 40–800 amu range at 1 scan/s. The ion source was operating in the EI mode. The samples (1 μL) were injected using the splitless sampling technique.

FTIR. FTIR of AG and CL were recorded using NaCl cells on a PerkinElmer Spectrum One spectrophotometer.

NMR. Proton (^{1}H NMR) and carbon NMR (^{13}C NMR) were recorded on a DRX500 Avance Bruker instrument equipped with probes for inverse detection and with *z* gradient-accelerated spectroscopy. Standard Bruker automation programs were used for 2-D NMR experiments. 2-D COSY experiments were performed using COSYDFTP (double-quantum-filtered phase-sensitive COSY) and COSYG (gradient-accelerated COSY) sequences. Inverse detected normal and long-range ^{1}H and ^{13}C heterocorrelated (HECTOR) 2-D NMR spectra were obtained by using the gradient sensitivity-enhanced pulse sequences INVIEAGSSI and INV4GPLRND, respectively. $CDCl_3$ was used as the solvent in all of the experiments. Residual ^{1}H and ^{13}C peaks of the solvent were used as internal standards to calculate chemical shifts referred to tetramethylsilane.

RESULTS AND DISCUSSION

General. Inspection of TLC of the extracts from the seeds of *P. cupana* and the commercial flour of guaraná showed that both were made of the same products, migrating at R_f 0.53 (AG) and 0.70 (CL), respectively. Accordingly, separation of each of the two extracts by CC afforded the AG and CL components, which amounted to 16% (commercial flour) and 28% (seeds) and to 5% (commercial flour) and 3% (seeds), respectively.

Purified AG and CL constituents from the total extract were further characterized by chromatographic, spectroscopic, and chemical means. Homogeneous results were obtained and, unless otherwise specified, reported chromatographic and spectroscopic data refer equally to AG and CL from both guaraná samples.

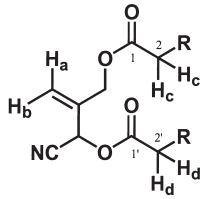
CL identification. IR spectra of plant CL appear to be distinct for each structural type I–IV (4,5). As expected, the guaraná fraction identified by us as the CL constituents showed the common absorption maxima found in acyl lipids spectra, that is, bands at 3003 (C–H olefins), 2924–2854 (aliphatic C–H stretching), 1748 (C=O stretching) broader and double, suggesting the possible presence of different CO, 1463 (aliphatic C–H bending), and 1149 (C–O stretching) cm^{-1}. In addition to those, the IR spectrum of the isolated CL exhibited weak and broad absorption bands at 1006 and 938 cm^{-1}; these are not normally found in the spectra of acyl lipids and are reported as diagnostic for type I CL (Fig. 1) (4). This first attempt to identify the type of CL contained in our extracts was also supported by the absence of nitrile absorption (2230 cm^{-1}). In fact, in the IR spectra of type I CL, the corresponding nitrile band should not be present owing to the quenching effect of the oxygen atom on the same carbon as the cyano group.

More structural information on the nature of the CL extracted from our samples of guaraná came from their ^{1}H and ^{13}C spectral data (Table 1). 2-D proton–proton and proton–carbon correlations also facilitated the assignments of the relative signals. The CL nature of the isolated molecules was indicated by the presence of specific resonances, that is, at δ 115.33, nitrile carbon, and at δ 61.28, carbon bearing the cyano function. Moreover, the singlet at δ 5.94 in the ^{1}H spectrum was assigned to the tertiary proton adjacent to the cyano group on the basis of the cross peak observed with the nitrilic carbon in the long-range ^{1}H-^{13}C 2-D heterocorrelated spectrum.

The study of the chemical shifts allowed the identification of type I CL as constituents of *P. cupana* seed oil. In fact, the resonances at δ 135.68 (vinyl carbon) and δ 121.42 (terminal vinyl carbon) together with the signal at δ 63.12 (allylic carbon) supported the presence of the expected dihydroxyisobutenyl cyanide moiety of this type of CL (Fig. 1). Two singlet signals assigned to the two hydrogens on the terminal vinyl carbon were found at δ 5.54 and 5.68, with the lower field resonance at δ 5.68 attributed to the proton closer to the nitrile group (Hb). As further evidence of this observation, in the ^{1}H-^{1}H 2-D COSY spectrum, this signal showed the expected stronger long-range coupling with the proton geminal to the nitrile group (δ 5.94) than with the two methylene protons, resonating at δ 4.64, adjacent to the oxygen. In the COSY spectrum, the vinyl proton at 5.54 (Ha) consistently showed a stronger long-range coupling with the methylene (δ 4.64) than with the methine proton (δ 5.94). Furthermore, distinct peaks for the two CO carbon atoms present in the structure of type I CL were detected in our spectra: C-1, δ 173.48 and C-1′, δ 171.94 (Table 1; Fig. 2). The upfield signal at δ 171.94 was assigned to the carboxyl group linked to the carbon bearing the nitrile function.

In contrast to what we observed in the ^{13}C spectra of the TAG (see below), the C-2 and the C-3 regions of the spectrum each showed two sharp distinct signals at δ 34.00 and 34.46

TABLE 1
^{1}H and ^{13}C NMR Data of Cyanolipids from Seed Oil of *Paullinia cupana* var. *sorbilis*



^{1}H	δ (J)	^{13}C	δ (J)
$C\underline{H}_2OCO$	4.64 *dd* (13.3)	$=\underline{C}$	135.68
$C\underline{H}$–CN	5.94 *s*	$\underline{C}H_2=$	121.42
$CH_2=$	5.54 Ha, *s*; 5.68 Hb, *s*	CN	115.33
$C\underline{H}_2C=O(O)$	2.31 Hc, *t* (7.5); 2.38 Hd, *dt* (2.2;7.7)	C-1; C-1′	173.48; 171.94
CH_3, ω1	0.85 *t* (6.6)	C-2; C-2′	34.46; 34.00
n-CH_2	1.25 *m*	C-3; C-3′	25.20; 25.02
CH_2–$C\underline{H}_2C\underline{H}_2$–C=O	1.59 (CH_2-3), *m*; 1.62 (CH_2-2), *m*	$\underline{C}H_2OCO$	63.12
–$C\underline{H}_2$–C=C–$C\underline{H}_2$– (*cis*)	1.98 *m*	$\underline{C}H$–CN	61.28
–C=C–$C\underline{H}_2$–C=C– (*cis*)	2.75 *m*	CH_3, ω1	14.52
			14.65 (18:3)
Olefinic (*cis*)	5.32 *m*	CH_2, ω2	22.98 (18:2)
			23.07/23.09 (ω7/Sat, ω9)
		CH_2, ω3	32.00 (18:2)
			32.19 (ω7)
			32.32/32.33 (ω9/Sat)
		n-CH_2	29.39-30.17
		–$\underline{C}H_2$–C=C–$\underline{C}H_2$–	27.62
		–C=C–$\underline{C}H_2$–C=C–	26.04
		C=CH[a]	128.29 (C-12 LA[a])
			128.51 (C-10 LA)
			130.07 (C-9 OL)
			130.22 (C-11 EI, VA)
			130.31 (C-12, C-13 PA)
			130.46 (C-9 LA; C-10 OL)
			130.35 (C-12 EI, VA)
			130.64 (C-13 LA)

[a]Identified unsaturated chains are as follows: LA, linoleic ; OL, oleic; EI, *cis*-11 eicosenoic; PA, paullinic acid; VA, vaccenic acid. Sat, saturated.

and at δ 25.02 and 25.20, which correlated in the ^{1}H-^{13}C 2-D heterocorrelated spectrum with the corresponding methylene signals at 2.38, 2.31, 1.62, and 1.59, respectively. From the long-range ^{1}H-^{13}C 2-D heterocorrelated spectra, cross peaks between the signal at ^{13}C δ 171.94 (C-1′) and resonances at ^{1}H δ 2.38 (CH_2-2′, Hd) and ^{1}H δ 1.62 (CH_2-3′) were evident. Consistently, the signal at δ 173.48 (C-1) showed correlation with proton chemical shifts at δ 2.31 (CH_2-2, Hc) and δ 1.59 (CH_2-3). Corresponding connectivities between CH_2-2, Hc and CH_2-3, as well as CH_2-2′, Hd and CH_2-3′ were found in the ^{1}H-^{1}H 2-D COSY spectrum (Fig. 2).

The signals at δ 2.31 (Hc) and at δ 2.38 (Hd) consisted of a triplet and a doublet of triplets, respectively. The multiplicity of the signal observed at δ 2.38 was interpreted as due to the diastereotopic splitting caused by the proton proximity to the chiral carbon bearing the nitrile group.

Proton and carbon resonances relative to the fatty acyl chains of the CL molecules are shown in Table 1. Examination of the frequency regions of the methyl (ω1) and the methylene carbons (ω2, ω3) of the esterified FA showed resonances at δ 14.52 and at δ 23.07/23.09 for ω1 and ω2 carbons (16,17). Both signals were associated with two very low intensity peaks at δ 14.65 and δ 22.98, consistent with ω1 and ω2 carbons of 18:3 and 18:2 fatty acyl chains, respectively. Peaks for ω3 carbons had chemical shifts at δ 32.00 (very low intensity), 32.19, and 32.32/32.33. GC–MS analyses of the CL fraction from guaraná oil (see below) indicated that *cis*-vaccenic acid ($C_{18:1}$ Δ11), and eicosenoic acids, namely *cis*-11-eicosenoic acid ($C_{20:1}$ Δ11) and *cis*-13-eicosenoic or paullinic acid ($C_{20:1}$ Δ13), are abundant constituents. Saturated acids and oleic acid ($C_{18:1}$ Δ9) were also present but in lesser amounts. ^{1}H and ^{13}C NMR spectra of the above compounds were also run to assign the relative chemical shifts; overall relative resonances were established as described in Table 1. Frequencies for ω1 to ω3 carbons generally follow a deshielding order, going from saturated to unsaturated depending on the unsaturation pattern and degree (16,17). Nevertheless, ω1 to ω3 reciprocal shifts are also influenced depending on the closeness of the double bond (17). On the basis of this evidence, it seems rational that *cis*-vaccenic and paullinic acids, both ω7 chains, give overlapping ω1 to ω3 signals, which differ from those of *cis*-11-eicosenoic acid, an

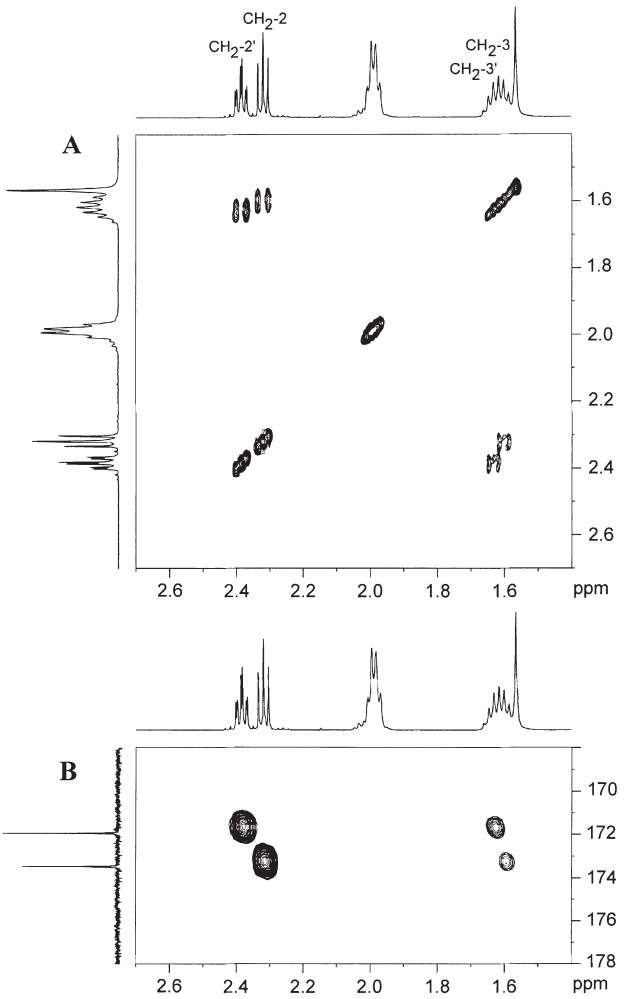


FIG. 2. (A) ^{1}H-^{1}H 2-D COSY spectrum and (B) corresponding long-range ^{1}H-^{13}C 2-D heterocorrelated spectrum of the cyanolipids from *Paullinia cupana* var. *sorbilis* in the region of the methylenes α (2, 2′) and β (3, 3′) to the carbonyls.

ω9 FA that is expected instead to pair with oleic acid. In good agreement with previous data recently reported for TAG of a plant oil rich in oleic-vaccenic acids (18), ω2 and ω3 peaks of esterified *cis*-vaccenic/paullinic acids (δ 23.07 and 32.19) and *cis*-11-eicosenoic/oleic acids (δ 23.09 and 32.32) were found to resonate as two peaks separated by 0.02 and 0.13 ppm, respectively. As shown in Table 1, in our sample, ω1 carbons resonated instead as a single peak, which is consistent, however, with the slight difference of 0.008 ppm previously described (18) for esterified oleate and vaccenate fatty acyl chains.

The olefin region in the ^{13}C spectrum of the CL fraction from guaraná presented two prominent peaks at δ 130.22 and 130.35, which can be referred to C-11 and C-12 of *cis*-11-eicosenoic and vaccenic acids, respectively (16–18). Paullinic acid resonated instead at δ 130.31 (16). Resonances of much lower intensities consistent with oleic and linoleic acids as esterified acyl chains were also present (Table 1).

To our knowledge, no detailed proton and carbon NMR assignments have appeared concerning those compounds. Nevertheless, NMR assignments relative to the dihydroxyisobutenyl cyanide moiety of CL are sometimes reported incorrectly (10–12). Therefore, we believe that the NMR data on guaraná seed oil reported here improve upon the data described earlier.

AG determination. IR spectra of AG compounds showed typical absorption bands at 3006 (C-H olefins), 2924, and 2854 (aliphatic C–H stretching), 1746 (C=O stretching), 1464 (aliphatic C–H bending), 1164 (stretching C–O), and 722 cm^{-1}. AG isolated from the seed oil of *P. cupana* var. *sorbilis* were also subjected to ^{1}H and ^{13}C NMR analysis (Table 2). Signals relative to glycerol α- and β-carbon atoms indicated that the isolated lipids were TAG (19,20).

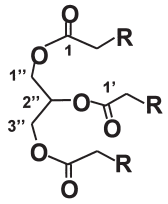
Carbon resonances of terminal methyls, ω1, along with ω2 and ω3 methylene signals were useful in determining the presence of ω9 (oleic and *cis*-11-eicosenoic acids), ω7 (vaccenic acid), ω6 (linoleic acid), and ω3 (linolenic acid) chains as AG constituents (Table 2). In the NMR spectrum of AG, ω2 and ω3 signals relative to 18:2 and 18:3 chains have a higher intensity than CL, suggesting a larger presence of those FA in the oil mixture. Nevertheless, in agreement with what is found for CL, ω2 to ω3 signals relative to saturated and monoenoic chains resonate as paired peaks, which can be assigned to ω7 (lower chemical shifts) and ω9 (higher chemical shifts) fatty acyl chains.

A closer examination of the spectral region between δ 127.5 and 132.4 in the carbon spectra (Table 2) allowed the detection of the following FA in the TAG structure (15,16, 20–22): linolenic acid, ($C_{18:3}$ Δ9,12,15), δ 127.50 (C_{15}), δ 128.20 (C_{10}), δ 128.60 (C_{13}), δ 128.70 (C_{12}), δ 130.64 (C_{9}), δ 132.40 (C_{16}), δ 14.68 (ω1), δ 20.96 (ω2); linoleic acid ($C_{18:2}$ Δ9,12), δ 128.30 (C_{12}), δ 128.51 (C_{10}), δ 130.40 (C_{9}), δ 130.64 (C_{13}), δ 14.48 (ω1), δ 22.98 (ω2), δ 31.93 (ω3); oleic acid ($C_{18:1}$, Δ9), δ 130.09/130.12 (C_{9}; 2- and 1(3)-positions, respectively), δ 130.42/130.43 (C_{10}; 1(3)- and 2-positions, respectively), δ 14.52 (ω1), δ 23.09 (ω2), δ 32.34 (ω3). On the basis of the NMR identification of the CL (see above), the two signals at δ 130.22 and 130.35 were attributed to *cis*-11 eicosenoic ($C_{20:1}$, Δ11) and vaccenic ($C_{18:1}$, Δ11) acids, respectively.

Carbon-13 spectra of model TAG mixtures of saturated (tripalmitin) and unsaturated (triolein and trilinolein) fats were also acquired to confirm our assignments. Published data were also evaluated for identification of the TAG acyl moieties (16,19,20,22–24).

FA composition. To corroborate NMR identification and to achieve good quantification of the FA in the seed oils from guaraná, AG and CL were submitted to derivatization before GC and GC–MS analyses. FA esterified to the nitrile moiety of the CL from guaraná were analyzed as their methyl, butyl ester, and DMOX derivatives by GC and GC–MS. In agreement with the NMR analyses, the bulk of the FA was represented by monounsaturated isomers with *cis*-11-octadecenoic (*cis*-vaccenic) and *cis*-11-eicosenoic acids contributing 30.4

TABLE 2
^{1}H and ^{13}C NMR Data of TAG from Seed Oil of *P. cupana* var. *sorbilis*



1H	δ (J)	^{13}C	δ
2, 2′	2.30 *m*	$\underline{C}H_3$, ω1	14.48 (18:2) 14.52 (Sat, ω7, ω9) 14.68 (18:3)
3, 4	1.61 *m*	$\underline{C}H_2$, ω2	20.96 (18:3) 22.98 (18:2) 23.07/23.09 (ω7/Sat, ω9)
–$C\underline{H}_2$–C=C–$C\underline{H}_2$– (*cis*)	2.00 *m*	CH_2, C-3	25.27
–C=C–$C\underline{H}_2$–C=C (*cis*)	2.75 *m*	–C=C–$\underline{C}H_2$–C=C–	25.94, 26.04
Olefinic (*cis*)	5.33 *m*	–$\underline{C}H_2$–C=C–$\underline{C}H_2$– (*cis*)	27.59, 27.62/27.63
Other (*n*-$C\underline{H}_2$)	1.29 *m*	*n*-CH_2	29.40–30.18
$C\underline{H}_3$, ω1	0.88 *t* (8)	CH_2, ω3	31.93 (18:2) 32.19 (ω7) 32.32/32.34 (ω9/Sat)
Glycerol α $C\underline{H}_2$ (1″a, 3″a)	4.11 *dd* (18.5)	Glycerol CH_2 (α carbons)	62.50
Glycerol α $C\underline{H}_2$ (1″b, 3″b)	4.28 *dd* (17.3)	Glycerol CH (β carbons)	69.29
Glycerol β $C\underline{H}$	5.23 *m*	–C=CH[a]	127.50 (C-15 LN) 128.29 (C-10 LN) 128.30 (C-12 LA) 128.51 (C-10 LA) 128.60 (C-13 LN) 128.70 (C-12 LN) 130.09/130.12 (C-9 OL *sn*-2/1,3) 130.22 (C-11 EI, VA) 130.35 (C-12 EI, VA) 130.40 (C-9 LA) 130.42/130.43 (C-10 OL, *sn*-1,3/2) 130.64 (C-13 LA, C-9 LN) 132.40 (C-16 LN)
		C-1, C-1′	173.70, 173.30
		C-2, C-2′	34.46, 34.59

[a]Identified unsaturated chains are as follows: LN, linolenic; LA, linoleic; OL, oleic; EI, *cis*-11 eicosenoic; PA, paullinic acid; VA, vaccenic acid; see Table 1 for other abbreviation.

and 38.7%, respectively (Table 3). High yields of *cis*-13-eicosenoic (paullinic acid; 7.0%) and oleic (7.0%) acids were also found.

Compositional data from guaraná AG are reported in Table 3. As shown, oleic (37.0%) and *cis*-vaccenic (20.9%) acids represented the main components. High amounts of linoleic (10.2%) and *cis*-11-eicosenoic (11.6%) acids were also present. In contrast to CL, the yield of paullinic acid in the AG lipid fraction from guaraná seed oils was reduced to 3.5% (Table 3). Data were in agreement with NMR findings.

Identification of the constituents in the AG and CL oil fraction was made by comparison of their GC retention times with those of reference compounds analyzed under the same conditions. Nevertheless, GC–MS of their DMOX derivatives proved to be particularly suitable for characterizing the unsaturated FA.

The following distinct MS fragmentation patterns were obtained for the main FA (i) *cis*-vaccenic, (ii) *cis*-11-eicosenoic, (iii) paullinic and (iv) oleic DMOX derivatives, respectively, *m/z*, (relative intensity): (i), 335, [M^+, $C_{22}H_{41}NO$ (13)], 320 (16), 278 (27), 264 (24), 236 (4), 224 (3), 126 [$M^+ - 209$, (74)], 113 [$M^+ - 222$, (100)]; (ii), 363, [M^+, $C_{24}H_{45}NO$ (11)], 348 (11), 278 (25), 264 (22), 236 (3), 224 (3), 126 [$M^+ - 237$, (73)], 113 [$M^+ - 250$, (100)]; (iii), 363, [M^+, $C_{24}H_{45}NO$ (4)], 348 (5), 278 (6), 264 [$M^+ - 99$, (7)], 252 (2), 126 [$M^+ - 237$, (40)], 113 [$M^+ - 250$, (100)]; (iv), 335, [M^+, $C_{22}H_{41}NO$ (8)], 320 (12), 278 (14), 250(18), 236 (22), 224 (1), 222 (16), 208 (4), 196 (5), 126 [$M^+ - 209$, (100)], 113 [$M^+ - 222$, (97)].

To our knowledge, this is the first detailed compositional study of the FA occurring in the oil lipid fractions, AG and CL, from *P. cupana* var. *sorbilis*. Two previous papers (6,12)

TABLE 3
FA Composition of the Acylglycerols (AG) and Cyanolipids (CL) from *P. cupana* Seed Oil

FA	AG (%)	CL (%)
Palmitic	5.2	2.4
Palmitoleic	Trace	0.6
Stearic	6.9	2.0
Oleic	37.0	6.9
cis-Vaccenic	20.9	30.4
Linoleic	10.2	6.1
Linolenic	1.0	—
Eicosanoic	3.7	1.8
cis-11-Eicosenoic	11.6	38.7
cis-13-Eicosenoic	3.5	7.0
Docosanoic	Trace	Trace

reported the total content of FA in guaraná seed oil; however, only a general description of the occurrence of homologs was given, without further characterization of their unsaturation position. Moreover, in both cases, only methyl ester derivatives were analyzed.

Previous detailed investigations on the total FA composition of seed oils from other members of the Sapindaceae (6,12) showed that they were very peculiar in that *cis*-vaccenic and paullinic acids were the principal components; these have been proposed as chemotaxonomic markers for the plant family. In fact, *cis*-vaccenic acid normally occurs in seed oils in low amounts, and paullinic acid appears to be very rare in the plant kingdom; it has been reported in some Proteaceae (25) and, within the Sapindaceae, in the oil of *Sapindus trifoliatus* (26).

Our data are in agreement with previous findings from other Sapindaceae, that is, a high yield of $C_{18:1}$ and $C_{20:1}$ FA is also characteristic of the seed oil from *P. cupana* var. *sorbilis*, with oleic, *cis*-vaccenic, and *cis*-11-eicosenoic acids as the dominant constituents (Table 3). Moreover, as reported for *P. elegans* (10), AG from guaraná had a higher amount of oleic acid (37.0%), which is a common compound of seed lipids, whereas eicosenoic acids were incorporated preferentially into the CL oil fraction (45.7 vs. 15.1% in CL and AG, respectively).

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FA Determination in Cold Water Marine Samples

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ABSTRACT: The determination of FA in cold water marine samples is challenging because of the presence of large proportions of a variety of labile PUFA. This study was undertaken to establish optimal methods for FA analysis in various sample types present in the marine environment. Several techniques used in FA analysis, including lipid fractionation, FAME formation, and picolinyl ester synthesis, were examined. Neutral lipids, acetone-mobile polar lipids, and phospholipids (PL) were readily separated from each other on columns of activated silica gel, but recoveries of PL were reduced. Deactivation of the silica gel with 20% w/w water produced variable recoveries of PL (66 ± 22%). FAME formation with BF_3 gave optimal recoveries, and a method to remove hydrocarbon contamination from these samples before GC analysis using column chromatography was optimized. Picolinyl derivatives of FA are useful in structural determinations with MS, and a new base-catalyzed transesterification method of their synthesis from FAME was developed. Finally, a series of calculations, combining FA proportions with acyl lipid class concentrations, was designed to estimate FA concentrations. In algae and animal samples, these estimates were in good agreement with actual FA concentrations determined by internal standards.

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FA analysis is important in fields such as biochemistry, oceanography, biogeochemistry, and aquaculture. For instance, in biogeochemical studies, FA may act as signature compounds of organisms to determine sources and sinks of organic material; such information enhances our knowledge of carbon cycling in the marine environment (1–4). In aquaculture the interest is in FA nutrition and the establishment of optimal levels of EFA in fish diets (5). Accurate quantification of these FA is a challenge, particularly with samples from cold-water environments that contain elevated levels of oxygen-sensitive PUFA. The widespread interest in FA applications has led to the development of a variety of techniques for their analyses, including, for example, several fractionation and methylation procedures. Ideally, all research groups conducting FA research should adopt standard methods. However, even uniformity in technique may not be sufficient to guarantee accurate results in separate laboratories. For example, Roose and Smedes (6) compared the efficiency of a lipid extraction technique in a number of laboratories and found that, although all groups claimed to follow the Bligh and Dyer (7) technique, very few actually used conditions that conformed to the original method. Modifications of this and other techniques so as to generate results more quickly and easily are commonplace. To allow comparisons of results acquired by different techniques, one must have some knowledge of the effects of variations in methodology on FA recovery.

Before FA analysis can be carried out, lipids must first be extracted from the matrix in which they are encountered. A number of studies have examined variations in lipid recovery with differing solvent systems (8–11) and further comparison is not necessary. In this report, the methods of both Folch *et al.* (12) and Bligh and Dyer (7), depending on sample type, are used. To determine FA qualitatively and quantitatively, methyl ester derivatives must be formed. A variety of methods to transesterify lipid extracts are available (13–17), and a quantitative evaluation of these transesterification methods will determine if all procedures, as assumed, produce equivalent results. Additional techniques, such as fractionation of the lipid extract (18), removal of contaminants, and formation of derivatives for MS (16,19), often are performed, and new methods for those steps are described and critically evaluated here. Finally, a simple method to approximate FA concentrations using acyl lipid data is discussed and applied to marine samples. In most cases, results of comparisons are evaluated by application to typical marine samples: *Nannochloropsis* sp. (green algae), *Isochrysis galbana* (flagellate), and *Mytilus edulis* (bivalve).

EXPERIMENTAL PROCEDURES

Samples. Blue mussels (*M. edulis*) were taken from stocks maintained at the Ocean Sciences Centre (OSC) in Logy Bay, Newfoundland, Canada. The shells were removed, and the bodies were blotted dry. Each mussel was immediately weighed and extracted. Algal samples of *Nannochloropsis* sp. and *I. galbana* were taken from cultures in logarithmic phase grown at the OSC. Known volumes of phytoplankton samples were filtered onto precombusted GF/C filters. Filters were then placed in $CHCl_3$ and stored under nitrogen in the dark at –20°C.

Extraction. Blue mussels were extracted according to the procedure of Bligh and Dyer (7). Typically, each sample of approximately 4 g wet weight was homogenized with a Trinitron homogenizer in 12 mL of 1:2 $CHCl_3$/MeOH. The homogenate was filtered, and the tissue and the filter were rehomogenized

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Abbreviations: AMPL, acetone-mobile polar lipid; NL, neutral lipid; NMID, nonmethylene-interrupted dienoic; OSC, Ocean Science Centre; PL, phospholipid; SE/WE, steryl ester/wax ester; TIC, total ion chromatogram.

with another 4 mL of $CHCl_3$. Following a second filtering, the filtrate was mixed with 4 mL of water, allowed to separate, and the $CHCl_3$ layer was recovered.

Phytoplankton samples were extracted with a simplified Folch *et al.* (12) procedure (20). Samples were homogenized with both sonication and grinding with a steel rod in 4 mL of 2:1 $CHCl_3$/MeOH and 0.5 mL of water. After centrifuging at $100 \times g$ for 2 min, the lower $CHCl_3$ layer was collected. At least three washes of the aqueous phase with 3 mL of $CHCl_3$ were carried out.

Quantification of lipid classes. Lipid classes were determined using the Iatroscan TLC-FID system according to the method described in Parrish (20). Samples of lipid extract were applied to Chromarods SIII, which were then developed twice in 99:1:0.05 hexane/diethyl ether/formic acid for 25 and 20 min. The rods were scanned from the top to just after the ketone peak. The second development was for 40 min in 80:20:0.1 hexane/diethyl ether/formic acid. Rods were then scanned to just after the DAG peak. Finally, the rods were developed twice in acetone (2×15 min), followed by a double development in 5:4:1 $CHCl_3$/MeOH/H_2O (2×10 min). The entire length of the rods was then scanned. This yielded three partial chromatograms that were joined to produce one full chromatogram.

Fractionation of lipid extract. Lipids were fractionated into neutral lipids (NL), acetone-mobile polar lipids (AMPL), and phospholipids (PL) using column chromatography on silica gel. A small amount of precombusted glass wool was placed in the tapered end of a Pasteur pipet, and the pipet was packed with approximately 0.8 g of silica gel that had been activated by heating at 110°C for 1 h. The column was then rinsed with 2 bed vol each of MeOH and $CHCl_3$. Approximately 5 mg of lipid extract in $CHCl_3$ was placed at the top of the column, and NL were recovered with 2 bed vol (approximately 6 mL) of 98:1:0.5 $CHCl_3$/MeOH/formic acid at a flow of approximately 1 mL min^{-1}. AMPL was eluted with 2 bed vol of acetone. One bed volume of $CHCl_3$ was then passed through the column to return it to a more neutral polarity, and PL were eluted with 2 bed vol of MeOH. Up to 20 mg of lipids were fractionated in this way but using proportionally greater solvent volumes.

FAME preparation. To evaluate the efficiency of FAME formation, five different procedures for their synthesis were applied to identical lipid extract samples (approximately 1 mg) of *I. galbana* with 23:0 FAME added as internal standard. In all cases, lipid extracts were evaporated to near dryness before any derivatization reagents were added. The five procedures are described briefly as follows: (i) BF_3 (14): hexane (0.5 mL) and 10% BF_3 in MeOH (1 mL) were added to the extract, the mixture was shaken, covered with N_2, and then heated at 80–85°C for 1 h. The samples were allowed to cool, 0.5 mL of water was added, and the samples were again shaken. Hexane (2 mL) was added and the mixture was shaken, then centrifuged. The upper hexane layer, containing the FAME, was removed and concentrated. (ii) HCl (16): acetyl chloride (2 mL) was added slowly to MeOH (18 mL) to make methanolic HCl. Hexane (1 mL) and methanolic HCl (2 mL) were added to the extract and heated for 2 h at 80°C. Samples were allowed to cool, and 3 mL of 5% aqueous NaCl was added. Hexane (2 mL) was added, the sample was shaken, and the hexane layer was withdrawn; (iii) H_2SO_4 (13): 2 mL of 6% H_2SO_4 in MeOH and 5 mg of hydroquinone were added to the sample and the mixture was heated at 70°C for 5 h. The samples were allowed to cool, and 1 mL of water and 1.5 mL of hexane were added. The mixture was shaken and centrifuged and the upper hexane layer was collected; (iv) AOCS Official Method Ce 1b-89 (17): to the sample was added 0.5 mL of 0.5 mol L^{-1} NaOH in MeOH; the mixture was heated at 100°C for 7 min and then allowed to cool. Next, 1 mL of 10% BF_3 in MeOH was added, and the mixture was heated at 100°C for 5 min. The sample was again allowed to cool, 0.5 mL of iso-octane was added, and the mixture was shaken for 30 s. Saturated NaCl solution (2 mL) was added, and the solution was shaken and centrifuged. The upper iso-octane layer was collected; (v) NaOMe (15): 2 mL of 0.25 mol L^{-1} sodium methoxide in 1:1 MeOH/diethyl ether was added to the sample, and the mixture was covered with nitrogen and heated at 100°C for 30 s. The sample was cooled, 1 mL of iso-octane and 5 mL of saturated NaCl were added, and the mixture was shaken vigorously for 15 s. The upper iso-octane layer was then collected.

Hydrocarbon cleanup. Several algal samples containing hydrocarbon pollutants from shipboard activity were collected. These hydrocarbon contaminants interfered with a number of FAME peaks and had to be removed before analysis. This was done using a Pasteur pipet packed with activated silica gel in a modification of a procedure recommended by Christie (16) for removal of sterols. The column was rinsed with 1 bed vol (approximately 3 mL) each of $CHCl_3$ and hexane, and the FAME sample (approximately 1 mg) was placed at the head of the column. Iso-octane (1 bed vol) was used to elute the contaminating hydrocarbons. The FAME were then recovered with 2 bed vol of 80:20 hexane/diethyl ether. In addition to hydrocarbon pollutants, this technique removed biogenic hydrocarbons, such as phytane and pristane.

Argentation TLC. Silver nitrate-impregnated plates were prepared by dipping silica gel-coated plates (coating thickness of 250 μm) in 20% $AgNO_3$ in acetonitrile. Plates were activated for 1 h at 110°C immediately prior to use. Approximately 10 μg of FAME was applied in a concentrated spot using a Hamilton syringe. Plates were then developed in a closed chamber in 90:10 hexane/diethyl ether (21) until the solvent front reached the top of the plate (approximately 20 min). Spots were visualized with 2′,7′-dichlorofluorescein under UV light, and the esters were recovered from the silica with 1:1 hexane/diethyl ether.

Picolinyl ester preparation. Picolinyl esters of FA were prepared following two separate procedures. FAME (approximately 10 mg) were hydrolyzed by reaction with 2 mL of 0.5 mol L^{-1} NaOH in MeOH at 70°C for 30 min, followed by acidification with 1 mol L^{-1} of HCl and extraction with hexane (22). The FA in hexane solution were evaporated to near dryness, 0.5 mL of trifluoroacetic anhydride was added, and the mixture was heated at 50°C for 30 min. Excess reagent was evaporated, and 0.5 mL of a solution containing both 20 mg of

3-(hydroxymethyl)pyridine and 4 mg of 4-dimethylaminopyridine in dichloromethane was added. The solution was then allowed to stand at room temperature for 3 h. The resulting picolinyl esters were extracted with hexane (16).

Alternatively, picolinyl esters were formed by transesterification based on the method of Roelofsen *et al.* (23), originally developed for the transesterification of methyl esters to butyl esters. Approximately 10 mg of freshly cut Na was dissolved in 10 mL of 3-(hydroxymethyl)pyridine by sonication, and 1 mL of this solution was added to the FAME sample (10 mg) that had been concentrated to near dryness. Twenty beads of precombusted molecular sieve (Type 3A) were added, and the mixture was covered with N_2 and heated at 80°C for 1 h. The sample was allowed to cool, and 2 mL of hexane was added to extract the picolinyl esters.

Chromatographic conditions. FAME were analyzed using a Varian 3400 gas chromatograph (GC) equipped with a temperature-programmable injector and a Varian 8100 autosampler. A flexible fused-silica column (30 m × 0.32 mm i.d.) coated with Omegawax 320 (Supelco, Mississauga, Canada) was used for general FAME separation. Hydrogen (flow rate 2 mL min^{-1}) was used as the carrier gas, and the gas line was equipped with an oxygen scrubber. The temperature program and flow rates used are described in Budge *et al.* (24). Theoretical response factors for FAME, as recommended by Craske and Bannon (25) and presented in Christie (16), were used in all analyses.

Picolinyl esters were analyzed on a Hewlett-Packard 5890/5971A GC–MS. A 70 eV ionization potential was used, and the mass range was 50–500 *m/z*. The esters were separated on a CP-Sil 5CB coated column (25 m × 0.25 mm i.d.; Chrompack, Middelburg, The Netherlands) using the following temperature program: 190°C for 0.5 min, followed by a ramp to 295°C at a rate of 3.0°C min^{-1} and hold at 295°C for 9.5 min. Helium was used as the carrier gas at a flow of 2 mL min^{-1}. The injector was isothermal at 250°C, and the mass analyzer interface was held at 280°C.

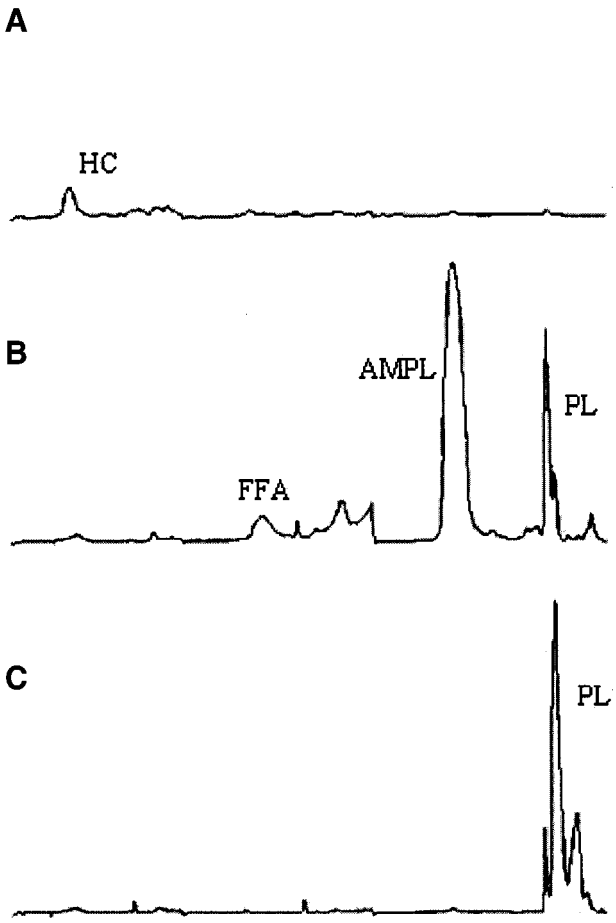


FIG. 1. TLC-FID chromatogram of fractionated lipid extracts of *Nannochloropsis* sp. (A) Neutral fraction; (B) acetone-mobile polar lipid (AMPL) fraction, (C) phospholipid (PL) fraction. Peak attentuation is the same for all chromatograms. HC, hydrocarbons.

RESULTS AND DISCUSSION

Fractionation of lipid extracts. The column chromatography separation of lipid extracts was evaluated by examining the resulting fractions by TLC-FID. Chromatograms of those fractions from typical algal and bivalve samples are shown in Figures 1 and 2. In both samples, both the NL and PL fractions contained predominantly the desired lipid classes with a small amount of pigment (AMPL) present in the NL fraction of *M. edulis*. However, the AMPL fraction in both samples appeared to contain a portion of the NL and PL fractions. For example, in *M. edulis*, sterols (ST) eluted from the column with acetone, suggesting that $CHCl_3$/MeOH/formic acid (98:1:0.5) was not polar enough to recover this mildly polar lipid within the NL fraction. DAG are slightly more polar than ST and can also be expected to elute from the column with acetone. DAG are rare in most marine samples and would normally make only a small contribution to total acyl lipids.

The other commonly encountered NL peak in the AMPL fraction was FFA. This was unexpected, as the 0.5% formic acid was included in the NL solvent specifically to recover FFA. The presence of FFA and acetone-insoluble PL in the AMPL fraction suggests that both peaks were the result of degradation of an AMPL species either on the silica gel column or during evaporation and concentration. Degradation of a glycolipid, for example, could produce a molecule of FFA and some more polar compound containing both a sugar moiety and an acylated FA. However, for the purposes of determining the FA composition of each fraction, this degradation will have little impact on the utility of the column separation if changes in FA structure with this breakdown are not apparent. In a separate study with sediments, where AMPL comprised more than 50% of total lipids, we found no significant differences in total FAME concentrations before and after fractionation. Of individual FA, only the concentrations of 16:1n-5, 17:0, and 20:1n-7, present in amounts less than 3% of total FA, were found to be significantly different ($P < 0.05$). Concentrations of the major FA remained unchanged. Thus, alteration of FA structure with breakdown of AMPL is not a concern.

Of the three fractions, recoveries of individual components in the NL and AMPL fractions were close to 100%. Recoveries of PL, however, were reduced on silica gel. PC, commonly

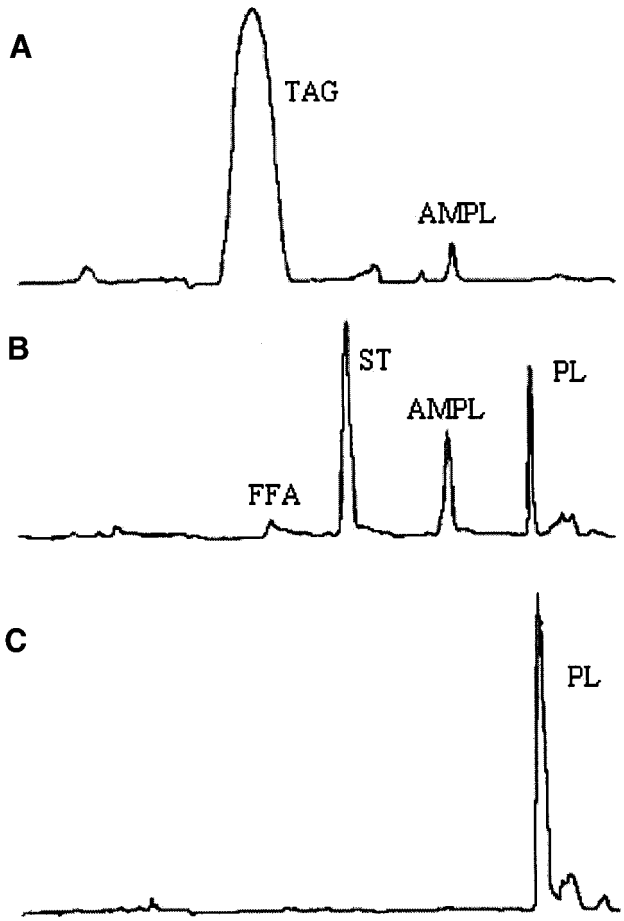


FIG. 2. TLC-FID chromatogram of fractionated lipid extracts of *Mytilus edulis*. (A) Neutral fraction; (B) AMPL fraction; (C) PL fraction. Peak attentuation is the same for all chromatograms. ST, sterols; for other abbreviations see Figure 1.

a major component of PL, was used as a model compound to determine the extent of this problem: Only 73 ± 4% of the PC was recovered. In an attempt to improve recovery, the silica gel was deactivated and equilibrated with 20% water by weight for 3 h. In one instance, this gave a recovery of 89%, but results were variable and, on average, only 66 ± 22% was recovered. It is generally assumed that recovery from silica gel is quantitative, and this on-column loss would certainly result in inaccurate PL and FA concentrations. However, these losses seem to be equivalent across the lipid class so that proportions of individual PL may still be accurately determined. In all situations, caution should be employed when using silica gel to separate PL from other lipid classes.

Methylation techniques. One-way ANOVA of the FAME concentrations (Table 1) revealed only a few significant differences in the results. Those differences were apparent in total branched, total monounsaturated, total polyunsaturated, and total FAME. With the AOCS method, significantly more ($P < 0.05$) branched-chain FAME were present than with any other method, suggesting that this method may be particularly useful in the esterification of FA with those structures. Of all five procedures, the NaOMe-catalyzed method produced the smallest concentration of total FAME. This was expected, as basic catalysts such as NaOMe are unable to methylate any FFA that are present, resulting in lower total FAME concentrations. While not statistically significant, the BF_3 method did produce the largest PUFA and total FAME concentrations of any acidic catalyst, despite highly cited reports that the use of BF_3 may lead to lowered PUFA yields (16,26).

Evaluated as percent total FAME (Table 2), the AOCS method produced significantly higher ($P < 0.001$) proportions of both branched-chain FAME, likely derived from bacteria, and monounsaturated FAME than all other methods. This method also generated significantly lower ($P = 0.008$) proportions of saturated FAME. These same trends were evident in the absolute concentration data, but the differences only became significant when expressed as proportions. These differences were due to differences in individual FAME proportions, specifically the presence of significantly more ($P < 0.001$) 16:1n-9 and significantly less ($P < 0.001$) 14:0 in the AOCS data. The higher yield of branched-chain FA obtained with the AOCS method and the suggestion that 16:1n-9 is derived from freshwater bacteria (27) implies that this method may be particularly effective at esterifying bacterial lipids. The only other technique producing 16:1n-9 methyl ester was the BF_3 method. A stronger catalyst such as BF_3 may be better able to esterify FA of those lipids. However, there were few significant differences in proportions among the other four methods, and if data are to be reported only as a percentage of the total, the most convenient method may be used.

Removal of hydrocarbon contamination. By using silica gel column chromatography, it was possible to remove hydrocarbon contamination from FAME samples and recover those FAME in proportions equivalent to the FAME levels prior to cleanup (Fig. 3). However, the proportion of diethyl ether in the solvent used to elute the FAME was critical. Table 3 contains ratios of peak areas before and after hydrocarbon removal in two mixed algal FAME samples for a variety of methyl esters relative to 16:0 as 1.0. Values near 1.0 represent close to 100% recovery, but values much less than 1.0 represent losses of FAME. Initial attempts to elute FAME with 99:1 hexane/diethyl ether did recover methyl esters, but proportions of PUFA were significantly reduced (Table 3). Clearly, recovery was a function of double bond number rather than chain length, and mixtures of 90:10 hexane/diethyl ether also suffered from reduced PUFA recovery. Mixtures of 80:20 hexane/diethyl ether, however, recovered all PUFA, yielding ratios of peak areas before and after cleanup that were very near unity (Table 3) with coefficients of variation less than 10% for all FAME. This equivalent recovery before and after cleanup is particularly important if an internal standard has been added before hydrocarbon removal.

Picolinyl ester preparation. The transesterification procedure reported here was applied to the methyl esters of *M. edulis* tissue, and examination of the total ion chromatogram (TIC) of the hexane-extractable reaction products revealed only picolinyl esters (Fig. 4A). There was no evidence of unreacted

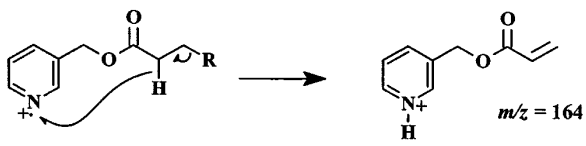
TABLE 1
Variation in FA Concentration (mg/mL culture) in Cultures of *Isochrysis galbana* with Differing Derivatization Procedures (mean ± SD, *n* = 4 or 5)[a]

	BF_3	HCl	H_2SO_4	AOCS	NaOMe
Branched					
i-15:0	0.06 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.02
ai-15:0	0.01 ± 0.03	0.04 ± 0.07	0.00 ± 0.00	0.11 ± 0.02	0.00 ± 0.00
ai-16:0	0.09 ± 0.05	0.09 ± 0.03	0.00 ± 0.00	0.20 ± 0.00	0.00 ± 0.00
Subtotal	0.16 ± 0.06[a]	0.13 ± 0.09[a]	0.00 ± 0.00[b]	0.32 ± 0.02[c]	0.01 ± 0.02[b]
Saturates					
14:0	3.10 ± 0.67	2.98 ± 0.49	2.95 ± 0.52	1.99 ± 0.30	2.07 ± 0.37
15:0	0.06 ± 0.02	0.03 ± 0.05	0.06 ± 0.01	0.04 ± 0.01	0.10 ± 0.03
16:0	1.49 ± 0.34	1.32 ± 0.29	1.38 ± 0.33	1.11 ± 0.21	1.03 ± 0.23
18:0	0.03 ± 0.03	0.05 ± 0.03	0.03 ± 0.02	0.03 ± 0.00	0.00 ± 0.00
Subtotal	4.68 ± 1.03	4.39 ± 0.80	4.42 ± 0.86	3.18 ± 0.52	3.21 ± 0.61
Monounsaturates					
16:1n-9	0.15 ± 0.09	0.00 ± 0.00	0.00 ± 0.00	0.55 ± 0.16	0.00 ± 0.00
16:1n-7	1.11 ± 0.24	1.03 ± 0.23	1.00 ± 0.19	0.84 ± 0.15	0.75 ± 0.12
18:1n-9	1.58 ± 0.25	1.54 ± 0.23	1.55 ± 0.26	1.48 ± 0.23	1.11 ± 0.23
18:1n-7	0.31 ± 0.03	0.32 ± 0.04	0.28 ± 0.03	0.16 ± 0.04	0.23 ± 0.11
Subtotal	3.16 ± 0.60[a]	2.89 ± 0.49	2.83 ± 0.47	3.03 ± 0.47	2.10 ± 0.30[b]
Polyunsaturates					
16:2n-4	0.23 ± 0.03	0.26 ± 0.03	0.21 ± 0.03	0.15 ± 0.04	0.15 ± 0.04
16:3n-4	0.06 ± 0.02	0.09 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.11 ± 0.07
16:4n-1	0.07 ± 0.01	0.04 ± 0.03	0.07 ± 0.01	0.05 ± 0.01	0.03 ± 0.03
18:2n-6	3.63 ± 0.59	3.39 ± 0.51	3.39 ± 0.55	2.97 ± 0.41	2.72 ± 0.36
18:3n-6	0.34 ± 0.06	0.32 ± 0.06	0.31 ± 0.06	0.27 ± 0.03	0.27 ± 0.04
18:3n-3	1.63 ± 0.19	1.51 ± 0.19	1.53 ± 0.20	1.36 ± 0.15	1.20 ± 0.11
18:4n-3	1.80 ± 0.25	1.62 ± 0.23	1.66 ± 0.25	1.43 ± 0.18	1.40 ± 0.17
20:2n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.04
20:4n-6	0.04 ± 0.02	0.01 ± 0.03	0.05 ± 0.01	0.05 ± 0.01	0.02 ± 0.03
20:5n-3	0.05 ± 0.02	0.02 ± 0.03	0.06 ± 0.01	0.05 ± 0.02	0.03 ± 0.03
22:5n-6	0.38 ± 0.04	0.38 ± 0.05	0.38 ± 0.07	0.36 ± 0.06	0.27 ± 0.06
22:6n-3	1.84 ± 0.13	1.78 ± 0.18	1.85 ± 0.22	1.80 ± 0.19	1.37 ± 0.24
Subtotal	10.09 ± 1.34[a]	9.42 ± 1.27	9.56 ± 1.41	8.53 ± 1.05	7.64 ± 0.96[b]
Total	18.12 ± 2.97[a]	16.83 ± 2.59	16.81 ± 2.73	15.05 ± 2.05	12.96 ± 1.81[b]

[a]*Note*: Means with different superscript roman letter designations (a,b,c) are significantly different ($P < 0.05$) from each other.

methyl esters, implying complete conversion of methyl esters to picolinyl esters. For comparison, a second TIC of reaction products generated by hydrolysis of methyl esters, followed by esterification (16), also is included (Fig. 4B). Although some picolinyl esters were formed, a variety of unreacted methyl esters were clearly present, indicating that incomplete hydrolysis was a problem. Undoubtedly, hydrolysis could be forced through the use of stronger base, higher reaction temperatures, and longer reaction times, but FA structures may be modified under such harsh conditions. However, the transesterification procedure developed here produced quantitative conversion of FAME to picolinyl esters without compromising lipid structure.

A fragmentation pattern of the picolinyl ester of an unusual FA is presented in Figure 5. This type of nonmethylene-interrupted dienoic (NMID) FA has been reported in bivalves (28), but confirmation of its structure is always difficult because authentic standards do not exist, making a mass spectral identification necessary. Harvey (19) has outlined mechanisms for the formation of various ions to be expected from a variety of picolinyl esters, but no information specifically applicable to FA containing double bonds separated by more than two methylene groups is provided. In Figure 5, a molecular ion at *m/z* 399 is obvious, allowing the FA to be assigned a length of 20 carbon atoms with two double bonds. A prominent ion, apparent at *m/z* 164, is the McLafferty rearrangement of the picolinyl ester, formed through the following mechanism:

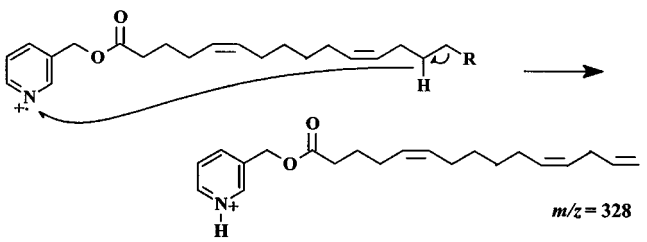


A series of diagnostic ions, such as those of *m/z* 328, 342, 356, and 370, is also generated by the following mechanism:

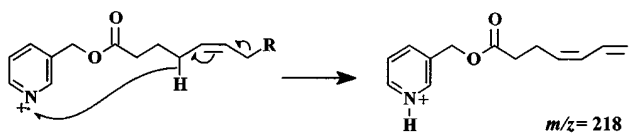
TABLE 2
Variation in Proportions of FA (% total FA) in Cultures of *Isochrysis galbana* with Differing Derivatization Procedures (mean ± SD, *n* = 4 or 5)[a]

	BF_3	HCl	H_2SO_4	AOCS	NaOMe
Branched					
i-15:0	0.33 ± 0.19	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.13
ai-15:0	0.07 ± 0.16	0.23 ± 0.37	0.00 ± 0.00	0.76 ± 0.10	0.00 ± 0.00
ai-16:0	0.48 ± 0.28	0.55 ± 0.13	0.00 ± 0.00	1.37 ± 0.18	0.00 ± 0.00
Subtotal	0.87 ± 0.31[a]	0.78 ± 0.47[a]	0.00 ± 0.00[b]	2.13 ± 0.19[c]	0.11 ± 0.13[b]
Saturated					
14:0	17.02 ± 1.17[a]	17.69 ± 0.37[a]	17.54 ± 0.70[a]	13.20 ± 0.38[b]	15.89 ± 0.82[a]
15:0	0.32 ± 0.05	0.17 ± 0.25	0.34 ± 0.03	0.29 ± 0.01	0.80 ± 0.21
16:0	8.17 ± 0.73	7.81 ± 0.62	8.13 ± 0.69	7.32 ± 0.52	7.91 ± 0.73
18:0	0.15 ± 0.15	0.30 ± 0.16	0.17 ± 0.10	0.22 ± 0.03	0.00 ± 0.00
Subtotal	25.70 ± 1.92[a]	25.97 ± 0.86[a]	26.18 ± 1.13[a]	21.04 ± 0.84[b]	24.60 ± 1.60[a]
Monounsaturated					
16:1n-9	0.82 ± 0.49[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	3.66 ± 0.79[b]	0.00 ± 0.00[a]
16:1n-7	6.12 ± 0.39	6.09 ± 0.54	5.91 ± 0.23	5.54 ± 0.32	5.79 ± 0.27
18:1n-9	8.76 ± 0.21	9.13 ± 0.10	9.20 ± 0.14	9.81 ± 0.37	8.57 ± 1.24
18:1n-7	1.72 ± 0.13	1.91 ± 0.10	1.69 ± 0.10	1.06 ± 0.30	1.83 ± 0.89
Subtotal	17.42 ± 0.90[a]	17.12 ± 0.49[a]	16.80 ± 0.12[a]	20.07 ± 0.63[b]	16.19 ± 0.66[a]
Polyunsaturated					
16:2n-4	1.29 ± 0.05	1.55 ± 0.14	1.23 ± 0.06	0.99 ± 0.30	1.18 ± 0.24
16:3n-4	0.34 ± 0.03	0.56 ± 0.11	0.35 ± 0.02	0.31 ± 0.03	0.85 ± 0.52
16:4n-1	0.40 ± 0.03	0.22 ± 0.20	0.44 ± 0.01	0.36 ± 0.02	0.21 ± 0.20
18:2n-6	20.09 ± 0.28	20.14 ± 0.24	20.15 ± 0.23	19.72 ± 0.14	21.00 ± 0.29
18:3n-6	1.88 ± 0.04	1.88 ± 0.07	1.85 ± 0.06	1.82 ± 0.04	2.11 ± 0.09
18:3n-3	9.06 ± 0.42	9.01 ± 0.32	9.15 ± 0.34	9.05 ± 0.24	9.32 ± 0.44
18:4n-3	9.99 ± 0.42	9.64 ± 0.24	9.87 ± 0.14	9.50 ± 0.13	10.84 ± 0.38
20:2n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.49 ± 0.33
20:4n-6	0.22 ± 0.12	0.07 ± 0.16	0.33 ± 0.02	0.30 ± 0.02	0.17 ± 0.24
20:5n-3	0.29 ± 0.07	0.12 ± 0.17	0.33 ± 0.03	0.32 ± 0.09	0.20 ± 0.20
22:5n-6	2.14 ± 0.18	2.28 ± 0.12	2.24 ± 0.08	2.41 ± 0.11	2.09 ± 0.36
22:6n-3	10.32 ± 1.06	10.67 ± 0.73	11.09 ± 0.70	11.98 ± 0.46	10.64 ± 1.74
Subtotal	56.04 ± 1.82[a]	56.13 ± 1.52[a]	57.02 ± 1.20[a]	56.76 ± 0.79[b]	59.10 ± 2.18[a]
Total	100	100	100	100	100

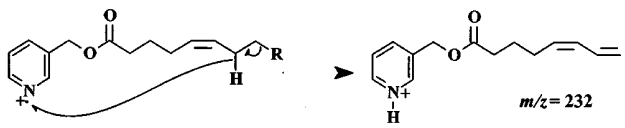
[a]*Note*: Means with different superscript roman letter designations (a,b,c) are significantly different ($P < 0.05$) from each other.



The 40-unit gap between *m/z* 178 and 218 and between 246 and 286 is due to suppression of the reaction in the first reaction and suggests that the double bonds are located in that area. The bonds are fixed at the Δ5 and Δ11 positions, identifying the FA as 20:2Δ5,11, by the presence of ions of *m/z* 218 and 300 generated by the following mechanism:



Ions at *m/z* 232 and 314 are also indicative of bond position owing to the following reaction:



Using these diagnostic ions and mechanisms, the identity of other NMID FA including 20:2Δ5,13, 22:2Δ7,13, and 22:2Δ7,15 was confirmed in samples of *M. edulis*.

Approximation of FA concentrations using acyl lipid data. Internal standards are not commonly used in FA analysis of marine samples because their FA composition is complex, and an initial GC analysis is necessary to ensure that the internal standard does not co-elute with any FAME. Because of this, it has become convention to report FA data as weight percentages of total FA, especially in biological samples, giving no information about actual concentrations. However, lipid class data are invariably expressed as concentrations, making it possible to

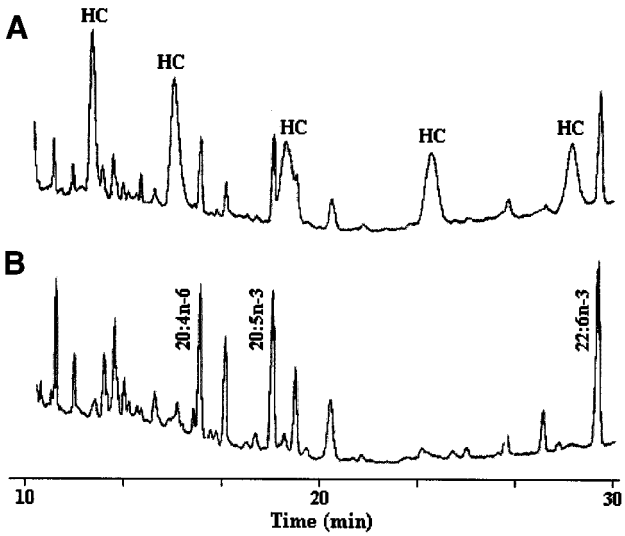


FIG. 3. GC-FID chromatograms of FAME before (A) and after (B) removal of HC contamination in a mixed algal sample. For abbreviation see Figure 2.

estimate FAME concentrations from acyl lipid data provided by TLC-FID. This concept of combining FAME and lipid data may be particularly relevant in light of the U.S. Food and Drug Administration's food labeling regulations insisting that a theoretical TAG, assembled from FA in the lipid extract, be defined as the "fat content" on food labels (29).

First, weight percent data of total FA are converted to mole percent data. These mole percent data allow calculation of an average number of double bonds and carbon atoms in a particular sample. This, in turn, allows calculation of an average FA M.W., which may then be applied to the acyl lipid data to determine a molar mass for each acyl lipid class. With that molar mass, the FA contribution to mass for each acyl lipid class may be determined. These calculations are performed within a spreadsheet, and Table 4 illustrates the results at each step. This calculation produces a very accurate approximation for lipid classes when the nonacyl portion of the molecule is known with certainty, such as with TAG, FFA, and PL. Inaccuracy increases with lipid classes such as steryl/wax esters (SE/WE) and AMPL. This is a particular problem with the AMPL group, which contains nonacylated pigments in addition to glycolipids and MAG. AMPL separations on Chromarods were originally conceived as a means of purifying the PL peak (30), but the AMPL peak itself has been important in some studies. For example, it was a dominant class in a cold ocean tunicate (31),

TABLE 3
Recoveries of FAME After Hydrocarbon Removal in a Mixed Algal Sample Using Different Proportions of Hexane/Diethyl Ether[a]

	Hexane/diethyl ether	
	99:1	80:20
Saturated		
14:0	1.05	0.99
16:0	1.00	1.00
22:0	1.02	—
Mean	1.02 ± 0.03	1.10 ± 0.10
Monounsaturated		
16:1n-9	0.95	1.06
16:1n-7	0.96	1.00
17:1	0.70	—
18:1n-9	0.97	—
18:1n-7	0.96	1.03
Mean	0.91 ± 0.12	1.04 ± 0.02
Polyunsaturated		
16:2n-6	0.62	0.98
16:3n-4	—	0.95
18:2n-6	0.85	1.08
18:3n-3	0.55	1.02
18:4n-3	0.23	1.02
20:5n-3	—	1.03
22:4n-6	0.37	—
22:6n-3	0.15	1.00
Mean	0.46 ± 0.26	0.99 ± 0.06
Mean total	0.76 ± 0.31	1.04 ± 0.08

[a]Expressed as normalized ratios of peak areas before and after cleanup. *Note*: Peak areas were normalized to the peak area of 16:0 by the following equation:

$$\frac{(\text{FA area})_{\text{after}}/(\text{FA area})_{\text{before}}}{(16:0\ \text{area})_{\text{after}}/(16:0\ \text{area})_{\text{before}}}$$

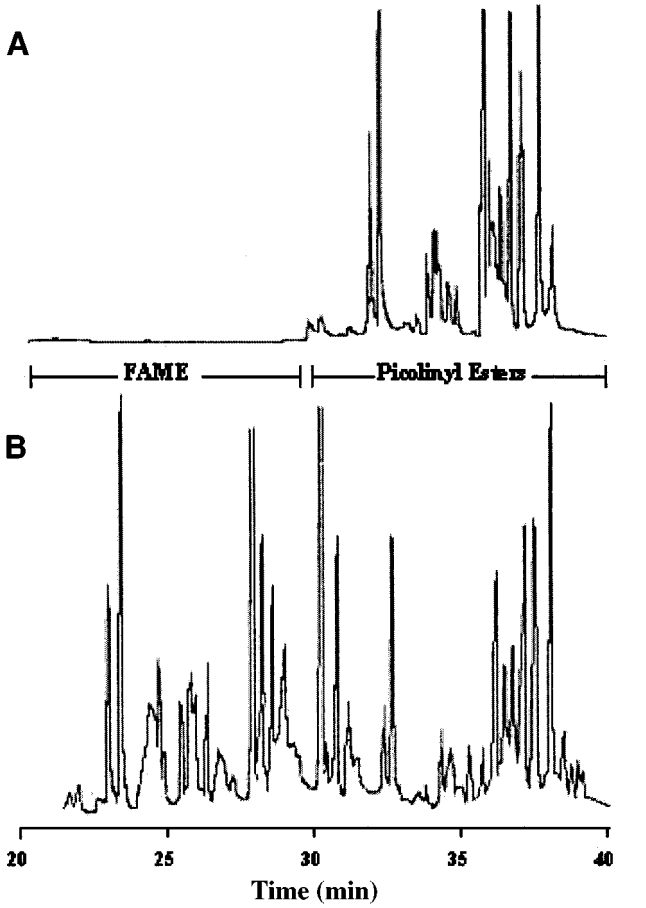


FIG. 4. Total ion chromatograms of the hexane-extractable material recovered after formation of picolinyl esters. (A) Picolinyl esters formed by transesterification of FAME; (B) picolinyl esters formed by hydrolysis of FAME, followed by reaction with 3-(hydroxymethyl)pyridine.

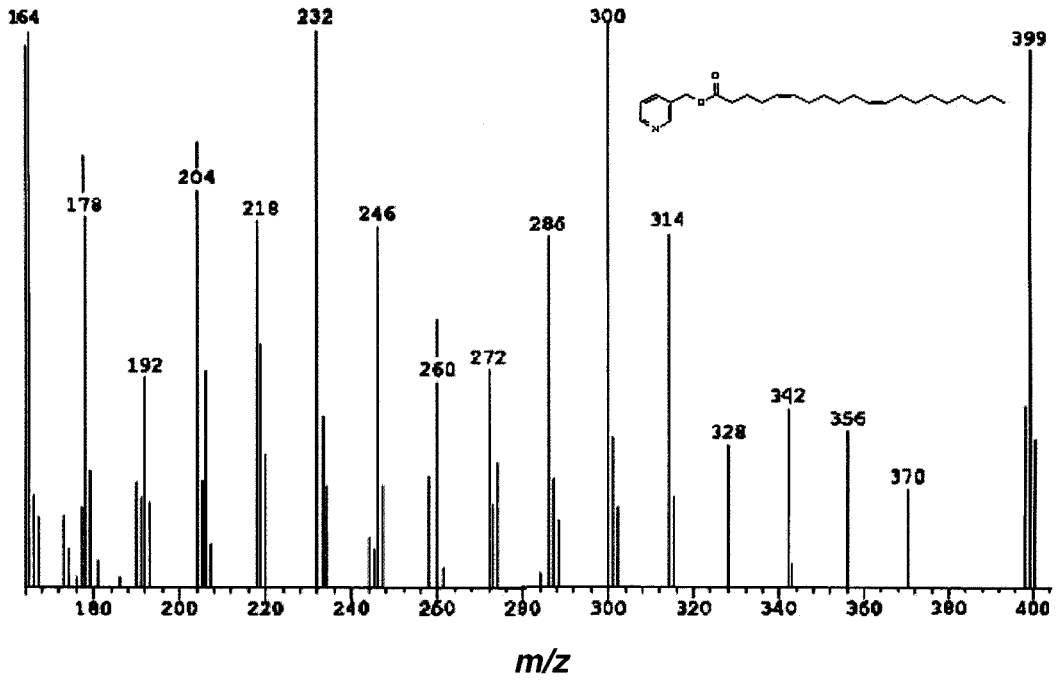


FIG. 5. Partial mass spectrum of the picolinyl ester of 20:2Δ5,11.

its contents were high in stressed scallops (32), and its concentrations in lake filtrates peaked during the decomposition of algal blooms (33). Likely structures for the backbones of AMPL can be proposed, but estimates of FA concentrations can be expected to be less reliable as AMPL portions increase.

In Table 5, the nonacyl lipid structures used to calculate molar mass are shown. In algae, approximately one-third of the AMPL peak was assumed to consist of digalactosyl diacylglycerol (34,35). A similar assumption was made with the calculation for animal tissue except that a cerebroside structure was substituted. SE/WE were determined as one peak and usually constituted a very small portion of total lipid (<5%). For ease of calculation, a 1:1 ratio of SE/WE was assumed with a 24-methylcholesta-5,22-dien-3β-yl ester in the SE portion (36). A C_{16} hydrocarbon chain in WE (36), primarily derived from zooplankton, was used to calculate the contribution of that class.

To evaluate the accuracy of these estimates, two marine samples (*Nannochloropsis* sp. and *M. edulis*) were analyzed for both lipid class concentrations and FAME proportions, and FAME concentrations were estimated from those data. FAME concentrations were also determined in the same samples using an internal standard (23:0 FAME). The results of these comparisons, as well as concentrations of TAG, FFA, and PL, are shown in Table 6. For both sample types, estimated and actual values were quite similar and, in fact, there was not a significant difference in the two values for the algal sample. The values for the animal tissue, however, were significantly different ($P = 0.002$), with the calculation slightly overestimating the actual amount. This suggests that some assumption within the calculation is incorrect. Better knowledge of the actual composition of the AMPL and PL fractions would undoubtedly improve the accuracy of this estimation. This calculation also makes the false assumption that FA structures are uniformly distributed throughout all lipid classes. Although this assumption simplifies the calculation, it must also be contributing to the error in the calculated method.

Before FA data can be applied to any problem, there must be some confidence that the results are accurate and reproducible. For that purpose, the methods and techniques typically used to generate FA data were critically evaluated in this work. A column chromatographic separation of the lipid extract can be performed to produce neutral, AMPL, and PL fractions. However, whereas deactivation of the silica gel with 20% w/w water improves recovery, only approximately 75% of PL can be recovered from these silica gel columns. Relative proportions of individual PL remain constant, but caution should be exercised when quantitative data are required. The extract can then be transesterified with the most convenient acid-catalyzed procedure to form FAME. In this laboratory, 10–14% BF_3 in MeOH is routinely used as a catalyst without any evidence of PUFA loss. A simple method to remove hydrocarbon contamination from FAME samples with column chromatography on silica gel also can be applied when necessary without selective loss of FAME on the column. A new transesterification method for the formation of picolinyl esters for use in the mass spectral identification of FA was also developed. This new method is quantitative and offers the advantage of avoiding hydrolysis of the lipid extract and formation of artifacts associated with that procedure.

TABLE 4
Results of Calculations to Produce FAME Concentrations from FAME Proportions and Lipid Class Concentrations in *Nannochloropsis* sp.

FAME	Molar mass	Weight %	Moles (×1000)	Mole %
14:0	242.400	5.61	23.14	6.94
14:1n-5	240.384	0.31	1.29	0.39
i-15:0	256.427	1.11	4.33	1.30
15:0	256.427	0.30	1.17	0.35
ai-16:0	270.454	1.40	5.18	1.55
16:0	270.454	16.20	59.90	17.95
16:1n-9	268.438	3.43	12.78	3.83
16:1n-7	268.438	19.35	72.08	21.61
i-17:0	284.481	0.19	0.67	0.20
ai-17:0	284.481	0.38	1.34	0.40
16:2n-4	266.422	0.29	1.09	0.33
17:0	284.481	0.22	0.77	0.23
16:3n-4	264.406	0.76	2.87	0.86
16:4n-1	262.391	0.24	0.91	0.27
18:0	298.508	0.33	1.11	0.33
18:1n-9	296.492	3.30	11.13	3.34
18:1n-7	296.492	0.50	1.69	0.51
18:2n-6	294.476	4.28	14.53	4.36
18:3n-6	292.461	0.43	1.47	0.44
18:3n-3	292.461	0.19	0.65	0.19
20:4n-6	318.498	4.14	13.00	3.90
20:5n-3	316.483	32.45	102.53	30.73
23:0				
Sum moles			333.63	100
Average chain length		17.41		
Average double bonds		2.14		
Average M.W.		285.97		

Lipid class	Amount (µg in extract)	FAME from	Amount (µg in extract)
Hydrocarbons	64		
Steryl/wax esters	0	Steryl/wax esters	0
Methyl esters	1	Methyl esters	1
Ketones	7		0
TAG	134	TAG	134
FFA	15	FFA	16
Alcohols	2		
Pink pigment	0		
Sterols	39		
DAG	6	DAG	6
AMPL[a]	370	AMPL	76
Polar lipids	335	Phospholipids	250
Total	973	Total lipids	483

[a]AMPL, acetone-mobile polar lipid.

FA data are rarely reported as absolute amounts, perhaps because there is hesitancy in many laboratories to add an internal standard that may coelute with a naturally occurring FA. Lipid class data, generated by TLC-FID, however, are commonly reported as concentrations. A series of calculations, designed to incorporate FA proportions with acyl lipid class concentrations to arrive at FA concentrations, was developed. These estimates, which will increase in accuracy as knowledge of the nonacyl structures of acyl lipids increases, were in good agreement with actual FA concentrations, determined using internal standards, in algae and animal samples.

TABLE 5
Nonacyl Structures of Acyl Lipids Used to Estimate FAME Concentrations from Acyl Lipid Data

Lipid class	Nonacyl structure
Steryl esters	24-Methylcholesta-5,22-dien-3β-yl ester
Wax esters	C_{16} alcohol
Methyl esters	—
TAG	Glycerol
FFA	Add CH_3
DAG	Glycerol
AMPL	DGDG[a] in algae, cerebroside in animal tissues
Phospholipids	Glycerol and choline

[a]DGDG, digalactosyl diacylglycerol; for other abbreviation see Table 4.

TABLE 6
Estimated and Actual FAME Concentrations[a] in Marine Samples (mean ± SD, *n* = 3 to 6)

		Nannochloropsis sp. (µg/40 mL culture)	*Mytilus edulis* (µg/mL extract)
Lipids	TAG	63 ± 48	1420 ± 160
	AMPL	318 ± 49	183 ± 24
	PL	307 ± 66	435 ± 86
FAME	Estimated	371 ± 95	2220 ± 210
	Actual	333 ± 59	1870 ± 160
% Discrepancy		11 ± 4	18 ± 1

[a]For abbreviations see Table 4.

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Determination of *c*9,*t*11-CLA in Major Human Plasma Lipid Classes Using a Combination of Methylating Methodologies

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ABSTRACT: Isomeric CLA exhibit several significant biological activities in animals and humans and are easily isomerized to their corresponding *t,t*-CLA isomers during methylation with various acid-catalyzed reagents. To minimize such isomerization and provide a valid quantification of human plasma CLA content, several methylation methods were tested. Plasma neutral lipid, nonesterified FA (NEFA), and polar lipid classes were separated into the following fractions: (i) cholesteryl ester (CE, 1.2 mg/12 mL, 37.5% lipids), (ii) TAG (0.8 mg/12 mL, 25% lipids), (iii) NEFA (0.2 mg/12 mL, 6.2% lipids), (iv) MAG/DAG/cholesterol (0.3 mg/12 mL, 9.4% lipids), and (v) phospholipid (PL, 0.5 mg/20 mL, 15.6% lipids). Data showed that *c*9,*t*11-CLA found in TAG, MAG/DAG/cholesterol, and PL fractions were converted to methyl esters with sodium methoxide within 2 h at 55°C. However, the *c*9,*t*11-CLA in the CE fraction could not be completely converted to methyl esters by sodium methoxide/acetylchloride in methanol or methanolic KOH; instead, CE was treated with sodium methoxide and methyl acetate in diethyl ether for 1 h. NEFA were converted to methyl esters with trimethylsilyldiazomethane (TMSDAM). All reaction mixtures were monitored by TLC prior to GLC analysis. The highest enrichment of *c*9,*t*11-18:2 (% FA) was in TAG (0.31%), followed by CE (0.14%) and PL (0.13%). The above methylation methods were then applied to a small subset ($n = 10$) of nonfasting plasma lipid fractions to confirm the applicability of these data. Results from this subset of samples also indicated that the greatest enrichment of *c*9,*t*11-CLA was present in the TAG fraction (0.39%), followed by CE (0.27%) and PL (0.22%). These data indicate that different plasma fractions have different *c*9,*t*11-CLA contents.

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CLA (*cis*-9,*trans*-11-CLA, 18:2), recently given the trivial name rumenic acid (RA; 1), is the main conjugated dienoic FA in human serum lipids (2). In general, the term CLA refers to a mixture of positional and geometric isomers of octadecadienoic acid (18:2) having a conjugated double bond. Animal studies have shown that CLA may inhibit carcinogenesis (3,4), lower body fat (5,6), increase lean body mass (7,8) decrease risk of atherogenesis (9,10), and exhibit antidiabetic characteristics (11). Conjugated linoleic isomers are present in human food and occur abundantly in dairy products (12) as well as in meat from ruminants (13), hydrogenated fats (14), and used frying oil (15). The distribution of CLA isomers has been examined using Ag^+-HPLC (16,17) in a number of natural products including milk, cheese, meat, adipose tissue, and tissues from animals fed commercial CLA mixtures. Biological activity has been attributed to *cis*-9,*trans*-11 dienoic acid (18) and *trans*-10,*cis*-12 dienoic acid (19), the two major CLA isomers.

Iversen *et al.* (20) and Harrison *et al.* (21) were the first to report that conjugated isomers of linoleic acid (LA) are associated with human plasma phospholipids (PL), and that a relatively uniform distribution of serum CLA exists among PL (36%), TAG (36%), and cholesteryl esters (CE) (28%). However, Fogerty *et al.* (22) found that only 16–34% of CLA was present in PL, whereas 58–78% of CLA was found in TAG and 2–8% in CE. Subsequent studies suggest that dietary CLA is incorporated into circulating TAG and PL (23,24), but investigations of the distribution of CLA into the other major lipid fractions are limited. Although the majority of human studies indicate that CLA accumulates predominantly in the TAG fraction, the discrepancy among study results remains unresolved.

CLA isomers are usually quantified by GLC analysis as their methyl esters, prepared by a variety of acid-/base-catalyzed reactions (25). Acid-catalyzed methylations using HCl/methanol and BF_3/methanol have been reported to change the isomer distribution of CLA and generate allylic methoxide from CLA (26), whereas base-catalyzed methods (i.e., those using sodium methoxide in methanol) are considered to be milder methods for methylating the TAG and PL fractions; however, they fail to methylate nonesterified FA (NEFA).

A growing literature shows that different isomers of CLA may have different physiologic effects. Therefore, it is very important to avoid modifying the original isomer when analyzing CLA in biologic fluids and tissues. The first objective (Experiment 1) of this set of studies was to develop methods for complete methylation of the *c*9,*t*11-CLA found in the major fractions of human plasma, followed by preliminary quantification of *c*9,*t*11-CLA in these fractions. Our second objective (Experiment 11) was to apply these methods to a small subset of human plasma samples to confirm the data collected in the initial methodological phase of the study. We hypothesized that different methodologies would be required

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Abbreviations: CE, cholesterol ester; MTBE, methyl *tert*-butyl ether; NEFA, nonesterified fatty acid; RA, rumenic acid; TMSDAM, trimethylsilyldiazomethane.

for different plasma fractions and that distribution of *c*9,*t*11-CLA among fractions would not be uniform.

MATERIALS AND METHODS

Materials. Phosphatidylcholine (PC), cholesteryl linoleate, and a mixture of CLA isomers (FFA) containing 80% *cis*-9,*trans*-11, 17% *cis*-9,*cis*-11, and 1% *trans*-9,*trans*-11 18:2 were purchased from Matreya (Pleasant Gap, PA). A FAME standard (C_8 to C_{24}), oxalic acid ($C_2H_2O_4$), sodium methoxide in methanol ($NaOCH_3$, 0.5 N), and trimethylsilyldiazomethane (TMSDAM) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Freshly opened reagents were redistributed to small amber glass bottles and sealed to prevent progressive deterioration during storage at 4°C. A vacuum manifold, silica Maxi-Clean™ cartridges (600 mg), and precoated silica gel plates (*ca.* 0.1 mm thick, 20 × 20 cm) were purchased from Alltech Associates, Inc. (Deerfield, IL). All chemicals and reagents used were of analytical grade.

Subjects and samples. For the initial methodological phase of the study, healthy women (25 to 38 yr; $n = 4$) donated blood that was collected in heparinized tubes in the morning (nonfasting sample). Blood was centrifuged at 1500 × *g* for 10 min at 4°C. Plasma was removed, pooled, and stored at −40°C. These samples were used to develop the various methylation methods. For the second phase of the study, nonfasting blood samples were collected from healthy women (19 to 38 yr; $n = 10$) and extracted as described above.

Lipid extraction. Plasma lipid was extracted using the modified method of Ingalls *et al*. (27). Briefly, plasma was thawed and vortexed for 15 s, then 700 µL was transferred into a microcentrifuge tube and centrifuged at 1500 × *g* for 2 min at 4°C to remove proteins; 200 µL duplicates were transferred into screw-capped glass tubes (15 mL) containing 300 µL of 0.005 N HCl and 3 mL chloroform/methanol (2:1, vol/vol). This mixture was vortexed until a complete emulsion had formed. Tubes were then centrifuged at 1500 × *g* for 15 min at 4°C. The chloroform/methanol layer was transferred to a Pasteur pipette column containing anhydrous Na_2SO_4 layered over a glass wool plug. The column was rinsed with an additional 1 mL of chloroform/methanol (2:1, vol/vol), and the samples were brought to dryness under a stream of nitrogen in a heated (45°C) water bath.

Column chromatography. Separation of lipid fractions was accomplished using silica Maxi-Clean™ cartridges (600 mg) and the method of Hamilton and Comai (28) modified to allow processing of several samples at a time using a vacuum pump. Briefly, the column was loaded with 1.5 mg of plasma lipid; CE and TAG fractions were eluted with a combination of hexane/methyl *tert*-butyl ether (MTBE) (200:3, 12 mL for CE followed by 12 mL for TAG). After removal of TAG, the column was acidified with 12 mL hexane/acetic acid (100:0.2, vol/vol). This fraction contained no lipid and was discarded after confirmation by TLC. NEFA were then eluted using 12 mL hexane/MTBE/acetic acid (100:2:0.2, by vol); MAG/DAG/cholesterol were eluted as a single fraction with 12 mL of hexane/acetic acid (100:0.2, vol/vol). Finally, the PL fraction was eluted with 20 mL MTBE/methanol/ammonium acetate (pH 8.6; 10:4:1, by vol). Each fraction was dried under nitrogen at 45°C for further analysis.

Thin-layer chromatography (TLC). TLC was performed on glass microscope plates coated with silica gel G (*ca.* 0.1 mm thick), and a mixture of *n*-hexane/diethyl ether/acetic acid (90:10:1, by vol) was used as the solvent. Lipid spots were visualized under UV light by spraying with a solution of 2′,7′-dichlorofluorescein in 95% ethanol to check the purity of each fraction; 50% H_2SO_4 in 95% ethanol was used to visualize spots after charring at 100°C.

Preparation of FAME by various methods (Experiment 1). For all the methylation procedures, 1 mL concentrated lipid solution (2 mg/mL hexane) was pipetted into a glass tube, and the following methylation procedures were performed.

(i) Method A: *$NaOCH_3$/methanol (26)*. A plasma lipid extract (2 mg) was transferred into a screw-capped glass tube (15 mL); 2 mL of 0.5 N sodium methoxide solution was added to the tube. The reaction mixture was heated for 2 h at 55°C. After the tube had been cooled to room temperature, 5 mL of 0.2 N HCl solution was added and extracted with MTBE/hexane (1:3, vol/vol; 3 × 2 mL). The reaction mixture was washed with water (2 × 2 mL) and passed through a Pasteur pipette column containing a glass wool plug, a 4-cm filter bed of silica gel, and 1 cm anhydrous $MgSO_4$. Sample was then concentrated under nitrogen to approximately 500 µL and transferred into a GLC vial.

(ii) Method B: *Acetyl chloride (CH_3COCl)/methanol-chloroform*. The reaction was carried out as previously described (29), except that benzene was substituted with chloroform. A plasma lipid extract (2 mg) was transferred into a screw-capped glass tube (15 mL) and dried under nitrogen. Anhydrous methanol (2 mL) and then 250 µL of CH_3COCl were added to the tube while the tube was gently shaken. The reaction mixture was heated at 100°C for 1 h. After the tube had been cooled in water at room temperature for 2 min, 5 mL 6% K_2CO_3 solution was slowly added to stop the reaction and neutralize the mixture before extraction. The reaction mixture was extracted with MTBE/hexane (1:3, vol/vol; 3 × 2 mL), and the solvent was passed through a Pasteur pipette column containing a glass wool plug, a 4-cm filter bed of silica gel, and 1 cm anhydrous $MgSO_4$. Sample was concentrated under nitrogen to approximately 500 µL and transferred into a GLC vial.

(iii) Method C: $NaOCH_3$/methanol followed by TMSDAM (30). Anhydrous methanol (0.5 mL) was added to plasma lipid extracts (2 mg), followed by the addition of 40 µL TMSDAM, and allowed to react overnight (12 h) at room temperature. Excess TMSDAM was decomposed with 2% acetic acid in hexane until colorless, followed by the addition of 1.5 mL 5% $NaHCO_3$ (wt/vol) and extraction with MTBE/hexane (1:3, vol/vol; 3 × 2 mL). The organic layer was removed and passed through a Pasteur pipette column containing a glass wool plug and a 4-cm silica gel overlaid with 1 cm anhydrous $MgSO_4$. Sample was concentrated under nitrogen to approximately 500 µL and transferred into a GLC vial.

(iv) Method D: Hydrolysis of plasma lipid/TMSDAM (31). Ethanolic KOH (1 mL) solution (0.5 M in 95% ethanol) was added to a plasma lipid extract (2 mg), and the reaction mixture was heated for 2 h at 55°C. After cooling to room temperature, water (5 mL) was added, the solution acidified with 6.0 N HCl and extracted with MTBE/hexane (1:3, vol/vol; 3 × 2 mL). Solvent was passed through a Pasteur pipette column containing a glass wool plug, a 4-cm filter bed of silica gel, and 1 cm anhydrous $MgSO_4$ and evaporated under nitrogen. The lipid sample was then treated with TMSDAM as described in Method C.

(v) Method E: *$NaOCH_3$/methyl acetate(CH_3COCH_3)/diethyl ether (32).* A plasma lipid extract (2 mg) was dried under nitrogen, and sodium-dried diethyl ether (1 mL) was added, followed by 20 µL methyl acetate and 40 µL of 0.5 N $NaOCH_3$. The reaction mixture was vortexed to ensure thorough mixing. After 1 h at room temperature, the reaction was stopped by adding 30 µL saturated oxalic acid in diethyl ether. The mixture was centrifuged at 1500 × *g* for 2 min and dried under a gentle stream of nitrogen. Hexane (1 mL) was added, and the mixture was passed through a Pasteur pipette column containing a glass wool plug and a 4-cm silica gel overlaid with 1 cm anhydrous $MgSO_4$. Sample was concentrated under nitrogen to approximately 500 µL and transferred into a GLC vial.

(vi) Method F: Analysis of CE in whole plasma (33). This method was used only for the methylation of the CE fraction of the whole plasma. Briefly, CE (1 mg) was dissolved in 2 mL petroleum ether; 1 mL of methanolic base ($KOCH_3$, 2 N) was added. The reactants were mixed by inversion for 1 min. An aliquot of the upper phase was used for TLC and GLC analyses.

Analysis of FAME. Samples were analyzed using GLC (model 6890; Agilent Technologies, Wilmington, DE) equipped with a capillary column (Quadrex 007-23-60-0.25F; 60 m, 0.25 mm i.d., with 0.25 µm film thickness; Cyanopropyl; Quadrex, New Haven, CT). Helium was used as carrier gas at a constant flow mode with linear velocity set at 22 cm/s. The FID was heated to 260°C, and detector gas flows were set at 40, 450, and 49 mL/min for hydrogen, compressed air, and nitrogen, respectively. Samples (1 µL) were injected in the splitless mode (injection temperature 260°C, purge at 0.75 min) with an initial oven temperature of 50°C with a 4-min hold time, increased at 10°C/min to 150°C and then 1°C/min to the final temperature of 191°C. Identities of selected CLA peaks and other FA peaks were established by comparing retention times to a 14-component C_8–C_{24} FAME standard mixture, a 9,11-CLA mixture containing 80% *cis*-9,*trans*-11; 17% *cis*-9,*cis*-11; and 1% *trans*-9,*trans*-11 18:2, respectively, and an anhydrous milk fat reference standard obtained from the Commission of the European Communities (CRM 164; European Community Bureau of Reference, Brussels, Belgium).

Preparation of FAME of major plasma lipid fractions of a small subset of samples (Experiment 11). To confirm the applicability of these data to a larger sample size, plasma lipids (nonfasting) from a subset of female subjects (19 to 38 yr; $n = 10$) were collected, fractionated, and then methylated according to the methods described above for the pool plasma samples. Because NEFA and MAG-DAG fractions were found not to contain much *c*9,*t*11-CLA, they were combined for analytical purposes.

RESULTS

Experiment 1. (i) Effect of the methylation procedure on FAME in whole plasma. Data in Table 1 can be used to compare the estimates of *c*9,*t*11-CLA concentration (% FAME) in human plasma lipid samples when analyzed by different methylating procedures. Compared to Method E ($NaOCH_3$/methyl acetate), use of Methods A, B, C, and D ($NaOCH_3$/methanol, CH_3COCl/methanol, $NaOCH_3$/TMSDAM, and KOH/TMSDAM) resulted in lower *c*9,*t*11-CLA content. The apparent loss of this CLA isomer was most pronounced in Methods A and C. Four other FA—16:0 (palmitic), 18:0 (stearic), 18:1 (oleic), and 18:2 (linoleic), which constitute 60 to 70% of the total plasma lipid—were also greatly influenced by these various esterification methods. For example, esterification of 16:0 and 18:2, the two plasma FA in greatest concentration, were considerably reduced in Method C as compared to Method D (Table 1). TLC analysis with hexane/diethyl ether/acetic acid (90:10:1, by vol) as the solvent system suggested incomplete FA methylation of the lipid samples obtained from Methods A, B, and C. To determine whether the CE and PL fractions were being completely methylated, known amounts (2 mg) of cholesteryl linoleate and PC were methylated as described in Methods A, B, C, and E and analyzed by TLC. Results indicated that PC was fully methylated with all methods, whereas cholesteryl linoleate was completely methylated only with Method E (Fig. 1). (Method D is not a transesterification method and therefore was excluded from this experiment.) However, methylation of the whole plasma with this method resulted in a chromatogram with many additional unidentified peaks after GLC analysis (Fig. 2A).

(ii) Effect of the methylation procedure on FAME in major lipid fractions. Results of column chromatographic separation of lipid fractions are as follows: CE, 1.2 mg, 37%; TAG, 0.8 mg, 25%; NEFA, 0.2 mg, 6.2%; MAG/DAG/cholesterol, 0.3 mg, 9.4%; and PL, 0.5 mg, 15.6%. Only 6.2% of the total lipid was not recovered following separation of fractions. The CE fraction was predominant, with TAG being the second major fraction. When the CE fraction (representing the major fraction of plasma) was treated with $NaOCH_3$ (0.5 N) at 55°C for 2 h, 4 h, or kept overnight at room temperature (Method A), the reaction was found to be incomplete by TLC; thus, Method A was unsuitable for methylation of the CE fraction. When the CE fraction was hydrolyzed with 0.5 M ethanolic KOH at 55°C for 2 h and then acidified with dilute 0.2 M HCl followed by overnight methylation with TMSDAM as described in Method D, many additional peaks (Fig. 2B) were detected by GLC analysis. No attempts were made to identify the unknown peaks. We then tested a methylation procedure

TABLE 1
FA (% identified FAME) Composition of Whole Human Plasma Using Various Methylation Methods

	Methylation method				
FA[a]	Method A $NaOCH_3$/methanol[b]	Method B CH_3COCl/methanol[c]	Method C $NaOCH_3$/TMSDAM[d]	Method D KOH/TMSDAM[e]	Method E $NaOCH_3$/methyl acetate[f]
14:0	0.87	1.15	0.59	0.70	1.02
14:1 (*c*9)	0.13	0.20	0.80	0.07	0.30
15:0	0.20	0.31	0.17	0.22	0.24
16:0	18.34	20.13	13.66	18.34	22.26
16:1 (*c*9)	1.26	1.30	1.20	1.34	0.30
18:0	6.40	7.39	5.36	7.52	5.90
18:1 (*c*9)	18.86	21.01	15.47	20.36	17.82
18:2 (*c*9,*c*12)	30.01	32.47	24.30	34.08	28.21
18:2 (*c*9,*t*11)[g]	0.19	0.21	0.19	0.20	0.36
18:3 (*c*9,*c*12,*c*15)	0.88	0.98	0.81	1.20	0.33
20:4 (*c*5,*c*8,*c*11,*c*14)	5.33	6.00	5.32	6.50	5.00

[a]These 11 FA (14:0, myristic acid; 14:1, myristoleic acid; 15:0, pentadecanoic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:2, CLA, 18:3, linolenic acid; and 20:4, arachidonic acid) accounted for 82, 91, 67, 90, and 81% of the total human plasma FA composition after methylation with $NaOCH_3$/methanol, CH_3COCl/methanol, $NaOCH_3$/TMSDAM, KOH/TMSDAM, and $NaOCH_3$/methyl acetate, respectively.
[b]Samples were treated with $NaOCH_3$ in methanol (0.5 N) for 2 h at 55°C.
[c]Samples were treated with acetyl chloride in methanol/chloroform for 1 h at 100°C.
[d]Samples were treated with $NaOCH_3$ in methanol (0.5 N) for 2 h at 55°C followed by TMSDAM overnight (12 h) at room temperature. TMSDAM, trimethylsilyldiazomethane.
[e]Samples were saponified with 0.5 N KOH in 95% ethanol for 2 h at 55°C and then methylated with TMSDAM overnight (12 h) at room temperature.
[f]Samples were treated with methyl acetate and sodium methoxide in methanol (0.5 N) in diethyl ether for 1 h at room temperature.
[g]Rumenic acid.

utilizing alcoholysis in an essentially nonalcoholic solution (33) (Method F). TLC analysis again revealed incomplete methylation; therefore, FAME were not analyzed on GLC. Despite such efforts, none of these methods was able to methylate the CE fraction completely. Finally, treatment with $NaOCH_3$/methyl acetate in diethyl ether (Method E) was

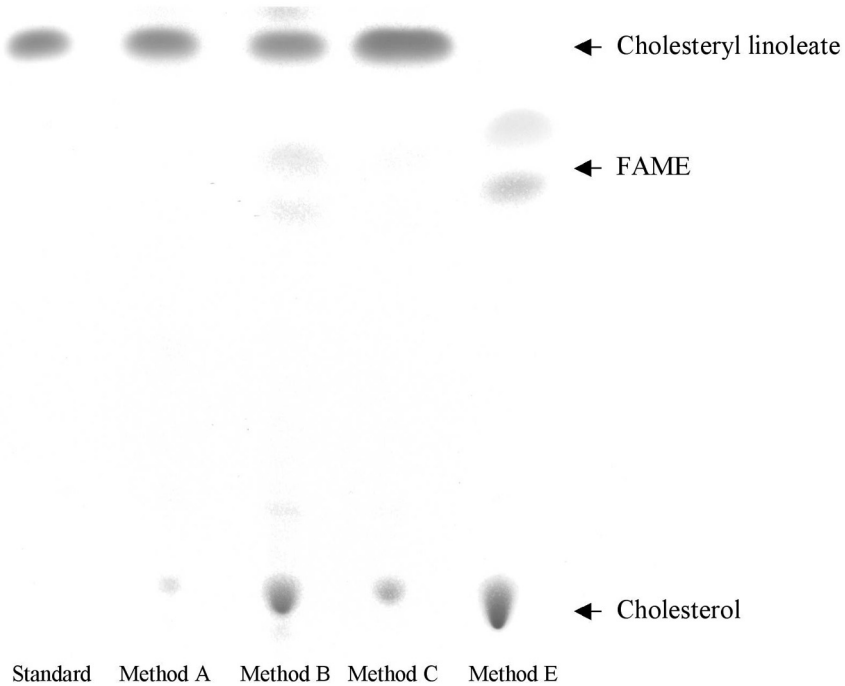

FIG. 1. Illustration of TLC plate with incomplete and complete methylation of cholesteryl linoleate (from left to right): cholesteryl linoleate standard; $NaOCH_3$ (0.5 N)/methanol (55°C, 2 h, Method A); CH_3COCl/methanol-chloroform (2:0.5 mL, vol/vol) (100°C, 1 h, Method B); $NaOCH_3$ (0.5 N)/methanol (55°C, 2 h)/TMSDAM (overnight at room temperature, Method C); $NaOCH_3$/methyl acetate/diethyl ether (room temperature, 1 h, Method E). The TLC plate was developed in hexane/diethyl ether/acetic acid (90:10:1, by vol), sprayed with 50% H_2SO_4 in 95% ethanol, and charred at 100°C. TMSDAM, trimethylsilyldiazomethane.

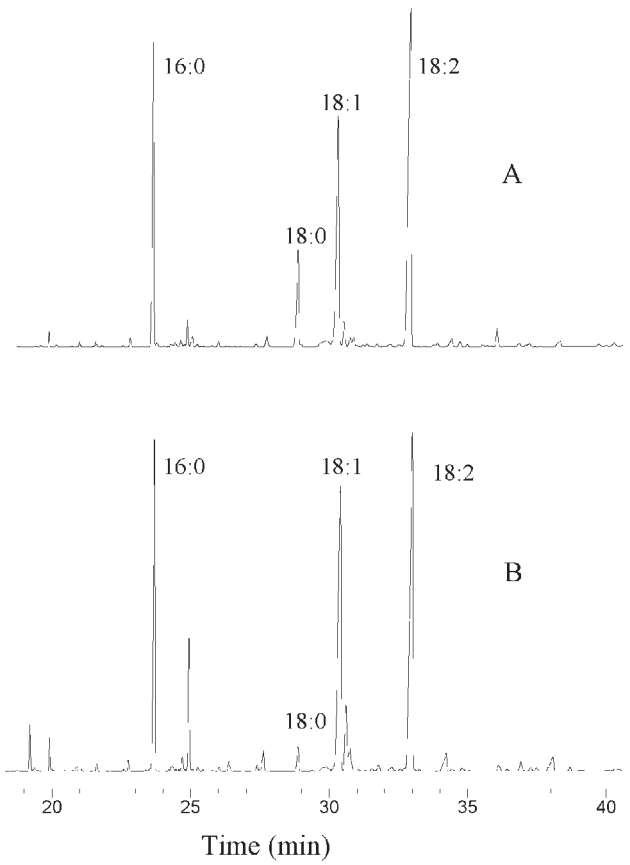

FIG. 2. Gas chromatograms of total plasma (A) and cholesterol ester fraction (B) after saponification with ethanolic KOH and methylation with TMSDAM (Method D); known peaks have been identified. For abbreviation see Figure 1.

found to fully methylate the CE fraction. Figure 1 shows the completeness of methylation of cholesteryl linoleate by this method. TAG, MAG/DAG/cholesterol, and PL were found to be completely methylated with Method A, whereas NEFA were methylated with Method C only. The FA composition of the plasma lipid classes are shown in Table 2. These data show that the greatest enrichment of *c*9,*t*11-CLA (% FAME) was in the TAG fraction (0.31%), followed by CE (0.14%) and PL (0.13%).

Experiment 11. Determination of c9,t*11-CLA in major lipid fractions of a small subset of plasma samples.* Results obtained from this experiment are shown in Table 3. These data indicate that the greatest enrichment of *c*9,*t*11-CLA is present in the TAG fraction (0.39%), followed by CE (0.27%) and PL (0.22%).

DISCUSSION

Results from this study confirm that, for the quantitative analysis of *c*9,*t*11-CLA in biological samples, care should be taken when choosing the methylation procedures. For example, methylation of the *c*9,*t*11-CLA isomer in CE is not achieved using sodium methoxide even under conditions of lengthy reaction times (2 h, 4 h, 55°C, or left overnight at room temperature) (Method A). Because a direct esterification method would be desirable when analyzing a large number of samples by GLC, such a method (Method B) was also investigated. Our data suggest that this direct esterification procedure is not suitable for *c*9,*t*11-CLA isomer quantification owing to incomplete methylation of the FA in whole plasma. Shantha *et al*. (34) have reported artifact formation

TABLE 2
FA (% identified FAME) Composition of Human Plasma Lipid Fractions in a Single Pooled Human Plasma Sample

	Lipid fraction				
FA[a]	CE[b]	TAG[c]	NEFA[d]	MAG/DAG/cholesterol[c]	PL[c]
14:0	0.50	1.42	2.36	1.00	0.70
14:1 (*c*9)	0.03	0.11	0.24	0.49	0.27
15:0	0.21	0.22	0.43	0.28	0.33
16:0	10.29	19.52	22.66	19.0	21.30
16:1 (*c*9)	1.79	1.87	0.60	2.58	0.22
18:0	9.60	4.96	35.47	33.31	11.21
18:1(*c*9)	17.18	32.32	7.93	4.56	6.95
18:2 (*c*9,*c*12)	51.93	21.28	4.47	3.03	17.91
18:2 (*c*9,*t*11)[e]	0.14	0.31	0.07	0.00	0.13
18:3 (*c*9,*c*12,*c*15)	0.54	1.82	0.14	1.68	0.20
20:4 (*c*5,*c*8,*c*11,*c*14)	6.10	0.82	0.37	0.21	6.25

[a]These 11 FA (14:0, myristic acid; 14:1, myristoleic acid; 15:0, pentadecanoic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:2, CLA, 18:3, linolenic acid; and 20:4, arachidonic acid) accounted for 98, 85, 75, 66, and 65% of the FA composition in cholesteryl esters (CE), TAG, nonesterified FA (NEFA), MAG/DAG/cholesterol, and phospholipid (PL) FA fractions, respectively.
[b]Samples were treated with $NaOCH_3$ in methanol/methyl acetate (Method E).
[c]Samples were treated with $NaOCH_3$ in methanol (0.5 N) for 2 h at 55°C (Method A).
[d]Samples were treated with $NaOCH_3$ in methanol (0.5 N) for 2 h at 55°C followed by TMSDAM overnight (12 h) at room temperature (Method C).
[e]*c*9,*t*11-CLA. For other abbreviation see Table 1.

TABLE 3
FA (% identified FAME) Composition of Human Plasma Lipid Fractions[a]

FA	Lipid fraction			
	CE[b]	TAG[c]	NEFA/MAG/DAG/chol[d]	PL[c]
14:0	0.53 ± 0.07	1.35 ± 0.29	2.81 ± 0.49	0.24 ± 0.02
14:1 (*c*9)	0.03 ± 0.03	0.15 ± 0.02	0.18 ± 0.02	0.18 ± 0.02
15:0	0.16 ± 0.01	0.26 ± 0.04	0.40 ± 0.05	0.17 ± 0.01
16:0	11.92 ± 0.44	19.75 ± 1.32	19.35 ± 1.22	29.15 ± 1.56
16:1 (*c*9)	3.48 ± 0.71	3.33 ± 0.62	0.72 ± 0.12	0.67 ± 0.10
18:0	1.08 ± 0.04	4.11 ± 0.51	23.18 ± 1.54	14.28 ± 0.23
18:1(*c*9)	19.12 ± 0.73	28.58 ± 1.31	9.51 ± 0.7	11.13 ± 0.54
18:2 (*c*9,*c*12)	49.64 ± 1.9	20.67 ± 1.93	4.02 ± 0.51	23.28 ± 1.32
18:2 (*c*9,*t*11)[e]	0.26 ± 0.03	0.39 ± 0.03	0.06 ± 0.01	0.22 ± 0.03
18:3 (*c*9,*c*12,*c*15)	0.42 ± 0.01	1.07 ± 0.17	0.50 ± 0.01	0.16 ± 0.14
20:4 (*c*5,*c*8,*c*11,*c*14)	4.98 ± 0.37	1.20 ± 0.29	0.89 ± 0.15	6.33 ± 0.77

[a]Values represent means ± SEM (n = 10).
[b]Samples were treated with $NaOCH_3$ in methanol/methyl acetate for 1 h at room temperature (Method E).
[c]Samples were treated with $NaOCH_3$ in methanol (0.5 N) for 2 h at 55°C (Method A).
[d]Samples were treated with $NaOCH_3$ in methanol (0.5 N) for 2 h at 55°C followed by TMSDAM overnight (12 h) at room temperature (Method C); NEFA and MAG/DAG/cholesterol fractions were combined together since they were found not to contain much *c*9,*t*11-CLA. chol, cholesterol.
[e]*c*9,*t*11-CLA; for other abbreviations see Tables 1 and 2.

when using this method. However, the formation of artifacts in our human plasma sample was not detected, perhaps because other FA present in trace amounts also eluted at that region. Method D is not a transesterification method and therefore was not applied to confirm the methylation on standard CE and PL. However, methylation of the whole plasma with this method resulted in a chromatogram with many additional peaks after GLC analysis (Fig. 2). No attempts were made to identify the unknown peaks. One possible explanation for these additional peaks and a lower *c*9,*t*11-CLA content could be the structural changes in double bonds during saponification. Indeed, Chahine *et al*. (35) observed structural changes while using this method to saponify unsaturated FA.

The data obtained from our pooled plasma lipid fractions (Table 2) were confirmed by analyzing a small subset of plasma samples (n = 10) (Table 3). These data are consistent in that both data sets suggest that the highest percentage of *c*9,*t*11-CLA is present in the TAG fraction, followed by CE and PL. The NEFA and DAG/MAG fractions were found to contain very little *c*9,*t*11-CLA. Fogerty *et al.* (22) also reported the highest percentage of *c*9,*t*11-CLA in the TAG fraction, followed by PL and CE in human blood serum. The levels of other major FA (e.g., 16:0, 18:0, 18:1, 18:2) in the PL and TAG fractions were also found to be similar to those reported by others (36,37).

Sodium methoxide in methanol is considered to be a good reagent that requires mild reaction conditions for transesterification. Bannon *et al.* (38) and Craske *et al.* (39) have discussed the merits of the $NaOCH_3$/methanol reagent, including low cost, reagent stability, and mild reaction conditions. However, the $NaOCH_3$/methanol reagent does not convert FFA to methyl esters, and strict anhydrous conditions are required. The methylation procedures tested in the present study using the $NaOCH_3$/methanol reagent gave little agreement with the reported values of the total plasma *c*9,*t*11-CLA content. For example, total plasma *c*9,*t*11-CLA concentrations ranged from 0.12 to 0.54% of total FA in Australian (n = 15), Swedish (n = 123), Finnish (n = 403), and American (n = 10) volunteeers (22,40–42). In these studies, the differences in total *c*9,*t*11-CLA content might be due to differences in the subjects' population/diet or in the methylation procedures. In most of the preceding studies, $NaOCH_3$/methanol, methanolic HCl, or BF_3-methanol have been used for transesterification, thereby ignoring the contribution of *c*9,*t*11-CLA in CE, as it would likely not be methylated but may be isomerized. Recently, Yamasaki *et al.* (43) and Park *et al.* (31) reported the isomerization of *t*,*c* and *c*,*t* isomers to *t*,*t* isomers of CLA while using methanolic H_2SO_4 or methanolic HCl and BF_3-methanol for transesterification. A similar phenomenon was also reported for oleic acid (44), and artifacts were produced from some lipids during BF_3 methylation (45).

In the current study, one possible reason for the *c*9,*t*11-CLA not being methylated in CE using sodium methoxide in methanol (Method A) could be the partial solubility of CE in methanol; the reverse occurs in Method E, where diethyl ether is used to dissolve the CE along with methyl acetate, thereby methylating the *c*9,*t*11-CLA completely. Longer reaction times required for methylating of CE and the partial solubility of CE in methanol also have been reported by Christie (25) and Stoffel *et al*. (46). Another explanation as to why whole plasma *c*9,*t*11-CLA content was estimated to be lower when methylating with methylation procedures other than Method E could be the presence of trace amounts of water from the atmosphere or absorbed on glassware, resulting in irreversible hydrolysis; the use of methyl acetate in Method E might have preferentially hydrolyzed the lipid itself, thus giving higher estimates of *c*9,*t*11-CLA content. Further, a rapid hydrolysis of FAME has been reported while transesterifying a variety of polar lipids (32).

Of all methods tested, we found no single method able to methylate *c*9,*t*11-CLA satisfactorily in the major fractions or the whole plasma lipid extract. Therefore, methylation of whole plasma lipid extract by any single method may result in incorrect estimation of *c*9,*t*11-CLA concentration. When methylation of the major plasma fractions is necessary, these results suggest that the CE fraction (which represents the major plasma lipid fraction) should be methylated with sodium methoxide and methyl acetate in methanol. A combination of methylating methods can be used (Methods E and C) when methylating the whole plasma sample to ensure complete methylation of all FA in plasma.

In conclusion, our data show that the highest enrichment of *c*9,*t*11-CLA is present in the TAG fraction, followed by CE and PL, and that different methodologies would be required for different plasma fractions. Further research is needed to determine whether physiologic state (fed vs. fasting) influences CLA distribution among fractions.

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Supplementation with CLA: Isomer Incorporation into Serum Lipids and Effect on Body Fat of Women

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ABSTRACT: Animal studies have suggested that CLA, a natural component of meat and dairy products, may confer beneficial effects on health. However, human studies using supplementation with CLA have produced contradictory results. The aim of the present study was to further investigate the effect of CLA supplementation on human body fat, serum leptin, and serum lipids, as well as the incorporation of CLA isomers into serum lipids classes. Sixteen young healthy nonobese sedentary women received 2.1 g of CLA (divided equally between the *cis,trans*-9,11 and *trans,cis*-10,12 isomers) daily for 45 d and placebo for 45 d in a randomized double-blind crossover design. Body fat was estimated (by measurement of skinfold thickness at 10 sites), and blood was sampled at the beginning, middle, and end of the entire intervention period; an additional blood sample was obtained 2 wk thereafter. No significant differences in energy, carbohydrate, lipid, or protein intake existed between the CLA and placebo intake periods. No significant differences were found in body fat or serum leptin, TAG, total cholesterol, HDL-cholesterol, and alanine aminotransferase between CLA and placebo. The CLA isomer content of serum TAG, phospholipids, and total lipids increased 2–5 times with CLA supplementation ($P < 0.05$). In contrast, the CLA content of cholesteryl esters did not change significantly. The period of 2 wk after the end of CLA supplementation was sufficient for its washout from serum lipids. These data indicate that supplementation with 2.1 g of CLA daily for 45 d increased its levels in blood but had no effect on body composition or the lipidemic profile of nonobese women.

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CLA is a family of constitutional isomers and stereoisomers of octadecadienoic acid having conjugated double bonds. Several of these isomers occur naturally, mainly in the fat of ruminants and dairy products, and two (*cis,trans*-9,11 and *trans,cis*-10,12) are known to possess biological activity. As has been shown in animals studies, both isomers inhibit carcinogenesis, whereas the latter lowers body fat and increases lean body mass (1). Other reported actions of CLA in animals include decrement of atherogenesis and prevention of the catabolic effects of immune stimulation (1).

Of the aforementioned physiological effects, that on body composition has been the one most extensively studied in humans so far. Results from the relevant articles are contradictory, with some (including one from our laboratory) indicating that supplementation with CLA causes body fat reduction (2–6) and others reporting no significant effect on body fat (7–9). Results with regard to the effect of CLA supplementation on human serum lipids are also equivocal (2–5,9,10). The aim of the present study was to further investigate the effect of CLA supplementation on body fat and serum biochemical parameters related to lipid metabolism by using a higher dose and longer supplementation compared to our previous study (3), as well as controlling for interindividual and gender differences. An additional aim was to determine the incorporation of individual CLA isomers into serum lipid classes.

MATERIALS AND METHODS

Subjects. Seventeen sedentary women, aged 19–24, who responded to a public invitation participated in the study initially. Subjects were eumenorrheic, not obese (body mass index < 30 kg/m^2), not suffering from any acute or chronic illness, and not taking any medication or dietary supplements. They were informed orally and in writing of the design and probable risks of the research and consented to participate. The study was designed and carried out according to the guidelines of the University of Thessaloniki Ethics Committee.

Design. Participants received CLA for 45 d and placebo for 45 d, with no washout period in between, in a randomized double-blind crossover design, thus forming two groups, CLA-placebo and placebo-CLA. The daily dose of CLA was 2.1 g in the form of six soft gelatine capsules from TrofoCell (Hamburg, Germany). Each capsule contained 500 mg of oil, 70% of which was CLA, divided equally between the *cis,trans*-9,11 and *trans,cis*-10,12 isomers. Placebo was in the form of identical-appearing capsules containing soybean oil. Subjects were asked not to modify their nutritional habits and physical activity for the duration of the study and to record their dietary intake as well as the quantity of experimental capsules taken daily. As an additional measure of compliance, they were asked to return any remaining capsules at the end of each supplementation period.

Measurements. The participants visited the laboratory at the onset, middle (change of regimen), and end of the entire intervention period, between 9 and 11 A.M., after an overnight fast. During each visit we measured body weight, height, and thickness of 10 skinfolds for the estimation of percentage

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Abbreviations: ALT, alanine aminotransferase; CE, cholesteryl esters; PL, phospholipids.

body fat (11). All skinfold thickness measurements were performed by the same highly experienced researcher using Harpenden metal calipers from British Indicators (West Sussex, United Kingdom). This method of estimating body fat has recently been reported to differ by 1.4 ± 2.2 (mean ± SD) percentage body fat units from the reference method, hydrodensitometry, in a large sample (n = 114) of women (12).

Blood sampling. At each of the three visits, the volunteers provided a blood sample from an antecubital vein into an evacuated test tube while seated. After clotting, serum was prepared by centrifugation and was stored at −20°C for the determination of TAG, total cholesterol, HDL cholesterol, leptin, and alanine aminotransferase (ALT), as well as for the FA analysis of lipid classes. In addition, the volunteers provided a blood sample during a follow-up visit to the laboratory 2 wk after the end of the intervention period for the examination of the washout of CLA from serum lipids.

Assays. TAG, cholesterol, and ALT were assayed by enzymic spectrophotometric methods through the use of reagent kits from Randox (Crumlin, Co. Antrim, United Kingdom). HDL cholesterol was determined after treatment of serum aliquots with a precipitant from Roche (Mannheim, Germany). Leptin was measured by ELISA through the use of a kit from DRG (Marburg, Germany).

Determination of the FA composition of serum lipid classes was initiated by the addition of a mixture of triheptadecanoin, diheptadecanoyl PC, and cholesteryl heptadecanoate (all from Sigma, St. Louis, MO) as internal standards to 0.5 mL of serum. Lipids were extracted and separated by TLC as described (3). Lipid spots were located under UV light after spraying with a solution of dichlorofluorescein in ethanol, and the spots corresponding to TAG, phospholipids (PL), and cholesteryl esters (CE) were excised and incubated with 0.5 mol/L $NaOCH_3$ in methanol (Sigma) at 50°C for 10 min (13). After extraction with hexane, the methyl esters were separated by GC in a Hewlett-Packard 5890 Series II chromatograph (Waldbronn, Germany) equipped with a 30-m-long BPX70 capillary column from SGE (Ringwood, Victoria, Australia). The column temperature was programmed from 140 to 240°C at 5°C/min. The carrier gas was helium at a flow rate of 0.67 mL/min (at 140°C). Methyl esters were identified by comparing their retention times to those of pure FAME purchased from Sigma. Additionally, *cis,trans*-9,11-, *trans,cis*-10,12-, and *trans,trans*-9,11-octadecadienoic acid were purchased from Cayman (Ann Arbor, MI). FAME in the samples were quantified by comparing the area under their peaks in the chromatogram to that of methyl heptadecanoate (derived from the internal standards) with the aid of the HP 3365 ChemStation software from Hewlett-Packard.

The FA composition of total serum lipids was determined by the addition of a mixture of the internal standards mentioned above to 10 μL of serum, followed by preparation of FAME and GC as described above.

Dietary analysis. Dietary records were analyzed in Microsoft® Access by the use of a food database created in our laboratory on the basis of published data (14).

Statistical analysis. Results are reported as the mean ± SD. Significant differences between supplementation with CLA and placebo were detected by ANOVA based on a counterbalanced Latin square design (15), which controls for possible carryover effects. The level of statistical significance was set at $\alpha = 0.05$. SPSS (version 10.0) was used for all analyses (SPSS Inc., Chicago, IL).

RESULTS

Before the end of the study, one volunteer withdrew owing to acute illness (influenza) and the demand of her physician that she discontinue the experimental capsules. No other adverse effect was reported by any of the participants. When the study was unblinded, the volunteer who had withdrawn was found to be member of the placebo-CLA group. Of the 16 volunteers who completed the study, 9 belonged to the CLA-placebo group and 7 to the placebo-CLA group. Compliance with the regimen was 99.1 ± 2.4% for CLA and 99.2 ± 3.2% for placebo.

The age of the participants was 22.3 ± 1.8 yr. There were no significant differences in energy, carbohydrate, lipid, or protein intake between the periods of CLA and placebo supplementation (data not shown). Daily energy intake averaged 1975 kcal and was derived from 43% carbohydrate, 41% fat, and 16% protein.

Anthropometric data are presented in Table 1. There were no significant differences between supplementation with CLA and placebo in regard to body mass, body mass index, sum of skinfold thickness, or percentage body fat. The observed power for these parameters was low, ranging from 0.05 to 0.14.

The results of the biochemical analyses except FA analysis are shown in Table 2. There was no significant effect of CLA supplementation on TAG, total cholesterol, HDL cholesterol, total/HDL cholesterol, leptin, or ALT.

TABLE 1
Anthropometric Data of Participants

	CLA-placebo group			Placebo-CLA group		
	Baseline	CLA	Placebo	Baseline	Placebo	CLA
Body mass (kg)	66.3 ± 9.5	66.2 ± 9.0	67.1 ± 9.9	66.7 ± 4.7	66.9 ± 5.1	67.4 ± 6.0
Height (m)	1.69 ± 0.08	1.69 ± 0.07	1.69 ± 0.07	1.68 ± 0.08	1.68 ± 0.08	1.68 ± 0.08
Body mass index (kg/m^2)	23.1 ± 2.4	23.2 ± 2.4	23.5 ± 2.5	23.7 ± 2.9	23.8 ± 3.0	24.0 ± 3.3
Sum of 10 skinfolds (mm)	189.4 ± 56.6	199.2 ± 40.0	186.0 ± 48.7	204.4 ± 44.1	223.8 ± 41.6	203.1 ± 50.4
Body fat (%)	28.1 ± 5.7	29.4 ± 3.6	28.0 ± 4.6	29.9 ± 3.5	31.5 ± 3.1	29.7 ± 4.0
Fat mass (kg)	19.0 ± 5.8	19.7 ± 4.6	19.1 ± 5.5	20.0 ± 3.4	21.1 ± 3.1	20.1 ± 4.0

TABLE 2
Biochemical Analyses

	CLA-placebo group			Placebo-CLA group		
	Baseline	CLA	Placebo	Baseline	Placebo	CLA
TAG (mg/dL)	65.0 ± 26.1	54.8 ± 18.0	51.8 ± 9.6	47.4 ± 14.2	41.2 ± 10.7	47.2 ± 6.7
Total cholesterol (mg/dL)	181.1 ± 17.3	172.0 ± 19.6	165.8 ± 17.0	168.2 ± 20.2	157.2 ± 18.3	159.3 ± 11.6
HDL cholesterol (mg/dL)	56.9 ± 11.1	55.2 ± 10.0	56.0 ± 11.9	56.2 ± 6.0	53.4 ± 6.5	50.9 ± 7.2
Total/HDL cholesterol	3.3 ± 0.6	3.2 ± 0.7	3.1 ± 0.6	3.0 ± 0.4	3.0 ± 0.4	3.2 ± 0.5
Leptin (ng/mL)	15.6 ± 8.9	17.7 ± 7.5	19.6 ± 16.3	24.4 ± 10.5	22.2 ± 13.1	25.5 ± 12.4
ALT[a] (U/L, 37°C)	9.6 ± 4.0	8.4 ± 3.8	12.6 ± 4.4	7.5 ± 4.3	7.8 ± 3.3	11.8 ± 4.4

[a]ALT, alanine aminotransferase.

GC revealed the presence, in considerable amounts, of 19 FA including the *cis,trans*-9,11, *trans,cis*-10,12, and the *trans,trans*-9,11 isomers of CLA, although a fourth isomer, *trans,trans*-10,12-CLA, has been reported to comigrate with the latter (16,17). The FA composition of serum TAG, PL, CE, and total lipids is listed in Tables 3–6.

No significant differences between placebo and CLA supplementation were found in the preceding FA except CLA. Compared to the placebo, CLA supplementation resulted in a significant increase in the concentration of all CLA isomers in serum TAG ($P < 0.05$), PL ($P < 0.001$), and total lipids ($P < 0.05$). The CLA content of CE increased to a lesser degree with CLA supplementation, and the difference from the placebo was not significant.

Concerning the individual CLA isomers, there was a significant increase in the percentage of *cis,trans*-9,11-CLA within the acyl groups of serum TAG (from 0.32 to 0.76, $P = 0.003$, mean of all 16 volunteers), PL (from 0.20 to 0.40, $P < 0.001$), and total lipids (from 0.18 to 0.40, $P = 0.001$) with CLA supplementation (Fig. 1). Likewise, the percentage of *trans,cis*-10,12-CLA increased significantly in TAG (from 0.05 to 0.24, $P = 0.021$), PL (from 0.07 to 0.15, $P < 0.001$), and total lipids (from 0.08 to 0.19, $P = 0.003$). Finally, the percentage of *trans,trans*-9,11-CLA increased significantly in TAG (from 0.20 to 0.30, $P = 0.001$) and PL (from 0.21 to 0.31, $P = 0.013$). There was no significant difference between placebo and CLA supplementation in the percentages of any of the three isomers or their sum in CE. On the contrary, the sum of the percentages of the three isomers increased significantly in TAG (from 0.57 to 1.29, $P = 0.004$), PL (from 0.48 to 0.87, $P < 0.001$), and total lipids (from 0.45 to 0.81, $P = 0.001$). Two weeks after the end of supplementation the CLA content of serum lipids had returned to the values before CLA supplementation (Fig. 1).

TABLE 3
Serum Concentrations (mmol/L) of TAG Acyl Groups

	CLA-placebo group			Placebo-CLA group		
FA	Baseline	CLA	Placebo	Baseline	Placebo	CLA
14:0	0.0337 ± 0.0344	0.0310 ± 0.0280	0.0204 ± 0.0127	0.0177 ± 0.0090	0.0113 ± 0.0011	0.0231 ± 0.0107
16:0	0.5366 ± 0.3030	0.4226 ± 0.1470	0.4331 ± 0.1211	0.3359 ± 0.0941	0.2817 ± 0.0801	0.3540 ± 0.0614
16:1n-7	0.0511 ± 0.0496	0.0379 ± 0.0288	0.0439 ± 0.0375	0.0300 ± 0.0124	0.0227 ± 0.0081	0.0269 ± 0.0068
18:0	0.1186 ± 0.0577	0.0955 ± 0.0264	0.0949 ± 0.0601	0.0700 ± 0.0286	0.0548 ± 0.0151	0.0722 ± 0.0132
18:1n-9	0.6244 ± 0.2887	0.5312 ± 0.1487	0.5889 ± 0.1813	0.4431 ± 0.1303	0.4089 ± 0.1067	0.4974 ± 0.0954
18:1n-7	0.0316 ± 0.0128	0.0279 ± 0.0109	0.0339 ± 0.0168	0.0218 ± 0.0052	0.0214 ± 0.0065	0.0227 ± 0.0036
18:2n-6	0.2448 ± 0.0779	0.2161 ± 0.0786	0.2379 ± 0.0495	0.1752 ± 0.0703	0.1873 ± 0.0330	0.1969 ± 0.0871
cis,trans-9,11-CLA[a]	0.0050 ± 0.0037	0.0109 ± 0.0091	0.0050 ± 0.0036	0.0041 ± 0.0018	0.0039 ± 0.0027	0.0100 ± 0.0076
trans,cis-10,12-CLA[b]	0.0006 ± 0.0003	0.0042 ± 0.0055	0.0005 ± 0.0003	0.0005 ± 0.0005	0.0006 ± 0.0005	0.0027 ± 0.0031
trans,trans-9,11-CLA[a]	0.0030 ± 0.0010	0.0039 ± 0.0025	0.0029 ± 0.0010	0.0024 ± 0.0009	0.0021 ± 0.0009	0.0039 ± 0.0010
CLA sum[a]	0.0086 ± 0.0044	0.0191 ± 0.0162	0.0084 ± 0.0043	0.0070 ± 0.0022	0.0066 ± 0.0032	0.0166 ± 0.0104
18:3n-6	0.0040 ± 0.0025	0.0023 ± 0.0016	0.0031 ± 0.0017	0.0028 ± 0.0023	0.0020 ± 0.0007	0.0024 ± 0.0020
18:3n-3	0.0047 ± 0.0016	0.0055 ± 0.0080	0.0043 ± 0.0020	0.0025 ± 0.0009	0.0022 ± 0.0012	0.0029 ± 0.0014
20:1n-9	0.0042 ± 0.0017	0.0038 ± 0.0015	0.0041 ± 0.0020	0.0029 ± 0.0007	0.0026 ± 0.0007	0.0034 ± 0.0007
20:3n-6	0.0028 ± 0.0010	0.0033 ± 0.0027	0.0031 ± 0.0019	0.0021 ± 0.0009	0.0018 ± 0.0004	0.0021 ± 0.0009
20:4n-6	0.0056 ± 0.0026	0.0050 ± 0.0029	0.0077 ± 0.0031	0.0055 ± 0.0018	0.0055 ± 0.0013	0.0054 ± 0.0023
20:5n-3	0.0002 ± 0.0002	0.0002 ± 0.0004	0.0003 ± 0.0006	0.0003 ± 0.0004	0.0002 ± 0.0003	0.0004 ± 0.0005
22:5n-3	0.0010 ± 0.0008	0.0009 ± 0.0007	0.0012 ± 0.0008	0.0009 ± 0.0008	0.0008 ± 0.0005	0.0007 ± 0.0005
22:6n-3	0.0008 ± 0.0005	0.0010 ± 0.0007	0.0014 ± 0.0008	0.0009 ± 0.0004	0.0009 ± 0.0009	0.0007 ± 0.0003
24:0	0.0008 ± 0.0007	0.0009 ± 0.0008	0.0009 ± 0.0007	0.0006 ± 0.0002	0.0008 ± 0.0003	0.0007 ± 0.0005
FA sum	1.6734 ± 0.7158	1.4041 ± 0.3845	1.4876 ± 0.3562	1.1193 ± 0.3146	1.0115 ± 0.2351	1.2287 ± 0.1664

[a]$P \leq 0.01$, significantly higher after CLA supplementation compared to placebo.
[b]$P < 0.05$, significantly higher after CLA supplementation compared to placebo.

TABLE 4
Serum Concentrations (mmol/L) of Phospholipid Acyl Groups

	CLA-placebo group			Placebo-CLA group		
FA	Baseline	CLA	Placebo	Baseline	Placebo	CLA
14:0	0.0171 ± 0.0167	0.0183 ± 0.0114	0.0139 ± 0.0073	0.0109 ± 0.0039	0.0075 ± 0.0033	0.0118 ± 0.0035
16:0	0.9696 ± 0.2966	0.8342 ± 0.1370	0.8657 ± 0.1865	0.8027 ± 0.1045	0.6840 ± 0.0914	0.7547 ± 0.1335
16:1n-7	0.0220 ± 0.0110	0.0191 ± 0.0079	0.0181 ± 0.0100	0.0183 ± 0.0047	0.0143 ± 0.0033	0.0149 ± 0.0019
18:0	0.9652 ± 0.5752	0.7739 ± 0.2016	0.8320 ± 0.4451	0.7454 ± 0.2606	0.6091 ± 0.0884	0.7063 ± 0.1968
18:1n-9	0.4260 ± 0.1144	0.3849 ± 0.0698	0.3701 ± 0.0553	0.3637 ± 0.0463	0.3267 ± 0.0531	0.3377 ± 0.0356
18:1n-7	0.0428 ± 0.0071	0.0408 ± 0.0098	0.0439 ± 0.0074	0.0340 ± 0.0048	0.0336 ± 0.0046	0.0305 ± 0.0027
18:2n-6	0.5902 ± 0.1009	0.5230 ± 0.1408	0.6206 ± 0.1489	0.4786 ± 0.1029	0.4689 ± 0.0746	0.5309 ± 0.1432
cis,trans-9,11-CLA[a]	0.0060 ± 0.0026	0.0123 ± 0.0038	0.0053 ± 0.0034	0.0048 ± 0.0019	0.0054 ± 0.0015	0.0093 ± 0.0020
trans,cis-10,12-CLA[a]	0.0021 ± 0.0017	0.0040 ± 0.0016	0.0022 ± 0.0013	0.0019 ± 0.0005	0.0016 ± 0.0010	0.0044 ± 0.0019
trans,trans-9,11-CLA[a]	0.0073 ± 0.0030	0.0082 ± 0.0026	0.0045 ± 0.0026	0.0048 ± 0.0023	0.0045 ± 0.0011	0.0084 ± 0.0038
CLA sum[a]	0.0154 ± 0.0063	0.0245 ± 0.0054	0.0120 ± 0.0061	0.0115 ± 0.0039	0.0115 ± 0.0028	0.0221 ± 0.0055
18:3n-6	0.0007 ± 0.0021	0.0001 ± 0.0003	0.0005 ± 0.0016	0.0011 ± 0.0017	0.0006 ± 0.0010	0.0004 ± 0.0004
18:3n-3	0.0114 ± 0.0152	0.0107 ± 0.0070	0.0122 ± 0.0118	0.0088 ± 0.0046	0.0078 ± 0.0042	0.0086 ± 0.0047
20:1n-9	0.0064 ± 0.0020	0.0066 ± 0.0011	0.0059 ± 0.0023	0.0050 ± 0.0017	0.0063 ± 0.0012	0.0063 ± 0.0014
20:3n-6	0.0608 ± 0.0224	0.0531 ± 0.0335	0.0704 ± 0.0309	0.0436 ± 0.0151	0.0425 ± 0.0154	0.0452 ± 0.0136
20:4n-6	0.1261 ± 0.0484	0.1205 ± 0.0592	0.1640 ± 0.0699	0.1213 ± 0.0605	0.1336 ± 0.0643	0.1295 ± 0.0552
20:5n-3	0.0044 ± 0.0018	0.0075 ± 0.0086	0.0082 ± 0.0055	0.0092 ± 0.0106	0.0053 ± 0.0040	0.0055 ± 0.0041
22:5n-3	0.0076 ± 0.0041	0.0129 ± 0.0079	0.0107 ± 0.0061	0.0077 ± 0.0040	0.0077 ± 0.0041	0.0081 ± 0.0036
22:6n-3	0.0325 ± 0.0246	0.0354 ± 0.0288	0.0427 ± 0.0276	0.0280 ± 0.0239	0.0290 ± 0.0273	0.0317 ± 0.0286
24:0	0.0104 ± 0.0059	0.0069 ± 0.0035	0.0085 ± 0.0038	0.0063 ± 0.0044	0.0107 ± 0.0100	0.0081 ± 0.0025
FA sum	3.3072 ± 0.9684	2.8695 ± 0.4466	3.0976 ± 0.7621	2.6949 ± 0.4246	2.3962 ± 0.2454	2.6497 ± 0.4752

[a]Significantly higher after CLA supplementation compared to placebo ($P < 0.001$).

DISCUSSION

In the present study we examined the effect of supplementation with CLA (2.1 g for 45 d) vs. a placebo on women's body fat, biochemical parameters of serum, and the CLA isomer content of individual serum lipid classes. We found no effect of CLA on body mass, body fat, or serum leptin, the latter considered an index of fat mass (18). These findings are in agreement with results published in three articles (7–9), which showed no significant change in body mass or body fat

TABLE 5
Serum Concentrations (mmol/L) of Cholesteryl Ester Acyl Groups

	CLA-placebo group			Placebo-CLA group		
FA	Baseline	CLA	Placebo	Baseline	Placebo	CLA
14:0	0.0212 ± 0.0079	0.0208 ± 0.0110	0.0165 ± 0.0122	0.0205 ± 0.0062	0.0155 ± 0.0054	0.0185 ± 0.0093
16:0	0.4424 ± 0.0650	0.3969 ± 0.0632	0.3919 ± 0.0382	0.3794 ± 0.0521	0.3513 ± 0.0582	0.3684 ± 0.0587
16:1n-7	0.0677 ± 0.0462	0.0634 ± 0.0506	0.0667 ± 0.0603	0.0584 ± 0.0132	0.0457 ± 0.0147	0.0461 ± 0.0098
18:0	0.0779 ± 0.0564	0.0588 ± 0.0300	0.0468 ± 0.0155	0.0446 ± 0.0182	0.0413 ± 0.0225	0.0658 ± 0.0458
18:1n-9	0.6136 ± 0.1334	0.6050 ± 0.1591	0.5744 ± 0.1107	0.5414 ± 0.0762	0.4956 ± 0.1006	0.4971 ± 0.0646
18:1n-7	0.0346 ± 0.0064	0.0324 ± 0.0110	0.0359 ± 0.0041	0.0287 ± 0.0078	0.0290 ± 0.0052	0.0282 ± 0.0055
18:2n-6	1.3732 ± 0.2956	1.2204 ± 0.1942	1.4136 ± 0.2294	1.2437 ± 0.1948	1.2054 ± 0.2182	1.3273 ± 0.2773
cis,trans-9,11-CLA	0.0020 ± 0.0020	0.0036 ± 0.0033	0.0024 ± 0.0026	0.0025 ± 0.0029	0.0032 ± 0.0027	0.0038 ± 0.0029
trans,cis-10,12-CLA	0.0014 ± 0.0010	0.0019 ± 0.0018	0.0015 ± 0.0010	0.0018 ± 0.0013	0.0020 ± 0.0013	0.0038 ± 0.0039
trans,trans-9,11-CLA	0.0051 ± 0.0027	0.0051 ± 0.0024	0.0045 ± 0.0022	0.0066 ± 0.0040	0.0044 ± 0.0019	0.0050 ± 0.0015
CLA sum	0.0085 ± 0.0052	0.0106 ± 0.0073	0.0084 ± 0.0056	0.0109 ± 0.0060	0.0096 ± 0.0050	0.0126 ± 0.0075
18:3n-6	0.0102 ± 0.0072	0.0070 ± 0.0048	0.0122 ± 0.0106	0.0110 ± 0.0076	0.0119 ± 0.0109	0.0084 ± 0.0040
18:3n-3	0.0074 ± 0.0079	0.0037 ± 0.0022	0.0059 ± 0.0024	0.0047 ± 0.0015	0.0063 ± 0.0045	0.0038 ± 0.0011
20:1n-9	0.0006 ± 0.0002	0.0004 ± 0.0001	0.0006 ± 0.0002	0.0005 ± 0.0002	0.0007 ± 0.0005	0.0006 ± 0.0006
20:3n-6	0.0096 ± 0.0041	0.0086 ± 0.0049	0.0115 ± 0.0041	0.0079 ± 0.0014	0.0073 ± 0.0026	0.0084 ± 0.0033
20:4n-6	0.0407 ± 0.0160	0.0368 ± 0.0101	0.0543 ± 0.0191	0.0447 ± 0.0112	0.0514 ± 0.0169	0.0475 ± 0.0170
20:5n-3	0.0042 ± 0.0015	0.0048 ± 0.0034	0.0069 ± 0.0019	0.0049 ± 0.0020	0.0039 ± 0.0013	0.0042 ± 0.0015
22:5n-3	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
22:6n-3	0.0044 ± 0.0020	0.0045 ± 0.0012	0.0052 ± 0.0033	0.0039 ± 0.0031	0.0038 ± 0.0030	0.0036 ± 0.0027
24:0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
FA sum	2.7169 ± 0.4874	2.4736 ± 0.4311	2.6508 ± 0.3696	2.4054 ± 0.3176	2.2787 ± 0.3820	2.4406 ± 0.4284

TABLE 6
Serum Concentrations (mmol/L) of Acyl Groups in Total Lipids

	CLA-placebo group			Placebo-CLA group		
FA	Baseline	CLA	Placebo	Baseline	Placebo	CLA
14:0	0.1010 ± 0.0828	0.0948 ± 0.0779	0.0733 ± 0.0521	0.0764 ± 0.0341	0.0587 ± 0.0303	0.0809 ± 0.0470
16:0	2.9500 ± 1.1592	2.6060 ± 0.9496	2.4593 ± 0.7720	2.3379 ± 0.6222	2.0516 ± 0.6210	2.0639 ± 0.6721
16:1n-7	0.2139 ± 0.1770	0.1939 ± 0.1417	0.2036 ± 0.1842	0.1636 ± 0.0591	0.1201 ± 0.0358	0.1220 ± 0.0316
18:0	1.5616 ± 1.0664	1.2220 ± 0.5900	1.0193 ± 0.4286	1.1376 ± 0.7004	0.9250 ± 0.3762	1.0267 ± 0.3785
18:1n-9	2.4941 ± 0.8858	2.3071 ± 0.6639	2.3250 ± 0.7348	1.9870 ± 0.5036	1.7843 ± 0.4916	1.9007 ± 0.4179
18:1n-7	0.1744 ± 0.0528	0.1610 ± 0.0737	0.1732 ± 0.0525	0.1422 ± 0.0417	0.1321 ± 0.0336	0.1236 ± 0.0400
18:2n-6	3.6316 ± 1.1366	3.3498 ± 1.1104	3.5880 ± 1.0820	3.1951 ± 1.2566	3.0691 ± 1.1100	3.2180 ± 1.3070
cis,trans-9,11-CLA[a]	0.0208 ± 0.0096	0.0401 ± 0.0228	0.0194 ± 0.0081	0.0205 ± 0.0105	0.0187 ± 0.0145	0.0386 ± 0.0266
trans,cis-10,12-CLA[a]	0.0091 ± 0.0029	0.0160 ± 0.0104	0.0073 ± 0.0045	0.0093 ± 0.0052	0.0069 ± 0.0049	0.0217 ± 0.0141
trans,trans-9,11-CLA[b]	0.0206 ± 0.0062	0.0204 ± 0.0066	0.0144 ± 0.0052	0.0211 ± 0.0087	0.0165 ± 0.0100	0.0212 ± 0.0101
CLA sum[a]	0.0504 ± 0.0117	0.0766 ± 0.0340	0.0410 ± 0.0118	0.0508 ± 0.0236	0.0422 ± 0.0266	0.0815 ± 0.0460
18:3n-6	0.0360 ± 0.0326	0.0260 ± 0.0226	0.0311 ± 0.0198	0.0464 ± 0.0355	0.0245 ± 0.0204	0.0369 ± 0.0366
18:3n-3	0.0268 ± 0.0163	0.0230 ± 0.0158	0.0312 ± 0.0250	0.0277 ± 0.0221	0.0236 ± 0.0133	0.0230 ± 0.0127
20:1n-9	0.0142 ± 0.0054	0.0137 ± 0.0049	0.0150 ± 0.0063	0.0114 ± 0.0028	0.0127 ± 0.0077	0.0145 ± 0.0059
20:3n-6	0.0972 ± 0.0650	0.0930 ± 0.0885	0.1139 ± 0.0932	0.0837 ± 0.0501	0.0670 ± 0.0414	0.0741 ± 0.0449
20:4n-6	0.2828 ± 0.1721	0.2830 ± 0.1906	0.3382 ± 0.2024	0.3065 ± 0.1616	0.3082 ± 0.2130	0.3104 ± 0.1962
20:5n-3	0.0169 ± 0.0065	0.0228 ± 0.0268	0.0288 ± 0.0166	0.0384 ± 0.0346	0.0259 ± 0.0161	0.0257 ± 0.0118
22:5n-3	0.0154 ± 0.0087	0.0158 ± 0.0129	0.0167 ± 0.0125	0.0164 ± 0.0092	0.0150 ± 0.0088	0.0244 ± 0.0207
22:6n-3	0.0446 ± 0.0397	0.0471 ± 0.0441	0.0660 ± 0.0630	0.0531 ± 0.0441	0.0430 ± 0.0351	0.0506 ± 0.0534
24:0	0.0170 ± 0.0158	0.0126 ± 0.0114	0.0112 ± 0.0070	0.0120 ± 0.0128	0.0114 ± 0.0112	0.0092 ± 0.0035
FA sum	11.7279 ± 3.6029	10.5482 ± 3.2376	10.5350 ± 2.8161	9.6862 ± 2.7535	8.7144 ± 2.2703	9.1862 ± 2.4528

[a]$P \leq 0.001$, significantly higher after CLA supplementation compared to placebo.
[b]$P < 0.05$, significantly higher after CLA supplementation compared to placebo.

after CLA supplementation in humans. On the other hand, our findings contrast those of five other articles (2–6), which have found that CLA reduced human body fat.

The equivocal findings on the effect of CLA on body fat may be attributed to differences in design, subject characteristics, dosage, and duration of supplementation. In particular, compared to our previous study (3), in which we found a significant reduction in body fat with 1.4 g of CLA for 28 d, the present study used a cautious increase in dosage (owing to concern about safety) and duration of supplementation. This, along with the use of the same method of body fat estimation, led us to expect a positive outcome in the present study as well. Our negative findings may be attributed to the different design of the present study, i.e., the use of each subject as a control of herself (to protect the validity of the results from differences in subject characteristics of different groups) and the use of an homogeneous sample in terms of gender. Interestingly, the participants in each of the studies that has found body fat reduction were of both sexes or men only (2–6), whereas two out of the four studies that showed no significant change in body fat (Ref. 8; present study) have used only women. Additionally, the effect of CLA may depend on the degree of fatness, as most of the studies that have found body fat reduction have used nonobese subjects (2,3,5,6), whereas two of the studies that have not have used obese subjects (7,8).

Concerning leptin, our data contrast the finding of a significant reduction after CLA supplementation despite no effect on body fat (19). This difference may be attributed to differences in dosage (3 vs. 2.1 g/d), supplementation period (64 vs. 45 d), and subject characteristics (obese vs. nonobese women) between the aforementioned and the present study.

The absence of a significant effect of CLA supplementation on the lipidemic profile in the present study is in agreement with the majority of the relevant articles. Specifically, studies have found no effect of CLA on human serum TAG (3–5,9,10), with the exception of the report by Noone *et al.* (20), who found decreased TAG levels after CLA supplementation. Likewise, studies have found no cholesterol lowering effect of CLA (3–5,9,10,20) except for that of Blankson *et al.* (2). Finally, HDL and LDL cholesterol have been reported to be unaffected by CLA supplementation (4,5,9,10,20) with two exceptions where reductions were found (2,3). As pointed out elsewhere, careful scrutiny of the literature suggests that at present it is premature to assign any beneficial role of CLA in terms of its ability to affect blood lipids (21).

Motivated by the implication of CLA in increased risk for liver tumor promotion in mice (22), we measured ALT as an index of liver damage. The finding of no significant change with CLA supplementation provides no evidence of such damage with the regimen used in the present study.

We have determined the incorporation of CLA isomers into lipid classes of human serum before and after CLA supplementation. To be certain that no modification of CLA isomers takes place we have used base-catalyzed transesterification of acyl groups. Isomer modification has been reported to occur with acid catalysis (13), although there is no agreement on the issue (16). Avoidance of possible artifacts was

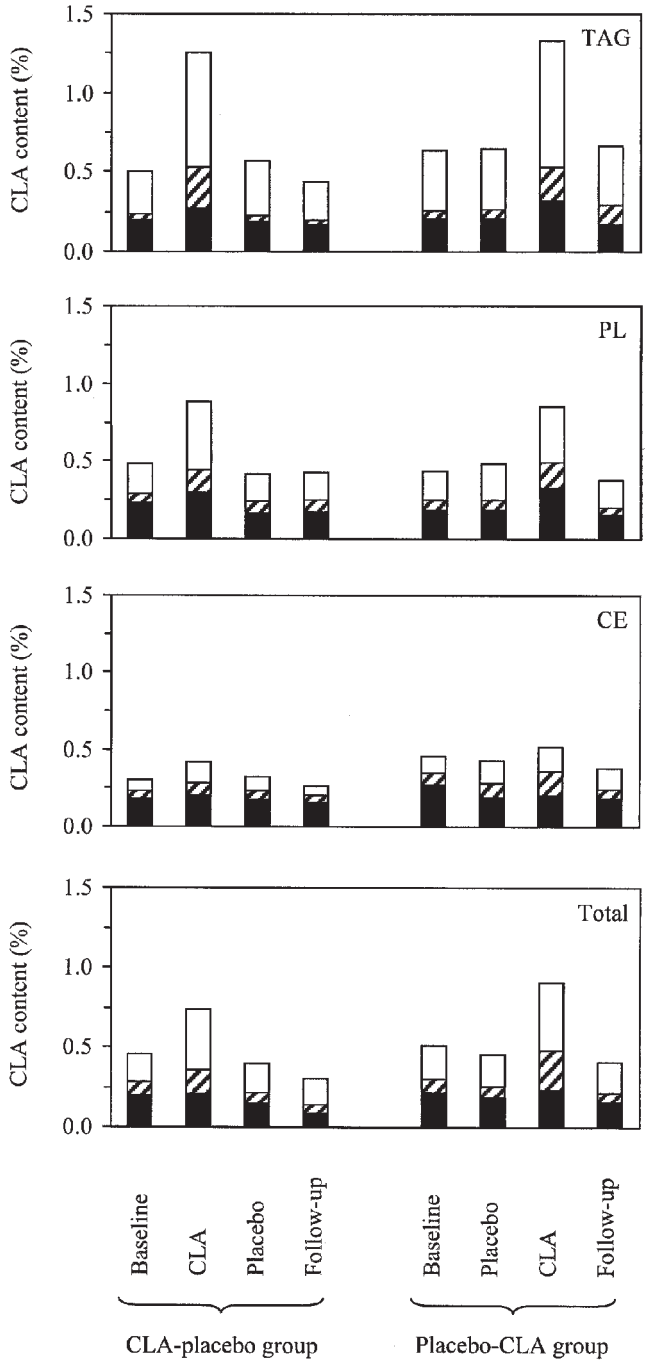

FIG. 1. Molar percentages of *cis,trans*-9,11-CLA (open bars), *trans,cis*-10,12-CLA (hatched bars), and *trans,trans*-9,11-CLA (solid bars) within the acyl groups of serum TAG, phospholipids (PL), cholesteryl esters (CE), and total lipids of the CLA-placebo and the placebo-CLA groups at baseline, end of CLA supplementation, end of placebo supplementation, and follow-up visit.

achieved at the cost of not determining two minor lipid components, nonesterified FA and sphingomyelin, which are not (trans)esterified through base catalysis (13).

The main CLA isomers of serum lipids at baseline were *cis,trans*-9,11 and *trans,trans*-9,11/10,12. These have been reported to exist in dairy products and ruminant fat (17, 23,24). CLA supplementation resulted in significant increases in the percentages of CLA isomers in serum lipids, in agreement with data from similar studies (3,5,10,20). TAG were the lipid class where the most remarkable increase occurred (from 0.57 to 1.29%) and the class with the highest CLA content. In accordance with our previous study (3), CLA was lowest in CE, where linoleate (the common isomer of CLA) is most abundant, thus reiterating the different metabolic fates of these FA.

The incorporation of the two CLA isomers of the experimental capsules (i.e., *cis,trans*-9,11 and *trans,cis*-10,12) into serum lipids increased two- to fivefold with supplementation (specifically, 2.4- and 4.8-fold in TAG, 2.0- and 2.1-fold in PL, and 2.2- as well as 2.4-fold in total lipids, respectively). Interestingly, the percentage of the *trans,trans*-9,11/10,12 isomers (which were not present in the supplement) also increased significantly, although only 1.5-fold and only in TAG and PL. This suggests that part of these isomers may be produced endogenously (probably from the other two isomers).

For the first time, we report on the washout of CLA supplement from human serum lipids. Our data show that a period of 2 wk after cessation of supplementation was sufficient for the return of CLA concentrations to baseline values (Fig. 1).

In conclusion, supplementation of healthy nonobese women with 2.1 g of CLA daily for 45 d caused a two- to fivefold increase in its incorporation into serum TAG, PL, and total lipids. In contrast, the CLA content of CE did not change significantly. Despite the significant increase of CLA levels in blood, there was no evidence of fat reduction. Additionally, there was no change in serum leptin and lipids. Further controlled studies should allow us to settle the discrepancies of the current literature on the effects of this interesting nutrient on human metabolism and the factors that may modulate these effects.

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Vitamin E Supplementation Increases Circulating Vitamin E Metabolites Tenfold in End-Stage Renal Disease Patients

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ABSTRACT: Vitamin E supplementation could elevate circulating vitamin E metabolites while modulating oxidative and inflammatory status in end-stage renal failure patients undergoing hemodialysis. Plasma concentrations of carboxyethyl-hydroxychromanols (α- and γ-CEHC), ascorbic acid, α- and γ-tocopherols, F_2-isoprostanes, and inflammatory biomarkers [tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), ferritin, and C-reactive protein (CRP)] were measured in blood samples obtained from patients (n = 11) before and after dialysis on two occasions prior to, and at 1 and 2 mon of daily vitamin E supplementation (400 IU *RRR*-α-tocopherol). Supplementation nearly doubled plasma α-tocopherol concentrations (from 18 ± 0.5 to 31 ± 1.7 μM, $P < 0.0001$), whereas γ-tocopherol concentrations decreased (from 2.8 ± 0.3 to 1.7 ± 0.2 μM, $P = 0.001$). Serum α-CEHC increased 10-fold from 68 ± 3 to 771 ± 175 nM ($P < 0.0001$), and γ-CEHC increased from 837 ± 164 to 1136 ± 230 nM ($P = 0.008$). Vitamin E supplementation also increased postdialysis hematocrits from 38 ± 1% to 41 ± 1% ($P < 0.001$). Dietary antioxidant intakes (vitamins E and C) were low in most subjects; plasma ascorbic acid levels (88 ± 27 μM) decreased significantly with dialysis (33 ± 11 μM, $P = 0.01$). Plasma IL-6, CRP, TNF-α, and free F_2-isoprostane concentrations were elevated throughout the study. There is a complex relationship between chronic inflammation and oxidative stress that is not mitigated by short-term vitamin E supplementation. Importantly, serum vitamin E metabolite concentrations that increased 10-fold within 30 d of supplementation did not increase further, suggesting routes other than urine for removal of metabolites.

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Renal failure patients undergo high physiological stress, and their compromised renal function results in elevated circulating metabolic by-products, altered lipoproteins, reactive oxidative species, and oxidation products (1–4). These patients are at increased risk for heart disease, cancer, diabetes, and other chronic diseases (5,6). There is a growing recognition that not only oxidative stress but also increased inflammation increase the incidence risk for chronic disease in chronic renal failure patients because inflammatory cytokines, including elevated levels of C-reactive protein (CRP) (5–7) and tumor necrosis factor-α (TNF-α) (7), have been reported.

Supplemental vitamin E in hemodialysis (HD) patients can counteract inadequate dietary intakes and increase antioxidant and anti-inflammatory responses by raising plasma α-tocopherol concentrations. Islam *et al.* (8) reported that α-tocopherol (800 IU/d) for 12 wk significantly decreased LDL susceptibility to oxidation in renal failure patients on dialysis therapy; however, the LDL remained proatherogenic (e.g., it stimulated monocyte-endothelial cell adhesion *in vitro*). However, supplemental vitamin E (800 IU daily) decreased cardiovascular disease risk and mortality in HD patients in the SPACE trial (secondary prevention with antioxidants of cardiovascular disease in end-stage renal disease) (9). Supplemented patients had significantly fewer primary and secondary cardiovascular disease end points and fewer myocardial infarctions than those in the placebo group (9).

Although vitamin E is fat soluble and is excreted in bile (10,11), vitamin E is metabolized to carboxyethyl-hydroxychromanol (CEHC), which is excreted in urine (12). CEHC are not a result of vitamin E oxidation, but rather are a metabolic end-product resulting from ω-oxidation by cytochrome (CYP) p450s, followed by β-oxidation (12–14). Both CYP3A and CYP4F have been implicated in vitamin E metabolism (13,15,16), and the nuclear orphan receptor, pregnane X receptor, that controls CYP3A expression is apparently up-regulated by vitamin E (17). CEHC production increases in response to vitamin E supplements (12); therefore, in HD patients circulating CEHC potentially may reach high plasma concentrations. Stahl *et al.* (18) found in normal subjects supplemented with 500 IU *RRR*-α-tocopherol that serum α-CEHC increased 40-fold. The consequences of elevated CEHC are unknown; however, Jiang *et al.* (19) found in an *in vitro* system that γ-CEHC, but not α-CEHC, has anti-inflammatory properties.

The present study was undertaken therefore to assess the ability of HD patients to metabolize vitamin E and dispose of circulating CEHC, as well as to assess the oxidative and inflammatory stress status of HD patients before and after vitamin E supplementation. Serum and urinary levels of vitamin E

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Abbreviations: α- and γ-CEHC, α- and γ-carboxyethyl-hydroxychromanols; CRP, C-reactive protein; CYP, cytochrome; DTPA, diethylene triamine pentaacetic acid; EPO, erythropoietin; HD, hemodialysis; hsCRP, high-sensitivity CRP; IL-6, interleukin-6; rHuEPO, recombinant human erythropoietin; TIBC, total iron-binding capacity; TNF-α, tumor necrosis factor-α.

metabolites (α- and γ-CEHC) were measured before and after dialysis on two occasions. Oxidative stress was also evaluated before and after the dialysis sessions by measuring plasma antioxidants (ascorbic acid, α- and γ-tocopherols) and a plasma marker of lipid peroxidation (free F_2-isoprostanes). Inflammatory biomarkers, including TNF-α, interleukin-6 (IL-6), ferritin, and CRP, were also assessed before and after dialysis. Subsequently, HD patients were supplemented daily with vitamin E (400 IU) for 2 mon and the above parameters reassessed.

MATERIALS AND METHODS

Subjects. The Oregon State University and the Good Samaritan Hospital, Corvallis, Institutional Review Boards for the Protection of Human Subjects approved the protocol for this study. Eleven subjects undergoing renal dialysis participated from Good Samaritan Hospital Dialysis Unit in Corvallis, Oregon. Each subject provided signed informed consent statements. Inclusion criteria included stable body weight (between 80 and 130% ideal body weight), resting blood pressure less than 160/105 mm Hg, fasting blood glucose concentration less than 7.77 mmol/L (140 mg/dL), and a routine consumption of fewer than three alcoholic beverage servings per day or less than 10 per week), as well as a willingness to maintain normal activity patterns.

Subject characteristics are shown in Table 1. Subjects were relatively healthy throughout this short study, did not have opportunistic infections or inflammatory episodes, and continued taking drugs to maintain normal lipids, blood pressure, and glucose, as necessary. On the Diet/Medical History form, subjects identified their current supplement use. Although multivitamin supplementation had been recommended to patients in the dialysis unit, only two subjects (#4 and #9) reported taking Nephro-vite (R&D Laboratories, Marina del Ray, CA). Nephro-vite contains 60 mg of ascorbic acid and no vitamin E.

Study Design. Blood was obtained on two occasions (Day 0 and Day 14) prior to vitamin E supplementation. Approximately 8 mL of arterial blood was removed from the dialysis tubing and collected into EDTA tubes (Becton Dickinson) before and after dialysis. Subjects were instructed beginning day 15 to consume one 400 IU *RRR*-α-tocopherol supplement (a gift from Archer Daniels Midland Company, Decatur, IL) daily with dinner for 60 d. EDTA blood samples were also obtained at 30 and 60 d after the start of supplementation. All patients were dialyzed 3 d per week using polysulfone, high-flux membranes (Hemoflow F80A Hollow Fiber Dialyzers; Fresenius AG, Dialysis Systems Division, Lexington, MA). The dialysis unit measures adequacy by the urea reduction ratio. Subjects maintained an average adequacy of 70% during the time of the study.

Dietary and supplement intakes were assessed using two 24-h recalls. Subjects were asked to identify their dietary intakes in a 24-h recall before and during supplementation. The 24-h recalls were analyzed by the Food Processor (Esha Research, Salem, Oregon). Data obtained from the analysis were used to establish dietary antioxidant intake of the study subjects.

Sample analyses. Plasma was separated from blood by centrifugation at $850 \times g$ and 4°C for 15 min. Plasma samples were stored at −80°C until analysis. Additional precautions (see below) were taken for ascorbic acid and F_2-isoprostane analyses.

Plasma tocopherols were extracted (20) and analyzed by HPLC with electrochemical detection (21). Peak areas were

TABLE 1
Subject Characteristics

Subject	Age (yr)	BMI[a] (kg/m^2)	Gender	Intravenous iron supplement[b]	Medications	Cause of renal failure
1	54	18.0	F	50 mg/wk	Thyroid USP 60 mg, insulin 70/30 28 u, quinidine sulfate 325 mg, verapamil 300 mg, Paracalcitol injection 2 µg IV	Diabetes
2	66	28.5	F	50 mg/wk	Quinapril 40 mg, levothyroxine 150 mg	Adverse reaction to medication
3	60	27.5	F	50 mg/wk	None	Diabetes
4	53	54.3	M	None	Quinapril 20 mg, minoxidil 10 mg, clopidogrel 75 mg, paracalcitol injection 2 µg IV	Diabetes
5	42	33.5	F	100 mg/wk	Furosemide 80 mg, paracalcitol injection 1 µg IV	Hypertension
6	70	18.3	F	50 mg/wk	Paracalcitol injection 2 µg IV, lisinopril 10 mg	Hypertension
7	73	22.5	M	None	Atenolol 12.5 mg Tues Thurs Sat, atorvastatin 10 mg	Hypertension
8	60	21.0	M	None	Levothyroxine 0.05 mg, paracalcitol injection 1 µg IV, lisinopril 20 mg	Polycystic
9	75	23.0	M	None	Bumetanide 0.5 mg, quinidine sulfate 325 mg, paracalcitol injection 1 µg IV	Hypertension
10	73	38.1	M	None	Losartan potassium 50 mg	Diabetes
11	81	22.3	M	50 mg/wk	Atorvastatin calcium 10 mg, metoprolol 50 mg, lisinopril 20 mg, paracalcitol injection 3 µg IV	Unknown
Mean ± SE	64 ± 4	27.9 ± 3.2	5F/6M			
SE						

[a]BMI, body mass index.
[b]Iron sucrose, $[Na_2Fe_5O_8(OH)\cdot 3(H_2O)_n]\cdot$ sucrose (Venofer; Luitpold Pharmaceutical, Inc., Shirley, NY).

integrated using a Shimadzu 4.2 class–VP automated software program (Columbia, MD), and tocopherol amounts were estimated by comparison to known amounts of authentic compounds.

For ascorbic acid and uric acid analyses, upon isolation, plasma (50 μL) was mixed with an equal volume of freshly prepared, chilled 5% (wt/vol) metaphosphoric acid in 1 mM diethylene-triamine-pentaacetic acid (DTPA) and centrifuged to remove the precipitated proteins. A portion of the supernatant was frozen at −80°C until the day of analysis, within 2 wk of sample collection. Plasma ascorbic acid and uric acid were measured using HPLC with electrochemical detection (22). Ascorbic acid and uric acid standards (in 1 mM DTPA in PBS) were used to quantify the ascorbic and uric acids in the samples.

For CEHC analysis, plasma was converted into serum using thrombin (23). Casual urine samples were collected from those subjects ($n = 7$) capable of producing urine. Serum (18) and urine (24) α- and γ-CEHC were extracted, then were quantified by HPLC with electrochemical detection by comparison to known amounts of Trolox used as an internal standard (24).

An enzyme immunoassay from Cayman Chemical (Ann Arbor, MI) was used to measure plasma isoprostanes. Plasma (1 mL) was frozen in liquid nitrogen and stored at −80°C until the time of analysis. Data were analyzed according to kit instructions.

Circulating levels of serum iron and total iron-binding capacity (TIBC) were determined on three occasions prior to dialysis by the Good Samaritan Hospital staff using standard laboratory methods; data for subject #4 were unavailable to us. The transferrin saturation index was calculated according to the following formula:

$$\text{saturation (\%)} = \text{serum iron/TIBC} \times 100 \quad [1]$$

Plasma ferritin was determined by using a commercial kit, Ferritin MAb (ICN Diagnostics, Inc., Costa Mesa, CA). Data were calculated as described by the kit instructions.

Cytokines IL-6, TNF-α, and high-sensitivity CRP (hsCRP) were measured using immunoassays as reported previously by Devaraj and Jialal (25). Plasma total cholesterol and TG were measured using standard clinical assays from Sigma Chemical (St. Louis, MO).

TABLE 2
Plasma Lipids[a] Are Unchanged by Dialysis or Vitamin E Supplements

Vitamin E	Dialysis	Cholesterol (mmol/L)	TG (mmol/L)
None	Before	3.97 ± 0.18	0.99 ± 0.13
	After	4.06 ± 0.32	0.95 ± 0.10
Supplement	Before	4.02 ± 0.14	1.13 ± 0.14
	After	4.61 ± 0.38	1.15 ± 0.16

[a]To convert to mg/dL, divide by 0.02586 for cholesterol or 0.01129 for TG.

Statistical analysis. Data are expressed as means ± SE. ANOVA with repeated measures of logarithmic transformed data was used to determine whether statistically significant differences occurred for each parameter between before and during supplementation, prior to and postdialysis, on the two occasions during baseline and vitamin E supplementation. All data were analyzed using SPSS statistical software (SPSS Inc., Chicago, IL) and $P < 0.05$ was considered to be statistically significant.

RESULTS

Dietary vitamins C and E. The subjects did not consume many antioxidant-rich foods. Daily median vitamin C intakes were 106 mg; mean intakes were 137 ± 42 mg with values ranging from 1 to 454 mg. Based on the two 24-h recalls, 41% of the subjects consumed less than 75% of the RDA for vitamin C (75 mg for women and 90 mg for men) (26). Daily median vitamin E intakes (excluding the supplement) were 8.2 mg; mean intakes were 10.6 ± 2.7 mg with values ranging from 1 to 38 mg; 68% of subjects consumed less than 75% of the RDA for vitamin E (15 mg α-tocopherol) (26).

α- and γ-Tocopherol, and α- and γ-CEHC concentrations. HD had no effect on circulating concentrations of lipids (Table 2), α-tocopherol, or α-tocopherol/lipids (Table 3). Vitamin E supplementation increased plasma α-tocopherol concentrations (averaged for the two baseline measurements, before and after HD) from 18 ± 0.5 to 31 ± 1.7 μmol/L ($P < 0.0001$) and decreased γ-tocopherol concentrations from 2.8 ± 0.3 to 1.7 ± 0.2 μmol/L ($P = 0.001$). Similar changes were observed when α-tocopherol was expressed per total lipids (sum of molar concentrations of cholesterol and TG).

TABLE 3
Circulating α-Tocopherol and α-CEHC Increase with Vitamin E Supplementation, but Free F_2-Isoprostanes Are Unchanged[a]

Vitamin E	Dialysis	α-Tocopherol (μmol/L)	α-Tocopherol/total lipids[b] (mmol/mol)	α-CEHC (nmol/L)	F2-Isoprostanes (pg/mL)
None	Before	17.9 ± 0.7	3.66 ± 0.23	73 ± 31	836 ± 263
	After	19.9 ± 0.9	3.77 ± 0.21	47 ± 15	602 ± 105
	Before	18.2 ± 0.8	4.34 ± 0.61	64 ± 16	1206 ± 419
	After	21.2 ± 1.5	4.51 ± 0.41	66 ± 25	748 ± 135
Supplement	Before	31.1 ± 2.2	6.09 ± 0.44	820 ± 197	1044 ± 391
	After	36.3 ± 3.0	6.04 ± 0.30	752 ± 176	897 ± 237
	Before	30.8 ± 1.7	6.92 ± 0.97	722 ± 159	1224 ± 410
	After	34.9 ± 2.1	6.78 ± 1.12	612 ± 152	687 ± 115

[a]In response to vitamin E supplementation, increased circulating concentrations of α-tocopherol (ANOVA, main effect, $P < 0.0001$), α-tocopherol/total lipids (ANOVA, main effect, $P < 0.0002$) and α-carboxyethyl-hydroxychromanols (α-CEHC) (ANOVA, main effect, $P < 0.0001$) were observed.
[b]Total lipids = cholesterol + TG.

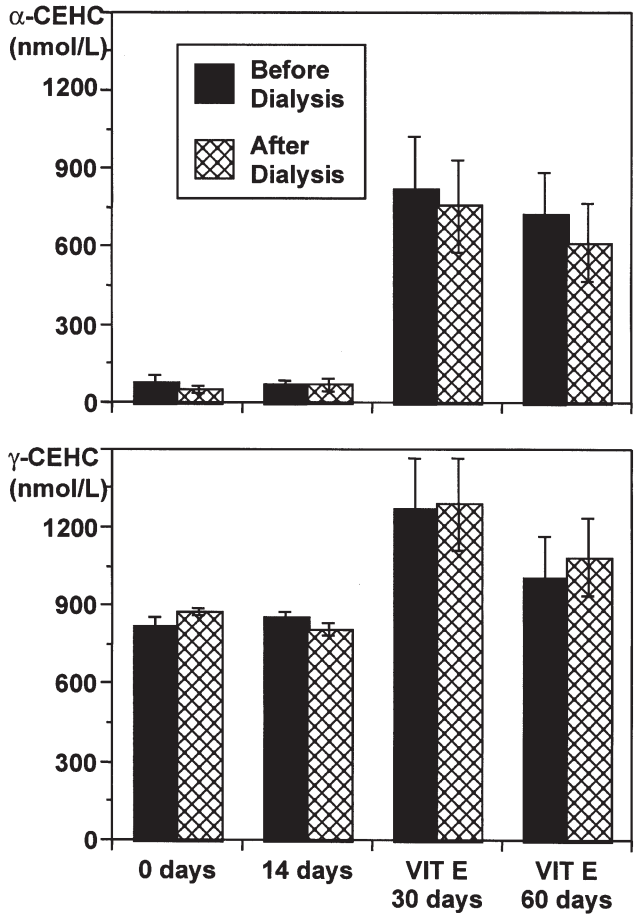

FIG. 1. Serum α-CEHC (upper panel) and γ-CEHC (lower panel) concentrations (mean ± SE) prior to and after dialysis, before and during vitamin E supplementation are shown. Serum α- and γ-CEHC concentrations increased with vitamin E supplementation (ANOVA, main effects, $P < 0.0001$ and $P < 0.007$, respectively). CEHC, carboxyethyl-hydroxychromanols.

Serum CEHC concentrations were measured before and after HD, twice before supplementation and twice after vitamin E supplementation (Table 3, Fig. 1). There were no significant differences between the two baseline samples or between the two supplementation samples. Serum α-CEHC concentrations increased following vitamin E supplementation (main effect, $P < 0.0001$). Serum γ-CEHC concentrations also increased with vitamin E supplementation ($P = 0.007$, Fig. 1).

Urine specimens were obtained before and during vitamin E supplementation from subjects ($n = 7$) capable of producing small amounts of urine. Vitamin E supplementation increased urinary α-CEHC/creatinine from 0.4 ± 0.1 to 1.2 ± 0.3 nmol/mg ($P = 0.04$), but urinary γ-CEHC/creatinine was unchanged (Fig. 2).

Erythrocytes are highly susceptible to hemolysis if they contain insufficient α-tocopherol (26); therefore, hematocrits were also measured. Hematocrits increased in response to both dialysis (ANOVA main effect, $P < 0.02$) and vitamin E supplementation (ANOVA main effect, $P < 0.02$; Fig. 3), but the latter increase was due to increased postdialysis hematocrits ($P < 0.001$).

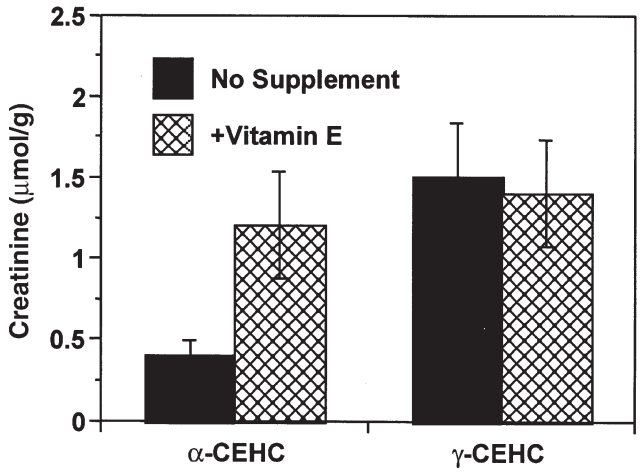

FIG. 2. Urinary α- and γ-CEHC/creatinine (μmol/g) are shown. Vitamin E supplementation increased urinary α-CEHC/creatinine ($P < 0.04$), but not γ-CEHC/creatinine. For abbreviation see Figure 1.

These data suggest additional α-tocopherol was needed for optimal erythrocyte membrane protection during HD.

Plasma ascorbic acid and uric acid concentrations. Plasma ascorbic acid concentrations varied widely between subjects. Prior to HD, four subjects had plasma ascorbic acid concentrations < 40 μM; after HD, all subjects except one had plasma ascorbic acid concentrations < 40 μM. HD decreased ($P < 0.01$, ANOVA main effect) ascorbic acid concentrations from 88 ± 27 to 33 ± 11 μM and during vitamin E supplementation from 59 ± 17 to 21 ± 4 μM.

Plasma uric acid levels were within the normal range (420 ± 25 μM) and decreased with HD (105 ± 6.8, $P < 0.0001$, ANOVA main effect) and vitamin E supplementation (328 ± 19, $P < 0.002$, ANOVA main effect).

Iron parameters and erythropoietin (EPO) dosage. Plasma ferritin concentrations were more than 2 SD above normal values (Table 4). Moreover, ferritin concentrations increased with

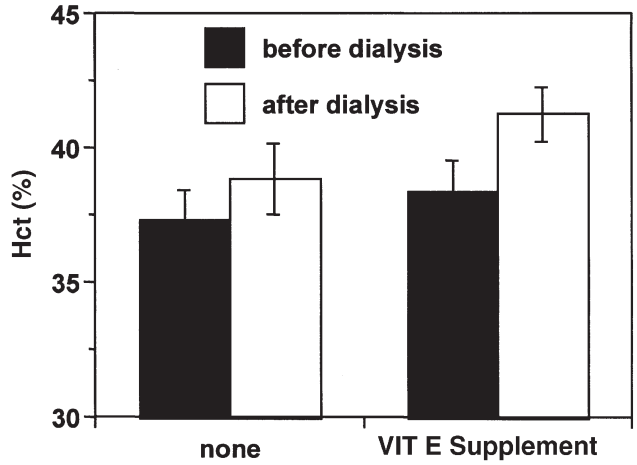

FIG. 3. Hematocrits (Hct %) increased in response to both dialysis (ANOVA main effect, $P < 0.02$) and vitamin E supplementation in postdialysis hematocrits ($P < 0.001$).

TABLE 4
Serum Ferritin Concentrations, Iron Parameters, and Erythropoietin (EPO) Dosage[a]

Parameters	Prior to supplement	Vitamin E supplement	
		1 mon	2 mon
Ferritin (μg/L)			
Prior to dialysis	439 ± 76[a]	381 ± 58[b]	412 ± 68[c]
Postdialysis	498 ± 89[a]	466 ± 75[b]	435 ± 78[c]
Serum iron (μmol/L)	9.2 ± 1.0	8.5 ± 0.9	9.4 ± 0.8
TIBC (μmol/L)	33.0 ± 1.9	33.4 ± 1.0	33.7 ± 1.1
Transferrin saturation (%)	28.2 ± 3.4	26.1 ± 3.1	28.3 ± 2.1
rHuEPO dose (unit)	2955 ± 512	3227 ± 672	3136 ± 765

[a]Abbreviations: TIBC, total iron-binding capacity; rHuEPO, recombinant human EPO. Values are means ± SE, $n = 11$, for EPO dose; all other data were from 10 subjects (subject #4 data were lost). [a,b,c]Significantly different from prior to dialysis session ($P < 0.02$).

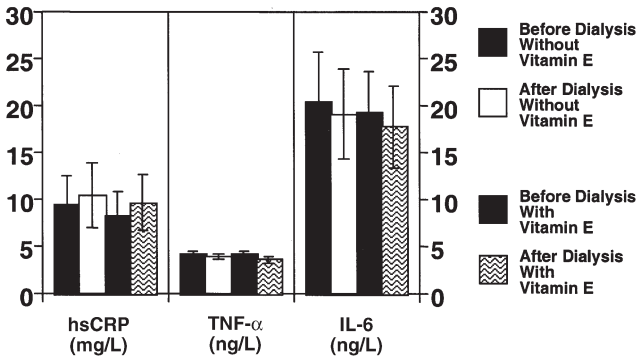

FIG. 4. Dialysis increased plasma hsCRP ($P < 0.002$, ANOVA main effect), decreased plasma TNF-α concentrations ($P < 0.01$, ANOVA main effect), and had no effect on IL-6 concentrations. Vitamin E supplementation had no effect on these parameters. hsCRP, high-sensitivity C-reactive protein; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6.

HD ($P < 0.001$, ANOVA main effect) but were unchanged by vitamin E supplementation.

Both serum iron and TIBC were 2 SD lower than normal values (Table 4) (27). Serum iron, transferrin saturation, TIBC, and recombinent human erythropoietin (rHuEPO) dosage were unchanged by vitamin E supplementation or HD. As noted in Table 1, some subjects received intravenous iron, but the amounts given were constant throughout the study.

Plasma markers of inflammation and oxidative stress. HD increased plasma hsCRP ($P < 0.002$, ANOVA main effect, Fig. 4) but decreased plasma TNF-α concentrations ($P < 0.01$, ANOVA main effect), whereas vitamin E supplementation had no effect on these parameters. IL-6 concentrations were unchanged by either HD or vitamin E supplementation.

Free F_2-isoprostane concentrations were elevated in the subjects, but were unaffected by HD or by vitamin E supplementation. Table 3 shows the values for each HD session, before and after vitamin E supplementation.

DISCUSSION

Circulating α-tocopherol concentrations in HD patients nearly doubled with vitamin E (400 IU) supplementation, whereas the vitamin E metabolite, α-CEHC, increased 10-fold (Table 3, Fig. 1). Schultz *et al.* (28) have suggested that vitamin E metabolites are markers of vitamin E adequacy, based on the observation that urinary α-CEHC increased when vitamin E supplements were consumed. We found in all subjects that both serum α- and γ-CEHC concentrations increased with supplementation. Prior to vitamin E supplementation, α-CEHC concentrations were ~150 nmol/L and increased to 1500–2000 nmol/L; γ-CEHC were at 1500 nmol/L and increased to 2000 nmol/L. Serum vitamin E metabolites in HD patients were higher than previously reported by Stahl *et al.* (18), who found in normal subjects that α- and γ-CEHC increased with 500 IU *RRR*-α-tocopherol supplementation from 5–10 to 200 nmol/L for α-CEHC and from 50 to 80 nmol/L for γ-CEHC. Similar changes in normal subjects were reported by Radosavac *et al.* (29). Galli *et al.* (30) reported that supplementation with γ-tocopherol increased serum γ-CEHC but not α-CEHC concentrations.

Urinary CEHC were measured in seven subjects who produced small amounts of urine [less than 1 cup (<240 mL)/per week]. α- and γ-CEHC (μmol/mg creatinine) were within the normal range we reported (31), and γ-CEHC were within the range that Swanson *et al.* (32) reported for normal subjects. These findings suggest that in HD patients, despite the increase in circulating α- and γ-CEHC, excretion by the kidney was limited. Moreover, circulating CEHC did not increase between 1 and 2 mon of vitamin E supplementation, suggesting that urinary excretion may not be the only route for removal of vitamin E metabolites. There is no information about biliary CEHC excretion in humans, but CEHC have been detected in rat bile (33,34). Thus, biliary excretion may also be a route of CEHC excretion in humans.

Before supplementation with vitamin E, plasma α-tocopherol concentrations in HD patients were at the low end of normal (26). Vitamin E supplementation may be beneficial in HD patients because erythrocyte α-tocopherol concentrations were reported to be low in HD patients, even with normal plasma α-tocopherol levels (35,36). Taccone-Gallucci *et al.* (37) suggested that this discrepancy may be due to impaired α-tocopherol transfer from HDL to erythrocytes. It is noteworthy that in our study, vitamin E supplementation increased postdialysis hematocrits, as has been reported previously (35).

The causes of anemia in HD patients are multiple, but the primary causes include insufficient production of EPO by the

diseased kidney, iron deficiency, and shortened erythrocyte survival (38). Several investigators (36,39,40) have reported that vitamin E supplementation in HD patients decreased the rHuEPO dosage and improved its therapeutic effect. In our limited patient population, all subjects were undergoing rHuEPO therapy; however, no change in the rHuEPO dose with vitamin E supplementation was noted (Table 4). Our HD patients had low serum iron and TIBC, but their plasma ferritin levels were high, suggesting that the amount of iron available for erythropoiesis was low. Nonetheless, vitamin E supplementation increased erythrocyte α-tocopherol concentrations (data not shown) and increased hematocrits (Fig. 3). These findings suggest that anemia in HD patients can partly be attributed to insufficient antioxidant protection, thereby allowing increased rates of lipid and protein oxidation and ultimately erythrocyte membrane destruction.

Plasma free F_2-isoprostanes in the HD patients we studied were elevated compared with those reported for normal subjects. Additionally, the low ascorbic acid status of the HD subjects may have contributed to elevated lipid peroxidation (41). Handelman *et al.* (6) also found HD patients' esterified F_2-isoprostanes were elevated. Ikizler *et al.* (42) reported that both free and esterified F_2-isoprostane concentrations were elevated in HD patients. Free F_2-isoprostanes might be expected to be cleared during HD, but we found no changes with HD, suggesting that they also may be generated during HD. It is surprising that in our HD patients, elevated free F_2-isoprostane concentrations were not decreased by vitamin E supplementation because the same dose and duration were effective in 184 normal subjects (43). It may be that the limited population size, as well as the short duration of vitamin E supplementation, was insufficient to decrease oxidative stress in HD patients, because 1-yr supplementation with vitamin E (200 mg daily) decreased F_2-isoprostanes in mildly hypercholesterolemic men (44).

The inflammatory status of HD patients was assessed using biomarkers (hsCRP, TNF-α, and IL-6). These biomarkers in diabetic subjects have been decreased by vitamin E supplementation when given at 1200 IU (25,45), but 400 IU vitamin E was insufficient to act as an anti-inflammatory agent (46). The HD patients studied here were found to have chronic inflammation; circulating IL-6 was elevated, and hsCRP concentrations were borderline high. Although TNF-α concentrations were not abnormal, HD did decrease TNF-α concentrations and increase CRP concentrations. Thus, the level of inflammation may have been too high to have been modified by 400 IU vitamin E given for only 60 d. Overall, the high level of oxidative and inflammatory stress found in this limited group of HD patients was not modified by vitamin E supplements.

In summary, HD patients had low vitamin E and vitamin C intakes, low circulating antioxidant levels, and high circulating levels of F_2-isoprostanes and biomarkers of inflammation. Supplementation with 400 IU vitamin E for 60 d did not alter markers of oxidation or inflammation but did increase both plasma α-tocopherol and vitamin E metabolite concentrations. Both α- and γ-CEHC increased to abnormally high concentrations in the plasma.

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A Sardine Oil-Rich Diet Increases Iron Absorption but Does Not Compensate the Hypoferremia Associated with Inflammation

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ABSTRACT: Inflammatory disorders are associated with a decrease in iron absorption because of the blockade of iron in storage organs. Fish oils, rich in n-3 PUFA, are used to prevent and treat inflammatory disorders and increase iron absorption in noninflamed rats. Here we examine whether n-3 PUFA prevent inflammation-related hypoferremia. Two groups of rats were fed isoenergetic semipurified diets (a standard diet and a sardine oil-rich diet). A carrageenan granuloma was induced in half of the rats of each dietary group. Ferrokinetic studies using ^{59}Fe, hematological analyses, and iron store evaluation were performed in noninflamed and inflamed rats. Although dietary n-3 PUFA increased ^{59}Fe absorption in carageenan-treated rats, they did not restore the hypoferremia associated with inflammation, which is due to several mechanisms. Among these mechanisms, the blockade of iron in storage organs is relevant. However, this blockade was less evident in the spleen of inflamed rats fed the sardine oil diet.

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The alterations in plasma iron status observed in inflammatory disorders are caused by a blockade of iron in storage organs (1,2), a decrease in iron absorption (3), and an increase in ferritin synthesis under the influence of interleukin-1 and tumor necrosis factor (4,5). n-3 PUFA have beneficial effects on inflammation (6) and rheumatoid arthritis (7), since they may reduce the production of inflammatory mediators by cyclooxygenase (8,9) or lipoxygenase (8), and reduce superoxide anion production by polymorphonuclear leukocytes from an inflammatory exudate (10). However, consumption of n-3 PUFA in inflammatory disorders may have adverse effects because of their susceptibility to oxidation.

A diet containing sardine oil as the source of n-3 PUFA (18.6% EPA, 20:5n-3; and 2.14% DHA, 22:6n-3) decreases iron stores in liver and spleen, which strongly increases iron absorption (11) when compared with rats fed a standard diet. These changes are due to an increased susceptibility of erythrocyte membranes to lipid peroxidation. However, little is known about the effect of an n-3 PUFA-rich diet on iron absorption in inflammatory disorders. When animals are fed diets supplemented with n-3 PUFA to reduce inflammation, an increase in iron absorption due to the high erythrocyte turnover has been reported (11). Moreover, inflammation in rats is associated with a reduction in iron absorption (3). Thus, two opposite effects on iron absorption would take place.

We aimed to study the effect of a sardine oil-rich diet on iron absorption in rats with an inflammatory syndrome and its repercussion on the associated hypoferremia, and on the oxidant and antioxidant parameters of erythrocytes. Results were compared with those obtained from rats fed a balanced-FA diet.

MATERIALS AND METHODS

Animals and diets. Sixty 4-wk-old male Sprague-Dawley rats were purchased from Harlan Ibérica (Barcelona, Spain) and housed in the Department of Animal Care at the Faculty of Biology at the University of Barcelona. The experimental protocols were reviewed and approved by the Ethical Committee of the Faculty of Biology, in accordance with the European Community guidelines.

For 18 wk, the rats were fed one of the following isoenergetic semipurified diets (g/kg): vitamin-free delipidated casein, 220; DL-methionine, 1; mineral mix, 45; vitamin mix, 10; cellulose, 20; corn starch, 436; and sucrose, 218. In addition, the standard diet contained 2.5% canola oil (25 g/kg) + 2.5% peanut oil (25 g/kg), and the n-3 PUFA-rich diet contained 5% sardine oil (50 g/kg) (Afampes 160; Afamsa, Vigo, Spain). Diets were stored in small packages at 4°C under nitrogen, and fresh food was given to the rats every day to avoid oxidation. Diets contained 48 mg iron/kg and 100 IU of all-*rac*-α-tocopherol/kg (50 mg/kg was provided by oils and 50 mg/kg was added in the vitamin mix) from Sigma Chemical (St. Louis, MO). Thus, the vitamin E concentration was the same in the two diets. No other antioxidants were present in the oils or diets, and rats were weighed weekly. The PV of the sardine oil diet was <10 meq/kg when ready for consumption. Room temperature was maintained at 21–23°C, with 40–60% relative humidity. The room was lit on a 12-h light/dark cycle.

A carrageenan granuloma (12) was induced randomly induced in half of the rats of each group at the end of the 16th week of the study diet by an injection of 6 mL of air under the skin on the back, followed by 4 mL of carrageenan 2% (wt/vol) 24 h later. Six days later, nine rats from each subgroup were used for ferrokinetic studies, which lasted 7 d, and the remaining six rats were used for the nonradioactive tests. Blood samples were withdrawn from diethyl-ether-anesthetized animals in the

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Abbreviations: IRE, iron responsive elements; IRP, iron regulatory proteins; SOD, Cu-Zn-superoxide dismutase; SELP, susceptibility of erythrocytes to lipid peroxidation; SOD, superoxide dismutase; TIBC, total iron-binding capacity.

presence of heparin. All rats were killed at the end of the 18th week of diet. Livers were perfused with 0.9% NaCl through the subhepatic vein, excised, and weighed. Spleens were also removed and weighed.

Ferrokinetic studies. At the end of the 17th week of diet, each rat received 74 kBq $^{59}FeSO_4$ provided by Dupont (Nemours, Germany) with specific radioactivity of 1.6 GBq ^{59}Fe/mg by gastric intubation together with 0.082 mg of $FeSO_4$. Rats were maintained in metabolic cages for a further week to measure the radioactivity in feces, erythrocytes, liver, and spleen with the 1282 CompuGamma CS gamma well-type scintillation counter from Pharmacia-Wallace (Turku, Finland). Feces were collected daily, and blood samples were taken from the retro-orbital sinus on day 7 to measure the radioactivity of erythrocytes from 0.5 mL of blood. At the end of this period, rats were killed, and the radioactivity in liver and spleen was also measured. Results are expressed as a percentage of iron administered as: ^{59}Fe absorbed as the mean of the difference between ^{59}Fe administered and ^{59}Fe eliminated in feces, and ^{59}Fe incorporated into erythrocytes, liver, and spleen. Counts were corrected for radioactive decay.

Hematological analyses and iron store evaluation. Blood was withdrawn by cardiac puncture at the end of the 18th week of diet and the following parameters were assessed: packed cell volume, hemoglobin concentration, plasma iron concentration, and total iron-binding capacity (TIBC) by a ferrozine technique (Boehringer Mannheim, Mannheim, Germany).

Erythrocytes were counted using a Coulter counter (Coulter Electronics, Hialeah, FL). The ferritin iron (13) and non-heme iron (14) were measured in liver and spleen homogenates. The susceptibility of erythrocytes to lipid peroxidation (SELP) was determined by measuring the TBARS (15). Cu- Zn-superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured in erythrocytes by a commercial kit (Oxis International, Portland, OR), and results were expressed as U/g hemoglobin. Catalase (EC 1.11.1.6) activity in erythrocytes was determined at 20°C by monitoring the decomposition of hydrogen peroxide at 240 nm (16), and the rate constant (k, $k = 10^7$ L/mol·s) for the first 30 s was calculated. Results were expressed as k/g hemoglobin.

Statistical analysis. Results are expressed as mean ± SEM of nine rats for ferrokinetic studies and six rats for the nonradioactive tests. Statistical analysis of the data was performed using SigmaStat statistical software, version 1.0 (Jandel Scientific Software, San Rafael, CA). The effect of the two main factors (diet and inflammation) on all variables was assessed by a two-way ANOVA, which allowed us to study its interaction. To isolate which group or groups differed from the others, results were compared using an all-pairwise multiple comparison procedure (Student–Newman–Keuls method). Differences were considered significant when $P < 0.05$.

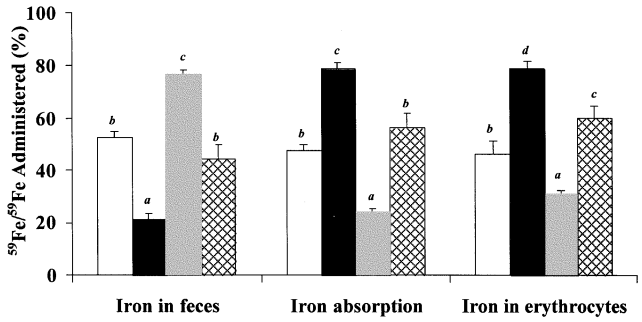

FIG. 1. Mean ^{59}Fe/^{59}Fe administered (%) eliminated in feces, absorbed and incorporated into erythrocytes in normal rats fed standard diet (open bar), or sardine oil diet (solid bar) and in carrageenan-treated rats fed standard diet (gray bar) or sardine oil diet (cross-hatched bar). Values are means of nine observations with their SEM indicated by vertical bars. Two-way ANOVA revealed that there was a diet effect ($P < 0.001$) and an inflammation effect ($P < 0.001$) on the three parameters, but there was no interaction between inflammation and diet ($P > 0.05$). Bars with different superscripts are significantly different ($P < 0.05$).

RESULTS

Ferrokinetic studies. There were no significant differences among groups in the mean final body weight (569 ± 14 g in noninflamed rats fed the standard diet).

Feeding rats with a diet rich in sardine oil resulted in increased ^{59}Fe absorption and ^{59}Fe in erythrocytes (Fig. 1). On the other hand, inflammation decreased ^{59}Fe absorption in both the sardine oil (29%) and standard diet (50%) groups. ^{59}Fe absorption by carageenen-treated rats fed a sardine oil diet was similar to that observed in the noninflamed control group (Fig. 1). No ^{59}Fe was found in plasma after 7 d of ^{59}Fe administration. Inflammation reduced ^{59}Fe incorporated into erythrocytes by 32 and 24% in rats fed the standard and sardine oil diets, respectively. However, the value in inflamed rats fed the sardine oil showed a 90% increase ($P < 0.05$) compared with those fed the standard diet (Fig. 1).

Whereas the incorporation of ^{59}Fe in liver was affected only by the diet, the ^{59}Fe present in spleen was affected by the two factors, diet and inflammation (Fig. 2). In both cases there was a significant interaction between the two factors. Thus, the ^{59}Fe in liver and spleen was increased by the sardine oil-rich diet, but inflammation reduced the values to levels similar to those observed in the noninflamed standard group.

Hematological analyses and iron store evaluation. Inflammation decreased the packed cell volume, the blood hemoglobin, and the plasma iron concentration, and it had no effect on the TIBC and the erythrocyte number (Table 1). In contrast, TIBC was the only parameter affected by diet ($P < 0.01$). There was not a significant interaction between inflammation and diet in any of the hematological parameters analyzed.

Although the relative weight of liver was similar among groups, the relative weight of spleen was increased in carageenen-treated rats fed the sardine oil diet, and there was a significant interaction between inflammation and diet (Table 2). Thus, whereas inflammation increased the relative weight of spleen in rats fed the standard diet by 12.5%, in carageenan-treated rats fed the sardine oil-rich diet the increase was 70%. Inflammation and diet had opposite effects on iron stores: Rats fed the sardine oil diet showed in general lower iron levels in storage organs (liver and spleen) than the standard group. Independently,

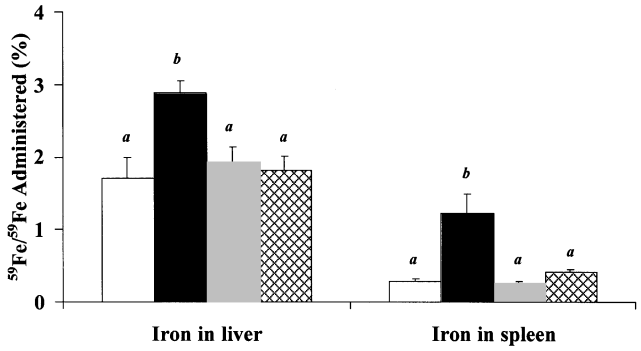

FIG. 2. Mean $^{59}Fe/^{59}Fe$ administered (%) present in liver and spleen 1 wk after ^{59}Fe administration in normal rats fed standard diet (open bar), or sardine oil diet (solid bar) and in inflamed rats fed standard diet (gray bar) or sardine oil diet (cross-hatched bar). Values are means of nine observations with their SEM indicated by vertical bars. Two-way ANOVA revealed that (i) there was a diet effect ($P < 0.05$) and an interaction between inflammation and diet ($P < 0.01$) on ^{59}Fe in liver, but inflammation had no significant effect, and (ii) there was a diet effect ($P < 0.01$), an inflammation effect ($P < 0.001$), and an inflammation × diet interaction ($P < 0.01$) in ^{59}Fe in spleen. Bars with different superscripts are significantly different ($P < 0.05$).

inflammation produced an increase in both iron stores. Significant interactions between inflammation and diet were observed in ferritin and non-heme iron in liver.

The dietary administration of sardine oil increased the SELP and Cu-Zn-SOD activity in erythrocytes (Table 3) but decreased the catalase activity. The activity of Cu-Zn-SOD was the only erythrocyte parameter influenced by inflammation.

DISCUSSION

Fish oil and n-3 PUFA are used in the treatment of inflammatory disorders, either in experimental models (17–20) or clinically (6,7,21–23). They reduce the synthesis of pro-inflammatory arachidonic acid-derived eicosanoids (8,9), tumor necrosis factor, and interleukin-1 production (24,25), and superoxide anion release by polymorphonuclear cells (10) *in vitro*.

Among the iron-related hematological parameters studied in this paper, only TIBC was reduced by the sardine oil-rich diet, while iron stores were low. This indicates that iron levels in the blood of rats fed the sardine oil-rich diet are maintained through iron stores that are reduced, and thus ^{59}Fe absorption

TABLE 1
Hematologic Indices and Iron-Related Parameters in Rats Given Various Diets and Treatments[a]

Diet	PCV (%)	Hemoglobin (g/100 mL)	Plasma iron (μmol/L)	TIBC (μmol/L)	Erythrocytes (10^{12}/L)
Standard	45.9 ± 0.86^{c}	17.3 ± 0.33^{d}	24.0 ± 1.67	106.8 ± 5.27^{c}	6.84 ± 0.33
Sardine	45.2 ± 0.72^{c}	17.1 ± 0.38^{c,d}	27.1 ± 2.82	81.6 ± 4.72^{b}	7.50 ± 0.43
Standard-inflamed	40.7 ± 0.79^{b}	15.6 ± 0.32^{b}	23.5 ± 1.40	109.0 ± 8.01^{c}	6.34 ± 0.15
Sardine-inflamed	42.2 ± 0.40^{b}	16.1 ± 0.19^{b,c}	19.3 ± 1.73	94.9 ± 1.42^{b,c}	6.81 ± 0.24
Multivariate analysis[b]					
Effect of:					
Diet	NS	NS	NS	0.01	NS
Inflammation	0.001	0.001	0.05	NS	NS
Diet x Inflammation	NS	NS	NS	NS	NS

[a]Results are expressed as mean ± SEM of six rats per group. Numbers in the same column with different superscripts are significantly different ($P < 0.05$). PCV, packed cell volume; TIBC, total iron-binding capacity.
[b]Values in the multivariate analysis table refer to *P* values. NS, not significant ($P > 0.05$).

TABLE 2
Ferritin Iron and Non-Heme Iron Contents in Liver and Spleen of Rats Given Various Diets and Treatments[a]

	Liver			Spleen		
	Relative weight (g/100 g)	Ferritin iron (μmol/g)	Non-hem-iron (μmol/g)	Relative weight (g/100 g)	Ferritin iron (μmol/g)	Non-heme iron (μmol/g)
Standard	2.39 ± 0.04	0.40 ± 0.02^{c}	1.85 ± 0.05^{c}	0.155 ± 0.017^{b}	3.64 ± 0.83^{b}	19.1 ± 1.97^{c}
Sardine	2.52 ± 0.11	0.18 ± 0.01^{b}	0.92 ± 0.07^{b}	0.142 ± 0.007^{b}	2.90 ± 0.41^{b}	13.3 ± 1.68^{b}
Standard-inflamed	2.57 ± 0.26	0.49 ± 0.06^{c}	2.37 ± 0.25^{d}	0.175 ± 0.014^{b}	6.35 ± 0.32^{c}	24.3 ± 1.28^{d}
Sardine-inflamed	2.40 ± 0.03	0.49 ± 0.02^{c}	2.32 ± 0.08^{d}	0.235 ± 0.004^{c}	4.46 ± 0.31^{b}	16.9 ± 1.02^{b,c}
Multivariate analysis[b]						
Effect of:						
Diet	NS	0.01	0.001	NS	0.05	0.001
Inflammation	NS	0.001	0.001	0.001	0.001	0.01
Diet × Inflammation	NS	0.01	0.01	0.01	NS	NS

[a]Results are expressed as mean ± SEM of six rats per group. Numbers in the same column with different superscript are significantly different ($P < 0.05$).
[b]Values in the multivariate analysis table refer to *P* values. NS, not significant ($P > 0.05$).

TABLE 3
Susceptibility to Lipid Peroxidation (SELP), Cu-Zn-Superoxide Dismutase, and Catalase Activity in Erythrocytes from Rats Given Various Diets and Treatments[a]

	SELP (nmol TBARS/g Hb)	SOD (U/g Hb)	Catalase (k/g Hb)
Standard	645 ± 19[b]	3124 ± 76[c]	233.9 ± 14.0[c]
Sardine	955 ± 72[c]	3202 ± 108[c]	178.6 ± 8.7[b]
Standard-Inflamed	746 ± 8[b]	2631 ± 146[b]	208.6 ± 9.2[c]
Sardine-Inflamed	940 ± 33[c]	3086 ± 96[c]	166.0 ± 5.1[b]
Multivariate analysis[b]			
Effect of:			
Diet	0.001	0.05	0.001
Inflammation	NS	0.05	NS
Diet × Inflammation	NS	NS	NS

[a]Results are expressed as mean ± SEM of six rats per group. Numbers in the same column with different superscript are significantly different ($P < 0.05$). Hb, hemoglobin; SELP, susceptibility of erythrocytes peroxidation; to lipid SOD, superoxide dismutase.
[b]Values in the multivariate analysis table refer to *P* values. NS, not significant ($P > 0.05$).

is increased. Inflammation induces hypoferremia, considered a host mechanism against invading organisms or malignant cells, which are deprived of vital iron. This hypoferremia is achieved by the action of multiple mechanisms, but it is mainly due to a negative balance between the destruction and production of erythrocytes and a decrease in erythrocyte survival time (26). The decrease in erythrocyte production is secondary to reduced iron availability because of decreased iron reutilization (1) and absorption (3), and to erythropoietin dysfunction (27). The proinflammatory cytokines generated in an inflammatory process play a key role in the synthesis of acute-phase proteins (28,29), which alter iron metabolism. However, the reduction in cytokines by n-3 PUFA (24,25) may also be involved in the decrease in iron stores in rats fed the sardine oil-rich diet.

In the present paper, we report that ^{59}Fe absorption increased in rats fed a sardine oil-rich diet, which is associated with low iron stores. However, the percentage of ^{59}Fe absorbed in inflamed rats fed the sardine oil reached the same level or even increased (136%) compared to normal or carageenan-treated rats fed the standard diet, respectively. It seems that dietary sardine oil compensates for the decrease in iron absorption observed in rats with inflammation, but all the ^{59}Fe was diverted mainly to erythrocytes. The SELP and the antioxidant defenses of erythrocytes are affected by the diet, and both are related to the FA composition of erythrocyte membranes (11). The decrease in catalase activity can be attributed to the diet, which, together with a high peroxidation tendency, favors increased erythrocyte turnover and explains the increase in ^{59}Fe in erythrocytes in regard to ^{59}Fe absorption. It is likely that in this situation only small amounts reached the liver and spleen, which can be regarded as indexes of reduced erythrocyte survival time. Thus, ^{59}Fe absorption is regulated by iron stores in liver and spleen, as expected (1). The hypoferremia induced by carrageenan inflammation may not have been followed by a detected decrease in TIBC because of the long time (13 d) between carrageenan injection and the sacrifice of the rats (1). It is accepted that macrophages present in liver and spleen, through processing hemoglobin iron from senescent cells, provide iron for erythropoiesis. Moreover, heme oxygenase facilitates the release of iron from hepatic and renal cells (30). The packed cell volume and hemoglobin in carageenan-treated rats fed the standard diet is low because of the reduced iron stores in liver and spleen, as reported elsewhere (1,2,28,31,32). However, iron stores are reduced in rats fed sardine oil, which do not have inflammation, and inflammation induced a significant reduction in the spleen compared to carageenan-treated rats fed the standard diet, the values remaining at the same level as in normal rats fed the standard diet. Thus, a sardine oil-rich diet is able to compensate to some extent for the increase in ferritin and non-heme iron in the spleen induced by inflammation. This fact, together with an increase in iron absorption, indicates that n-3 PUFA can have implications for altering the hypoferremia observed in a more chronic inflammatory process and in the anemia of chronic diseases such as infectious or neoplastic disorders where a blockade of iron in organ stores has been described (33). In this regard different treatments have been tested, but their effectiveness remains unclear (34).

Intracellular iron homeostasis is controlled by cytoplasmic iron regulatory proteins (IRP), which regulate several mRNA containing iron-responsive elements (IRE) in their untranslated regions (35). IRP1 is a bifunctional protein that can react either as a cytoplasmic aconitase or as an IRE-binding protein (36), depending on iron stores, and we have demonstrated that IRP1 activity and expression in liver and spleen were responsive to fish oil-feeding and iron stores (37). In addition to iron availability, other signals such as nitric oxide, which is critically dependent on the levels of nitric oxide synthase, and oxidative stress can modulate the activity of IRP and thus influence cellular iron metabolism (38–40). n-3 PUFA affect the production of eicosanoids (8,9,41) and cytokines (7) and may modulate protein kinase C activity (42). Thus, they may alter the generation of reactive oxygen species and nitric oxide. We have observed that resident peritoneal macrophages from rats fed a fish oil-rich diet, stimulated with phorbol 12-myristate 13-acetate,

show an increase in superoxide anion and nitric oxide generation (43). The regulation of iron metabolism in rats with inflammation that are fed a fish oil-rich diet, in which nitric oxide production is increased, is even more complex.

The increase in iron absorption in carageenan-treated rats fed the sardine oil-rich diet is due to the alteration of iron metabolism, owing to erythrocyte oxidative stress, and it could be anticipated to restore the decrease in iron absorption induced by inflammation. However, although stored iron is reduced to a lesser degree in the spleen, it did not compensate for the hypoferremia associated with inflammation under our study conditions.

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Hepatic Δ9, Δ6, and Δ5 Desaturations in Non-Insulin-Dependent Diabetes Mellitus eSS Rats

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ABSTRACT: Both diabetes mellitus type 1 and diabetes mellitus type 2 are widespread diseases that alter carbohydrate and lipid metabolism. e Stilmann-Salgado (eSS) rats are experimental animals that spontaneously evolve to a state similar to that of young people affected by non-insulin-dependent diabetes mellitus (NIDDM; type 2). Using 6-mon-old eSS rats that, according to the literature [Martinez, S.M., Tarrés, M.C., Montenegro, S., Milo, R., Picena, J.C., Figueroa, N., and Rabasa, S.R. (1988) Spontaneous Diabetes in eSS Rats, *Acta Diabetol. Lat. 25*, 303–313], had already developed insulin resistance, we investigated the changes evoked on Δ9, Δ6, and Δ5 liver desaturases. The abundance of mRNA and enzymatic activities were measured, as well as the FA composition of liver microsomal lipids. Compared to control rats, the mRNA content and activity of SCD-1 (stearoyl CoA-desaturase, isoform of the Δ9 desaturase) were significantly higher, whereas the mRNA and activities of Δ6 and Δ5 desaturases were not significantly modified. Correspondingly, the proportion of 18:1n-9 and the ratios of 18:1n-9/18:0 and 16:1/16:0 in lipids were significantly increased, whereas the proportion of 20:4n-6 was unaltered. These effects were found while glycemia was constant or increased. The results are completely opposite those described in insulin-dependent diabetes mellitus (type 1), in which a depression of all the desaturases is found. They suggest that in eSS rats, the activities of the desaturases were not modified by an insulin-resistance effect. Moreover, we suggest that the enhancement of SCD-1 activity might be considered as another typical sign of the NIDDM syndrome, because it has also been found in other animal models of NIDDM, for example, the ones evoked by the sucrose-rich diet and in the Zucker rat.

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The two main types of diabetes are insulin-dependent diabetes mellitus (IDDM), characterized principally by its dependence on insulin secretion, and non-insulin-dependent diabetes mellitus (NIDDM), recognized fundamentally by its insulin resistance. Of the two diseases, the latter is the more common form of diabetes, and its prevalence is increasing at present. NIDDM is considered to have a polygenetic origin associated with environmental effects. Several animal models have been used to study NIDDM experimentally. Most of them are based on genetic modifications (1)—e.g., the ones called the Agouti mouse, tubby mouse, fat mouse, Zucker rat, and eSS (e Stilmann-Salgado) rat (2)—and some others are produced by dietary manipulations, e.g., the administration of a sucrose-rich diet (3).

NIDDM alters carbohydrate and lipid metabolism, and the alterations progress with aging. Plasmatic changes are generally represented by hypertriglyceridemia, an increase in FFA, increased or normoinsulinemia, and increased or normoglycemia, accompanied by glucose intolerance.

However, the effect of NIDDM on the FA desaturases and PUFA enzymatic biosynthesis has been investigated less; only in the last decade were the first studies initiated (4). In contrast, research into both monounsaturated FA and PUFA enzymatic biosynthesis in experimental IDDM started as early as the 1960s. In 1964 (5) insulin-dependent Δ9 desaturase modulation of monounsaturated FA biosynthesis was demonstrated, and in 1966 (6) a similar modulation of Δ6 desaturase activity and PUFA biosynthesis was shown.

In the present experiments we used the rat model called the eSS strain, obtained in Rosario, Argentina, by genetic manipulations (2,7). The generation of eSS rats was described in detail by Martínez *et al.* (2). These rats spontaneously progress to a diabetic state that resembles the NIDDM of young people (2,7), but the clinical repercussions are moderate. They specifically show diabetic blood glucose levels and low glucose tolerance as early as the second month of age, followed by hypertriglyceridemia. Obesity is not regularly associated with this model. Insulinemia is increased early on (7), but the production of insulin decreases with aging (2). Whereas the pancreatic islets are normal in 1-mon-old eSS rats, 6-mon-old eSS animals show disruption of the islet architecture, and the volume density of endocrine tissue and the percentage of β-cells are diminished (8).

That Δ9 desaturase modulates the biosynthesis of monounsaturated FA is well known, and by altering the ratio of 18:1n-9/18:0 FA in membrane phospholipids, it may modify cell membrane structure and fluidity. This enzyme has not been studied until now in eSS rats. On the other hand, it has been fully proved that the Δ6 and Δ5 desaturases are key enzymes in the biosynthesis and regulation of PUFA of the n-6 and n-3 families, which are relevant in mammalian physiology.

In consequence, we decided to investigate the changes

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Abbreviations: ABCA-1, ATP binding cassette transporter A-1; eSS rats, e Stilmann-Salgado rats; IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus; PtdCho, phosphatidylcholine; SCD-1, stearoyl-CoA desaturase-1.

evoked in eSS rats on the gene expression and catalytic activity of rat liver Δ9, Δ6, and Δ5 desaturases, as well as the corresponding FA compositions of liver microsomal lipids. The results obtained were compared to data previously published using (i) the same type of rats (4,9); (ii) sucrose-fed rats (10), which also show a time-dependent NIDDM syndrome with an increase in all the desaturase activities; and (iii) streptozotocin-treated rats (11,12), which show an IDDM syndrome with a decay in all the desaturase activities.

MATERIALS AND METHODS

Materials. [1-^{14}C]Stearic acid (56 mCi/mmol, 98% radiochemically pure) and [1-^{14}C]linoleic acid (55 mCi/mmol, 99% radiochemically pure) were purchased from Amersham Life Science (Buckinghamshire, United Kingdom). [1-^{14}C]Eicosa-8,11,14-trienoic acid (52 mCi/mmol, 98% radiochemically pure) was provided by New England Nuclear (Boston, MA). Unlabeled FA were provided by Nu-Chek-Prep (Elysian, MN). Cofactors used for enzymatic reactions were obtained from Sigma Chemical Co. (St. Louis, MO). Analytical grade solvents were purchased from Carlo Erba (Milan, Italy).

Rat cDNA of SCD-1 (stearoyl-CoA desaturase-1, isoform of the Δ9 desaturase), Δ6 desaturase, and Δ5 desaturase were kind gifts from Dr. Juris Ozols (Department of Biochemistry, University of Connecticut, Central Health, Farmington, CT), Dr. Tsunehiro Aki (Department of Molecular Biotechnology, Hiroshima University, Higashi-Hiroshima, Japan), and Drs. Reza Zolfaghari and A. Catharine Ross (Department of Nutritional Sciences, The Pennsylvania State University, University Park, PA), respectively. Restriction enzymes and other molecular biology reagents were obtained from Promega (Madison, WI). They were used for further Northern blot determinations of mRNA (13). TLC plates of silica gel 60 were from Merck (Darmstadt, Germany).

Animals. The study was performed under the international rules for animal care. Six-month-old male eSS rats were provided by the University of Rosario, School of Medicine (Rosario, Argentina). eSS and control rats were fed on a complete commercial diet (Cargill, Buenos Aires, Argentina) and water *ad libitum*. The percent FA composition (wt%) of the food was 22.5% 16:0, 1.3% 16:1, 13.7% 18:0, 25.9% 18:1n-9, 2.5% 18:1n-7, 30.7% 18:2n-6, and 3.4% 18:3n-3.

Blood samples. Blood samples, obtained by cardiac puncture, were centrifuged rapidly at 4°C and plasma was immediately stored at −20°C until further analysis. Blood glucose and TG levels were measured by commercial enzymatic methods (Wiener Lab. Test, Rosario, Argentina).

Liver subcellular fractionation. Animals were killed by decapitation without anesthesia and exsanguinated. The livers were excised rapidly and placed in an ice-cold homogenizing solution (1:3, wt/vol) composed of 0.25 M sucrose, 0.15 M KCl, 0.1 mM EDTA, 1.41 mM *N*-acetyl cysteine, 5 mM $MgCl_2$, and 62 mM phosphate buffer (pH 7.4). Microsomes were obtained by differential ultracentrifugation at 100,000 × *g* (Beckman Ultracentrifuge) as described elsewhere (14). The microsomal fractions were stored at −80°C. Protein concentration was measured according to the procedure of Lowry *et al.* (15).

Lipid analysis. Lipids were extracted from microsomes according to the procedure of Folch *et al.* (16). Phosphatidylcholine (PtdCho) was separated from other microsomal lipids by TLC using chloroform/methanol/acetic acid/water (50:37.5:3.5:2, by vol).

FA compositions from both total lipids and PtdCho were determined by GLC of their methyl esters in a Hewlett-Packard HP 6890 apparatus. They were injected into an OMEGAWAX 250 (Supelco, Bellefonte, PA) capillary column of 30 m, 0.25 mm i.d., and 0.25 μm film. The temperature was programmed to obtain a linear increase of 3°C/min from 175 to 230°C. The chromatographic peaks were identified by comparison of their retention times with those of authentic standards.

Δ9, Δ6, and Δ5 desaturation activity determinations. Δ9, Δ6, and Δ5 desaturations were measured in hepatic microsomes using as substrates 50 μM [1-^{14}C]stearic acid, 50 μM [1-^{14}C]-linoleic acid, and 30 μM [1-^{14}C]eicosa-8,11,14-trienoic acid, respectively. Substrates were incubated with 2.5 mg of microsomal protein, in a final volume of 1.5 mL at 36°C. The reaction consisted of 0.25 M sucrose, 0.15 M KCl, 1.41 mM *N*-acetyl-L-cysteine, 40 mM NaF, 60 μM CoA (sodium salt), 1.3 mM ATP, 0.87 mM NADH, 5 mM $MgCl_2$, and 40 mM potassium phosphate buffer (pH 7.4). After 1 min preincubation at 36°C, the reaction was started by the addition of microsomal protein, and the mixture was incubated in open tubes for 15 min in a thermoregulated shaking water bath. The desaturation reaction was stopped with 10% (wt/vol) KOH in ethanol, followed by saponification. The extracted FFA were dissolved in methanol/water/acetic acid (85:15:0.2, by vol) and fractionated by RP-HPLC. Separations were performed on an Econosil C_{18}, 10 μm particle size, reversed-phase column (250 × 4.6 mm) (Alltech Associates, Inc., Deerfield, IL), coupled to a guard column (10 × 4 mm) filled with pellicular C_{18}. The mobile phase consisted of methanol/water/acetic acid (90:10:0.2, by vol) at a flow rate of 1 mL/min, and a Merck-Hitachi L-6200 solvent delivery system (Darmstadt, Germany), was used. The column eluate was monitored by a UV spectrometer at 205 nm for FA identification on the basis of their retention times. The effluent was mixed with Ultima Flo-M scintillation cocktail (Packard Instruments, Downers Grove, IL) at a 1:3 ratio, and the radioactivity was measured by passing the mixture through an on-line Radiomatic Instruments Flo-One-β detector fitted with a 0.5-mL cell at a rate of 3 mL/min.

Measurements of mRNA desaturases. Total liver RNA of different animals tested was isolated with Wizard RNA Isolation System (Promega) according to the manufacturer's instructions. Twenty micrograms of total RNA was size-fractionated on a 1% agarose-formaldehyde gel and then transferred to a Zeta-Probe nylon membrane (Bio-Rad, Richmond, CA). The SCD-1, Δ6 and Δ5 desaturases, and β-actin probes were prepared by incorporating [^{32}P]dCTP by random prime labeling. Northern blot hybridization analyses were performed as described by Sambrook *et al.* (13). The autoradiographic signals for SCD-1, Δ6 desaturase, and Δ5 desaturase mRNA were quantified using 1D Image Analysis Software (Kodak, Rochester, NY) from multiple exposures. They were normalized to mRNA for β-actin, with all the mRNA probed on the

same gel. Northern blot analyses were performed using an unpaired *t*-test.

Statistical analyses. Results are expressed as means ± SD. Statistical significance was determined by Student's *t*-test. $P < 0.05$ was accepted as statistically significant.

RESULTS

Experiment 1. In a first experiment, the fasting glucose concentration of 6-mon-old male eSS rats revealed a glycemic profile similar to but statistically higher than ($P < 0.05$) that of control rats (controls 1.19 ± 0.08 g/L; eSS rats 1.36 ± 0.05 g/L), equivalent to that found by Martínez *et al.* (2) in eSS rats of that age. At age 6 mon this type of rat has already developed mild NIDDM with an abnormal glucose tolerance test, as described by Martínez *et al.* (2,9).

(i) FA desaturation activity. Δ9 desaturation activity, which may be considered to measure the eSS-1 isoform, and Δ6 desaturation activity of liver microsomes of the eSS rats compared to controls are displayed in Table 1. Results showed a 6.7-fold increase in Δ9 desaturase activity, as measured by conversion of labeled stearic acid to oleic acid, but no statistically significant change in the Δ6 desaturation of linoleic acid was observed.

(ii) mRNA of SCD-1 and Δ6 and Δ5 desaturases. Changes in the abundance of mRNA of the SCD-1 isoform of Δ9 desaturase and Δ6 desaturase in eSS rat livers compared to controls are shown in Figure 1. In this assay the comparative increase in the SCD-1 mRNA found in eSS rats correlates quite well with the enhancement of liver Δ9 desaturase activity, as shown in Table 1. However, the Δ6 desaturase mRNA was higher in eSS rats (Fig. 1), whereas the enzymatic activity of this desaturase (Table 1) was constant. Therefore, it did not increase correlatively as expected. The Δ5 desaturase mRNA was not modified.

(iii) FA composition of liver microsomes. From the preceding results, it might be expected that the proportions of palmitoleic (16:1) and oleic (18:1n-9) acids and the ratios of 16:1/16:0 and 18:1n-9/18:0 in the microsomal liver lipids of eSS rats would be enhanced owing to the increase in Δ9 desaturase. These results are shown in Table 2, which gives FA compositions of liver microsomes. The FA 16:1 and 18:1n-9 increased by 1.6- and 0.6-fold, respectively, in the total lipid fraction of eSS diabetic rats compared to controls. Additionally, the ratios of 16:1/16:0 and 18:1n-9/18:0 were 1.4- and 0.7-fold higher, respectively, in the diabetic rats compared to normal control rats.

On the other hand, the absence of a statistically significant change in arachidonic acid (20:4n-6) (Table 2) correlates with the unchanged Δ6 and Δ5 desaturase activities. Notwithstanding, a small increase in the minor FA 20:3n-6 was found in eSS rats, and this enhancement correlated with a decrease in 18:2n-6, which might suggest an activation of Δ6 desaturation. A significant increase (38%) in DHA (22:6n-3) of the n-3 family is shown in Table 2, indicating a different type of effect compared to the 20-carbon PUFA generally found in diabetic rats (11,12).

Experiment 2. In this experiment, a new lot of 6-mon-old eSS rats was studied to check the previous results. This lot showed a glycemia that was not statistically significant compared to control animals (controls 1.25 ± 0.09 g/L, eSS rats

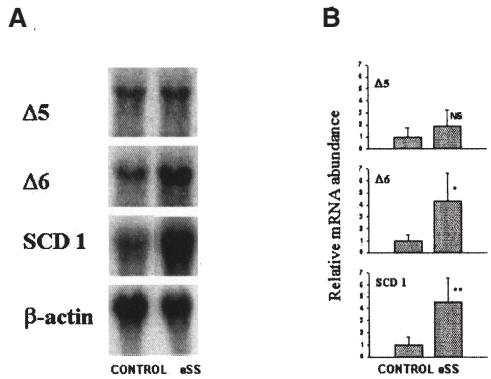

FIG. 1. mRNA levels of liver stearoyl-CoA desaturase-1 (SCD-1) and Δ6 and Δ5 desaturases in Experiment 1. (A) Representative autoradiographs of a Northern blot analysis. Total liver mRNA were electrophoresed on a 1% agarose formaldehyde gel, blotted to nylon membrane and probed with ^{32}P random-primed cDNA. mRNA levels were compared to β-actin samples. (B) The signals of Northern blots representing the ratio of the intensities of desaturase mRNA to β-actin mRNA were quantified by 1D Image Analysis Software (Kodak, Rochester, NY) and normalized. Results are the mean ± SD, $n = 3$. Statistical significance was analyzed by Student's *t*-test. $^{**}P < 0.01$, $^{*}P < 0.05$, NS, not significant. eSS, e Stilman-Salgado rats.

TABLE 1
Δ9 and Δ6 Desaturation Activities of Liver Microsomes in Experiment 1[a]

Desaturases	Control	eSS
Δ9	0.013 ± 0.001	0.100 ± 0.015***
Δ6	0.174 ± 0.067	0.265 ± 0.035

[a][1-^{14}C]Stearic acid and [1-^{14}C]linoleic acid, respectively, were used as substrates. Results, expressed as nmol product/min·mg protein, are the mean ± SD, $n = 3$. They were evaluated by Student's *t*-test. $^{***}P < 0.001$. eSS, e Stilmann-Salgado rats.

TABLE 2
FA Composition (g/100 g) of Liver Microsomes in Experiment 1[a]

FA	Control	eSS
16:0	17.80 ± 0.23	18.76 ± 1.12
16:1	0.26 ± 0.14	0.67 ± 0.04*
18:0	24.95 ± 1.12	22.68 ± 0.18
18:1n-9	4.30 ± 0.54	6.78 ± 0.72**
18:1n-7	1.78 ± 0.51	2.39 ± 0.07
18:2n-6	13.87 ± 1.15	10.28 ± 0.09*
20:3n-6	0.32 ± 0.03	0.61 ± 0.01***
20:4n-6	28.75 ± 1.04	27.42 ± 1.07
22:4n-6	0.38 ± 0.05	0.35 ± 0.02
22:5n-6	0.14 ± 0.04	0.20 ± 0.05
22:5n-3	0.95 ± 0.11	0.85 ± 0.05
22:6n-3	6.50 ± 0.41	9.01 ± 0.50**
16:1/16:0	0.015	0.036
18:1n-9/18:0	0.172	0.299
20:4n-6/18:2n-6	2.073	2.667

[a]Only the principal FA were considered. Data are the mean ± SD, $n = 3$. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$, evaluated by Student's *t*-test. For abbreviation see Table 1.

TABLE 3
Δ6 and Δ5 Desaturation Activities of Liver Microsomes in Experiment 2[a]

Desaturases	Control	eSS
Δ6	0.174 ± 0.036	0.181 ± 0.026
Δ5	0.147 ± 0.039	0.096 ± 0.020

[a][1-^{14}C]Linoleic acid and [1-^{14}C]eicosatrienoic n-6 acid, respectively, were used as substrates. Results, expressed as nmol product/min·mg protein, are the mean ± SD, $n = 4$. Differences were not significant when evaluated by Student's *t*-test. For abbreviation see Table 1.

1.34 ± 0.07 g/L). Gómez Dumm *et al.* (9) found similar results for eSS rats of the same age.

The triglyceridemia measured in these eSS rats (four animals) indicated a statistically significant ($P < 0.005$) increase (eSS rat TG 2.14 ± 0.61 g/L vs. control rat TG 0.49 ± 0.10 g/L), typical of the NIDDM syndrome.

(i) FA desaturation activity and abundance of mRNA. Δ6 and Δ5 desaturations of [1-^{14}C]linoleic and [1-^{14}C]eicosa-8,11,14-trienoic acids, respectively, found in the eSS rat liver are shown in Table 3. The activity of both enzymes was not modified significantly in eSS rats compared to the controls. The results for Δ6 desaturase reproduced those found in the first experiment (Table 1). They correlated well with the absence of alteration in the level of mRNA of both Δ6 and Δ5 desaturases in the liver (Fig. 2), indicating the absence of a change in their expression.

In the second experiment (Fig. 2), the eSS animals again showed a significant, approximately fourfold enhancement of liver SCD-1 mRNA.

(ii) FA composition of liver microsomal lipids. The FA compositions of liver microsomal total lipids and PtdCho of eSS rats are listed in Table 4. They showed, first, that oleic acid (18:1n-9) and the ratio of 18:1n-9/18:0 were increased in both types of lipids when compared to control rats (*cf.* Table 2). Palmitoleic acid (16:1n-7) was also increased in PtdChol and the ratio of 16:1/16:0 was increased in both lipids. Moreover, microsomal PtdCho also showed a statistical increase of 18:1n-7, which would be the elongated product of 16:1n-7. Therefore, the changes in 18:1n-9, 16:1, and 18:1n-7 were in accordance with an increase in Δ9 desaturation activity.

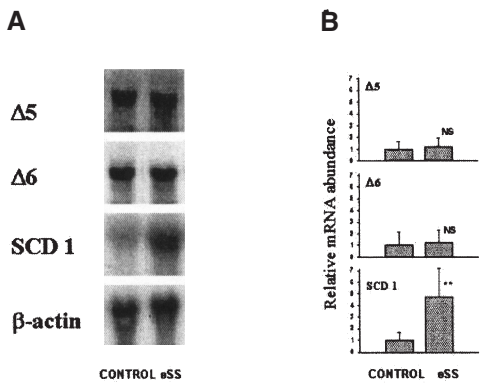

FIG. 2. mRNA levels of liver SCD-1 and Δ6 and Δ5 desaturases in Experiment 2. (A) Representative autoradiographs of a Northern blot analysis; (B) quantified signals. Procedures are as described in Figure 1. Results are the mean ± SD, $n = 3$. Statistical significance was analyzed by Student's *t*-test $^{**}P < 0.01$, NS, not significant; for other abbreviations see Figure 1.

TABLE 4
FA Composition (g/100 g) of Liver Microsomal Lipids of Experiment 2[a]

	Total lipids	
FA	Control	eSS
16:0	20.79 ± 4.04	19.95 ± 1.14
16:1	0.93 ± 0.64	1.03 ± 0.24
18:0	25.69 ± 0.42	24.17 ± 0.16***
18:1n-9	4.69 ± 0.81	6.32 ± 0.22**
18:1n-7	1.95 ± 0.65	2.24 ± 0.11
18:2n-6	13.44 ± 1.33	14.16 ± 0.98
20:3n-6	0.49 ± 0.06	0.96 ± 0.14***
20:4n-6	25.04 ± 3.56	25.16 ± 1.24
22:4n-6	0.31 ± 0.12	0.27 ± 0.02
22:5n-6	0.02 ± 0.05	0.14 ± 0.02**
22:5n-3	0.71 ± 0.14	0.74 ± 0.07
22:6n-3	5.94 ± 1.44	4.86 ± 0.37
16:1/16:0	0.045	0.052
18:1n-9/18:0	0.183	0.261
20:4n-6/18:2n-6	1.863	1.777
	Phosphatidylcholine of microsomal lipids	
FA	Control	eSS
16:0	21.57 ± 1.17	21.83 ± 0.17
16:1	0.79 ± 0.23	1.26 ± 0.09**
18:0	27.26 ± 0.63	25.73 ± 0.35**
18:1n-9	3.83 ± 0.19	5.08 ± 0.36***
18:1n-7	1.68 ± 0.01	2.24 ± 0.17***
18:2n-6	13.82 ± 1.10	13.71 ± 1.24
20:3n-6	0.63 ± 0.08	0.90 ± 0.16*
20:4n-6	25.58 ± 1.98	25.51 ± 1.39
22:4n-6	0.20 ± 0.14	0.11 ± 0.13
22:5n-6	0.01 ± 0.03	0.09 ± 0.02**
22:5n-3	0.46 ± 0.09	0.41 ± 0.07
22:6n-3	4.16 ± 0.55	3.13 ± 0.52*
16:1/16:0	0.036	0.058
18:1n-9/18:0	0.140	0.197
20:4n-6/18:2n-6	1.851	1.861

[a]Only the principal FA were considered. Data are the mean ± SD, $n = 4$. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$, evaluated by Student's *t*-test. For abbreviation see Table 1.

The lack of change in the amounts of arachidonic acid (20:4n-6) and the invariant 20:4n-6/18:2n-6 ratio shown in microsomal lipids and PtdCho (Table 4) were also in accordance with the unaltered Δ6 and Δ5 desaturations and mRNA levels of both enzymes, as illustrated in Table 3 and Figure 2. However a small increase was found in the minor acids 20:3n-6 and 22:5n-6.

Unlike the microsomal FA composition shown in Table 2, the amount of the n-3 FA DHA (22:6n-3) remained almost unchanged in the second experiment.

DISCUSSION

Previous studies (2,7) have shown that eSS rats develop fasting hyperglycemia, abnormal glucose tolerance, and hyperinsulinemia from a rather early age. The percentage of male eSS animals showing hyperglycemia increased from 16% at 1 mon to 64.7% at 2.5 mon and to 90.5% at 10 mon. Marked glucose intolerance was found at 4, 6–8, 10–13, and 15–18 mon of age, together with slowly declining hyperinsulinemia measured at the same ages, which was no longer significant at 15–18 mon.

In addition, a sequential morphological study of pancreatic islets (8) did not reveal any significant changes in 1-mon-old eSS rats. But at 6 mon they showed a disruption of the islet architecture and fibrosis in the stroma, suggesting a hyperplasia of β-cell mass. At 18 mon eSS rats also showed conspicuous islet lesions, which might indicate that the capacity for β-cell replication was much lower or exhausted. All these results led to the conclusion that eSS rats developed insulin resistance rather early, as evidenced by their reduced capacity to normalize glucose homeostasis. This insulin ineffectiveness was partially compensated for early on by an increase in insulin secretion. However, in older rats insulin secretion was also diminished.

In the present study we chose 6-mon-old eSS rats because previously published data (8) indicated they would be in a rather early period of NIDDM, but with a net NIDDM syndrome. In 5-mon-old rats, Gómez Dumm *et al.* (9) also found another typical sign of NIDDM syndrome in the eSS rats, i.e., an increase in triglyceridemia, but they also found normoglycemia.

The eSS rats used in the present experiments were raised in the same laboratory where this line of animals was first bred and that provided the rats for earlier investigations (2,7–9). Similar to the results of Martínez *et al.* (2) and Gómez Dumm *et al.* (9), our results obtained using eSS rats showed hypertriglyceridemia and normo- or hyperglycemia compared to control rats. These data and others reported by the authors mentioned in previous works (2,9) indicated that 6-mon-old eSS rats showed insulin resistance in the regulation of glucose homeostasis.

In experimental IDDM, it was shown long ago (5,6), and since then fully and repeatedly confirmed (12,17,18), that insulin promotes the recovery of SCD-1, Δ6 and Δ5 desaturase mRNA, and enzymatic activities depressed in IDDM. However, in the 6-mon-old eSS rats described in previous publications (2,7) that were considered to have developed insulin resistance, we found a very significant increase in both mRNA and the enzymatic activity of the liver SCD-1 isoform of Δ9 desaturase. Moreover, liver Δ6 and Δ5 desaturase mRNA and enzymatic activities, instead of being decreased as would be expected, were unmodified except for one case in which the Δ6 desaturase mRNA was increased (Fig. 1).

It is important to remark that the enhancement of mRNA and the activity of liver SCD-1 shown in this study were correlated with increases in oleic acid (18:1n-9), in the ratios of 18:1n-9/18:0 and 16:1/16:0 in liver microsomal lipids (Tables 2 and 4), and in microsomal PtdCho (Table 4). In this phospholipid an increase in 18:1n-7 was also shown. Since Brenner (11,12) and others (19) have repeatedly shown that the changes in unsaturated FA composition of liver microsomal phospholipids and, more precisely, of PtdCho are good sensors of changes in FA desaturation, the data in Tables 2 and 4 would confirm the increase in activity of Δ9 desaturase (SCD-1 isoform). Also, they indicate the increase in activity of this enzyme and a relevant effect on the lipid composition and the structural and biophysical properties of liver membranes, as well as other possible and outstanding effects such as the enhancement of TG biosynthesis or an increase in plasma TG, as shown by Ntambi's group (20). In this respect, according to Listenberger *et al.* (21), the increase in oleic acid (18:1n-9) at the cellular level that leads to the accumulation of TG rescues palmitate-induced apoptosis by channeling palmitate into TG pools and away from pathways leading to apoptosis. Moreover, an increase in the ratios of monounsaturated/saturated FA in the membrane phospholipids of eSS rats, as mentioned above, would favor an increase in the proportion of disordered liquid crystalline domains of the bilayers at the expense of ordered membrane regions, evoking an enhancement of fluidity and permeability (22). In that respect, Sun *et al.* (22) demonstrated that whereas overexpression of SCD-1 and SCD-2 activity increased passive cholesterol efflux in cells, it inhibited ATP-binding cassette transporter A-1 (ABCA-1) transporter-mediated cholesterol efflux.

The results of this study for NIDDM rats are quite opposite those detected in IDDM experimental animals (18,23), in which the ratios of monounsaturated/saturated FA are depressed by the effect of decrease in Δ9 desaturation of the saturated FA, producing a decline in the disordered liquid crystalline domains of membranes. Similarly, increases in arachidonic acid and the ratio of 20:4n-6/18:2n-6 were not found in the same microsomal lipids (Table 4), which is in good accordance with the unchanged Δ6 and Δ5 desaturase activities (Table 3) and the corresponding mRNA (Fig. 2).

In consequence, all these data regarding Δ9, Δ6, and Δ5 desaturases cannot be ascribed to and are apparently opposite to an insulin-resistance effect.

However, research done in 1993 by Gómez Dumm *et al.* (4) investigating the activities of the Δ6 and Δ5 desaturases in 10-mon-old eSS rats found that the animals were slightly hyperglycemic, that they showed a diabetic glucose-tolerance profile, and that the hepatic Δ6 and Δ5 *in vitro* desaturations of linoleic and eicosa-8,11,14-trienoic acids, respectively, were decreased. Therefore, these results might be attributed to an insulin-resistance effect. On the other hand, we found (10) that a sucrose-rich diet provoked the NIDDM syndrome in normal rats after 6 mon as characterized by hyperglycemia, hypertriglyceridemia, and high plasma FFA but normoinsulinemia. In these animals we also found increased mRNA of the hepatic SCD-1 and Δ6 desaturase, which correlated with increased activities of both desaturases. In addition, Δ5 desaturation activity was also enhanced. In those animals, which also showed the NIDDM syndrome, the independence of desaturase activities from an insulin-resistance effect was found to be similar to the eSS rats in our present investigation. Waters and Ntambi (18) also found an increase in SCD-1 gene expression independent of insulinemia in streptozotocin diabetic mice when using a fructose diet.

Therefore, in comparing all these results, we may conclude that eSS rats exhibit an increase in liver SCD-1 gene expression and enzymatic activity along with the development of the NIDDM syndrome. Moreover, Δ9, Δ6, and Δ5 desaturase activities are not altered by an insulin-resistance effect, and a direct effect of insulinemia is doubtful. They would show that in eSS rats and in animals receiving a sucrose-rich diet (10), liver insulin resistance is selective. It would impair the suppression of gluconeogenesis and glycemic homeostasis without an impairment of Δ9 desaturation of FA.

In addition, our present results with the eSS rats, with Zucker rats (24,25), and with the effect of a sucrose-rich diet (10),

together with the results of Ntambi's group (20), lead us to suggest that an increase in Δ9 desaturase activity may be added as a typical and very important signal to the other previously known alterations of carbohydrate and lipid metabolism found in the NIDDM syndrome. This increase in Δ9 desaturase may even be a means by which cells react in an attempt to protect themselves from apoptosis and other deleterious effects evoked by FFA.

In consequence, we reached the interesting conclusion that, in our experimental group of eSS rats and in rats fed a sucrose-rich diet (10), both showing the NIDDM syndrome, other factors different from insulin level may have been altered and are modulating the activity of the two groups of desaturases, the Δ9 and the Δ6 and Δ5. With respect to this conclusion, we have to consider that many biological factors such as peroxisome proliferative-activated receptor-α and -γ, sterol regulatory element-binding proteins, leptin, and ABCA-1 transporters are known to be involved in modulation of FA desaturation; moreover, they are apparently related to insulin effects and therefore may be involved in the effects described in eSS rats.

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Influence of Temperature and High Dietary Linoleic Acid Content on Esterification, Elongation, and Desaturation of PUFA in Atlantic Salmon Hepatocytes

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ABSTRACT: The esterification, desaturation, and elongation of [1-^{14}C]18:3n-3, [1-^{14}C]18:2n-6, and [1-^{14}C]20:5n-3 at 5 and at 12°C were studied using cultivated hepatocytes from Atlantic salmon. The salmon were fed diets, in which 0, 50, or 100% of the supplementary fish oil had been replaced by soybean oil, for 950 day-degrees at 5 and 12°C. The endogenous percentage of 18:2n-6 in hepatocyte lipids was 2% in cells from fish fed a diet with 100% of the supplemental lipid from fish oil, and it was slightly less than 25% in cells from fish fed the diet with 100% of the supplemental lipid from soybean oil. Furthermore, the percentages of 20:3n-6 and 20:4n-6 were significantly higher in hepatocytes from fish fed on soybean oil than they were in those of fish fed on fish oil. The percentages of 20:5n-3 and 22:6n-3, on the other hand, were lower. The endogenous levels of n-6 FA were not significantly correlated with the total amounts of radiolabeled FA esterified in hepatocyte lipids. The main radiolabeled products formed from 18:2n-6 were 20:2n-6 and 20:3n-6. The level of the important eicosanoid precursor 20:4n-6 was twice as high in hepatocyte phospholipids from fish fed the 100% soybean oil diet as it was in hepatocytes from fish fed the diet with 100% of supplemental lipid from fish oil. The main products formed from 18:3n-3 were 20:4n-3, 20:5n-3, and 22:6n-3. High levels of dietary 18:2n-6 do allow, or even seem to increase, the production of 22:6n-3 from 18:3n-3 in hepatocytes. The main products formed from 20:5n-3 were 22:5n-3 and 22:6n-3. The production of 22:6n-3 from 20:5n-3 was higher at 5°C than at 12°C. The percentage of 24:5n-3 was higher at 5°C than it was at 12°C, as was the ratio of 24:5 to 22:5. These results suggest that the elongation rate of 22:5n-3 to 24:5n-3 is higher at the lower temperature.

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The FA composition of fish tissues is determined not only by the dietary lipid composition but also by the ability of a species to modify the dietary FA input. Membranes become more rigid in the cold, and fish respond to cold by increasing the degree of FA unsaturation of phospholipids (PL) to restore the fluidity of the membranes (1,2). The regulatory enzymes involved in this important response have not been clearly identified. It is generally believed that the activities of desaturases increase in cold conditions, and this may be a widespread feature of membrane restructuring (1,3). Cold acclimation induces the enzymatic activation of acyl CoA-Δ9 desaturase in carp liver (4).

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Abbreviations: FO, fish oil; PL, phospholipids; SO, soybean oil.

Atlantic salmon (*Salmo salar*) are able to elongate and desaturate C_{18} PUFA to C_{20} and C_{22} PUFA (5–7). The FA in the n-9, n-6, and n-3 families may compete for the same desaturation and elongation enzymes, and thus the rate of conversion may be influenced not only by the capacities of the enzymes but also by the balance between the different FA present. The rates of desaturation and elongation of 18:2n-6 in isolated salmon hepatocytes are strongly influenced by the prior dietary balance of n-3 FA (7). Tocher *et al.* (8) found that the products of Δ6-desaturase from 18:3n-3 increased in hepatocytes from salmon fed on diets that contained high levels of n-6 FA. We have recently shown that Atlantic salmon grow well on high-energy fish meal-based diets, even when the supplementary oil in the diet is exclusively soybean oil (SO) (9), and have observed that the SO diet had no detrimental effects on the health of the salmon. On the other hand, some studies on salmonid species have led to the suggestion that the high level of 18:2n-6 in vegetable oils may inhibit the conversion of 18:3n-3 to the essential 20:5n-3 (EPA) and 22:6n-3 (DHA) FA, thereby leading to EFA deficiency in the fish (10). The uncertain supply and variable price of fish oil (FO) have led to a search for other sources of energy in Atlantic salmon diets, and vegetable oils are one alternative source that is being considered. This makes it necessary to obtain more basic knowledge about dietary-induced changes in the balance between FA of the different PUFA families and about how these changes influence the production of essential n-3 and n-6 FA in salmon at different water temperatures.

We have investigated the effects of increasing the amount of SO in high-energy diets for Atlantic salmon on the metabolism of n-3 and n-6 PUFA. Our focus was on esterification into neutral and polar lipid fractions and on the desaturation and elongation of n-3 and n-6 PUFA at different temperatures.

MATERIALS AND METHODS

Materials. The radiolabeled FA [1-^{14}C]18:2n-6, [1-^{14}C]-18:3n-3, and [1-^{14}C]20:5n-3 were obtained from Amersham (Buckinghamshire, United Kingdom), and nonlabeled FA, EFA-free BSA, HEPES, 2′,7′-dichlorofluorescein, Leibowitz-15 culture medium, and collagenase type 1 were obtained from Sigma Chemical Co. (St. Louis, MO). Acetic acid, chloroform, petroleum ether, diethyl ether, and methanol were all obtained from Merck (Darmstadt, Germany). Benzene was obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland). Methanolic HCl and 2,2-dimethoxypropane were

purchased from Supelco Inc. (Bellefonte, PA). Glass plates covered with silica gel K6 were obtained from Whatman International Ltd. (Maidstone, England).

Fish and feeding. The feeding study was performed as described by Grisdale-Helland *et al*. (9). In brief, Atlantic salmon in tanks containing seawater at 5 or 12°C (three tanks per diet at each temperature and 40 fish per tank) were fed for approximately 950 day-degrees on one of three test diets. The diets were based on fish meal and differed only in the type of supplemental oil that they contained (Table 1). The diet called "100% FO" contained 100% of the supplemental lipid as capelin oil; the diet called "100% SO" contained 100% of the supplemental lipid from crude SO; and the diet called "50% SO" contained a 50:50 mixture of the two sources of supplemental lipid. The feeds were kept frozen (–20°C) until needed, and batches of feed sufficient for approximately 1 wk were withdrawn to the feed room. The salmon grew from an initial weight of 113 ± 5 g to a final weight of 338 ± 19 g.

Preparation of salmon hepatocytes. At the end of the growth trial, three fish from each tank were used for isolation of hepatocytes. The fish were anesthetized with metacain. The abdominal cavity was exposed and the vena porta cannulated. The liver was perfused, and parenchymal cells were prepared using a two-step collagenase perfusion procedure as described by Seglen (11) and modified by Dannevig and Berg (12). After collagenase digestion, the parenchymal cells were easily isolated by gently shaking the liver in a buffer (300 mOsm) containing 137 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.35 mM KH_2PO_4, 0.81 mM $MgSO_4$, 2 mM $CaCl_2$, 40 mM HEPES at pH 7.5, and 1% BSA. The suspension of parenchymal cells was filtered through a 100-µm nylon filter. Hepatocytes were isolated from this suspension of parenchymal cells by conducting three sedimentations each of 2 min at 50 × *g*. The hepatocytes were resuspended in Leibowitz-15 culture medium containing 2% FBS, 2 mM L-glutamine, and 0.1 mg/mL gentamycin. Cell viability was assessed by staining with Trypan blue (0.4%). The protein content of the cell suspension was determined using the method described by Lowry *et al.* (13).

TABLE 1
Formulation and Chemical Composition of Diets

	Diets[a]		
	100% FO	50% SO	100% SO
Ingredients (g·kg^{-1})			
Fish meal	409.40	409.40	409.40
Capelin oil	278.00	139.00	
Soybean oil		139.00	278.00
Maize gluten	215.00	215.00	215.00
Wheat	76.40	76.40	76.40
Lysine (78%)	11.83	11.83	11.83
Mineral + vitamin + pigment	9.28	9.28	9.28
Yttrium oxide	0.11	0.11	0.11
Chemical composition			
Dry matter (%)	97.2	97.5	96.1
Crude protein	51.9	51.9	51.3
Crude fat	29.6	28.8	30.8
Ash	6.1	6.1	5.9
Gross energy (MJ/kg)	26.13	26.10	25.94

[a]100% FO = 100% fish oil diet; 50% SO = 50% fish oil and 50% soybean oil diet; 100% SO = 100% soybean oil diet.

Approximately 5×10^6 hepatocytes (approximately 8 mg of protein) were plated onto 75-mL Nunc flasks and left to attach overnight at either 5 or 12°C.

Incubations with radiolabeled FA. The cells were thoroughly washed with Leibowitz-15 medium without additional serum before incubation with radiolabeled FA. All cell flasks with hepatocytes were incubated for 24 h with 0.5 µmol radiolabeled FA (final concentration 0.1 mM) and 10 mM lactate in a total volume of 5 mL Leibowitz-15 medium. The specific radioactivity of the FA was 7 mCi/mmol (3.5 µCi of radioactive FA substrate was added to each cell flask). The radiolabeled FA were added to the medium in the form of their potassium salts bound to BSA. Hepatocytes isolated from fish kept in water at 5°C were incubated at 5°C, and hepatocytes isolated from fish kept in water at 12°C were incubated at 12°C. Blank incubations were performed for each radiolabeled FA substrate. Blanks were treated in the same way as the samples, except that the cell protein was denaturated by heating to 80°C prior to the incubations. After the 24-h incubation, the culture medium was transferred from the culture flasks to vials and centrifuged for 5 min at 50 × *g*. The supernatants (culture media) were immediately frozen at –40°C and stored until analysis of radiolabeled lipids. Hepatocytes (attached to the culture flask) were washed twice in PBS. The cells were then harvested in PBS, frozen at –40°C and stored until analysis of radiolabeled lipids.

Lipid extraction and FA analysis. Total lipids were extracted from homogenized cells and culture media using the method described by Folch *et al.* (14). The solutions used for lipid extraction contained 2,6-di(*tert*-butyl)-*p*-cresol (50 mg/L) as an antioxidant, and the lipid extracts were stored under nitrogen in the dark at –50°C to prevent the oxidation of unsaturated FA. The chloroform phase produced by Folch extraction was dried under nitrogen and dissolved in hexane. The level of radioactivity in an aliquot of the total lipid was measured with a scintillation spectrometer. Polar lipids were separated from neutral lipids by TLC with a mixture of petroleum ether, diethyl ether, and acetic acid (113:20:2 by vol) as the mobile phase. The lipids were visualized by spraying the TLC plates with 0.2% (wt/vol) 2′,7′-dichlorofluorescein in methanol and were identified by comparison with known standards under UV light. The spots corresponding to TG, DG, and MG were pooled and defined as the nonpolar lipid fraction (MG + DG + TG). The level of radioactivity in the spots corresponding to phospholipids (PL), nonpolar lipids, and FFA was measured with a scintillation spectrometer. The spots corresponding to PL and nonpolar lipids were scraped into glass tubes and transmethylated overnight with 2,2-dimethoxypropane, methanolic HCl, and benzene at room temperature, as described by Mason and Waller (15). The radioactive FA compositions of the PL fraction and of the nonpolar lipid fraction were determined by RP-HPLC as described by Narce *et al.* (16). The mobile phase was acetonitrile/H_2O (80:20 vol/vol, isocratic elution). The flow rate was 1 mL·min^{-1} and the temperature 30°C. A reversed-phase Symmetry 3.5 µm C_{18} HPLC column from Waters was used. Levels of radioactive FA were measured in a radioactive flow detector A100 (Packard 1900TR Tri-Carb; Radiomatic Instruments & Chemicals, Tampa, FL). Absorbance of

TABLE 2
FA Composition of Diets[a] (% of total FA)

FA	100% FO	50% SO	100% SO
14:0	5.7	3.1	0.9
16:0	14.0	13.2	12.7
18:0	1.6	2.7	3.7
16:1n-7	7.5	4.2	1.3
18:1n-7	2.8	2.2	1.6
18:1n-9	10.3	15.4	20.3
20:1 (sum isomers)	12.8	6.8	1.1
22:1 (sum isomers)	15.9	8.4	1.0
18:2n-6	4.4	25.3	45.4
18:3n-3	0.8	3.1	5.3
18:4n-3	3.2	1.8	0.5
20:5n-3	9.0	5.5	2.1
22:5n-3	0.6	0.4	0.2
22:6n-3	7.0	4.9	2.8
∑ Others	4.4	3.0	1.1
∑ EPA + DHA	16.0	10.4	4.9
∑ n-6	5.3	25.7	45.4
∑ n-3	21.3	16.2	10.9

[a]For diet compositions see Table 1.

nonradioactive FA was measured in a UV detector (SPD-6AV UV-vis spectrophotometric detector; Shimadzu) at 215 nm. The FA were identified by comparing the sample retention volumes with the retention volumes of known FA standards. The total FA composition of hepatocytes was determined basically as described by Røsjø *et al.* (17). The methyl esters of FA were separated in a gas chromatograph (PerkinElmer Autosystem GC equipped with an autoinjector, programmable split/splitless injector) with a CP Wax 52 column (length = 25 m, i.d. = 0.25 mm, depth of film = 0.2 μm), FID, and 1022 data system. The carrier gas was He, and the injector and detector temperatures were 280°C. The oven temperature was raised from 50 to 180°C at the rate of 10°C min^{-1}, and then raised to 240°C at the rate of 0.7°C min^{-1}. The relative quantity of each FA present was determined by measuring the area under the peak corresponding to a particular FA. The FA profiles in the diets (Table 2) were determined as described by Grisdale-Helland *et al.* (9).

Statistics. The data were analyzed by two-way ANOVA for the factors diet and water temperature. Significant differences between means were analyzed using Duncan's test in the software package UNISTAT (London, England). The significance levels (*P*-values) from the statistical tests are presented together with means and SEM for each variable. The significance level was set at 5%.

RESULTS

Endogenous FA composition of hepatocytes. The total endogenous FA composition of hepatocytes of fish kept at 5°C was approximately the same as that of fish kept at 12°C (Table 3). Diet, on the other hand, affected FA composition significantly. The hepatocyte percentage of 18:2n-6 in the total lipids was almost six times greater in fish fed the 50% SO diet than in fish fed the 100% FO diet, and it was nearly 10 times greater in fish fed the 100% SO diet. Total n-6 FA increased from approximately 5% in the 100% FO group to approximately 18% in the 50% SO group and 31% in the 100% SO group. A notable feature of the data in Table 3 is that, whereas the percentage of 20:5n-3 + 22:6n-3 in the total lipid fraction

TABLE 3
FA Compositions with Temperature and Diet of Total Lipid Fraction of Hepatocytes[a]

	5°C			12°C		
	100% FO	50% SO	100% SO	100% FO	50% SO	100% SO
FA						
14:0	2.5 ± 0.09^{b}	2.1 ± 0.16^{b}	0.9 ± 0.29^{a}	2.5 ± 0.01^{b}	2.0 ± 0.01^{b}	1.9 ± 0.22^{b}
16:0	16.9 ± 0.71	13.4 ± 1.28	14.2 ± 1.83	15.5 ± 0.02	16.4 ± 1.13	16.2 ± 1.18
18:0	4.9 ± 0.24^{a}	5.3 ± 0.20^{a}	7.3 ± 0.08^{b}	4.9 ± 0.22^{a}	5.8 ± 0.25^{a}	7.9 ± 0.31^{b}
16:1n-7	3.5 ± 0.23^{b}	3.4 ± 0.38^{b}	1.2 ± 0.12^{a}	3.8 ± 0.21^{b}	3.4 ± 0.45^{b}	1.1 ± 0.19^{a}
18:1n-7	3.4 ± 0.05^{b}	3.0 ± 0.20^{b}	1.4 ± 0.09^{a}	3.6 ± 0.13^{b}	3.2 ± 0.27^{b}	1.5 ± 0.18^{a}
18:1n-9	13.5 ± 0.44	21.5 ± 3.17	16.2 ± 2.13	13.9 ± 0.68	15.6 ± 1.18	15.9 ± 2.18
∑ 20:1 (isomers)	4.8 ± 0.10^{b}	4.6 ± 0.03^{b}	3.7 ± 0.38^{a}	5.0 ± 0.11^{b}	4.1 ± 0.15^{b}	3.2 ± 0.37^{a}
22:1n-11	0.8 ± 0.06^{b}	0.9 ± 0.08^{b}	0.4 ± 0.01^{a}	0.9 ± 0.12^{b}	0.6 ± 0.1^{a,b}	0.4 ± 0.06^{a}
18:2n-6	2.4 ± 0.10^{a}	13.8 ± 0.72^{b}	23.6 ± 2.10^{c}	2.3 ± 0.18^{a}	12.6 ± 1.23^{b}	22.4 ± 1.99^{c}
20:2n-6	0.5 ± 0.03	2.2 ± 0.20	2.3 ± 1.59	0.5 ± 0.00	2.3 ± 0.03	2.4 ± 0.71
18:3n-3	ND	ND	ND	ND	ND	ND
20:3n-6	0.6 ± 0.18^{a}	1.3 ± 0.17^{a}	3.4 ± 0.71^{b}	0.2 ± 0.20^{a}	1.3 ± 0.15^{a}	3.2 ± 0.54^{b}
18:4n-3	0.2 ± 0.02^{a}	0.4 ± 0.05^{b}	0.4 ± 0.11^{b}	0.5 ± 0.11^{b}	0.4 ± 0.25^{b}	0.2 ± 0.23^{a,b}
20:4n-6	1.6 ± 0.07^{a,b}	1.3 ± 0.34^{a}	2.9 ± 0.10^{b}	1.4 ± 0.22^{a}	1.3 ± 0.51^{a}	2.9 ± 0.15^{b}
20:4n-3	0.9 ± 0.11	0.7 ± 0.30	3.0 ± 2.19	0.8 ± 0.15	1.0 ± 0.16	2.9 ± 1.65
20:5n-3	8.4 ± 0.29^{b}	3.9 ± 0.94^{a}	3.2 ± 0.61^{a}	8.5 ± 0.40^{b}	3.9 ± 0.85^{a}	3.3 ± 0.32^{a}
22:5n-3	2.9 ± 0.19^{b}	1.5 ± 0.34^{a}	1.5 ± 0.15^{a}	3.0 ± 0.25^{b}	1.6 ± 0.21^{a}	1.5 ± 0.22^{a}
22:6n-3	28.5 ± 0.22^{b}	17.1 ± 2.52^{a}	16.1 ± 2.13^{a}	26.5 ± 0.33^{b}	17.8 ± 3.31^{a}	16.6 ± 1.11^{a}
∑ Others	3.9 ± 0.51	3.6 ± 0.66	0.7 ± 0.04	6.2 ± 0.60^{a}	6.7 ± 0.72^{b}	0.2 ± 4.76^{c}
∑ EPA + DHA	36.9 ± 0.23^{b}	21.0 ± 3.46^{a}	19.4 ± 2.73^{a}	35.0 ± 0.31^{b}	21.7 ± 4.52^{a}	19.9 ± 2.70^{a}
∑ n-3	40.8 ± 0.28^{b}	23.8 ± 3.64^{a}	24.0 ± 5.15^{a}	39.3 ± 0.31^{b}	24.7 ± 2.50^{a}	24.5 ± 5.21^{a}
∑ n-6	5.1 ± 0.52^{a}	18.6 ± 0.56^{b}	32.2 ± 5.71^{c}	4.5 ± 0.60^{a}	17.5 ± 1.72^{b}	31.0 ± 4.76^{c}

[a]The quantity of each FA is given as a percentage of the total FA. Data are means ± SEM. ND, not detectable. Values with different roman superscripts are significantly different ($P \leq 0.05$; $n = 3$). See Table 1 for abbreviations.

TABLE 4
Percentage Distributions[a] of Radioactivity from [1-^{14}C]20:5n-3, [1-^{14}C]18:3n-3, and [1-^{14}C]18:2n-6 in Lipid Fractions of Hepatocytes at 5 and 12°C

	5°C			12°C		
	100% FO	50% SO	100% SO	100% FO	50% SO	100%SO
[1-^{14}C]20:5n-3						
PL	45.6 ± 2.17^{a,b}	44.1 ± 1.18^{a,b}	39.3 ± 2.97^{a}	50.6 ± 5.64^{a,b}	52.2 ± 3.34^{b}	46.2 ± 4.71^{a,b}
Nonpolar (MG + DG + TG)	31.2 ± 1.36^{b}	28.8 ± 1.26^{b}	30.0 ± 2.88^{b}	9.7 ± 1.74^{a}	10.0 ± 0.80^{a}	9.8 ± 0.68^{a}
FFA	23.1 ± 2.58^{a}	27.0 ± 1.54^{a,b}	30.6 ± 5.88^{a,b}	39.8 ± 4.40^{b,c}	38.0 ± 2.65^{b,c}	44.2 ± 4.61^{c}
Total nmol	101.7 ± 14.99	80.9 ± 9.76	70.0 ± 10.79	108.4 ± 12.78	118.0 ± 16.15	110.0 ± 20.70
[1-^{14}C]18:3n-3						
PL	37.9 ± 2.99^{a,b}	31.8 ± 2.49^{a}	31.3 ± 3.74^{a}	50.6 ± 12.10^{b}	48.7 ± 1.64^{a,b}	45.6 ± 5.11^{a,b}
Nonpolar (MG + DG + TG)	38.5 ± 3.79^{c}	24.6 ± 3.64^{b}	29.2 ± 3.42^{b}	11.0 ± 2.67^{a,b}	12.7 ± 1.57^{a,b}	9.6 ± 0.76^{a}
FFA	23.6 ± 4.41^{a}	43.6 ± 4.22^{b}	42.5 ± 6.57^{b}	38.4 ± 9.78^{a,b}	38.6 ± 2.14^{a,b}	44.8 ± 5.49^{b}
Total nmol	82.2 ± 3.93^{a,b}	74.0 ± 11.63^{a}	62.7 ± 14.18^{a}	133.3 ± 4.17^{b}	145.0 ± 17.84^{b}	145.4 ± 41.70^{b}
[1-^{14}C]18:2n-6						
PL	37.9 ± 2.60^{a,b}	31.2 ± 3.50^{a}	31.7 ± 5.19^{a,b}	47.3 ± 8.53^{a,b}	49.0 ± 0.82^{b}	35.8 ± 2.42^{a,b}
Nonpolar (MG + DG + TG)	33.2 ± 2.91	25.5 ± 2.85	22.8 ± 8.52	31.7 ± 7.57	28.1 ± 4.50	31.9 ± 0.29
FFA	29.1 ± 4.71^{a,b,c}	43.6 ± 4.04^{c}	42.9 ± 16.14^{b,c}	21.7 ± 3.40^{a}	23.1 ± 3.81^{a,b}	36.5 ± 2.36^{a,b,c}
Total nmol	81.6 ± 4.84	73.9 ± 5.45	68.9 ± 14.18^{a}	94.1 ± 4.60	105.8 ± 19.17	96.4 ± 14.61

[a]Each entry is the average of three experiments ± SEM. Values marked with different roman superscripts are significantly different. Total nmol = nmol of ^{14}C FA recovered in lipid fractions of hepatocytes after 24 h incubation. PL, phospholipids; for other abbreviations see Table 1.

of hepatocytes decreased from approximately 36% in the 100% FO group to 21% in the 50% SO group, it remained at almost 20% in the 100% SO group. On the other hand, the percentage of 20:4n-6 was approximately the same in the 100% FO as it was in the 50% SO group, but 20:4n-6 and 20:3n-6 were significantly higher in the 100% SO group.

Recovery of [1-^{14}C] FA in lipid classes of hepatocytes and culture media. Twenty-four hours after the start of the incubation, we recovered 15–20% of the added radioactivity in the total lipid fraction of the hepatocytes at 5°C and 20–30% at 12°C (Table 4), whereas 60–80% of the radioactivity was recovered in the culture media (Table 5). The amounts of radioactive FA incorporated into hepatocyte lipids were the same for the three dietary groups, but radiolabeled n-3 FA were incorporated to a greater extent than radiolabeled 18:2n-6 at 12°C.

Table 4 shows a comparison between the proportional distributions of radioactivity from [1-^{14}C]20:5n-3, [1-^{14}C]-18:3n-3, and [1-^{14}C]18:2n-6 in PL, nonpolar lipids (MG + DG + TG) and FFA from hepatocytes, whereas Table 5 shows the corresponding results for culture media. Approximately 50% of the total radioactivity incorporated in the hepatocytes at 12°C was esterified in the PL fraction with all three substrates, about 10% was esterified in nonpolar lipids with the n-3 substrates, and about 30% with the n-6 substrate. As much as 25 to 40% of all three substrates was not esterified but recovered as FFA in the hepatocyte lipids. About 35% of the total radioactivity incorporated in the hepatocytes at 5°C was esterified in the PL fraction for the C_{18} FA substrates, and 40–45% for the 20:5n-3 substrate. About 30% of the total radioactivity was esterified in (MG + DG + TG), and about 25 to 40% was

TABLE 5
Percentage Distributions[a] of Radioactivity from [1-^{14}C]20:5n-3, [1-^{14}C]18:3n-3, and [1-^{14}C]18:2n-6 in Lipid Fractions of Culture Media of Hepatocytes at 5 and 12°C

	5°C			12°C		
	100% FO	50% SO	100% SO	100% FO	50% SO	100% SO
[1-^{14}C]20:5n-3						
PL	9.9 ± 1.49^{b}	9.1 ± 0.59^{b}	3.3 ± 0.17^{a}	13.7 ± 1.73^{b}	14.2 ± 2.65^{b}	12.2 ± 2.30^{b}
Nonpolar (MG + DG + TG)	39.4 ± 3.71^{b}	44.8 ± 3.42^{b,c}	53.4 ± 4.47^{c}	31.1 ± 3.36^{a,b}	23.1 ± 1.36^{a}	37.2 ± 5.12^{b}
FFA	50.7 ± 3.00^{a}	46.1 ± 3.65^{a}	43.4 ± 4.31^{a}	55.2 ± 1.63a^{a}	62.7 ± 3.91^{b}	50.6 ± 2.89^{a}
Total nmol	348.0 ± 11.82	369.1 ± 12.40	380.4 ± 20.01	342.0 ± 33.42	332.3 ± 24.00	340.0 ± 30.22
[1-^{14}C]18:3n-3						
PL	5.5 ± 0.35	4.6 ± 0.07	4.5 ± 0.52	5.7 ± 0.61	6.2 ± 1.28	4.6 ± 1.15
Nonpolar (MG + DG + TG)	44.2 ± 0.49^{a}	50.6 ± 4.22^{a,b}	48.2 ± 0.77^{a,b}	43.8 ± 2.87^{a}	40.9 ± 4.70^{a}	56.8 ± 3.39^{b}
FFA	50.3 ± 0.55^{b}	44.8 ± 4.17^{a,b}	47.3 ± 0.73^{a,b}	50.5 ± 1.35^{b}	52.9 ± 3.60^{b}	38.6 ± 2.62^{a}
Total nmol	368.0 ± 18.82^{b}	376.1 ± 15.01^{b}	387.1 ± 31.42^{b}	317.3 ± 33.41^{a,b}	305.1 ± 25.20^{a}	305.0 ± 9.42^{a}
[1-^{14}C]18:2n-6						
PL	3.7 ± 0.26^{a,b}	3.2 ± 0.34^{a,b}	4.5 ± 0.52^{b}	3.1 ± 0.23^{a,b}	3.3 ± 0.31^{a,b}	2.7 ± 0.56^{b}
Nonpolar (MG + DG + TG)	44.4 ± 1.12	48.6 ± 2.80	48.2 ± 0.78	42.1 ± 2.42	40.6 ± 3.31	44.1 ± 3.89
FFA	51.9 ± 0.87	48.2 ± 3.07	47.3 ± 0.73	54.8 ± 2.65	56.1 ± 3.11	53.2 ± 4.35
Total nmol	368.3 ± 15.58	376.1 ± 17.57	381.2 ± 22.81	356.0 ± 14.12	344.2 ± 21.21	354.1 ± 54.00

[a]Each entry is the average of three experiments ± SEM. Values marked with different roman superscripts are significantly different. Total nmol = nmol of ^{14}C FA recovered in lipid fractions of culture media after 24 h incubation. For abbreviations see Tables 1 and 4.

as FFA. Diet did not have a significant effect on the distribution of radiolabeled FA into lipid classes.

Approximately 50% of the total radioactivity recovered in the total lipid fraction of the culture media was in the form of FFA. Approximately 4–5% of the total radioactivity was recovered in the PL fraction with the C_{18} FA substrates, and 40–50% was recovered in nonpolar lipids (Table 5). About 10% was found esterified in the PL fraction with 20:5n-3 as substrate. The radioactivities found in the PL and nonpolar lipids of culture media are most probably represented by radioactive FA, which were incorporated into lipoproteins in the hepatocytes and then further secreted to the culture media.

[1-^{14}C]PUFA esterified in the PL of hepatocytes. The main radiolabeled FA esterified in the PL fraction from [1-^{14}C]-20:5n-3 were, in addition to 20:5n-3 itself, the elongation and desaturation products 22:5n-3 and 22:6n-3. The high levels of 18:2n-6 in hepatocytes of fish fed the 100% SO diet did not affect the production of 22:6n-3 (Table 6). The level of unmetabolized 20:5n-3 substrate was slightly lower at 12°C than it was at 5°C. Interestingly, the percentage of the first elongation product (22:5n-3) was higher at 12°C (30–35%) than at 5°C (20%). The percentage of 22:6n-3 was, in spite of this, significantly higher at 5°C than at 12°C. This suggests that the further conversion from 22:5n-3 to 22:6n-3 is significantly more efficient at 5°C than at 12°C. Interestingly, both the percentage of 24:5n-3 and the ratio of 24:5n-3 to 22:5n-3 are higher at the lower temperature. The ratio of 24:5n-3 to 22:5n-3 can be used as an indicator of the activity of the final elongation step, and thus suggests that the rate of this elongation is substantially higher at 5°C than at 12°C (Fig. 1).

The main metabolites in the PL fraction esterified from [1-^{14}C]18:3n-3 were, in addition to itself, the desaturation and elongation products 20:4n-3, 20:5n-3, and 22:6n-3. Substantial amounts of the dead-end elongation product 20:3n-3 were, however, also formed (Table 6).

Elongation and desaturation products also appeared in the PL fraction after 24 h of incubation with [1-^{14}C]18:2n-6 as substrate, both at 5 and at 12°C. However, more than 50% of the radioactivity recovered in the PL fraction was as unchanged substrate at both temperatures. The main radiolabeled FA metabolites formed were the eicosanoid precursors 20:3n-6 and 20:4n-6, and the dead-end elongation product 20:2n-6. The Δ5-desaturase activity, calculated as (20:4 + 22:4 + 24:5)/(20:3 + 20:4 + 22:4 + 24:5), was higher in hepatocytes from fish fed the SO diets than it was in hepatocytes from fish fed the FO diet. The percentage of 20:4n-6 was somewhat higher at the higher temperature than it was at the lower temperature, and it increased with increased dietary SO at both temperatures (Fig. 2).

The distributions of radiolabeled FA in the pooled nonpolar lipid fraction (MG + DG + TG) revealed essentially the same patterns as those seen in the PL fraction (Table 7). About 50% remained as the unchanged substrate when 20:5n-3 was used as as substrate, and about 25% formed the elongated product 22:5n-3. Temperature effects were less clear in the nonpolar lipid fraction than in the PL fraction, but

TABLE 6
Percentage Distributions[a] with Temperature and Diet of Radioactivity from [1-^{14}C]20:5n-3, [1-^{14}C]18:3n-3, and [1-^{14}C]18:2n-6 in PUFA of PL of Hepatocytes

	5°C			12°C		
	100% FO	50% SO	100% SO	100% FO	50% SO	100% SO
[1-^{14}C]20:5n-3						
20:5n-3	44.8 ± 0.90	45.0 ± 1.94	43.6 ± 1.87	39.5 ± 2.48	40.1 ± 1.67	40.5 ± 2.83
22:5n-3	21.6 ± 0.14^{a}	21.9 ± 1.86^{a}	22.7 ± 2.28^{a}	35.9 ± 2.81^{b}	29.1 ± 2.02^{b}	33.4 ± 4.33^{b}
22:6n-3	26.4 ± 0.85	25.3 ± 3.76	22.2 ± 2.72	18.2 ± 3.33	24.3 ± 1.55	20.9 ± 5.94
24:5n-3	5.9 ± 0.36^{b}	5.9 ± 0.44^{b}	9.3 ± 0.23^{c}	3.9 ± 0.98^{a,b}	4.2 ± 1.14^{a,b}	2.8 ± 0.15^{a}
24:6n-3	1.4 ± 0.41	1.9 ± 0.18	2.1 ± 0.93	2.6 ± 0.89	2.3 ± 0.56	2.5 ± 0.20
DI	0.55 ± 0.01	0.55 ± 0.02	0.56 ± 0.03	0.61 ± 0.04	0.60 ± 0.02	0.59 ± 0.02
[1-^{14}C]18:3n-3						
18:3n-3	58.1 ± 0.91	56.0 ± 2.79	58.9 ± 6.23	53.4 ± 2.42	50.2 ± 0.92	49.5 ± 3.98
18:4n-3	1.3 ± 0.19^{a}	1.9 ± 0.22^{a}	3.8 ± 1.33^{b}	2.1 ± 0.36^{a,b}	2.3 ± 0.13^{a,b}	2.6 ± 0.51^{a,b}
20:3n-3	11.5 ± 0.94	8.5 ± 1.96	8.4 ± 3.74	12.0 ± 1.43	13.1 ± 1.03	10.9 ± 0.78
20:4n-3	18.3 ± 1.54	16.5 ± 2.01	13.8 ± 2.15	16.2 ± 0.66	17.5 ± 1.00	15.2 ± 0.55
20:5n-3	4.6 ± 0.55^{a,b}	7.0 ± 0.53^{b,c}	4.3 ± 0.55^{a}	7.8 ± 0.21^{c}	7.7 ± 0.81^{c}	9.0 ± 1.54^{c}
22:5n-3	0.4 ± 0.11^{a}	0.6 ± 0.24^{a,b}	1.2 ± 0.08^{a,b}	1.9 ± 0.31^{b,c}	1.4 ± 0.08^{b,c}	2.2 ± 0.80^{c}
22:6n-3	5.9 ± 0.32	9.5 ± 1.74	10.1 ± 2.95	7.1 ± 1.23	8.2 ± 0.42	10.5 ± 3.26
DI	0.34 ± 0.02	0.39 ± 0.04	0.35 ± 0.05	0.39 ± 0.02	0.42 ± 0.01	0.44 ± 0.01
[1-^{14}C]18:2n-6						
18:2n-6	53.6 ± 1.80	52.6 ± 1.48	60.5 ± 2.35	60.3 ± 3.56	54.5 ± 1.89	57.9 ± 2.30
18:3n-6	0.7 ± 0.11^{a}	0.9 ± 0.06^{a}	1.1 ± 0.35^{a}	1.2 ± 0.26^{a}	1.5 ± 0.07^{a,b}	2.1 ± 0.50^{b}
20:2n-6	18.7 ± 1.16	17.5 ± 1.27	14.8 ± 0.28	14.3 ± 3.96	18.4 ± 2.51	13.2 ± 0.40
20:3n-6	22.2 ± 2.22^{b}	20.4 ± 1.39^{a,b}	15.9 ± 1.16^{a}	17.0 ± 0.20^{a}	17.6 ± 0.84^{a}	15.9 ± 0.42^{a}
20:4n-6	2.8 ± 0.46^{a}	6.3 ± 0.18^{b}	6.0 ± 0.97^{a,b}	6.0 ± 0.54^{a,b}	6.7 ± 1.49^{b}	11.8 ± 2.11^{c}
22:4n-6	0.2 ± 0.14	0.8 ± 0.44	0.2 ± 0.16	0.2 ± 0.19	0.3 ± 0.27	0.5 ± 0.49
24:5n-6	1.7 ± 0.46	1.6 ± 0.29	1.5 ± 0.55	1.1 ± 0.60	1.1 ± 0.59	0.3 ± 0.32
DI	0.33 ± 0.03	0.36 ± 0.02	0.29 ± 0.03	0.30 ± 0.02	0.33 ± 0.02	0.33 ± 0.03

[a]Each entry is the average of three experiments ± SEM. Values marked with different roman superscript letters are significantly different ($P \leq 0.05$). DI, desaturation index; for other abbreviation see Table 4.

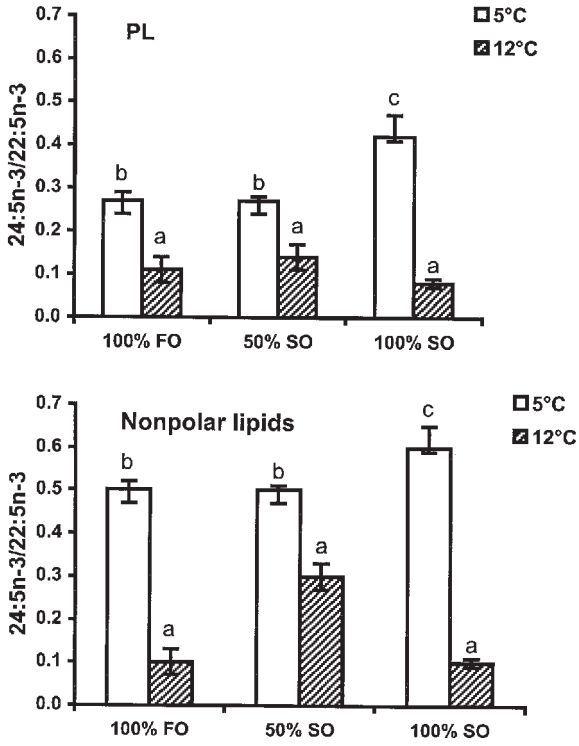

FIG. 1. Relative elongase activities (measured as the ratio of 24:5n-3 to 22:5n-3) in hepatocytes incubated for 24 h with [1-^{14}C]20:5n-3 at 5 and 12°C. The hepatocytes were from fish fed the 100% FO (= 100% fish oil), 50% SO (= 50% fish oil and 50% soybean oil), and 100% SO (= 100% soybean oil) diets. PL = phospholipids. Data are means ± SEM (n = 3). Different letters within each graph indicate significant differences ($P \leq 0.05$) between values.

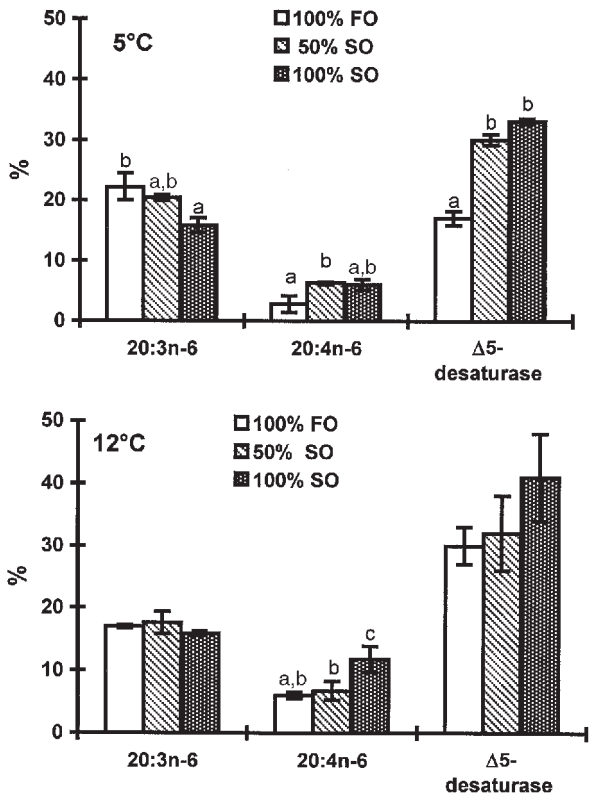

FIG. 2. Percentage radiolabeled 20:3n-6 and 20:4n-6 and relative activity of Δ5-desaturase activity in PL of hepatocytes incubated with [1-^{14}C]18:2n-6 for 24 h at 5 and 12°C. The hepatocytes were from fish fed the 100% FO, 50% SO, or 100% SO dietary groups. Data are means ± SEM (n = 3). Different letters within each graph indicate significant differences ($P \leq 0.05$) between values. For abbreviations see Figure 1.

even here a slightly higher ratio of 24:5n-3 to 22:5n-3 was evident at the lowest temperature. A significantly higher percentage of 24:5n-3 at 5°C than at 12°C was also evident. Diet did not have any significant effect on the distributions.

The nonpolar lipid fraction was dominated by the unchanged substrates (≈70%) when 18:3n-3 and 18:2n-6 FA were used as substrates, and relatively high amounts of dead-end elongation products formed in this case. On the other hand, 15–20% of the lipids were 20:4n-3 when 18:3n-3 was used, and very little longer or more unsaturated products formed. Neither diet nor temperature had any significant effect on the distributions. Only small amounts of the longer, more unsaturated products were formed when 18:2n-6 was used as substrate. However, the percentage of 20:4n-6 increased significantly when dietary SO increased. The distribution patterns of elongated and desaturated products in culture media were similar to those in the hepatocytes (results not shown).

DISCUSSION

The FA composition of total hepatocyte lipids was influenced by the composition of the dietary oils. This agrees with several studies using different types of vegetable oils in salmonid diets. Generally, the replacement of FO with SO results in higher levels of n-6 FA and lower levels of n-3 FA in the tissues of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (18–21). Bell *et al.* (22) found that the liver 18:1n-9 level was related to the diet level of rapeseed oil. Our results for 18:2n-6 agree with this, whereas the changes observed for the longer, more unsaturated FA such as 20:4n-6 and 22:6n-3 are more complex. The levels of these important FA are probably also controlled by more specific regulatory mechanisms. It has been postulated that mechanisms exist in the rat that promote the selective retention of EFA, especially in PL (23). This is supported by our previous observation of a very slow decline in liver PL 22:6n-3 in Atlantic salmon given diets deficient in n-3 and n-6 PUFA (7).

More radioactivity was found in the hepatocytes at 12°C than at 5°C with all three (18:2n-6, 18:3n-3, and 20:5n-3) substrates. This is not surprising, since cellular metabolic rates usually increase with increasing temperature. Slightly more of the n-3 substrates, and especially 18:3, were incorporated than the n-6 substrate 18:2 at 12°C. This agrees with earlier reports on rainbow trout hepatocytes (24). The same results for C_{18} FA also have been reported from rat studies, probably related to the higher activity in this species of acyl-CoA synthetase with 18:3n-3 than with 18:2n-6 (25). The lipid class distribution of cell-associated radioactivity was affected by the degree of unsaturation of the FA substrates. Relatively more 18:3n-3 and

TABLE 7
Percentage Distributions[a] of Radiolabeled FA in the Nonpolar Lipid Fraction (MG + DG + TG) of Hepatocytes Incubated with [1-^{14}C]20:5n-3, [1-^{14}C]18:3n-3, and [1-^{14}C]18:2n-6 at 5 or 12°C

	5°C			12°C		
	100% FO	50% SO	100% SO	100% FO	50% SO	100% SO
[1-^{14}C]20:5n-3						
20:5n-3	50.3 ± 1.84^{a,b}	50.4 ± 1.41^{a,b}	55.1 ± 1.29^{b,c}	50.9 ± 2.38^{a,b}	48.7 ± 0.42^{a}	57.8 ± 1.98^{c}
22:5n-3	25.2 ± 0.97^{a,b,c}	23.7 ± 0.75^{a,b}	21.5 ± 1.61^{a}	29.6 ± 1.69^{c}	27.6 ± 2.15^{b,c}	28.1 ± 1.49^{b,c}
22:6n-3	9.7 ± 0.39	9.2 ± 1.56	8.0 ± 1.21	6.7 ± 1.40	10.1 ± 1.04	7.1 ± 2.52
24:5n-3	12.0 ± 0.81^{c}	12.6 ± 0.71^{c}	12.2 ± 1.91^{c}	3.7 ± 0.98^{a}	7.6 ± 1.25^{b}	4.0 ± 0.59^{a}
24:6n-3	2.9 ± 0.31^{a}	4.0 ± 0.39^{a}	3.3 ± 0.93^{a}	9.2 ± 0.86^{c}	6.0 ± 0.46^{b}	3.0 ± 0.56^{a}
[1-^{14}C]18:3n-3						
18:3n-3	66.1 ± 1.0	69.2 ± 2.24	62.3 ± 2.69	67.0 ± 12.50	56.6 ± 18.54	69.7 ± 9.70
18:4n-3	2.5 ± 1.00	3.8 ± 1.18	4.3 ± 1.55	2.8 ± 0.76	ND	3.1 ± 1.65
20:3n-3	15.8 ± 2.22	10.1 ± 1.99	14.3 ± 1.81	9.1 ± 4.73	19.0 ± 1.47	4.8 ± 2.98
20:4n-3	15.6 ± 1.97	15.0 ± 1.81	18.1 ± 2.31	15.1 ± 2.16	24.4 ± 5.36	17.6 ± 3.34
20:5n-3	ND	0.82 ± 0.82	ND	6.0 ± 5.85	ND	3.0 ± 2.15
22:5n-3	ND	ND	ND	ND	ND	ND
22:6n-3	ND	1.1 ± 1.14	1.0 ± 1.02	ND	ND	1.8 ± 0.91
[1-^{14}C]18:2n-6						
18:2n-6	73.2 ± 0.92^{a,b}	75.1 ± 0.89^{b}	74.2 ± 2.48a^{a,b}	71.8 ± 1.65^{a,b}	71.7 ± 1.07^{a,b}	70.4 ± 0.16^{a}
18:3n-6	2.4 ± 0.22^{a,b}	2.6 ± 0.10^{a,b}	1.6 ± 0.43^{a}	2.1 ± 0.09^{a,b}	1.9 ± 0.11^{a,b}	3.0 ± 0.70^{b}
20:2n-6	15.9 ± 0.46	13.1 ± 1.08	13.0 ± 1.67	14.8 ± 1.97	15.4 ± 1.53	13.3 ± 2.52
20:3n-6	8.1 ± 0.73	8.7 ± 1.09	10.4 ± 0.98	10.0 ± 0.31	9.2 ± 0.75	10.3 ± 1.30
20:4n-6	0.5 ± 0.15^{a}	0.5 ± 0.10^{a,b}	0.7 ± 0.27^{a,b}	1.4 ± 0.62^{a,b}	1.8 ± 0.14^{b}	3.0 ± 0.51^{c}
22:4n-6	ND	ND	ND	ND	ND	ND
24:5n-6	ND	ND	ND	ND	ND	ND

[a]Each entry is the average of three experiments ± SEM. Values marked with different roman superscript letters are significantly different ($P \leq 0.05$).

20:5n-3 were esterified in PL than 18:2n-6 at the lower temperature. No such preference for incorporation of the n-3 FA into PL relative to nonpolar lipids was observed at the higher temperature, consistent with results from a similar study with rainbow trout (1). Sellner and Hazel (1) showed that cells from cold-acclimated trout incorporated radiolabeled FA with a high degree of unsaturation into their polar lipids to a greater extent than they incorporated FA with a lower degree of unsaturation. Cells from warm-acclimated trout, on the other hand, showed no such preference for the more unsaturated FA.

We used the ratio of 24:5 to 22:5 as a measure of the rate of elongation of 22:5 to 24:5, using 20:5n-3 as substrate. This rate was substantially higher at 5°C than it was at 12°C. This higher elongation rate may explain to a certain extent the increased production of 22:6 from 18:3 at the lower temperature. These results agree with results obtained with rainbow trout (1), which showed that hepatocytes from cold-acclimatized trout were more efficient at producing 22:6 from 18:3n-3 at 5°C than cells from warm-acclimatized trout. Interestingly, we found that hepatocytes from fish fed diets containing high levels of 18:2n-6 had an even greater elongation rate at 5°C than hepatocytes from fish in the fish oil group. Elongation reactions are not usually considered as major control steps in FA metabolism. However, Luthria and Sprecher (26) suggested that the last chain-elongation step in the unsaturated FA biosynthesis in rat liver may be equally regulatory in governing the synthesis of FA as is the Δ6-desaturation. Our results suggest that the final elongation step in fish is also an important point of regulatory control.

The production of 20:4n-6 from 18:2 increased with increasing levels of n-6 FA in the hepatocytes, which may be caused by an increase in Δ5-desaturase activity. The activity of Δ5-desaturase can be measured by calculating (20:4 + 22:4 +24:5)/(20:3 + 20:4 + 22:4 +24:5). Activities calculated in this way show that the activity increases in hepatocytes from fish fed the SO diets, in agreement with results from mammals (27,28). The results also agree with recent studies published by Bell *et al.* (22) showing that Δ5-desaturase activity increases in hepatocytes from salmon given increasing levels of palm oil or rapeseed oil. Several nutritional studies in higher vertebrates have shown that hepatic Δ5-desaturase activity is suppressed by diets rich in marine oils (28–30). Thus, the increased Δ5-desaturase activity may be due not only to the increased levels of n-6 FA in hepatocyte lipids but also to the removal of product inhibition as a result of decreased levels of EPA and DHA in the hepatocytes.

The production of 22:6n-3 from 18:3 also increased with increasing levels of n-6 FA in the hepatocytes. This increase may also be partially explained by the reduced product inhibition that results from reduced endogenous levels of 20:5 and 22:6 since 24:5 (produced from 20:5) probably competes with 18:3 for the same Δ6-desaturase. The endogenous percentage of 20:5n-3 is low in hepatocytes from fish fed an SO diet, and the desaturation and elongation of 18:3 may proceed unhindered in this case.

In summary, diets containing high levels of soybean oil lead to high endogenous levels of n-6 FA in Atlantic salmon hepatocytes, and these in turn lead to an enhanced production of the longer-chain n-3 and n-6 FA from C_{18} FA. This increase may have been due to an increase in Δ5-desaturase activity. Furthermore, the production rate of 22:6n-3 from 20:5n-3 was higher at 5°C than at 12°C probably due to an increased elongation rate of 22:5 to 24:5 at the lower temperature.

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Pharmacological Modulation of Fatty Acid Desaturation and of Cholesterol Biosynthesis in THP-1 Cells

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ABSTRACT: In THP-1 cells, simvastatin decreases, in a concentration-dependent manner, cholesterol synthesis and increases linoleic acid (LA) conversion to its long-chain derivatives, in particular to arachidonic acid, activating Δ6 and Δ5 fatty acid (FA) desaturases. The intermediates in cholesterol synthesis, mevalonate and geranylgeraniol, partially reverse the effects of simvastatin on the LA conversion. The aims of this work were to evaluate: (i) the correlation between cholesterol synthesis and desaturase activity and (ii) the possible involvement of protein isoprenylation in desaturase activity, assessed through pharmacological treatments. THP-1 cells were incubated with [1-^{14}C]LA or with [1-^{14}C]di-homo-γ-linolenic acid (DHGLA) and treated with simvastatin or with curcumin and nicardipine, inhibitors of desaturases. Curcumin was more active than nicardipine in inhibiting LA and DHGLA conversion: 20 μM curcumin, alone or with simvastatin, totally inhibited Δ6 and Δ5 desaturation steps; 10 μM nicardipine only partially inhibited the enzymes, being more active on Δ5 desaturase. Simvastatin treatment decreased the incorporation of acetate in cholesterol (–93.8%) and cholesterol esters (–70.2%), as expected. Curcumin and nicardipine also decreased cholesterol synthesis and potentiated simvastatin. Finally, the isoprenylation inhibitors (perillic acid and GGTI-286) neither affected the conversion of LA nor inhibited the Δ5 desaturase activity. In conclusion, our results indicate that there is no direct relationship between cholesterol synthesis and desaturase activity. In fact, simvastatin decreased cholesterol synthesis and enhanced LA conversion (mainly Δ5 desaturation), whereas curcumin and nicardipin decreased Δ5 desaturation, with a limited effect on cholesterol synthesis.

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Long-chain PUFA (LC-PUFA) present in animal tissues are ultimately derived from the diet, either provided directly as preformed compounds or produced from the 18-carbon PUFA that constitute the classical EFA. Linoleic (18:2, LA) and α-linolenic (18:3, α-LNA) acids are the EFA precursors of the n-6 and n-3 FA, respectively. LC-PUFA, i.e., arachidonic acid (20:4n-6, AA), EPA (20:5n-3), and DHA (22:6n-3), are produced in the two FA series through elongation and desaturation reactions.

The desaturation reactions are catalyzed by different enzymes: the conversion of 18:2n-6 into 18:3n-6 by Δ6 desaturase and that of 20:3n-6 into 20:4n-6 by Δ5 desaturase. These enzymes are modulated by various factors. They are inhibited by desaturase products (1,2) and cholesterol-rich diets (3), whereas Δ6 desaturase activity is induced by low EFA intakes, i.e., in the presence of low substrate concentrations, and is suppressed by diets rich in vegetable or marine oils (4,5), i.e., by the excess of substrates. The enzymatic activities are also modulated by hormones—activated by insulin and reduced by adrenaline, glucagon, and steroids (6). Different classes of xenobiotics, e.g., peroxisome proliferators (7), calcium antagonists (8), and statins (9), also affect the activity of desaturases.

Among the desaturase inhibitors, curcumin, the major component of a spice obtained from *Curcuma longa* (L.), is a nonspecific and noncompetitive inhibitor in rat liver microsomes (10). The Ca^{2+} antagonist nicardipine is a specific inhibitor of Δ5 desaturase, whereas nifedipine, another Ca^{2+} channel blocker, is a specific inhibitor of Δ6 desaturase. Since other Ca^{2+} antagonists, e.g., verapamil, flunarizine, and diltiazem, do not show inhibitory effects (8), the inhibition of desaturase reactions apparently is not dependent on intracellular Ca^{2+} levels.

In THP-1 cells, simvastatin increases the conversion of LA to AA, acting in particular on Δ5 desaturase, and this effect is correlated with a marked reduction in cholesterol synthesis. The treatment of cells with intermediates in cholesterol synthesis, such as mevalonate and geranylgeraniol, restores the normal metabolic conditions, underlining the complexity of the interactions between cholesterol synthesis and FA metabolism (9). The direct isoprenylation of the Δ5 desaturase can be excluded: The characteristic CAAX motif (C, cysteine; A, aliphatic amino acid; X, any amino acid) of isoprenylation (11) is not present in the amino-acidic sequence of this enzyme (GeneBank, accession number AAF29378).

This study investigates the involvement and regulation of Δ5 and Δ6 desaturases in the effects of simvastatin on the synthesis of AA from LA or from its immediate precursor dihomo-γ-linolenic acid (20:3n-6), in THP-1 cells. THP-1, derived from monocytes, have a very active FA metabolism and may provide information on lipid metabolism in circulating cells. First, to better characterize the pharmacological modulation of desaturases, the inhibitors curcumin and nicardipine were used. The relationships between the effects of simvastatin on PUFA metabolism (increased desaturase activity) and cholesterol synthesis are also described. Finally, we investigated the possible involvement of prenylated proteins, other than desaturases, in the elevation of FA desaturation by simvastatin.

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Abbreviations: α-LNA, α-linolenic acid; AA, arachidonic acid; CE, cholesterol esters; FC, free cholesterol; LA, linoleic acid; LC-PUFA, long-chain PUFA; PL, phospholipids.

Perillic acid, an inhibitor of both farnesyltransferase and geranylgeranyltransferase types I and II (12,13), and the compound GGTI-286, a specific inhibitor of geranylgeranyltransferase type I (14), were studied.

MATERIALS AND METHODS

Materials and reagents. The materials for cell cultures (RPMI 1640 medium, penicillin, and streptomycin), curcumin, nicardipine and (–)-perillic acid were from Sigma (Sigma-Aldrich S.r.l., Milan, Italy); GGTI-286 was from Calbiochem (Darmstadt, Germany); FCS was from Life Technologies (Paisley, United Kingdom). Solvents and TLC plates were from E. Merck (Darmstadt, Germany). The radiolabeled compounds, [1-^{14}C]LA (specific activity 55 mCi/mmol) and [1-^{14}C]acetic acid sodium salt (specific activity 58 mCi/mmol), were purchased from Amersham (Amersham Pharmacia Biotech, Little Chalfont, England). [1-^{14}C]20:3n-6 (specific activity 52 mCi/mmol) was from NEN (NEN Life Science Products, Zaventem, Belgium). Simvastatin in lactone form (Merck, Sharp & Dohme Research Laboratories, Woodbridge, NJ) was dissolved in 0.1 M NaOH to give the active, open β-hydroxy acid form.

Cell culture. THP-1 cells, a human monocytic cell line (15), were grown in RPMI medium containing 10% FCS, 100 μg/mL penicillin, 100 IU/mL streptomycin, and 2 mM L-glutamine.

Experimental design. At the time of the experiments, cells were centrifuged at 200 × *g* for 10 min, and the pellet obtained was resuspended in RPMI medium without FCS; the concentration of the cells was adjusted to 10^6/mL.

Simvastatin (5 μM) was added (9) at the start of the experiments, whereas the desaturase inhibitors, curcumin (10) and nicardipine (8), were added after 23 h. The radiolabeled substrates, [1-^{14}C]LA (0.1 μCi/mL), [1-^{14}C]acetic acid sodium salt (1 μCi/mL) or [1-^{14}C]20:3n-6 (0.03 μCi/mL), were added to the medium, at the final concentrations indicated, after 24 h from the start of the experiment and were incubated for an additional 24 h. In the experiments on protein prenylation, THP-1 cells were incubated with the inhibitors of transferases (–)-perillic acid or GGTI-286 at the start and for an additional 48 h; [1-^{14}C]LA (0.1 μCi/mL) was added to the medium for the last 24 h.

At the end of the experiments, the cells were centrifuged, resuspended in PBS, and counted by means of a cell counter.

Lipid extraction was carried out according to Folch *et al.* (16); the lipid concentration in the samples was evaluated by microgravimetry (17), whereas radioactivity, expressed as cpm/μL, was evaluated by the use of a β-counter.

Dose–response curves for the activity of desaturase inhibitors were obtained in experiments using 2.5, 5, 10, and 20 μM curcumin or 5, 10, 20, and 40 μM nicardipine, with or without 5 μM simvastatin. Concentrations of the inhibitors that resulted in maximal inhibition without cytotoxicity were used in these studies.

Analysis of radiolabeled FA by HPLC. FAME were prepared by transesterification with 3 N CH_3OH/HCl for 1 h at 90°C; after FA methylation, the radioactivity associated with the different FA was determined by HPLC equipped with an on-line radiodetector (Flo-One Beta A-200, Radiomatic Instruments and Chemical, Tampa FL), as previously reported (9).

Separation of lipid classes. The lipid classes (phospholipids, PL; TG; cholesterol esters, CE; DG; and FFA) were separated by one-dimensional TLC using silica gel plates and a mobile phase of hexane/diethyl ether/acetic acid (70:30:1.5, by vol). To separate free cholesterol (FC) and MG, a second solvent system was used: chloroform/methanol (98:2, vol/vol). Lipids were detected on plates by iodine vapor exposure; spots were scraped off and the radioactivity was detected after addition of 1 mL methanol/water (1:1, vol/vol) and 10 mL of scintillation fluid (Ultima Gold^{TM}) in a scintillation counter.

Statistical analysis. Significance of differences, when comparing values in control and treated cells, was assessed by the use of Student's *t*-test.

RESULTS

We initially evaluated the possible cytotoxic effects of drug treatments on THP-1 cells. At the end of the experiments, the cell number in 5 μM simvastatin samples vs. controls was not significantly different (2–3%). Treatment of THP-1 cells with 20 μM curcumin or 10 μM nicardipine decreased the number of cells by about 15 and 7%, respectively, in comparison to controls. After incubation with labeled substrates, the total radioactivity recovered in lipids, extracted from each sample, was similar.

The total conversion of [1-^{14}C]LA (expressed as the sum of all n-6 FA derived from LA) in control cells was about 30% of the total radioactivity provided in the medium, whereas after treatment with 5 μM simvastatin for 48 h, the conversion increased up to 60% (Fig. 1). Curcumin and nicardipine were

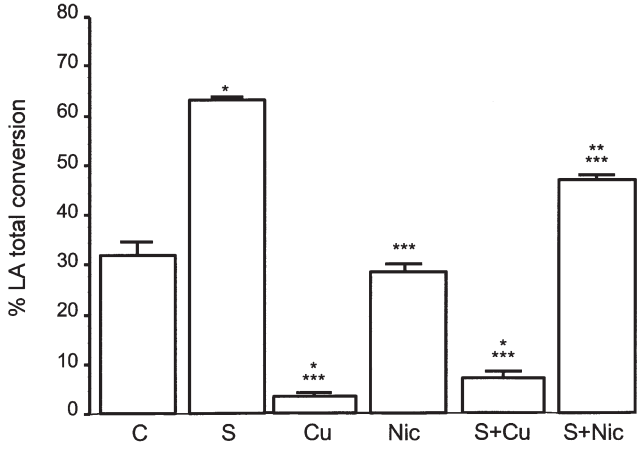

FIG. 1. Linoleic acid (LA) total conversion to n-6 FA in THP-1 cells incubated with [1-^{14}C]LA in the absence (control: C) or presence of simvastatin (S) (5 μM), curcumin (Cu) (20 μM), and nicardipine (Nic) (10 μM). Values are the mean ± SE of four different experiments. Values significantly different from C at $*P < 0.002$; $**P < 0.006$; significantly different from S at $***P < 0.001$.

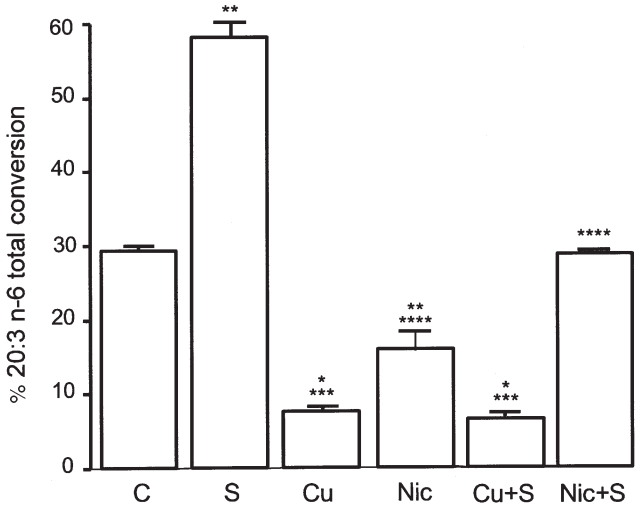

FIG. 2. Total conversion of 20:3n-6 to n-6 FA in THP-1 cells incubated with [1-^{14}C]20:3 n-6 in the absence (control: C) or presence of simvastatin (S) (5 μM), curcumin (Cu) (20 μM), and nicardipine (Nic) (10 μM). Values are the mean ± SE of four different experiments. Values significantly different from C at $*P < 0.001$; $**P < 0.006$; significantly different from S at $***P < 0.001$; $****P < 0.005$.

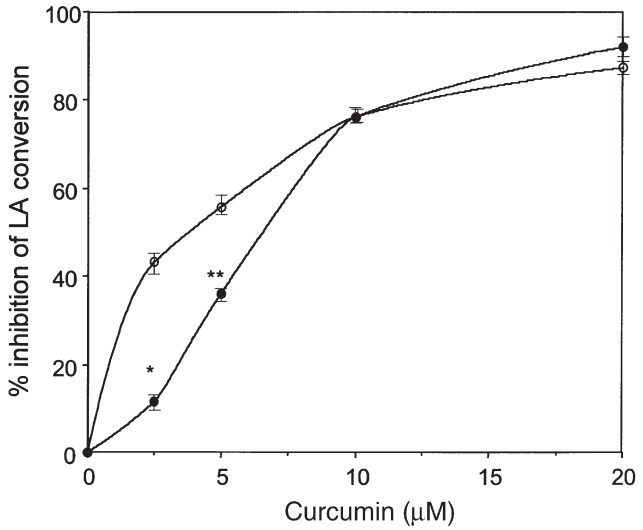

FIG. 3. Dose–response curves of curcumin (Cu) (0–20 μM), without or with 5 μM simvastatin (S), for the inhibition of linoleic acid (LA) total conversion. Values are the mean ± SE of three different experiments. (○) Cu, (●) Cu + S. % Inhibition = (activity without curcumin – activity with curcumin)/(activity without curcumin). Values significantly different from Cu at $*P < 0.00001$; $**P < 0.005$.

used at the highest noncytotoxic concentrations that inhibit desaturase, as reported in the literature. Curcumin (20 μM), a nonspecific Δ5 and Δ6 desaturase inhibitor (10), markedly reduced LA total conversion to very low levels (about 3%), whereas 10 μM nicardipine, a Δ5 desaturase inhibitor (8), did not significantly affect LA total conversion vs. control after 24 h of incubation (Fig. 1).

When both simvastatin and desaturase inhibitors were used, curcumin reversed the effect of simvastatin, whereas nicardipine significantly decreased the LA total conversion enhanced by simvastatin, but not below control values (Fig. 1).

The conversion of [1-^{14}C]20:3n-6 to AA and then to longer-chain and more unsaturated FA involves the Δ5 desaturation step. Similar to the effect on LA total conversion, the total conversion of [1-^{14}C]20:3n-6 was enhanced by simvastatin from 28.2% in controls to about 57.4% (Fig. 2). The radioactivity recovered in AA from [1-^{14}C]20:3n-6 was 25.5 ± 2.0% in control cells and 49.6 ± 0.8% in simvastatin-treated cells ($P < 0.006$), whereas the radioactivity in 22:4n-6 was 2.7 ± 0.1 and 5.3 ± 0.1% ($P < 0.005$), respectively. Conversely, curcumin and nicardipine decreased the total conversion of 20:3n-6 from 28.2 to 7.4 ($P < 0.001$) and 15.9% ($P < 0.006$), respectively, and again curcumin was more effective than nicardipine (Fig. 2). In addition, curcumin and nicardipine totally inhibited the elongation of AA to 22:4 (data not shown).

We further investigated the pharmacological interactions between the desaturase inhibitors and simvastatin by evaluating the changes in the dose–response curves for these compounds. The curves showing the inhibition of LA total conversion, obtained with different concentrations of curcumin without or with 5 μM simvastatin, are reported in Figure 3. LA total conversion was inhibited by 43 and 87% with 2.5 and 20 μM of curcumin in the medium, respectively. When THP-1 cells were preincubated with simvastatin, the same inhibitory effect of curcumin was obtained at a concentration more than double that required in the absence of simvastatin, for concentrations of curcumin up to 10 μM. Figure 4 shows the dose–response curves for nicardipine effects on LA total conversion. At elevated concentrations, the inhibitory effect of nicardipine, in the

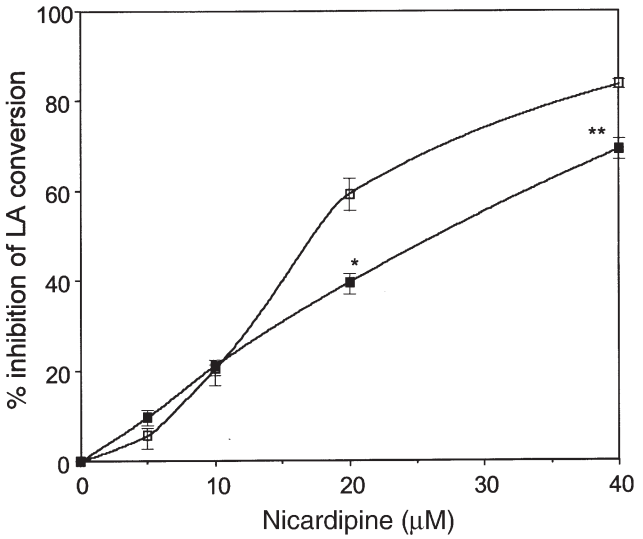

FIG. 4. Dose–response curves of nicardipine (Nic) (0–40 μM), without or with 5 μM simvastatin (S), for the inhibition of linoleic acid (LA) total conversion. Values are the mean ± SE of three different experiments. (□) Nic, (■) Nic + S. % Inhibition = (activity without nicardipine – activity with nicardipine)/(activity without nicardipine). Values significantly different from Nic at $*P < 0.001$; $** P < 0.005$.

TABLE 1
Percent Distribution[a] of Radioactivity in Lipid Classes of THP-1 Cells Incubated with [1-^{14}C]Acetic Acid Sodium Salt[a]

	C	S	Cu	Nic	Cu + S	Nic + S
PL	56.90 ± 5.8	66.60 ± 7.3	48.60 ± 4.3	49.90 ± 1.6	55.40 ± 2.1	52.21 ± 1.7
TG	31.40 ± 7.6	30.40 ± 6.6	41.40 ± 4.1	43.80 ± 2.4	42.20 ± 2.0	46.00 ± 1.5
FFA	0.60 ± 0.1	0.70 ± 0.2	0.51 ± 0.0	0.53 ± 0.2	0.65 ± 0.2	0.56 ± 0.1
CE	1.78 ± 0.3	0.52 ± 0.1[a]	2.38 ± 0.9	0.46 ± 0.1[a]	0.47 ± 0.2[a]	0.33 ± 0.3[b]
FC	8.10 ± 2.2	0.50 ± 0.0[a]	5.60 ± 1.1[d]	3.69 ± 1.1[d]	0.23 ± 0.0[a,c]	0.18 ± 0.0[a,c]
MG	0.34 ± 0.1	0.54 ± 0.3	0.58 ± 0.2	0.47 ± 0.0	0.52 ± 0.1	0.22 ± 0.0
DG	0.89 ± 0.2	0.75 ± 0.4	0.92 ± 0.3	1.21 ± 0.3	0.54 ± 0.1	0.49 ± 0.1

[a]PL, phospholipids; CE, cholesterol esters; FC, free cholesterol; C, control; S, simvastatin (5 μM); Cu, curcumin (20 μM); Nic, nicardipine (10 μM). Values refer to average ± SE of three different experiments. Significantly different from C at [a]$P < 0.03$; [b]$P < 0.06$; significantly different from S at [c]$P < 0.01$; [d]$P < 0.05$.

presence of simvastatin, is smaller than that of nicardipine alone.

The effects of simvastatin and of the desaturase inhibitors on cholesterol synthesis from acetate were also evaluated. The incorporation of [1-^{14}C]acetate into individual lipid classes (Table 1) revealed that there were reduced incorporations in FC and CE of approximately 93.8 and 70.2%, respectively, after treatment with simvastatin. Curcumin (20 μM) and nicardipine (10 μM) marginally affected acetate incorporation in FC, with reductions—with respect to the control—of 30.9 and 54.4%, respectively. In addition, curcumin and especially nicardipine appeared to further reduce the incorporation of acetate in FC and CE induced by simvastatin.

To investigate whether inhibition of isoprenylation was involved in the effects of simvastatin on LA conversion, we used inhibitors of this process. Figure 5 shows the LA total conversion after treatment with 5 μM simvastatin or with 0.5, 1.0, or 3.0 mM (–)-perillic acid (12,13), in THP-1 cells. Simvastatin increased but 0.5 mM or 1.0 mM (–)-perillic acid did not significantly affect LA total conversion. Only at the highest concentration (3 mM), was there decreased [^{3}H]thymidine incorporation into cells, indicating cytotoxic effects (data not shown).

The specific inhibitor of geranylgeranyltransferase I, GGTI-286, in a concentration range of 0.5–3 μM, was also studied (14). At these GGTI-286 concentrations, LA total conversion was not affected (Fig. 6); moreover, the product/precursor ratios, an indicator of the Δ6 (18:3/18:2) and Δ5 (20:4/20:3) desaturation, were not different in treated cells with respect to controls.

DISCUSSION

Statins enhance LA conversion to long-chain n-6 FA, increasing AA levels *in vitro* (9,18) and *in vivo* (19,20). Data from *in vitro* studies indicate that the two desaturation steps, from 18:2n-6 to 18:3n-6 and from 20:3n-6 to 20:4n-6, are affected by statins, and the effects on LA conversion are more potent than those on cholesterol synthesis (21); in particular, the second step, involving the Δ5 desaturase, is markedly increased. The product/precursor ratios show that the activity of these enzymes (Δ6 and Δ5) is significantly increased after 48 h of cell incubation with simvastatin.

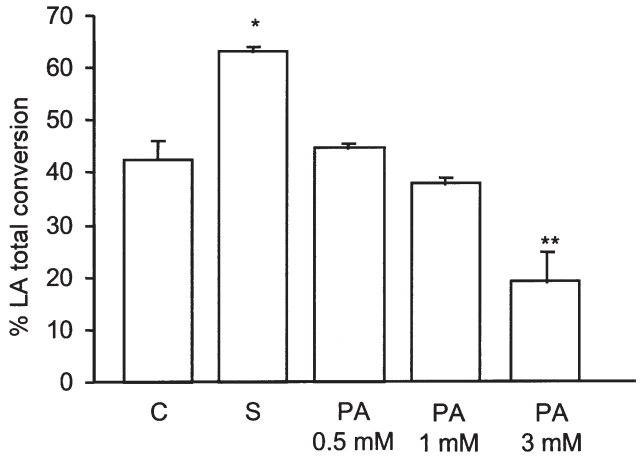

FIG. 5. Linoleic acid (LA) total conversion in THP-1 cells incubated with simvastatin (S) (5 μM), *S*(–) perillic acid (PA) (0.5, 1, and 3 mM), and [1-^{14}C]LA. Values are the mean ± SE of four different experiments. Values significantly different from Control C at $*P < 0.005$; $**P < 0.008$.

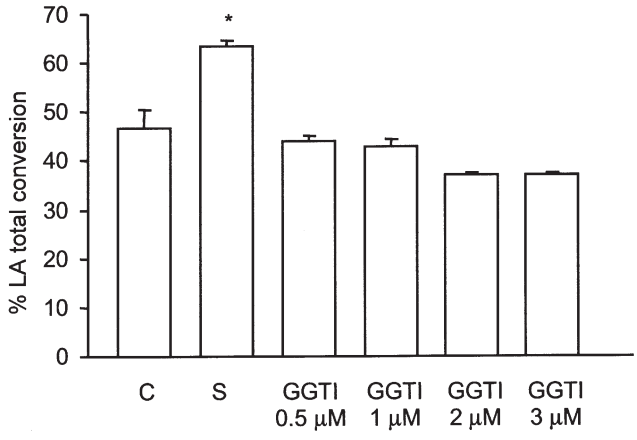

FIG. 6. Linoleic acid (LA) total conversion in THP-1 cells incubated with simvastatin (S) (5 μM), GGTI-286 (0.5, 1, 2, 3 μM), and [1-^{14}C]LA. Values are the mean ± SE of four different experiments. Values significantly different from Control C at $*P < 0.008$.

The most important action of statins is to inhibit the HMG-CoA reductase and consequentely the synthesis of cholesterol and the formation of isoprenylated compounds. The increased LA conversion and the inhibition of cholesterol synthesis would appear to be metabolically related also because of the reversal of the effects of statins by precursors of cholesterol synthesis, such as geranylgeraniol and mevalonate, (9).

As mentioned in the introduction, the Δ6 and Δ5 desaturases do not appear to be prenylated proteins, and their activity does not involve any farnesylated or geranylgeranylated proteins. In fact, both perillic acid, an inhibitor of farnesyl and geranylgeranyltransferases, and the synthetic compound GGTI-286, a specific inhibitor of geranylgeranyltransferase, did not affect LA conversion and desaturase activity in this study.

In THP-1 cells incubated with [1-^{14}C]acetate, simvastatin drastically decreased the incorporation of radiolabeled acetate in FC and CE fractions, as expected and previously reported (9). Curcumin and nicardipine decrease plasma and liver total cholesterol levels in rats (22,23). In our experiments, however, curcumin and nicardipine alone were marginally effective in inhibiting cholesterol synthesis from acetate in THP-1 cells and enhanced only to a small extent the effect of simvastatin when incubated together (Table 1).

In contrast, curcumin and nicardipine alone inhibit LA total conversion and decrease or nullify the effects of simvastatin. Curcumin was more potent than nicardipine in inhibiting LA total conversion (IC_{50} for curcumin of 3.75 μM and of 17.1 μM for nicardipine).

When cells were incubated with curcumin and nicardipine for 48 h and then simvastatin was added to the cells (for a final 24 h), the statin was unable to modify the effects of the desaturase inhibitors, unlike the results from experiments previously described (data not shown). This suggests that curcumin and nicardipine may be irreversible inhibitors of the desaturases or that simvastatin, curcumin, and nicardipine act on different sites of the desaturases.

The effects of the two desaturase inhibitors on cholesterol synthesis, qualitatively similar but much smaller than those of simvastatin, in contrast with the divergent effects on the Δ5 and Δ6 desaturases and the exclusion of prenylated protein involvement, suggest that there is no direct interaction between desaturase activity and cholesterol synthesis.

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Comparison of *ex vivo* Inhibitory Effect Between 2-Hydroxyestradiol and Its 17-Sulfate on Rat Hepatic Microsomal Lipid Peroxidation

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ABSTRACT: Two endogenous antioxidants that are speculated to be defense substances against preeclampsia, 2-hydroxyestradiol (2-OH-E_2) and its 17-sulfate, 2-hydroxyestradiol 17-sulfate (2-OH-E_2-17-S), were administered to rats to compare their inhibitory effects on hepatic microsomal lipid peroxidation, and the lipid peroxides were determined in NADPH- and ascorbic acid-dependent systems. The two catechols showed a strong inhibitory effect on lipid peroxidation in both systems, and the effect was dose dependent. However, a large difference was observed in their inhibition patterns. After administration of 2-OH-E_2, the effect appeared immediately and decreased gradually with time. In contrast, the effect of 2-OH-E_2-17-S appeared some time after administration and persisted for a longer time. Both catechols also showed a striking difference in their dynamics. After administration, 2-OH-E_2 was detected in the blood together with its metabolites, 2-methoxyestradiol and 2-methoxyestrone, and they disappeared immediately. In contrast, 2-OH-E_2-17-S was present in the blood for a longer time together with its O-methylated product, 2-methoxyestradiol 17-sulfate, but disappeared from liver microsomes within 2 h after administration. The results imply no occurrence of a direct inhibition effect of 2-OH-E_2-17-S.

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Preeclampsia, a serious syndrome whose main symptom is pregnancy-induced hypertension, occurs during the second and third trimesters of pregnancy (1). The mechanism of its occurrence is recognized to be the result of a quantitative imbalance between placental lipid peroxides and antioxidants, with the accumulation of the former causing endothelial dysfunction (2). As placental antioxidants, catechol estrogens such as 2-hydroxyestradiol (2-OH-E_2, Scheme 1) or 2-hydroxyestrone (2-OH-E_1, Scheme 1) were proposed as candidates because of the increase in the amounts produced during pregnancy (3) and their strong inhibitory effect on lipid peroxidation (4). However, there remains a problem regarding the antioxidant effect of free catechol estrogens in humans, because they are so rapidly metabolized that their plasma levels become too low to exert any antioxidant effect.

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Abbreviations: 2-OH-E_1, 2-hydroxyestrone; 2-OH-E_2, 2-hydroxyestradiol; 2-OMe-E_1, 2-methoxyestrone; 2-OMe-E_2, 2-methoxyestradiol; 4-OMe-E_2, 4-methoxyestradiol; 2-OH-E_2-17-S, 2-hydroxyestradiol 17-sulfate; 2-OMe-E_2-17-S, 2-methoxyestradiol 17-sulfate; 4-OMe-E_2-17-S, 4-methoxyestradiol 17-sulfate; AsA, ascorbic acid; COMT, catechol *O*-methyl transferase; TBA, 2-thiobarbituric acid.

Recently, we proposed 2-hydroxyestradiol 17-sulfate (2-OH-E_2-17-S, Scheme 1) as a placental antioxidant based on the following lines of evidence: the increase in plasma (5) or urinary (6) 2-OH-E_2-17-S concentration with the progress of gestation; a significant difference in the amount present between healthy and preeclamptic pregnancies (6); its strong inhibitory effect on lipid peroxidation (7); and the capacity of the placenta to produce 2-OH-E_2-17-S from estradiol 17-sulfate (8). To prove our proposal, the antioxidant effect of 2-OH-E_2-17-S was investigated by conducting *ex vivo* experiments.

In this paper, we describe the inhibitory effect of 2-OH-E_2-17-S on *ex vivo* rat hepatic microsomal lipid peroxidation and the comparison of the effect with that of 2-OH-E_2.

EXPERIMENTAL PROCEDURES

Steroids and chemicals. The following steroids were synthesized in our laboratory by known methods (9): 2-OH-E_2, 2-methoxyestrone (2-OMe-E_1, Scheme 1), 2-methoxyestradiol (2-OMe-E_2, Scheme 1), 4-methoxyestradiol (4-OMe-E_2), 2-OH-E_2-17-S, 2-methoxyestradiol 17-sulfate (2-OMe-E_2-17-S, Scheme 1), and 4-methoxyestradiol 17-sulfate (4-OMe-E_2-17-S). 2-Hydroxyestrone (2-OH-E_1) was purchased from Steraloids (Newport, RI). ADP and NADPH were obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan), and 2-thiobarbituric acid (TBA) was from Merck (Darmstadt, Germany). Heparin sodium (1000 units/mL) was purchased from Takeda Chemical Industries, Ltd. (Osaka, Japan). Triton N-101 was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Sep-Pak C_{18} cartridges and Millex 4 mm HV were purchased from Waters (Milford, MA) and Millipore (Tokyo, Japan), respectively. All other reagents including ascorbic acid (AsA) and solvents were of reagent grade.

Animals and their treatment. Wistar rats (male, body weight: 230–240 g) were used. A solution of steroid (2-OH-E_2 or 2-OH-E_2-17-S) dissolved in saline containing 5% ethanol was administered by abdominal injection into the animals such that the dose injected was 200, 275, or 350 nmol per 100 g body weight. Control rats received an equal amount of vehicle. At appropriate time intervals after steroid treatment, rats were anesthetized with ether and sacrificed by exsanguination. The hepatic microsomal fraction was prepared as described previously (6). The animal studies were performed in adherence to the "Guiding Principles for the Care and Use of Experimental Animals in Hokkaido College of Pharmacy" (1988; revised 2001).

Conditions for lipid peroxidation. (i) NADPH-dependent microsomal lipid peroxidation. Into ice-cooled reaction vessels were added microsomal protein (0.25 mg/mL), NADPH (0.05 mM), and ADP-Fe^{3+} complex (0.15 mM ADP/0.0075 mM $FeCl_3$). The mixture was diluted with 50 mM Tris-HCl buffer solution (pH 7.4) to 2 mL as the final volume and incubated for 10 min at 37°C under aerobic conditions.

(ii) AsA-dependent microsomal lipid peroxidation. The same reaction vessels were prepared except that AsA (0.25 mM) was used instead of NADPH (0.05 mM), followed by dilution with the same buffer solution and finally incubation under the same conditions.

Determination of lipid peroxidation. Lipid peroxidation was assayed as TBARS (10). To 1 mL of each microsomal reaction mixture were added 30 μL of 2% BHT and 2 mL of TCA-TBA-HCl reagent (15% TCA/0.375% TBA/0.25 N HCl). The mixture was heated for 15 min in boiling water. After cooling in water, the mixture was centrifuged for 10 min at 1500 × *g*. The absorbance of the supernatant was determined at 535 nm using a UV-vis spectrophotometer (model 200-20; Hitachi, Tokyo) against a blank that contained water.

HPLC apparatus. HPLC was carried out using a model CCPS high-performance liquid chromatograph (Tosoh, Tokyo) equipped with an EC-8011 electrochemical detector (Tosoh) at 0.7 V vs. a Ag/AgCl reference electrode (11). A Mightysil RP-18GP column packed with 5-μm particles (250 × 3.0 mm, i.d.; Kanto, Tokyo) was used as the stationary phase and maintained at 40°C in a column heater. A Model C-R6A Chromatopac (Shimadzu, Kyoto) was used for data processing.

Calibration curves. Calibration curves were obtained by injecting a methanolic solution of each catechol or guaiacol estrogen containing a quantitative amount of 4-OMe-E_2 or 4-OMe-E_2-17-S as an internal standard for free or conjugated steroids, respectively.

Preparation of steroid-free plasma. Steroid-free plasma was prepared according to the method of Heyns *et al.* (12). Charcoal (50 mg) was added to 1 mL of a pooled plasma sample, and the mixture was stirred vigorously for 30 min at room temperature. The mixture was centrifuged at 1500 × *g* for 10 min to remove the charcoal, and the supernatant was filtered through a membrane filter to obtain the steroid-free plasma.

Measurement of plasma catechol and guaiacol estrogens. Blood samples, collected from each animal before excision of the liver and heparinized, were centrifuged at 1500 × *g* for 20 min to obtain the plasma. Each plasma sample (2 mL) was passed through a Sep-Pak C_{18} cartridge, followed by washing with 2 mL of water and finally eluting with 4 mL of methanol. The methanolic eluate was concentrated under a stream of nitrogen at 40°C to obtain the sample as residue, which was thereafter dissolved in methanol containing 0.01% AsA (100 μL). After being treated once with a Millex 4-mm HV, the solution was subjected to HPLC. The following solvent systems were used as the mobile phase at a flow rate of 0.6 mL/min: system A: 0.5% $NH_4H_2PO_4$ (pH 3.0)—methanol (55:45, vol/vol); and system B: 0.5% $NH_4H_2PO_4$ (pH 3.0)—methanol (45:55, vol/vol), for the analyses of conjugated and free steroids, respectively.

Recovery of free and conjugated estrogens from plasma. A known amount (0.5, 1.0, and 5.0 nmol) of free (2-OH-E_1, 2-OH-E_2, 2-OMe-E_1, or 2-OMe-E_2) or conjugated steroids (2-OH-E_2-17-S or 2-OMe-E_2-17-S) was added to 1 mL of the steroid-free plasma, and the mixture was passed through a Sep-Pak C_{18} cartridge, followed by the same procedure as described above. Triplicate experiments were carried out on each steroid.

Determination of microsomal estrogens. The microsomal fraction was obtained from rats injected with 2-OH-E_2 or 2-OH-E_2-17-S in the amount of 350 nmol per 100 g body weight. To this fraction (5 mg protein) was added a solubilizer composed of 1 mM DTT, 1 mM EDTA, 1.5% Triton N-101, and 50 mM Tris-HCl buffer solution (pH 7.4), followed by dilution with 0.01% AsA to make a final volume of 1 mL. The solution was stirred for 1 h at 4°C, and a quantitative amount of 4-OMe-E_2 was added as an internal standard, followed by treatment with a Sep-Pak C_{18} cartridge. After washing with water, the steroid-containing fraction was eluted with methanol containing 0.01% AsA (2 mL). The methanolic eluate was concentrated under a stream of nitrogen to give the residue, which was subjected to HPLC by using system A as the mobile phase at the same flow rate. A microsomal fraction from nonsteroid-injected rats was used as a control. A recovery test was carried out on 2-OH-E_2 and its metabolites (2-OH-E_1, 2-OMe-E_1, and 2-OMe-E_2) and on 2-OH-E_2-17-S and 2-OMe-E_2-17-S using a control microsomal fraction, followed by the same treatment as described above. Triplicate experiments were carried out on each steroid.

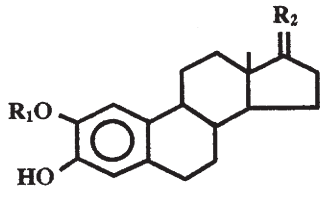
Data analysis. Data expressed as means ± SD were obtained from five experiments (n = 5), except for the recovery test (n = 3), and analyzed by Bonferroni's test. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Effect of 2-OH-E_2 and 2-OH-E_2-17-S on rat hepatic microsomal lipid peroxidation. The curves in Figure 1, made by plotting TBARS as the ordinate and time as the abscissa, show the inhibitory effect of 2-OH-E_2 and 2-OH-E_2-17-S on a rat hepatic microsomal NADPH-dependent system at 2, 5, 12, 24, and 72 h after administration of 200, 275, or 350 nmol catechols per 100 g body weight for the same period. When 5% ethanol in saline was used as the vehicle, no inhibition was observed in this system.

An important difference was observed in the inhibition patterns between 2-OH-E_2 and 2-OH-E_2-17-S. For 2-OH-E_2, the inhibitory effect appeared immediately within at least 2 h after administration, and TBARS recovered quickly. When the dose administered was 200 nmol per 100 g body weight, TBARS reverted to the control level 24 h after administration. When the doses given were 275 and 350 nmol per 100 g body weight, the inhibition was approximately 15 and 40%, respectively, at 72 h after administration.

In contrast, in the group administered 2-OH-E_2-17-S, the inhibitory effect appeared slightly later but persisted for a longer time. When a dose of 200 nmol per 100 g body weight was



2-OH-E_2 : R_1 = H, R_2 = 

2-OMe-E_2 : R_1 = Me, R_2 = 

2-OH-E_1 : R_1 = H, R_2 = O

2-OMe-E_1 : R_1 = Me, R_2 = O

2-OH-E_2-17-S : R_1 = H, R_2 = 

2-OMe-E_2-17-S : R_1 = Me, R_2 = 

SCHEME 1

injected, about 20% inhibition remained for more than 72 h after administration. It was natural that higher doses of 275 and 350 nmol per 100 g body weight showed stronger inhibition: 40 and 65% at 72 h, respectively.

Similar results on lipid peroxidation were obtained in the AsA-dependent system (Table 1).

Plasma catechol and guaiacol estrogens. (i) HPLC analysis. It was speculated that the difference in the persistence of the inhibitory effect between 2-OH-E_2 and 2-OH-E_2-17-S may be related to their metabolic clearance from the blood. To confirm this, plasma catechol and guaiacol estrogens were measured at various intervals after their administration. After confirmation of the complete separation of free estrogens (2-OH-E_2, 2-OH-E_1, 2-OMe-E_2, and 2-OMe-E_1) as well as conjugated ones (2-OH-E_2-17-S and 2-OMe-E_2-17-S), calibration curves for these steroids were prepared. Satisfactory linearity was observed on calibration curves for each estrogen over the concentration range from 5 to 50 pmol when 10 pmol of internal standard was used.

(ii) Plasma estrogen levels. Figure 2A shows an HPLC chromatogram of a rat plasma estrogen fraction from the group that received a dose of 350 nmol 2-OH-E_2 per 100 g body weight. The peaks at *ca.* 11 and 22 min were identical to those of authentic 2-OH-E_2 and 2-OMe-E_2, respectively. Similarly, the peaks at *ca.* 9 and 20 min corresponded to 2-OH-E_1 and its O-methylated product, 2-OMe-E_1, respectively. These estrogens disappeared within 1 h after administration of 2-OH-E_2.

Figure 2B shows the chromatogram of a plasma estrogen fraction from rats given 2-OH-E_2-17-S. The peak at *ca.* 10 min, corresponding to 2-OH-E_2-17-S, appeared as soon as it was administered. The peak height decreased with time and disappeared within 5 h. The peak at *ca.* 21 min was assigned as 2-OMe-E_2-17-S.

Recovery test for plasma estrogens. Recoveries of the above catechol and guaiacol estrogens from steroid-free plasma were obtained through the entire cleanup procedure, the results of which are shown in Table 2. Each steroid was recovered to a satisfactory extent regardless of the amount added.

Time course of plasma catechol and guaiacol estrogen levels. To elucidate the difference between the two catechols in the rate of disappearance from the blood, we measured the plasma levels of 2-OH-E_2, 2-OH-E_2-17-S, and their metabolites at appropriate time intervals after administration, the results of which are shown in Figure 3.

For 2-OH-E_2 (Figs. 3A and 3A′), plasma 2-OH-E_2 was already slightly observable at 15 min after administration; its metabolites, 2-OMe-E_2, 2-OH-E_1, and 2-OMe-E_1, however, were observable depending on the dose administered. The level of plasma 2-OH-E_1 was almost the same as that of plasma 2-OH-E_2. The plasma 2-OMe-E_2 and 2-OMe-E_1 levels were approximately five to six times and two times higher, respectively, than the level of plasma 2-OH-E_2, but they disappeared within 60 min.

In contrast, the level of plasma 2-OH-E_2-17-S depended on the dose administered, and its O-methylated product, 2-OMe-E_2-17-S, was measured (Fig. 3B). The level of plasma 2-OH-E_2-17-S was about 20 times higher at 15 min than that of plasma 2-OH-E_2. The conjugates 2-OH-E_2-17-S and 2-OMe-E_2-17-S

TABLE 1
Inhibitory Effect of 2-Hydroxyestradiol (2-OH-E_2) and 2-Hydroxyestradiol 17-Sulfate (2-OH-E_2-17-S) on Ascorbic Acid-Dependent Rat Hepatic Microsomal Lipid Peroxidation at Times (h) After Injection

		TBARS (%) at				
Estrogen	Dose	2 h	5 h	12 h	24 h	72 h
2-OH-E_2	200	88.4 ± 1.9	90.9 ± 3.4	92.7 ± 2.4	96.2 ± 2.3	102.2 ± 3.2
2-OH-E_2-17-S	200	94.5 ± 4.7 *	89.5 ± 3.3	74.3 ± 3.2 **	87.1 ± 1.7 *	92.4 ± 3.2 *
2-OH-E_2	275	50.6 ± 1.0	53.4 ± 1.0	54.2 ± 1.0	66.0 ± 1.4	82.3 ± 1.8
2-OH-E_2-17-S	275	65.7 ± 1.0 **	63.9 ± 1.0 *	59.8 ± 1.2	65.8 ± 2.1	76.4 ± 3.8
2-OH-E_2	350	36.0 ± 1.0	39.7 ± 3.2	44.1 ± 2.6	52.8 ± 1.0	69.9 ± 3.3
2-OH-E_2-17-S	350	40.9 ± 2.2	37.7 ± 1.0	32.6 ± 1.4 **	44.7 ± 1.0	49.5 ± 1.7 **

[a]Values are expressed as inhibitory activity (%) for the control group (39 nmol/mg protein/10 min) as 100%, means ± SD (n = 5). Dose is expressed as nmol per 100 g body weight. *P < 0.05, **P < 0.01.

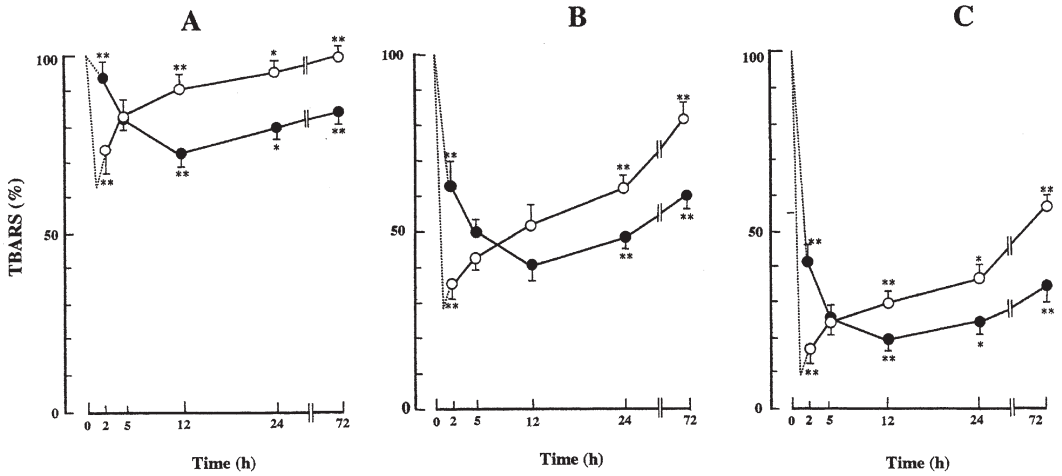


FIG. 1. Time course of the inhibitory effect of 2-hydroxyestradiol (2-OH-E_2) (○) and 2-hydroxyestradiol 17-sulfate (2-OH-E_2-17-S) (●) on NADPH-dependent lipid peroxidation in the presence of rat liver microsomes. The dose of catechol administered was 200 (A), 275 (B), and 350 (C) nmol per 100 g body weight. Data represent the means ± SD of five determinations. Control value (36 nmol/mg protein/10 min) obtained from vehicle-injected rats was expressed as 100%. $^*P < 0.05$, $^{**}P < 0.01$: TBARS between 2-OH-E_2 and 2-OH-E_2-17-S at the same time after administration.

could be detected at 1 and 2 h after administration in proportion to the dose administered, but after 5 h they could no longer be detected.

Microsomal estrogen levels. To investigate whether the inhibition of 2-OH-E_2 and 2-OH-E_2-17-S on *ex vivo* TBARS might have originated in the estrogens that remained in the microsomal fraction, their contents were determined by HPLC. The recovery experiments carried out beforehand on catechol and guaiacol estrogens are shown in Table 3. One can observe that all steroids tested were recovered satisfactorily through the solubilization treatment.

For 2-OH-E_2, only 2-OMe-E_1 was detected, the amount being *ca.* 50 pmol per 5 mg of protein at 15 min after administration (Fig. 4A). On the other hand, for 2-OH-E_2-17-S (Fig. 4B),

TABLE 2
Recoveries of Estrogens from Plasma

Estrogen	Amount (nmol) Added	Detected	Recovery (%)	CV (%)
2-OH-E_2	0.5	0.47 ± 0.05	95.4 ± 0.8	0.8
	1.0	0.96 ± 0.10	96.1 ± 1.8	1.8
	5.0	4.77 ± 0.19	95.3 ± 0.5	0.5
2-OMe-E_2	0.5	0.48 ± 0.05	95.7 ± 0.9	0.5
	1.0	0.94 ± 0.13	94.2 ± 2.8	1.6
	5.0	4.78 ± 0.34	95.5 ± 1.3	0.8
2-OH-E_1	0.5	0.48 ± 0.03	96.3 ± 0.6	0.4
	1.0	0.95 ± 0.07	94.8 ± 2.1	1.5
	5.0	4.95 ± 0.15	99.1 ± 3.1	2.2
2-OMe-E_1	0.5	0.48 ± 0.01	96.3 ± 1.0	0.6
	1.0	0.96 ± 0.02	95.5 ± 2.0	1.2
	5.0	4.90 ± 0.11	98.0 ± 2.1	1.2
2-OH-E_2-17-S	0.5	0.48 ± 0.03	96.7 ± 0.5	0.3
	1.0	0.96 ± 0.05	96.5 ± 5.1	2.9
	5.0	4.86 ± 0.17	97.2 ± 1.4	0.8
2-OMe-E_2-17-S	0.5	0.48 ± 0.08	96.0 ± 1.8	1.0
	1.0	0.95 ± 0.02	95.2 ± 1.9	1.1
	5.0	4.86 ± 0.06	97.3 ± 1.3	0.7

[a]Values are expressed as means ± SD (n = 3). 2-OMe-E_2, 2-methoxyestradiol; 2-OH-E_1, 2-hydroxyestrone; 2-OMe-E_1, 2-methoxyestrone; 2-OMe-E_2-17-S, 2-methoxyestradiol 17-sulfate; for other abbreviations see Table 1.

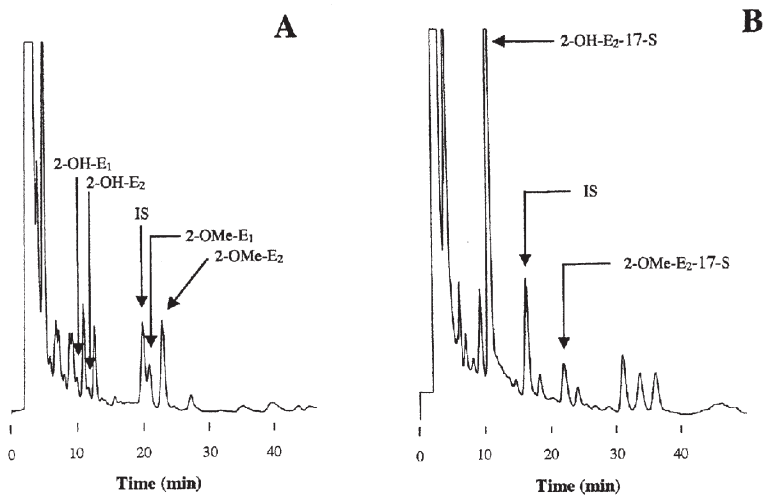


FIG. 2. HPLC chromatograms of plasma steroid fractions at 15 min after administration of 2-OH-E_2 (A) and 2-OH-E_2-17-S (B). The amount injected was 350 nmol per 100 g body weight. Internal standards (IS) of A and B are 4-methoxyestradiol (4-OMe-E_2) and 4-methyoxyestradiol 17-sulfate (4-OMe-E_2-17-S), respectively. 2-OH-E_1, 2-hydroxyestrone; 2-OMe-E_1, 2-methoxyestrone; 2-OMe-E_2, 2-methoxyestradiol; 2-OMe-E_2-17-S, 2-methoxyestradiol 17-sulfate; for other abbreviations see Figure 1.

only 2-OMe-E_2-17-S was detected, with an amount of less than 10 pmol per 5 mg of microsomal protein.

Similarly, the estrogen contents were determined at 2 h after the administration. For 2-OH-E_2, only 2-OMe-E_1 was detected in the microsomal fraction, an amount of approximately one-seventh that at 15 min. For 2-OH-E_2-17-S, no estrogen peak appeared.

DISCUSSION

Figure 1 and Table 1 demonstrate that both 2-OH-E_2 and 2-OH-E_2-17-S inhibited TBARS in proportion to the dose administered. The inhibitory effect of 2-OH-E_2 at 2 h after administration was roughly twice that of 2-OH-E_2-17-S at all doses administered. In the *in vitro* experiment, on the other hand,

TABLE 3
Recoveries of Estrogens from Solubilized Microsomes[a]

Estrogen	Amount (nmol)		Recovery (%)	CV (%)
	Added	Detected		
2-OH-E_2	0.5	0.47 ± 0.02	94.5 ± 3.8	2.1
	1.0	0.97 ± 0.02	97.4 ± 1.6	0.9
	5.0	4.94 ± 0.11	98.8 ± 2.2	1.3
2-OMe-E_2	0.5	0.49 ± 0.02	97.6 ± 4.6	2.6
	1.0	0.98 ± 0.02	97.7 ± 2.4	1.4
	5.0	4.94 ± 0.14	98.7 ± 2.9	1.7
2-OH-E_1	0.5	0.49 ± 0.01	97.5 ± 2.1	1.2
	1.0	1.04 ± 0.07	104 ± 7.4	4.3
	5.0	4.90 ± 0.14	97.9 ± 2.8	1.6
2-OMe-E_1	0.5	0.50 ± 0.01	99.7 ± 2.3	1.3
	1.0	1.00 ± 0.05	99.9 ± 4.6	2.6
	5.0	4.86 ± 0.11	97.3 ± 2.3	1.3
2-OH-E_2-17-S	0.5	0.48 ± 0.06	96.7 ± 1.2	0.7
	1.0	1.03 ± 0.07	103 ± 7.7	4.4
	5.0	4.93 ± 0.06	98.7 ± 1.3	0.7
2-OMe-E_2-17-S	0.5	0.48 ± 0.01	96.7 ± 2.4	1.4
	1.0	0.99 ± 0.02	99.3 ± 2.0	1.1
	5.0	4.86 ± 0.11	97.2 ± 2.2	1.3

[a]Values are expressed as means ± SD (n = 3). For abbreviations see Tables 1 and 2.

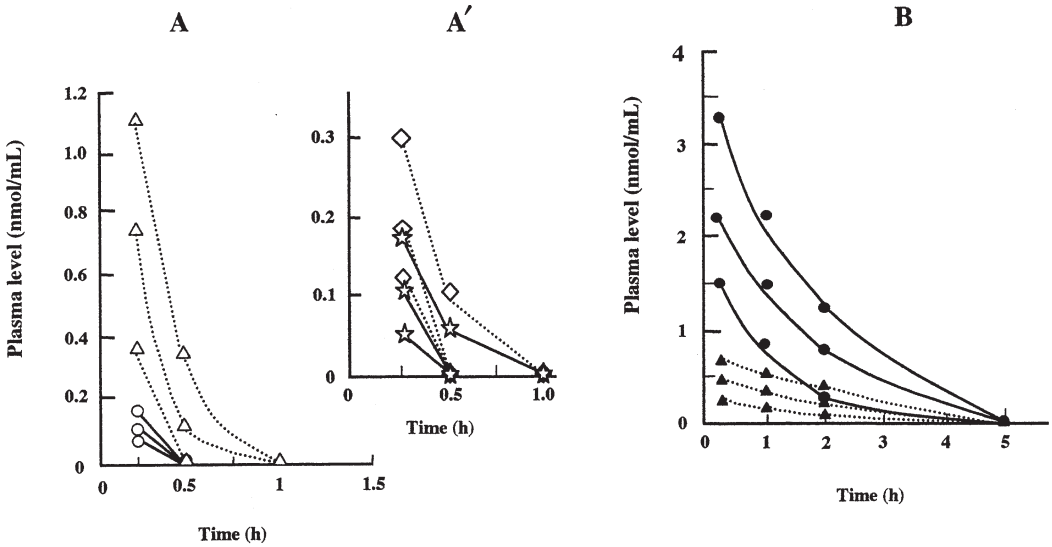


FIG. 3. Plasma estrogen levels after administration of 2-OH-E_2 (A) or 2-OH-E_2-17-S (B). Open circles (○), open triangles (△), closed circles (●), and closed triangles (▲) show 2-OH-E_2, 2-OMe-E_2, 2-OH-E_2-17-S, and 2-OMe-E_2-17-S, respectively. Graph A′ shows the amounts of 2-OH-E_1 (☆) and 2-OMe-E1 (◇). The upper, middle, and lower curves in each curve represent the dose administered, 350, 275, and 200 nmol per 100 g body weight, respectively. For abbreviations see Figures 1 and 2.

2-OH-E_2 showed a 1.8-times greater inhibitory effect than 2-OH-E_2-17-S (6); thus, *in vitro* inhibition was reproduced well by the present *ex vivo* study. The inhibition of both catechols became roughly equal at 5 h after administration, and completely reversed at 12 h after administration. On the basis of these findings, the manner in which the antioxidant effects of 2-OH-E_2 and 2-OH-E_2-17-S were expressed was clearly determined to be different; namely, the former demonstrated its antioxidant effect rapidly, whereas the latter dose had a delayed effect.

One possible reason for this difference was the difference in residual levels of the two catechols in the blood, namely, the difference in their half-lives ($t_{1/2}$). The blood levels of both catechols were therefore measured and compared over time. As shown in Figure 3, in contrast to the $t_{1/2}$ of 2-OH-E_2-17-S of roughly 80 min, the $t_{1/2}$ of 2-OH-E_2 was so short that it was difficult to measure. Probably it is no more than a few minutes, as reported by Kono *et al.* (4).

The difference between these two catechols in their elimination rates from the blood is presumably related to the difference in their metabolism. We can speculate from Figure 2 that a part of 2-OH-E_2 is oxidized in the body into 2-OH-E_1, and both catechols are then O-methylated to 2-OMe-E_2 and 2-OMe-E_1, respectively. On the other hand, 2-OMe-E_2-17-S was detected as the sole metabolite of 2-OH-E_2-17-S. The blood level ratios of 2-OH-E_2 to 2-OMe-E_2 and of 2-OH-E_2-17-S to 2-OMe-E_2-17-S were 1:8 and 4:1, respectively. This difference is considered to be due to a difference between these two catechols

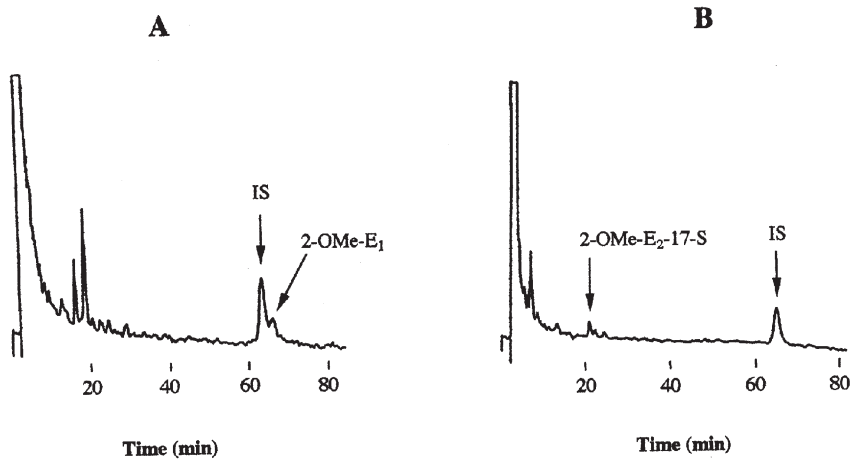


FIG. 4. HPLC chromatograms of microsomal fraction at 15 min after administration of 2-OH-E_2 (A) and 2-OH-E_2-17-S (B). IS: 4-OMe-E_2 in A and B. For abbreviations see Figures 1 and 2.

in their permeability through the erythrocytic membrane (4,13,14). Namely, although 2-OH-E_2 is converted into 2-OMe-E_2 by catechol *O*-methyltransferase present in erythrocytes, it is able to penetrate the erythrocyte membrane easily. In contrast, 2-OH-E_2-17-S is hardly O-methylated—it does not penetrate the erythrocytic membrane easily due to its low membrane permeability, thus resulting in a large difference between them in their elimination rates from the blood. This hypothesis is supported by our previous report on the plasma metabolic clearance rate of 2-OH-E_2-17-S in rats (13) and a report by Ball *et al*. (14) regarding 2-OH-E_2. The difference in polarity between these two catechols can also be said to result in the difference in their membrane permeability.

In this study, clear differences were observed regarding the duration of the antioxidant effects of the two catechols. In contrast to the levels of lipid peroxides in the hepatic microsomes of the NADPH-dependent system for a 2-OH-E_2 dose of 200 nmol per 100 g body weight, which returned to control levels in 24 h, 20% of the antioxidant effect still remained when an equal dose of 2-OH-E_2-17-S was administered. In addition, at a dose of 275 nmol per 100 g body weight, 15 and 40% of the antioxidant effect of 2-OH-E_2 and 2-OH-E_2-17-S, respectively, were retained at 72 h, and when the dose was increased to 350 nmol per 100 g body weight, roughly 40 and 65% of their respective effects remained. Similar trends were observed in the inhibition of AsA-dependent lipid peroxidation.

The O-methylated product of 2-OH-E_2, 2-OMe-E_2, has a considerably strong antioxidant effect (6), and because it is bound to testosterone–estradiol-binding globulin during transport in the blood (15), its metabolic clearance rate is lower than that of 2-OH-E_2 (16). However, 2-OMe-E_2 is known to be excreted as a result of high-speed glucuronic acid or sulfate conjugation (17). As shown in Figure 3A, this is understandable based on the fact that 2-OMe-E_2 is eliminated rapidly from the blood. In addition, although a pathway is known to exist in the liver by which 2-OH-E_2 is regenerated from 2-OMe-E_2 (18,19), since the elimination rate of 2-OMe-E_2 from the blood is high, the proportion of the O-demethylation metabolism that contributes to the duration of the antioxidant effect is considered to be low. On the other hand, as is clearly demonstrated in Figure 3B, in the case of 2-OH-E_2-17-S, since its O-methylated product, 2-OMe-E_2-17-S, is present in the blood for a longer period of time than 2-OMe-E_2, even though its antioxidant effect is weak (6), it is surmised to contribute to the antioxidant effect to some extent. Thus, the difference between these guaiacols in their retention in the blood is surmised to affect the difference in duration of the antioxidant effect.

In spite of the complete disappearance of 2-OH-E_2 or 2-OH-E_2-17-S from plasma within 1 or 5 h after administration, respectively, the inhibition of TBARS continued for far longer. As one possibility, this longer time effect may be derived from the catechols tightly bound to the microsomal membrane. Thus, the steroids estimated to remain in the membrane were measured by solubilization of the microsomal fraction, followed by confirmation with the recovery test. However, amounts of both catechols and their O-methylated metabolites sufficient to affect the inhibition were not detected in the microsomal fractions. We speculate from the present results that catechol estrogens, especially 2-OH-E_2-17-S, do not participate directly in the inhibition of microsomal lipid peroxidation.

Although the mechanism of inhibition is not clear at the present time, 2-OH-E_2-17-S is obviously superior to 2-OH-E_2 in terms of the duration of its antioxidant effect.

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Chemical and Enzymatic Transacylation of Amide-Linked FA of Buttermilk Gangliosides

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ABSTRACT: The goal of this work was to alter the composition of amide-linked FA of bovine buttermilk gangliosides, particularly the disialoganglioside GD_3, to adjust lipid sources to special food specifications and pharmacological or cosmetic applications. The chemical transacylation of amide-linked FA of buttermilk gangliosides with free arachidic acid (20:0) by a combination of basic hydrolysis and diethylphosphorylcyanide/triethylamine- catalyzed reacylation was compared to an enzymatic sphingolipid ceramide N-deacylase (EC 3.5.1.23)-catalyzed FA exchange by GC analysis and nano electrospray ionization–MS. The buttermilk predominantly contained the disialoganglioside GD_3 and the monosialoganglioside GM_3. The heterogeneity of FA that are incorporated into gangliosides, mainly palmitic acid (29.4 wt%), stearic acid (16.9 wt%), oleic acid (17.8 wt%), and myristic acid (8.5 wt%), was effectively altered by both transesterification techniques. Arachidic acid, which was not integrated into the initial buttermilk gangliosides, was transacylated to total gangliosides with 23.2 wt% (GD_3, 6.7 wt%) by the chemical process and with 8.7 wt% (GD_3, 13.8 wt%) when catalyzed enzymatically. Mainly behenic acid and lignoceric acid of GD_3 were exchanged chemically, and stearic acid was exchanged by the enzymatic process. This observation might depend on hydrolytic sensitivities of amide-linked very long chain saturated FA or specific enzyme subtrate affinities, respectively. Results of chemical hydrolysis indicated there was a risk of sialic acid decomposition and unspecific degradations. Regarding specificity and avoidance of critical agents, the enzymatic transesterification is recommended for industrial-scale production of consumer goods.

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Lipids from plants or animals are not necessarily ideal for use by humans and may have to be modified. The goal of this research was to alter the composition of amide-linked FA of bovine buttermilk gangliosides. Gangliosides are a group of sialic acid-containing glycosphingolipids that appear widely in vertebrate tissues, body fluids, and milk and are important constituents of neural membranes (1), immune-cell membranes (2), and membranes of the small intestine brush border (3). They consist of a hydrophobic ceramide moiety and a hydrophilic, sialylated oligosaccharide chain linked to the hydroxyl group at carbon atom 1 (4). Ceramide consists of a sphingoid base and an amide-linked FA that varies in chain length from 14 to 30 carbon atoms, in degree of saturation, and in the presence or absence of hydroxylation. Usually, the amide-linked FA is saturated (5). Nevertheless, exceptional hydroxylated, unsaturated, and branched FA of sphingolipids have been detected in skin (6).

Concerning biochemical and physiological properties, the oligosaccharide chain serves as a binding site for several microorganisms, microbial toxins, and viruses, and for cell–cell interactions (7,8). Gangliosides are uniformly distributed on the cell surface. Together with sphingomyelin and cholesterol, they can segregate into membrane domains as physiological surroundings of membrane proteins. Therefore, the FA composition of the ceramide moiety is thought to influence membrane fluidity (9,10) and the formation of sphingolipid clusters in the cell membrane (11,12). These microdomains are obviously important for the action of growth factors, membrane-associated enzymes (12), receptor–ligand interactions, and cell signaling both within and outside of pathways (13). Therefore, the transduction of signals through the membrane may be influenced by the alkyl-chain properties of gangliosides. Moreover, ceramide is introduced by sphingomyelinase into a complex, intracellular messenger metabolism, thus being a substrate for enzymes like ceramidase, sphingosine transacylase, and sphingosine kinase. The FA composition may determine the affinity of these enzymes for the substrate and therefore increase or inhibit the second messenger properties of the ceramide mediators (14). Additionally, immune-modulating properties of gangliosides, as a function of the carbon chain lengths of the constituent FA, have been described recently (15).

The adjustment of ganglioside sources to special food specifications or the increase of the physiological potential of gangliosides for pharmacological or cosmetic applications might require the modification of the sphingoid base FA composition. For dietary applications buttermilk is a potential source of glycosphingolipids. In milk, gangliosides are integrated into the membrane composite bilayer made of milk fat globules, which contain a TAG core surrounded by a lipoprotein, glyco-, phospholipid, and cholesterol (16). The technological process for buttermilk implies the removal of milk fat TAG and the simultaneous accumulation of water-soluble polar lipids derived from the globule membrane. Depending on the mammalian species, each milk has its own particular ganglioside content and profile (17). Approximately 1–2% of the total lipid content of bovine buttermilk is gangliosides, mainly the disialoganglioside GD_3 and the

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Abbreviations: GD disialoganglioside; GM, monosialoganglioside; HPTLC, high performance TLC; nano ESI-MS^n, nano electrospray ionization-MS; RT, retention time.

monosialoganglioside GM_3 (18,19). In human colostrum GD_3 is the main ganglioside, whereas in mature human milk GM_3 and several highly polar gangliosides are predominant, depending on the maturation status (20). In addition, the FA composition of gangliosides is species specific. In bovine milk, the FA pattern of TAG, phospholipids, and gangliosides depends on the stage of lactation and may be an adjustment to specific metabolic demands (21). In human milk the concentration of constituent arachidic acid (20:0) is markedly higher compared to bovine milk (21,22). Thus, an increase of amide-linked arachidic acid could be relevant in adapting bovine buttermilk gangliosides to the human milk composition for infant food.

The amide-acyl linkage between FA and sphingoid bases of glycosphingolipids is specifically hydrolyzed by the sphingolipid ceramide N-deacylase (EC 3.5.1.23) in aqueous systems (23–25). The enzyme also catalyzes the condensation reaction between FFA and sphingolipids (26). Likewise, the FA exchange of gangliosides is made possible by chemical hydrolysis combined with a diethylphosphorylcyanide/triethylamine-catalyzed transacylation (27). In the biosynthesis of ganglioside, the first step in the formation of ceramide is the reduction of 3-ketosphinganine followed by the acylation of sphinganine to dihydroceramide (4). The amide-group acylation is catalyzed by dihydroceramide synthase using activated FA with chain lengths of 16–26 carbon atoms. The goal of this study was to modulate this native pattern of FA occurring in buttermilk gangliosides by a chemical and enzymatic transacylation with free arachidic acid (Fig. 1). Here, the specificity and effectiveness of both methods were considered by nano electrospray ionization–MS (nano ESI-MS^n) and FAME analysis by GC.

MATERIALS AND METHODS

Isolation of gangliosides from buttermilk. Pure buttermilk, Staudenhof brand (TMA GmbH, Aretsried, Germany), with 0.51% total fat content was lyophilized. The glycosphingolipids were extracted according to Ladisch and Gillard (28).

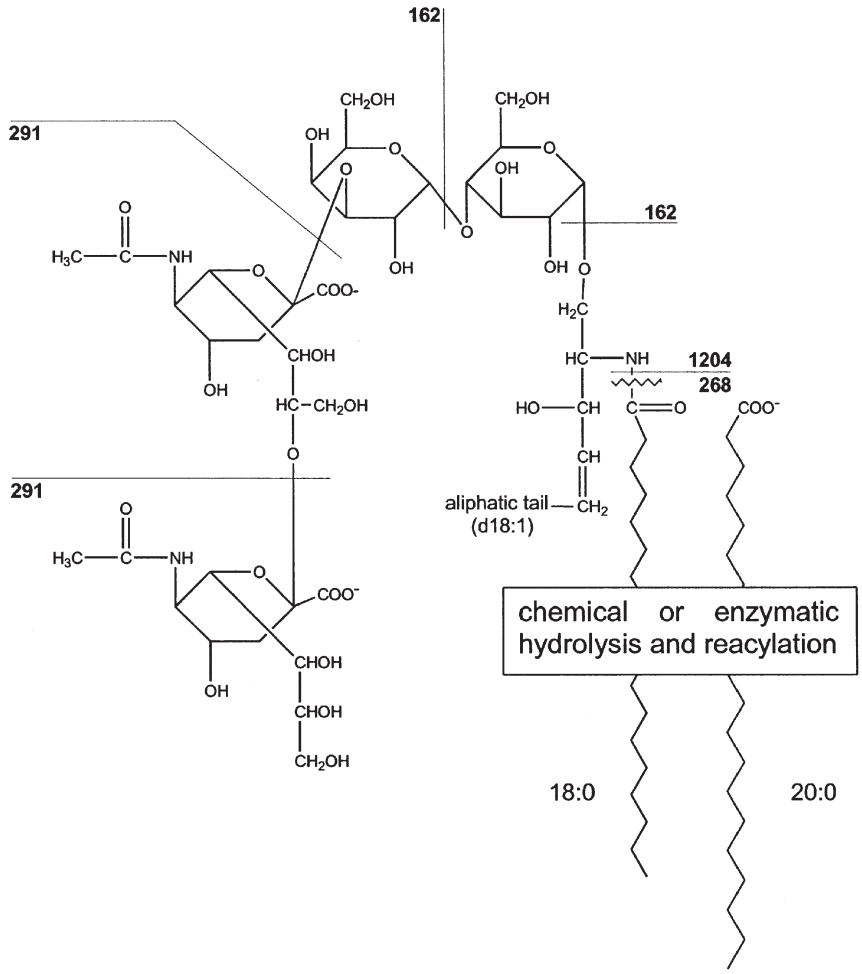

FIG. 1. Transesterification of GD_3 with free arachidic acid illustrated by MS^n experiment. Fragmentation pattern: 1564 Da, GD_3 sphingoid 18:1 with tetracosanoic acid; 291 Da, sialic acid; 162 Da, hexose; 1204 Da, lysoform GD_3; 269 Da, cleaved free stearic acid. GD, disialoganglioside; MS^n, mass spectrometry, n times.

Buttermilk powder (1 g) was dissolved in 15 mL methanol plus 15 mL chloroform. The solution was incubated for 15 min at 4°C and centrifuged at 456 × *g* for 10 min at 4°C. The supernatant was separated, and the pellet was extracted again. Both supernatants were assembled, and the volume was reduced to one-fourth of the initial volume. The solution was incubated at –20°C overnight and centrifuged at 456 × *g* at 4°C for 10 min. Finally, the supernatant was lyophilized.

For partition, the dried supernatant was dissolved in 12 mL diisopropyl ether/1-butanol (60:40, vol/vol) and 6 mL distilled water and centrifuged at 456 × *g* for 10 min at 4°C. The organic (upper) phase was rejected. The aqueous phase was washed with diisopropyl ether/1-butanol (60:40, vol/vol) and lyophilized. One gram of buttermilk powder resulted in 7–10 mg extracted gangliosides.

Chemical transacylation–hydrolysis. The amide-linked FA of the buttermilk gangliosides were hydrolyzed with potassium hydroxide/methanol solution according to Sonnino *et al.* (29) or by acetonitrile/hydrochloric acid based on the method of Kadowaki *et al.* (30). For the basic hydrolysis 3 µmol (calculated for MG: 1500 Da) extracted gangliosides were dissolved in 1 mL 1 N potassium hydroxide in methanol/water (1:1, vol/vol). The solution was incubated for 14 h at 60°C. To stop the reaction, 1 mL 1 N HCl was added. The solution was evaporated with nitrogen.

For the acid hydrolysis 3 µmol (calculated for MG: 1500 Da) extracted gangliosides were dissolved in 1 mL 0.1–0.5 N HCl in acetonitrile. The solution was incubated for 20–40 min at 60°C. To stop the reaction 100 µL 1–5 N KOH was added. The solution was evaporated with nitrogen.

For a solid-phase extraction with an aminopropyl cartridge (500 mg cartridge; ICT, Bad Homburg, Germany), the sample was dissolved in chloroform/methanol (2:1, vol/vol). The cartridge was conditioned with 4 mL *n*-hexane, and the sample was applied. In a three-step process neutral lipids were eluted with 5 mL isopropanol/chloroform (1:2, vol/vol) and FFA with 5 mL 2% acetic acid in diethyl ether. Finally, the hydrolyzed gangliosides were eluted with methanol/chloroform/distilled water (10:5:4 ,by vol). The fractions were evaporated with nitrogen.

Chemical transacylation–reacylation. To establish a new specific amide-linked FA composition of glycosphingolipids from buttermilk, the lysogangliosides were reacylated with arachidic acid according to Anand *et al.* (27). In a glass tube 3 µmol lyso-gangliosides, 6 µmol diethylphosphorylcyanide, and 9 µmol freearachidic acid were dissolved in 2 mL dimethylformamide/dichloromethane (1:3, vol/vol), and 2.5 µmol triethylamine was added. The solution was covered with nitrogen, and the tube was sealed and then incubated at 20°C for 45 min. The reacylated gangliosides were extracted by a solid-phase extraction with an aminopropyl cartridge as mentioned in the previous paragraph.

Enzymatic transacylation–hydrolysis and reacylation. The amide-linked FA of buttermilk gangliosides were hydrolyzed and reacylated within one step by the substrate-specific sphingolipid ceramide N-deacylase EC 3.5.1.23 (Takara Shuzo Co. Ltd. Biomedicals, Shiga, Japan). Three micromoles (calculated for MG: 1500 Da) of extracted gangliosides were dissolved in 200 µL of 10 mM, pH 6 sodium acetate buffer system, and 3 µmol free arachidic acid (ratio: 1 mol gangliosides to 10 mol FFA) and 100 mU enzyme were added. The solution was incubated at 37°C for 28 h. The sodium acetate buffer system was adjusted at pH 5.5 for all hydrolysis experiments.

For reversed solid-phase extraction, 200 mg of evaporated sample was dissolved in chloroform/methanol (2:1, vol/vol). The C18-cartridge (2 g cartridge; ICT) was washed with 10 mL methanol and 10 mL methanol/chloroform (2:1, vol/vol) and conditioned with 10 mL methanol/distilled water (2:1, vol/vol). The sample was applied onto the cartridge. In a two-step process the cartridge was washed with 20 mL distilled water. The ganglioside lysoforms were eluted with 10 mL methanol/distilled water (2:1, vol/vol.). The gangliosides were eluted with 5 mL methanol and 10 mL chloroform/methanol (2:1, vol/vol) (31). Furthermore, a solid-phase extraction with an aminopropyl cartridge was prepared to separate the FFA, as discussed previoiusly. The fractions were evaporated with nitrogen.

Separation of gangliosides by high-performance TLC (HPTLC). The samples were dissolved in chloroform/ methanol (2:1, vol/vol), and 10 µL of the solution was applied on a Silica gel 60 plate (Merck Eurolab, Darmstadt, Germany). The mobile phase was chloroform/methanol/20 mM calcium chloride (11:9:2, by vol) according to Müthing *et al.* (32). The gangliosides were separated within 2 h in a gas-saturated chamber. After drying the plate, gangliosides were specifically detected with resorcinol/HCl spray reagent by heating at 120°C for 15 min (33). As standards, lyso-GM_1, GM_1, GM_2, GM_3, $GD_{1a/b}$, and GD_3 from Sigma (Chemical (St. Louis, MO) were used.

Preparation of gangliosides by HPLC. The extracted buttermilk gangliosides were separated by an HPLC Alliance 2695 Separation module (Waters GmbH, Eschborn, Germany) using a LiChrosorb-NH_2 column (250 × 4 mm i.d., 5 µm particle diameter; Merck Eurolab) as previously described (34). Before HPLC separation, the extract was purified with a C18 reverse solid-phase extraction, as mentioned above. The purified extract was dissolved in chloroform/methanol (1:1, vol/vol). From a 500 µg/mL stock solution, 20 µL (10 µg) was introduced into the system. The order of ganglioside elution depended on the charge and the degree of sialylation. The eluent system was (A) acetonitrile/5 mM phosphate buffer (K_2HPO_4/KH_2PO_4), pH 5.6 (83:17, vol/vol) and (B) acetonitrile/20 mM phosphate buffer, pH 5.6 (1:1, vol/vol). All solvents used were HPLC grade or supra-solvent quality (Merck). The solvent gradient system was as follows: 0–7 min A/B (%) 100:0, 7–59 min, A/B (%) 66:34, 59–73 min, A/B (%) 36:64. The flow rate was 1 mL/min, and the elution profile was detected by UV absorbance at a wavelength of 197 nm. The distinct lipid species were fractionated with an automatic fraction-sampler by peak signal recognition. The fractions were sampled in 5-mL sealed glass tubes. The standards used for HPTLC were also used for HPLC.

Structural analysis of gangliosides by nano ESI-MS^n. Mass spectra were acquired on an LC–MS system (LCQ Deca, ThermoFinningan, San Jose, CA) equipped with an on-line nano electrospray ionization source. The tip voltage was set at 2.3 kV in the positive mode or 2.0 kV in the negative mode. The tip diameter was 30 µm. The capillary voltage was 12 V, and the capillary temperature was held at 160°C. The ion collision energy in the MS^n experiments was adjusted between 30 and 45%. The samples were dissolved in chloroform/methanol/distilled water (2:3:1, by vol) at a concentration of about 50 ng/µL. The samples were applied by a syringe with a flow of 2 µL/min.

FAME analysis by capillary GC. The buttermilk gangliosides were evaporated under nitrogen to dryness. For a direct methylation according to Lepage and Roy (35), the samples were dissolved in 2 mL methanol/hexane (4:1, vol/vol) plus 0.5% pyrogallol as antioxidant and 200 µL of acetyl chloride. The samples were incubated at 100°C for 1 h. The reaction was stopped with 4.85 mL of potassium carbonate. The upper hexane phase was removed after 10 min of centrifugation at 2200 × *g* and was evaporated with disodium sulfate. The FAME were analyzed by capillary GLC on an LS 32 Varian Chrompack system (Varian Chrompack, Middelburg, The Netherlands) equipped with a cold split/splitless injector to avoid FA discrimination. A chemically bound 50% cyanopropyl-methylpolysiloxane capillary column DB 23, 40 m Fisons (Varian, Chrompack), was used to separate the FA species. The FA were identified according to their retention times (RT) by an FID at 280°C (36). The chromatographic conditions were as follows: injector (programmable temperature vaporization): 65 to 270°C; split ratio 15:1; carrier gas: helium at 40 cm/s flow; column oven: initial temperature 60°C for 1 min, from 60 to 180°C at 40°C/min, 180°C for 2 min, from 180 to 210°C at 2°C/min, 210°C for 3 min, from 210 to 240°C at 3°C/min, and 240°C for 10 min.

RESULTS AND DISCUSSION

The buttermilk that was used and that was characterized by HPTLC and HPLC analysis with qualitative UV (λ = 197 nm) detection contained mainly the disialoganglioside (NeuAc2-9NeuAc2-3Galb1-4GlcCer) GD_3 (R_f value 0.45; RT 33.4 min) as well as the monosialogangliosides (NeuAc2-6Galb1-4GlcCer) GM_3 (R_f value 0.74; RT 9.3 min) according to the literature (19) (Figs. 2, 3).

Regarding the FA composition of total gangliosides from buttermilk, oleic acid (18:1), palmitic acid (16:0), and stearic acid (18:0) are predominant (18). In this study, the heterogeneity of ganglioside-integrated FA—mainly palmitic acid, oleic acid, stearic acid, myristic acid (14:0), and linoleic acid (18:2n-6)—reflected the FA pattern of total buttermilk. However, erucic acid (22:1n-9) was not detectable in buttermilk but integrated within gangliosides (Table 1). Hauttecoeur and colleagues (19) have already described two subclasses of GD_3 defined by their FA composition. In this study, such a FA profile was detected, containing monounsaturated FA with a chain length of 22 to 25 carbon atoms, indicating the subgroup $GD3_{C22–C25}$, whereas FA of

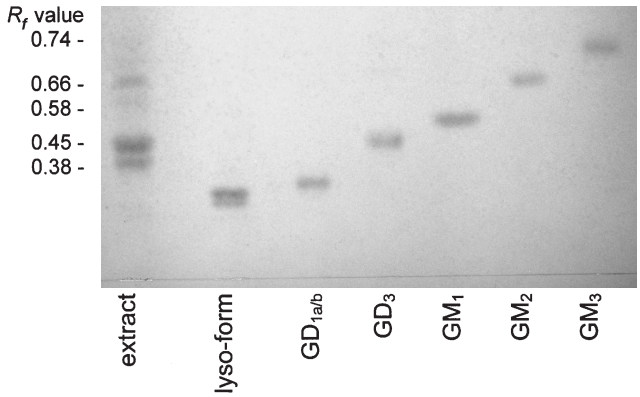

FIG. 2. High-performance TLC separation (HPTLC) of native buttermilk gangliosides in comparison to GM_1, GM_2, GM_3, $GD_{1a/b}$, GD_3, and lysoganglioside standards. For HPTLC the samples were applied on a Silica gel 60 plate. The mobile phase was chloroform/methanol/20 mM calcium chloride (by vol) 11:9:2. The gangliosides were visualized with resorcin-HCl spray reagent at 120°C for 15 min. GM, monosialoganglioside; for other abbreviations see Figure 1.

chain lengths between 16 and 18 carbon atoms belonged to the subgroup $GD3_{C16–C18}$. Arachidic acid was not detected at all. Therefore, the possibility of modulating the FA composition of isolated buttermilk gangliosides by chemical or enzymatic transacylation with free arachidic acid was examined. Concerning infant nutritional needs, it is worthwhile to adapt raw material so as to produce products as close as possible to breast milk. The concentration of ganglioside-integrated arachidic acid is significant higher compared to breast milk (21,22); thus, a transesterification with this FA could be relevant.

The chemical FA exchange of gangliosides was established by a basic hydrolysis (29) combined with a diethylphosphorylcyanide/triethylamine-catalyzed reacylation with free arachidic acid, based on the method of Anand *et al.* (27). The heterogeneous FA composition of native gangliosides was reduced to mainly palmitic acid and stearic acid. Arachidic acid, which did not appear in the initial buttermilk gangliosides, was transacylated to total gangliosides with 23 wt% by the chemical process (Table 1). As GD_3 predominates in buttermilk, arachidic acid was chemically transacylated to this species at 6.7 wt%. Remarkably, behenic acid (22:0) and lignoceric acid (24:0) of GD_3 were the main acids that were exchanged. Owing to the long acyl chain length, the amide-linked FA could be very sensitive to basic hydrolysis in this system.

The buttermilk-derived GD_3 and the resulting lysoforms after hydrolysis were analyzed by nano ESI-MS^n to characterize possible degradations or unspecific reactions during the hydrolysis (Figs. 2, 4, and 5). ESI experiments with glycosphingolipids are available with mainly tandem or triple-stage quadrupole MS applications (37,38). The on-line nano ESI technology, combined with ion trap detection, used in this study provides an enhanced ionization of amphiphilic glycolipids and the possibility to fragment defined ions, thus enabling a detailed molecular structural analysis *via* sequenced MS^n experiments (39,40). The total ion scan in the positive mode of native

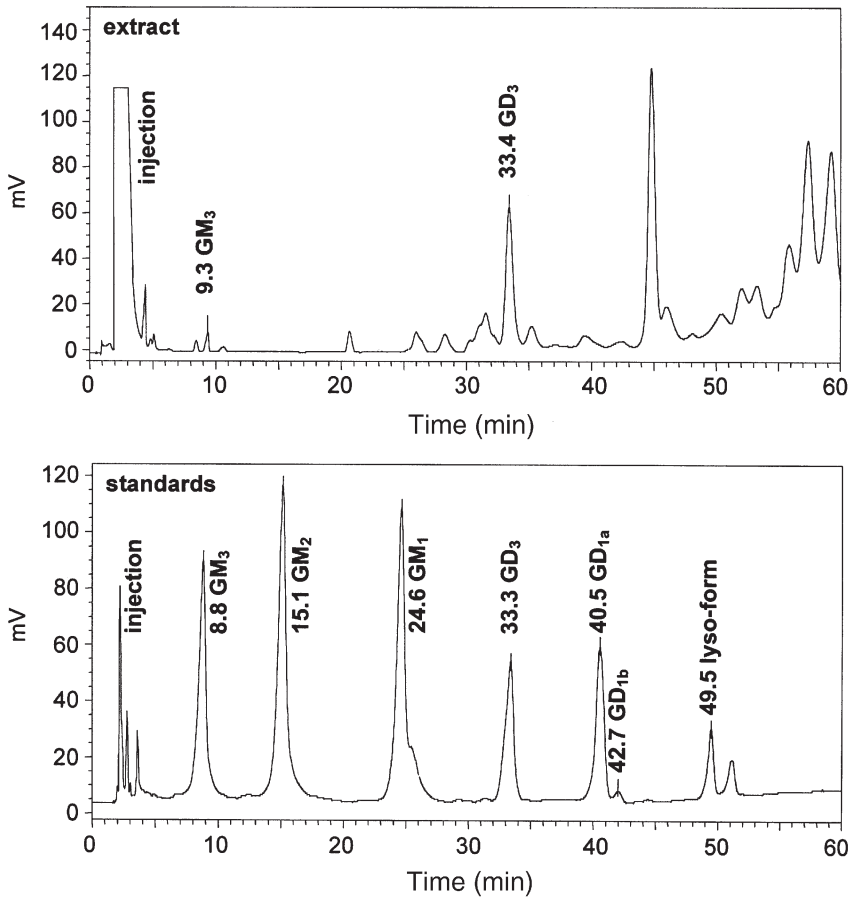

FIG. 3. HPLC separation of native buttermilk gangliosides in comparison to GM_1, GM_2, GM_3, $GD_{1a/b}$, GD_3, and lysoganglioside standards. For HPLC the samples were separated by a LiChrosorb-NH_2 column (Merck, Darmstadt, Germany; 250 × 4 mm i.d., 5 µm particle diameter). The eluent system was: (A) acetonitrile/5 mM phosphate buffer, pH 5.6 (83:17 vol/vol); (B) acetonitrile/20 mM phosphate buffer, pH 5.6 (1:1, vol/vol) with the solvent gradient: 0–7 min A/B (%) 100:0, 7–59 min A/B (%) 66:34, 59–73 min A/B (%) 36:64. The flow rate was 1 mL/min and detection was by UV absorbance at 197 nm wavelength.

GD_3 indicated a mass per charge (*m/z*) value of 1564.6 with a pattern of 28 Da (dimethyl fragment) mass differences that were dependent on the FA heterogeneity of the gangliosides. MS^n experiments with this parent ion indicated a sequential fragmentation pattern of two sialic acids (291 Da) and two hexoses (162 Da) (Figs. 1, 4).

A previous study suggested that GM_1 can be deacylated by tetramethylammonium hydroxide or KOH (29). To deacylate the amide-linked FA while reducing the incidence of unspecific/unintended degradations, a 14-h incubation at 60°C with 1 N KOH, according to Sonnino *et al.* (29), provided the most practicable hydrolysis of buttermilk gangliosides. Alternatively, acid-catalyzed hydrolysis was considered. However, the oligosaccharide part of the buttermilk GD_3 disappeared totally when using 0.1–0.5 N HCL in acetonitrile for 20–60 min at 60°C based on a method of Kadowaki *et al.* (30) (data not shown). The hydrolysis experiments were measured in the nano ESI^n negative mode for optimal signal intensities. Native GD_3 measured in the positive mode with *m/z* 1564.7 represented a (GD_3 sphingoid d18-18:0) + $4Na^+$ adduct ion. However, in the negative mode the same molecule with *m/z* 1540.8 was exclusively observed as a (GD_3 sphingoid d18-18:0)$^+3Na^+$ adduct ion. In comparison to *m/z* 1540.8 of GD_3, the chemically induced lysoform had a *m/z* value of 1271.1 (lyso-GD_3 sphingoid d18:0)$^+3Na^+$, indicating a mass difference of 269 Da corresponding to the M.W. of cleaved stearic acid. The mass difference of 582 Da (2 × 291 Da) between *m/z* 1541 and 958 revealed sialic acid fragmentation, and *m/z* 723.7 and *m/z* 677.9, unspecific degradation products (Fig. 5).

Recent studies have indicated that the amide-acyl linkage between FA and the sphingoid bases of sphingolipids is specifically hydrolyzed by the sphingolipid ceramide N-deacylase (EC 3.5.1.23) in aqueous systems. This enzyme, derived from *Pseudomonas* sp. strain TK4, reacts with different acidic and neutral glycosphingolipids and sphingomyelin, but not with ceramide (24,25). The enzyme also catalyzes the condensation reaction between FFA and sphingolipids as well as the transacylation between FFA and sphingolipids. The cleavage

TABLE 1
Efficiency of Arachidic Acid Exchange: Fatty Acids Content (wt%) of Total Buttermilk, Gangliosides and GD_3, Either Native or After Transesterification as Measured by GC FAME Analysis[a]

		Native		After chemical transacylation		After enzymatic transacylation	
Fatty acid	Total	Gangliosides	GD_3	Gangliosides	GD_3	Gangliosides	GD_3
6:0	1.4	1.2					0.6
8:0	1.0	1.2				0.4	0.8
10:0	2.1	2.7	5.6			1.5	1.3
11:0		0.3				0.3	
12:0	2.6	2.5	1.8		0.7	1.7	2.3
13:0		0.9			1.3	0.8	9.7
14:0	10.0	8.5	4.3	3.0	3.9	4.2	6.0
14:1n-5	0.6	0.6				0.3	0.6
15:0	1.3	1.8				0.6	
16:0	30.3	29.4	32.1	23.5	32.0	28.4	29.8
16:1n-7	1.4	1.1					
17:0	0.9	0.7			1.5	0.9	
18:0	12.4	16.9	19.9	23.9	46.4	35.0	29.5
18:1n-9	25.9	17.8	2.6	4.0	2.3	7.9	4.5
18:1n-7	0.9						
18:2n-6	3.7	2.3				1.5	
18:3n-3	1.1	1.2					
20:0				23.2	6.7	8.7	13.8
20:1n-9			7.1			0.8	1.1
20:2n-6							
22:0	0.9	1.0	10.3	6.4		3.0	
22:1n-9		3.5		4.2	5.1		
22:2n-6			3.4				
23:0	1.5	1.3		5.1		2.5	
24:0	1.1	0.9	8.0			1.7	
24:1n-9			5.0				
25:0	0.6						
SAFA	66.3	69.5	82.0	91.8	92.5	89.4	93.8
MUFA	28.9	27.0	14.7	8.3	7.4	9.0	6.2
PUFA	4.8	3.5	3.4			1.6	

[a]SAFA, saturated FA; MUFA, monounsaturated FA; GD3, disialoganglioside (NeuAc2-9 NeuAc2-3Galb1-4GlcCer).

reaction has a pH optimum of 5.0, whereas the reverse reaction has its optimum at pH 7.0 (26). In this study the FA exchange was established in a one-step process at pH 6.0. As the reaction kinetic depends on the substrate concentrations, the FA exchange was enhanced by a 1:10 ratio of gangliosides to FFA. Arachidic acid was enzymatically transacylated to total gangliosides in the amount of 8.5 wt% and to GD_3 at 13.8 wt%. The heterogeneity of the constituent FA was also reduced by both the enzymatic and the chemical processes (Table 1). In contrast to the chemical process, mainly stearic acid of GD_3 was transacylated enzymatically, but palmitic acid was not. These data suggest that the sphingolipid ceramide N-deacylase might possess substrate-specific affinities relative to incorporated FA. The content of ganglioside-linked short- (6–8 carbon atoms) and medium-chain FA (10–12 carbon atoms) was reduced less than with chemical transesterification.

The reaction specificity of the enzymatic transacylation of GD_3 was characterized by MS^n experiments. The ions observed had a *m/z* value of 1204, indicating lyso-GD_3 sphingoid d18:1, which is comparable to the chemically induced lysoform. In contrast to the chemical hydrolysis, the decomposition of only one sialic acid moiety was detected by a mass difference of 291 Da between *m/z* 1204 and 913. No nonspecific fragmentations were observed in the enzymatic process. MS^n experiments with the parent ion of *m/z* 1226 (1204 + Na^+) indicated mass differences of 291 Da between 1226 and *m/z* 935 or 913 (935 – Na^+) as evidence of a cleaved N-acyl linkage of GD_3 without the disappearance of sialic acids (Fig. 5).

In conclusion, the distinct heterogeneity of incorporated FA was effectively modified by both transesterification techniques without affecting the oligosaccharide part. The chemical and enzymatic modification effectively integrated free arachidic acid, which was not detected in the initial buttermilk gangliosides and GD_3. As the enzymatic transacylation is specific, the risk of undesirable molecule fragmentations seems to be much lower than when applying the chemical method. The method described in this study offers the possibility of modifying the FA composition of glycosphingolipids, not only to adapt plant or animal sources to human needs but also to meet technical needs such as texture properties or flavor. Also, the synthesis of gangliosides with physiologically relevant FA like CLA, medium-chain PUFA, or very long chain PUFA to combine glycolipid and FA properties are conceivable.

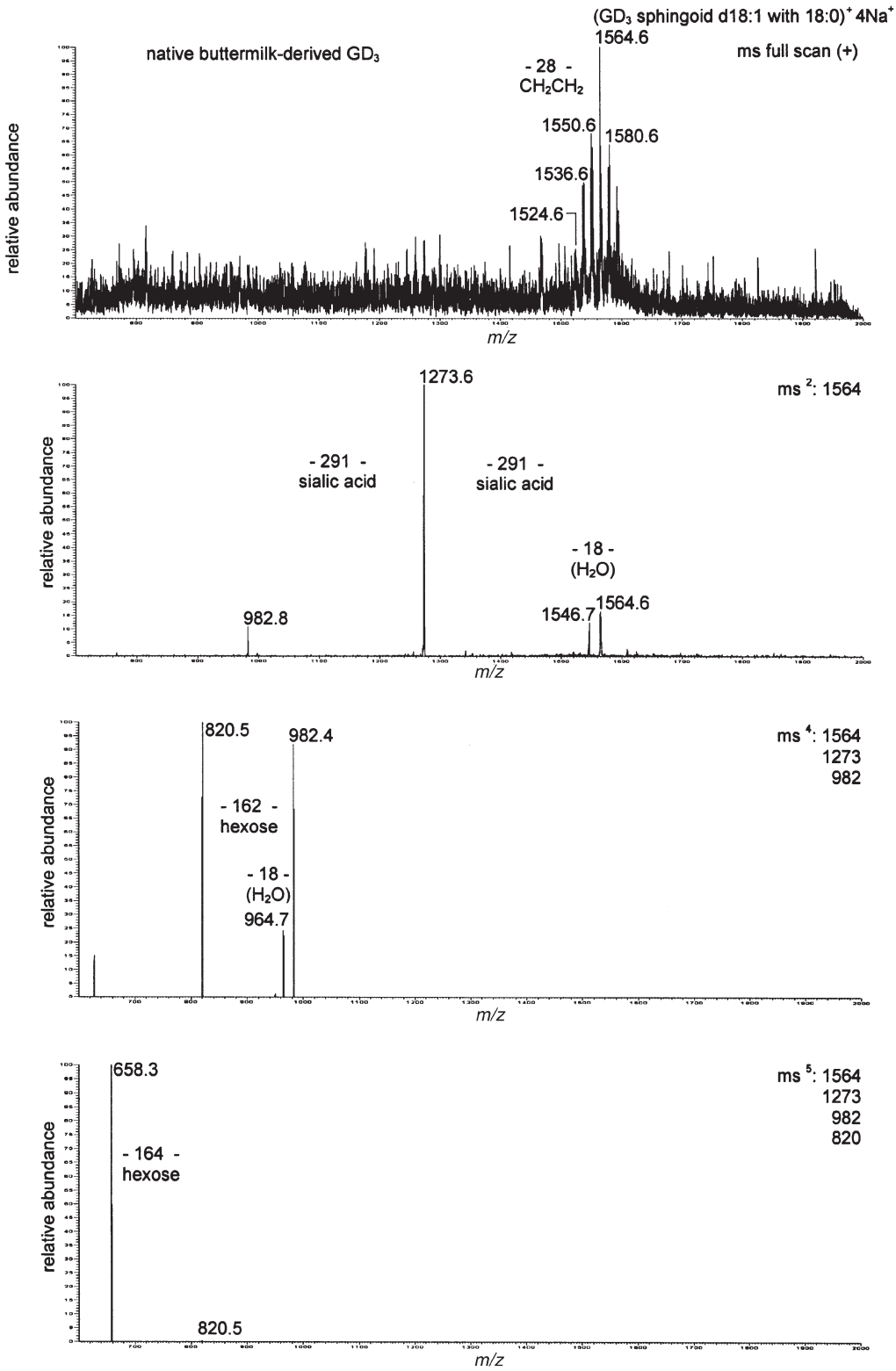

FIG. 4. Nano ESI-MS^n spectra of native buttermilk GD_3. The specific fragmentation is illustrated in Figure 1. Mass spectra were acquired on an MS system with a nano electrospray ionization source. The tip voltage was set at 2.3 kV in the positive mode. The ion collision energy in the MS/MS experiments was adjusted between 30 and 45%. The data are presented as *m/z*. Nano ESI-MS^n, nano electrospray ionization-MS^n; MS/MS, tandem MS; for other abbreviation see Figure 2.

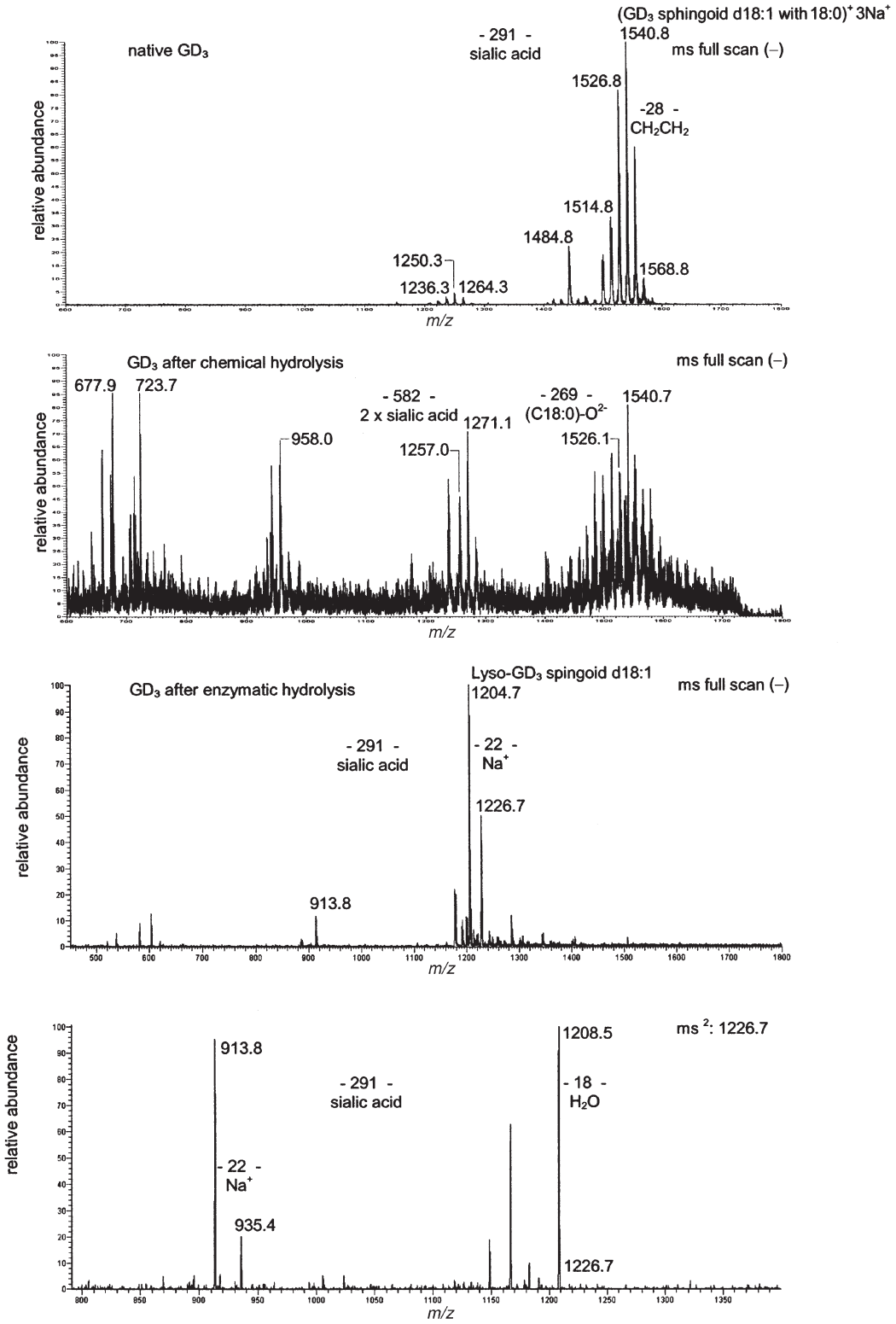

FIG. 5. Nano ESI-MS^n spectra of buttermilk GD_3 lysoforms after chemical and enzymatic hydrolysis. The tip voltage was set at 2.0 kV in the negative mode. The ion collision energy in the MS/MS experiments was adjusted between 30 and 45%. The data are presented as *m/z*. For abbreviations see Figures 2 and 4.

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Synthesis of Fluorinated Analogs of Myristic Acid as Potential Inhibitors of Egyptian Armyworm *(Spodoptera littoralis)* Δ^{11} Desaturase

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ABSTRACT: To study the activity of the different desaturases present in the pheromone biosynthetic pathway of the Egyptian armyworm, *Spodoptera littoralis,* we prepared a series of mono- and *gem*-difluorinated analogs of myristic acid with halogen substitution at the C8–C11 positions of the aliphatic chain *via* specifically positioned dithiane precursors. Thus, transformation of dithianes by treatment with *N*-bromosuccinimide in the presence of H_2O followed by reduction with $LiAlH_4$ afforded the appropriate alcohols, which reacted with diethylaminosulfur trifluoride to give rise to the corresponding monofluoroderivative intermediates. Alternatively, the introduction of the *gem*-difluoro functionality was carried out by reaction of the appropriate dithiane intermediate with 1,3-dibromo-5,5-dimethylhydantoin in the presence of HF/pyridine. The activity of these fluorinated FA as substrates and inhibitors of the desaturases involved in the biosynthesis of the sex pheromonal blend of *S. littoralis* has been studied. In this case, 11-fluorotetradecanoic acid elicited a moderate inhibitory activity of Δ^{11} desaturase.

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Biosynthesis of unsaturated FA in living organisms occurs by the direct introduction of double bonds into a saturated precursor in a reaction that is catalyzed by specific desaturases. In insects, some moth pheromone glands contain additional desaturases that transform unsaturated FA to conjugated dienoic acids. To gain insight into general aspects of the mechanism of desaturase enzyme catalysis, studies on the activities of desaturases are desirable. In this context, we recently demonstrated some important features of that desaturation mechanism in the FA dienoic functionality of the Egyptian armyworm *(Spodoptera littoralis)* sex pheromone by studying the stereochemistry (1–3) and cryptoregiochemistry (4,5) of the desaturation process. However, our ongoing interest in these desaturases has led us to undertake the search for specific enzyme inhibitors that could be useful in such studies. In this sense, replacement of hydrogen by fluorine in those positions of the aliphatic chain closer to the interaction with the enzyme-active center of Δ^{11} and Δ^{9} desaturases might be effective. Previous work in our laboratory showed that different mono- and difluoropalmitic acids can inhibit the β-oxidation step of the biosynthesis of (*Z*,*E*)-9,11-tetradecadienyl acetate, the major component of *S. littoralis* sex pheromone blend (6–9). In insect sex pheromones we (10–12) and others (13,14) demonstrated that replacements of hydrogen atom(s) by fluorine mimic, potentiate, or inhibit the action of the natural products.

Recently, Behrouzian *et al.* (15,16) studied the desaturation of both enantiomers of 9-fluoroderivatives of stearic acid by stearoyl acyl carrier protein Δ^{9} desaturase isolated from castor seed oil and gained valuable insights into the enantioselectivity of this enzyme in dehydrogenation and oxidation reactions. In our case, we were interested in studying the activity of those mono- and *gem*-difluoroderivatives of myristic acids bearing the fluorine substitution at the C8–C11 positions. For this, a versatile synthetic route was required that could also eventually be adapted to the preparation of the corresponding enantiomeric monofluorinated derivatives.

In the present communication we report on this synthetic route and on the preliminary results of the activity of the above fluorinated analogs with the Δ^{11} desaturase of *S. littoralis*.

EXPERIMENTAL PROCEDURES

General methods. Commercially available reagents and solvents were used directly as supplied with the following exceptions: diethyl ether and THF were distilled over Na/benzophenone under an argon atmosphere. Reactions sensitive to moisture and oxygen were carried out under an argon or nitrogen atmosphere. Unless otherwise stated, organic solutions obtained from workup of crude reaction mixtures were dried over anhydrous $MgSO_4$, and purification procedures were carried out by flash chromatography on silica gel (230–400 mesh); products were mostly obtained as oils, and they were at least 98% pure (GC). Visualization of UV-inactive materials was accomplished by soaking the TLC plates in a ethanolic solution of anisaldehyde and sulfuric acid (96:2:2, by vol) or in an ethanolic solution of phosphomolybdic acid (5%).

All ^{1}H NMR spectra were acquired at 300 MHz; ^{13}C NMR spectra were acquired at 75 MHz in $CDCl_3$ solutions. Chemical shifts are given in ppm downfield from $Si(CH_3)_4$ for ^{1}H, and $CDCl_3$ for ^{13}C. In the same way, ^{19}F NMR spectra were

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Abbreviations: DAST, diethylaminosulfur trifluoride; DEPT, distortionless enhancement by polarization transfer; FA, fatty acid; MTBE, methyl *tert*-butyl ether; NBS, *N*-bromosuccinimide; SIM, selected ion monitoring.

acquired at 282 MHz and are reported in ppm downfield from $CFCl_3$. Assignment of critical signals in the ^{13}C NMR spectra was carried out on the basis of distortionless enhancement by polarization transfer (DEPT). GC–MS was performed by electron impact at 20 eV using the equipment and conditions described below. All IR spectra were run on a Michelson Bomem MB-120 spectrometer. Elemental analyses were obtained by the Microanalysis Service of IIQAB-CSIC.

In vitro *gland culture procedure.* Inhibition of Δ^{11} desaturase activity by the fluorinated derivatives synthesized was also investigated by using d_3-tetradecanoic acid as substrate and determining the amounts of d_3-(*E*)-11-tetradecenoic acic formed in controls and experimental glands. These experiments were carried out using round-bottomed 96-well plates. To each well, a 10-μL drop of incubation medium was added. The incubation medium consisted of the commercial Grace's insect medium (17) (135 μL) and a DMSO solution (15 μL) of a 1:1:1 mixture of d_3-16:acid/d_3-14:acid/fluoroacid (10 mg/mL each) for treated tissues or a DMSO solution of d_3-14:acid (10 mg/mL) for controls; these deuterated acids were obtained from IC Chemicalien (Munich, Germany). One-day-old virgin *S. littoralis* females were briefly anesthetized on ice, and the pheromone glands were excised, carefully cleaned, and immersed individually into a drop of the incubation medium. Plates were sealed with adherent plastic covers, and incubations proceeded for 3 h at 25°C. After this time, to obtain the methyl ester derivatives of the gland lipids for analysis, pheromone glands were collected and soaked in 0.5 M KOH for 30 min, and then the organic solution was neutralized with 1 N HCl and extracted with hexane containing methyl pentadecanoate (10 ng/gland) as internal standard for quantification. Five glands were used for each sample.

Instrumental analysis of the biological extracts. The extracts were analyzed by GC-coupled to MS (GC–MS) at 70 eV on a Fisons gas chromatograph (8000 series) coupled to a Fisons MD-800 mass selective detector. The system was equipped with a nonpolar Hewlett-Packard HP-1 capillary column (30 m × 0.20 mm i.d.) using the following program: from 120 to 180°C at 5°C/min and then 260°C at 2°C/min after an initial delay of 2 min. Analyses were carried out under selected ion monitoring (SIM) mode. Selected ions were 245 (trideuterated methyl tetradecanoate, $M^{\bullet+}$), 242 (methyl tetradecanoate, $M^{\bullet+}$), 243 (trideuterated methyl (*Z*)-11 and (*E*)-11 tetradecenoates, $M^{\bullet+}$), 240 (methyl (*Z*)-11 and (*E*)-11 tetradecenoates, $M^{\bullet+}$). To investigate the conversion of the fluoro-FA into their unsaturated derivatives, the analyses of the corresponding methyl esters were carried out under the full scan method.

*Preparation of products **4a–d**, **5a–d**, and **6a–d**.* These syntheses were carried out according to previously described procedures (4,18).

*Synthesis of dithianes **7a–d**. General procedure.* These products were obtained following the procedure reported by Seebach and Corey (19). To a solution of the dithiane **6** (10 mmol) in 15 mL of dry THF, kept at −20°C, was added 12 mmol of a hexane BuLi solution (7.5 mL, 1.6 M). The light yellow reaction mixture was stirred for 30 min, cooled at −78°C, and held for 10 min, and then product **5** (8 mmol) was added dropwise and stirring was continued at −78°C for 2 h. The resulting solution was allowed to warm to room temperature, and the solvent was then evaporated. The residue was suspended in 50 mL of H_2O, extracted with CH_2Cl_2, dried, and concentrated to dryness. The residue was purified by flash chromatography on silica gel using a gradient of 0–10% methyl *tert*-butyl ether (MTBE) in hexane to give the pure dithiane **7** in 74–80% yields.

*(i) 2-Propyl-2-(11,13-dioxatetradecyl)-1,3-dithiane (**7a**).* (2.15 g, 74% yield); IR 2930, 2855, 1465, 1275, 1150, 1110, 1045, 920 cm^{-1}; ^1H NMR δ 4.62 (*s,* 2H, OCH_2O), 3.52 (*t, J* = 6.5 Hz, 2H, CH_2O), 3.36 (*s,* 3H, OCH_3), 2.81 (4H, SCH_2), 2.02–1.90 (2H, CH_2CH_2S), 1.90–1.80 (4H, $SSCCH_2$), 1.66–1.50 (2H, CH_2CH_2O), 1.50–1.40 (2H, CH_2CH_3), 1.42–1.23 (14H, CH_2), 0.94 (*t, J* = 7 Hz, 3H, CH_3); ^{13}C NMR δ 96.3 (OCH_2O), 67.8 (CH_2O), 55.0 (OCH_3), 53.3 (CSS), 40.4 (CH_2CSS), 38.2 (CH_2CSS), 29.8 (CH_2S), 29.7 (CH_2S), 29.5 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 29.4 (CH_2), 26.2 (CH_2), 26.0 (CH_2), 25.6 (CH_2), 24.0 (CH_2), 17.4 (CH_2CH_3), 14.3 (CH_3); MS *m/z* 362 ($M^{\bullet+}$, 15), 319 (20), 287 (15), 161 (100); anal. calcd. for $C_{19}H_{38}O_2S_2$: C, 62.93; H, 10.56; S, 17.68. Found: C, 62.84; H, 10.47; S, 17.56.

*(ii) Synthesis of ketones **8a–d** from dithianes **7a–d**. General procedure.* To a solution of NBS (5.45 g, 30 mmol) in 47 mL of acetone and 2.5 mL of H_2O held at −30°C was added dropwise 1.81 g of dithiane **7** (5 mmol) dissolved in 50 mL of the same solvent mixture. Stirring was continued for 5 min, and a 10% $Na_2S_2O_3$ water solution was added until the orange color of the solution disappeared. The solvent was evaporated at reduced pressure, and the residue extracted with CH_2Cl_2, dried, and concentrated to dryness. The residue was purified by flash chromatography on silica gel using a gradient of 0–10% MTBE in hexane to give the pure ketones in 87–92% yields.

*(iii) 15,17-Dioxa-4-octadecanone (**8a**).* (1.25 g, 92% yield); IR 2930, 2855, 1715 (CO), 1465, 1410, 1370, 1150, 1110, 1045, 920 cm^{-1}; ^1H NMR δ 4.62 (*s,* 2H, OCH_2O), 3.51 (*t, J* = 6.5 Hz, 2H, CH_2O), 3.36 (*s,* 3H, OCH_3), 2.38 (*t, J* = 7.5 Hz, 4H, CH_2CO), 1.67–1.46 (6H, CH_2CH_2CO and CH_2CH_2O), 1.40–1.18 (12H, CH_2), 0.91 (*t, J* = 7.5 Hz, 3H, CH_3); ^{13}C NMR δ 211.6 (CO), 96.3 (OCH_2O), 67.8 (CH_2O), 55.0 (OCH_3), 44.7 (CH_2), 42.8 (CH_2), 29.7 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 29.2 (CH_2), 26.2 (CH_2), 23.8 (CH_2), 17.3 (CH_2), 13.7 (CH_3); MS *m/z* 257 ($M^{\bullet+} - CH_3$, 1), 241 (2), 227 (5), 167 (10), 149 (15), 99 (20), 83 (35), 71 (100), 69 (40), 55 (45); anal. calcd. for $C_{16}H_{32}O_3$: C, 70.54; H, 11.84. Found: C, 70.60; H, 11.68.

*Preparation of alcohols **9a–d** from ketones **8a–d**. General procedure.* A solution of the corresponding ketone **8** in Et_2O, maintained under argon and at room temperature, was treated with 4 molar equiv of $LiAlH_4$, and the mixture was stirred until the reaction was completed. Reagent excess was carefully quenched with water and after the usual workup, the residue obtained was purified by flash chromatography on silica gel using hexane/MTBE 80:20 to give the corresponding pure alcohols **9** in 92–96% yields.

*(i) 15,17-Dioxa-4-octadecanol (**9a**).* This alcohol was isolated (1.05 g, 96% yield) starting from 1.09 g (4 mmol) of ketone **8a**. IR 3450 (OH), 2930, 2855, 1465, 1385, 1215, 1150, 1110, 1045, 920 cm^{-1}; ^1H NMR δ 4.61 (*s,* 2H, OCH_2O), 3.59 (*bs,* 1H, OH), 3.51 (*t, J* = 6.5 Hz, 2H, CH_2O), 3.36 (*s,* 3H, OCH_3), 1.59 (*m,* 2H, CH_2CH_2O), 1.52–1.22 (21H), 0.92 (*t, J* = 6.5 Hz, 3H, CH_3); ^{13}C NMR δ 96.3 (OCH_2O), 71.7 (CHOH), 67.8 (CH_2O), 55.0 (OCH_3), 39.6 (CH_2), 37.5 (CH_2), 29.7 (CH_2), 29.7 (CH_2), 29.6 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 29.4 (CH_2), 26.2 (CH_2), 25.6 (CH_2), 18.8 (CH_2), 14.1 (CH_3); MS *m/z* 225 ($M^{\cdot+} - H_2O - OCH_3$, 1), 200 (10), 169 (5), 149 (12), 109 (15), 95 (35), 81 (25), 69 (30), 55 (60), 45 (100); anal. calcd. for $C_{16}H_{34}O_3$: C, 70.02; H, 12.49. Found: C, 69.94; H, 12.35.

*Preparation of monofluoride derivatives **10a–d**. General procedure.* To a stirred solution of the corresponding alcohol **9** in 2 mL of dry CH_2Cl_2 at −78°C diethylaminosulfur trifluoride (DAST) was added dropwise (1.15 equiv) with a syringe under a nitrogen atmosphere. Stirring was continued for 2 h, and then the reaction mixture was allowed to warm to room temperature and carefully poured over a cold saturated $NaHCO_3$ solution. The mixture was extracted with hexane, and the organic layer was washed with brine, dried, and concentrated to obtain a residue that was purified by flash chromatography on silica gel using a hexane/MTBE gradient (0–8%) to give the corresponding pure fluoroderivatives in 72–76% yields.

*(i) 15,17-Dioxa-4-fluorooctadecane (**10a**).* This product was isolated (207 mg, 75% yield) starting from 274 mg (1 mmol) of alcohol **9a**. IR 2930, 2855, 1465, 1385, 1150, 1110, 1045, 920 cm^{-1}; ^1H NMR δ 4.62 (*s,* 2H, OCH_2O), 4.47 (*dm,* J_1 = 49.5 Hz, 1H, CHF), 3.52 (*t, J* = 6.5 Hz, 2H, CH_2O), 3.36 (*s,* 3H, OCH_3), 1.72–1.22 (22H, CH_2), 0.93 (*t, J* = 7 Hz, 3H, CH_3); ^{13}C NMR δ 96.4 (OCH_2O), 94.3 (*d, J* = 166.5 Hz, CHF), 67.8 (CH_2O), 55.1 (OCH_3), 35.1 (*d, J* = 20.5 Hz, CH_2CHF), 34.8 (*d, J* = 21 Hz, CH_2CHF), 29.7 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 29.4 (CH_2), 26.2 (CH_2), 25.1 (*d, J* = 4.5 Hz, CH_2), 18.4 (*d, J* = 4.5 Hz, CH_2–CH_3), 14.0 (CH_3);^{19}F NMR δ −180.8; MS *m/z* 275 ($M^{\cdot+} - 1$, 1), 256 (1), 224 (5), 211 (8), 165 (15), 151 (10), 137 (22), 123 (40), 111 (60), 109 (65), 95 (90), 82 (100), 69 (85), 55 (70); anal. calcd. for $C_{16}H_{33}FO_2$: C, 69.52; H, 12.03; F, 6.87. Found: C, 69.60; H, 11.86; F, 6.69.

Synthesis of gem*-difluoro derivatives **11a–d** from dithianes **7a–d**. General procedure.* These reactions were performed according to the procedure developed by Sondej and Katzenellenbogen (20). 1,3-Dibromo-5,5-dimethylhydantoin was dissolved in 12 mL of dry CH_2Cl_2 and stirred under nitrogen. The mixture was cooled at −90°C, and 0.5 mL of pyridinium poly(hydrogen fluoride) was added *via* a plastic syringe, followed by the dropwise addition of the corresponding dithiane (1 mmol). The reaction mixture was stirred overnight at −78°C, then poured over 10 mL of hexane, quenched with 10 mL of a saturated $NaHCO_3$ solution, and extracted with hexane (2 × 10 mL). The organic fractions were combined, dried, and concentrated. The residue was purified by flash chromatography on silica gel using a hexane/MTBE gradient (0–4%) to give the corresponding *gem*-difluoro derivatives in 55–65% yields.

*(i) 15,17-Dioxa-4,4-difluorooctadecane (**11a**).* This product was isolated (160 mg, 55% yield) starting from 362 mg (1 mmol) of dithiane **7a**. IR 2930, 2855, 1470, 1385, 1150, 1110, 1045, 920 cm^{-1}; ^1H NMR δ 4.62 (*s,* 2H, OCH_2O), 3.52 (*t, J* = 6.5 Hz, 2H, CH_2O), 3.36 (*s,* 3H, OCH_3), 1.92–1.67 (4H, CH_2CF_2), 1.66–1.39 (6H, $CH_2CH_2CF_2$ and CH_2CH_2O), 1.40–1.20 (12H), 0.96 (*t, J* = 7 Hz, 3H, CH_3); ^{13}C NMR δ 125.4 (*t, J* = 240 Hz, CF_2), 96.4 (OCH_2O), 67.8 (CH_2O), 55.1 (OCH_3), 38.3 (*t, J* = 25.5 Hz, CH_2CF_2), 36.3 (*t, J* = 25.5 Hz, CH_2CF_2), 29.7 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 26.2 (CH_2), 22.3 (*t, J* = 4.5 Hz, $CH_2CH_2CF_2$), 15.8 (*t, J* = 5 Hz, CH_2CH_3), 14.0 (CH_3); ^{19}F NMR δ −98.12 (*quint, J* = 16.5 Hz); MS *m/z* 244 ($M^{\cdot+} - F - OCH_3$, 2), 230 (4), 209 (4), 202 (3), 163 (15), 149 (20), 135 (25), 123 (40), 109 (44), 95 (50), 82 (100), 75 (80), 69 (75), 55 (96); anal. calcd. for $C_{16}H_{32}F_2O_2$: C, 65.27; H, 10.95; F, 12.91. Found: C, 65.19; H, 10.84; F, 12.71.

*Methoxymethane deprotection to alcohols **12a–d** and **13a–d**. General procedure.* Products were deprotected to the corresponding alcohols by treatment with a MeOH/HCl solution (0.5 M) for 24 h at room temperature. Solvent was evaporated, and the crude product was treated with 2 mL of water, extracted with CH_2Cl_2, dried, and concentrated to a residue, which was purified by flash cromatography on silica gel using a hexane/AcOEt gradient (0–10%) to give the corresponding pure alcohol derivatives in 85–95% yields.

*(i) 11-Fluoro-1-tetradecanol (**12a**).* This alcohol was isolated (40 mg, 87% yield) from 55 mg (0.2 mmol) of the protected alcohol. IR 3305 (OH), 2920, 2850, 1470, 1065 cm^{-1}; ^1H NMR δ 4.47 (*dm, J_1* = 49.5 Hz, 1H, CHF), 3.64 (*t, J* = 6.5 Hz, 2H, CH_2OH), 1.72–1.22 (22H, CH_2), 0.93 (*t, J* = 7 Hz, 3H, CH_3); ^{13}C NMR δ 94.3 (*d, J* = 166.0 Hz, CHF), 63.1 (CH_2OH), 37.1 (*d, J* = 21.0 Hz, CH_2CHF), 35.2 (*d, J* = 21 Hz, CH_2CHF), 32.8 (CH_2), 29.5 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 25.7 (CH_2), 25.1 (*d, J* = 4.5 Hz, CH_2CH_2CHF), 18.4 (*d, J* = 4.5 Hz, CH_2CH_3), 14.0 (CH_3); ^{19}F NMR δ −180.8; MS *m/z* 194 ($M^{\cdot+} - F - H_2O - 1$, 2), 166 (5), 151 (5), 139 (15), 123 (20), 109 (35), 96 (65), 82 (100), 81 (70), 68 (60), 55 (70).

*(ii) 11,11-Difluoro-1-tetradecanol (**13a**).* This alcohol was isolated (42 mg, 86% yield) from 59 mg (0.2 mmol) of the protected alcohol. IR 3330 (OH), 2930, 2850, 1460, 1210, 1180 cm^{-1}; ^1H NMR δ 3.63 (*t, J* = 6.5 Hz, 2H, CH_2OH), 1.90–1.67 (4H, CH_2CF_2), 1.66–1.39 (6H, $CH_2CH_2CF_2$ and CH_2CH_2O), 1.40–1.20 (12H), 0.95 (*t, J* = 7 Hz, 3H, CH_3); ^{13}C NMR δ 125.4 (*t, J* = 240 Hz, CF_2), 63.0 (CH_2OH), 38.3 (*t, J* = 25.5 Hz, CH_2CF_2), 36.3 (*t, J* = 25.5 Hz, CH_2CF_2), 29.5 (CH_2), 29.4 (CH_2), 25.7 (CH_2), 22.3 (*t, J* = 4.5 Hz, $CH_2CH_2CF_2$), 15.8 (*t, J* = 5 Hz, CH_2CH_3), 13.9 (CH_3); ^{19}F NMR δ −98.11 (*quint, J* = 16.5 Hz); MS *m/z* 167 ($M^{\cdot+} - 2F - CH_2CH_2OH$, 3), 149 (10), 124 (12), 114 (20), 96 (25), 82 (65), 81 (35), 69 (50), 55 (100).

*Preparation of carboxylic acids **1a–d** and **2a–d**.* Alcohols were dissolved in a solution of 4 mL of acetone and 350 μL of H_2SO_4 at −5°C and then 350 mg of CrO_3 dissolved in 700

μL of water was added dropwise. The reaction mixture was stirred at −10°C for 1 h and then allowed to warm to room temperature and stirred for 6 h. The reaction mixture was concentrated and 2 mL of HCl (1 M) added, extracted with CH_2Cl_2, dried, and concentrated to a residue that was purified by flash chromatography on silica gel using hexane/MTBE 85:15 to give the corresponding acids in 60–68% yields.

*(i) 11-Fluorotetradecanoic acid (**1a**).* This acid was isolated (17 mg, 68% yield) from 23 mg (0.1 mmol) of the starting alcohol **12a**. IR 2930, 2850, 1700 (CO), 1465, 1295, 935 cm^{-1}; ^1H NMR δ 4.47 (*dm,* J_1 = 49.5 Hz, 1H, CHF), 2.35 (*t, J* = 7.5 Hz, 2H, CH_2CO), 1.76–1.22 (22H, CH_2), 0.93 (*t, J* = 7 Hz, 3H, CH_3); ^{13}C NMR δ 178.4 (CO), 94.3 (*d, J* = 166.0 Hz, CHF), 37.2 (*d, J* = 20.5 Hz, CH_2CHF), 35.1 (*d, J* = 21 Hz, CH_2CHF), 33.9 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 29.0 (CH_2), 25.1 (*d, J* = 4.5 Hz, CH_2CH_2CHF), 24.6 (CH_2), 18.4 (*d, J* = 4.5 Hz, CH_2CH_3), 14.0 (CH_3); ^{19}F NMR δ −180.8; MS *m/z* (–OMe ester) 238 ($M^{\cdot+}$ – F – CH_3O – 2, 1), 206 (3), 189 (2), 164 (5), 150 (5), 123 (10), 109 (10), 95 (15), 87 (20), 82 (65), 74 (100), 69 (20), 55 (40); anal. calcd. for $C_{14}H_{27}FO_2$: C, 68.25; H, 11.05; F, 7.71. Found: C, 68.18; H, 11.01; F, 7.69.

*(ii) 10-Fluorotetradecanoic acid (**1b**).* This acid was isolated (15 mg, 60% yield) from 23 mg (0.1 mmol) of the starting alcohol **12b**. IR 2930, 2850, 1700 (CO), 1465, 1295, 935 cm^{-1}; ^1H NMR δ 4.46 (*dm,* J_1 = 49.0 Hz, 1H, CHF), 2.35 (*t, J* = 7.5 Hz, 2H, CH_2CO), 1.72–1.52 (4H, CH_2CHF), 1.52–1.20 (18H, CH_2), 0.91 (*t, J* = 7 Hz, 3H, CH_3); ^{13}C NMR δ 179.8 (CO), 94.6 (*d, J* = 166.5 Hz, CHF), 35.1 (*d, J* = 21 Hz, CH_2CHF), 34.8 (*d, J* = 21 Hz, CH_2CHF), 33.9 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.1 (CH_2), 29.0 (CH_2), 27.3 (*d, J* = 4.5 Hz, CH_2CH_2CHF), 25.1 (*d, J* = 4.5 Hz, CH_2CH_2CHF), 24.6 (CH_2), 22.6 (CH_2CH_3), 14.0 (CH_3); ^{19}F NMR δ −180.5; MS *m/z* (–OMe ester) 240 ($M^{\cdot+}$ – F – CH_3O, 1), 208 (5), 190 (2), 166 (15), 124 (10), 111 (15), 97 (30), 87 (35), 83 (35), 74 (100), 69 (50), 55 (75); anal. calcd. for $C_{14}H_{27}FO_2$: C, 68.25; H, 11.05; F, 7.71. Found: C, 68.28; H, 11.04; F, 7.60.

*(iii) 9-Fluorotetradecanoic acid (**1c**).* This acid was isolated (15 mg, 60% yield) from 24 mg (0.1 mmol) of the starting alcohol **12c**. IR 2935, 2855, 1700 (CO), 1470, 1295, 940 cm^{-1}; ^1H NMR δ 4.46 (*dm,* J_1 = 49.0 Hz, 1H, CHF), 3.64 (*t, J* = 6.5 Hz, 2H, CH_2CO), 1.72–1.22 (22H, CH_2), 0.89 (*t, J* = 7 Hz, 3H, CH_3); ^{13}C NMR δ 180.0 (CO), 94.6 (*d, J* = 166.5 Hz, CHF), 35.1 (*d, J* = 21 Hz, CH_2CHF), 32.7 (CH_2), 31.7 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 25.7 (CH_2), 25.1 (*d, J* = 4.5 Hz, CH_2CH_2CHF), 24.8 (*d, J* = 4.5 Hz, CH_2CH_2CHF), 22.5 (CH_2), 14.0 (CH_3); ^{19}F NMR δ −180.5; MS *m/z* (–OMe ester) 240 ($M^{\cdot+}$ – F – CH_3O, 1), 208 (2), 190 (2), 166 (15), 124 (10), 111 (15), 97 (30), 87 (35), 83 (35), 74 (100), 69 (50), 55 (40); anal. calcd. for $C_{14}H_{27}FO_2$: C, 68.25; H, 11.05; F, 7.71. Found: C, 68.24; H, 11.00; F, 7.63.

*(iv) 8-Fluorotetradecanoic acid (**1d**).* This acid was isolated (16 mg, 65% yield) from 24 mg (0.1 mmol) of the starting alcohol **12d**. IR 2930, 2850, 1700 (CO), 1465, 1295, 935 cm^{-1}; ^1H NMR δ 4.46 (*dm,* J_1 = 49.0 Hz, 1H, CHF), 3.64 (*t, J* = 6.5 Hz, 2H, CH_2CO), 1.74–1.20 (22H, CH_2), 0.89 (*t, J* = 7 Hz, 3H, CH_3); ^{13}C NMR δ 180.1 (CO), 94.6 (*d, J* = 166.5 Hz, CHF), 35.2 (*d, J* = 21 Hz, CH_2CHF), 35.1 (*d, J* = 21 Hz, CH_2CHF), 34.0 (CH_2), 31.7 (CH_2), 29.2 (CH_2), 29.1 (CH_2), 28.9 (CH_2), 25.1 (*d, J* = 4.5 Hz, CH_2CH_2CHF), 25.0 (*d, J* = 4.5 Hz, CH_2CH_2CHF), 22.6 (CH_2CH_3), 14.1 (CH_3); ^{19}F NMR δ −180.5; MS *m/z* (–OMe ester) 238 ($M^{\cdot+}$ – F – CH_3O – 2, 1), 206 (3), 189 (2), 164 (5), 150 (5), 123 (10), 109 (10), 95 (15), 87 (20), 82 (65), 74 (100), 55 (40); anal. calcd. for $C_{14}H_{27}FO_2$: C, 68.25; H, 11.05; F, 7.71. Found: C, 68.13; H, 11.02; F, 7.55.

*(v) 11,11-Difluorotetradecanoic acid (**2a**).* This acid was isolated (10 mg, 63% yield) from 15 mg (0.06 mmol) of the starting alcohol **13a**. IR 2930, 2850, 1700 (CO), 1465, 1295, 935 cm^{-1}; ^1H NMR δ 3.63 (*t, J* = 6.5 Hz, 2H, CH_2CO), 1.90–1.67 (4H, CH_2CF_2), 1.66–1.39 (6H, $CH_2CH_2CF_2$ and CH_2CH_2CO), 1.40–1.20 (12H, CH_2), 0.95 (*t, J* = 7 Hz, 3H, CH_3); ^{13}C NMR δ 179.8 (CO), 125.4 (*t, J* = 240 Hz, CF_2), 38.3 (*t, J* = 25.5 Hz, CH_2CF_2), 36.3 (*t, J* = 25.5 Hz, CH_2CF_2), 34.0 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 29.2 (CH_2), 29.0 (CH_2), 24.6 (CH_2), 22.3 (*t, J* = 4.5 Hz, $CH_2CH_2CF_2$), 15.8 (*t, J* = 5 Hz, CH_2CH_3), 14.0 (CH_3); ^{19}F NMR δ −98.11 (*quint, J* = 16.5 Hz); MS *m/z* (–OMe ester) 208 ($M^{\cdot+}$ – 2F – CH_3 – 2, 5), 190 (2), 166 (15), 137 (10), 124 (20), 111 (20), 97 (30), 87 (35), 82 (35), 74 (100), 69 (50), 55 (70); anal. calcd. for $C_{14}H_{26}F_2O_2$: C, 63.61; H, 9.91; F, 14.37. Found: C, 63.62; H, 9.79; F, 14.17.

*(vi) 10,10-Difluorotetradecanoic acid (**2b**).* This acid was isolated (11 mg, 60% yield) from 18 mg (0.07 mmol) of the starting alcohol **13b**. IR 2930, 2850, 1700 (CO), 1465, 1295, 935 cm^{-1}; ^1H NMR δ 3.63 (*t, J* = 6.5 Hz, 2H, CH_2CO), 1.90–1.68 (4H, CH_2CF_2), 1.66–1.51 (2H, CH_2CH_2CO), 1.50–1.20 (16H, CH_2), 0.92 (*t, J* = 7 Hz, 3H, CH_3); ^{13}C NMR δ 180.0 (CO), 125.5 (*t, J* = 240 Hz, CF_2), 36.2 (*t, J* = 25.5 Hz, CH_2CF_2), 36.0 (*t, J* = 25.5 Hz, CH_2CF_2), 34.0 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 29.1 (CH_2), 29.0 (CH_2), 24.6 (CH_2), 24.4 (*t, J* = 4.5 Hz, $CH_2CH_2CF_2$), 22.5 (CH_2), 22.3 (*t, J* = 4.5 Hz, $CH_2CH_2CF_2$), 13.8 (CH_3); ^{19}F NMR δ −98.09 (*quint, J* = 16.5 Hz); MS *m/z* (–OMe ester) 238 ($M^{\cdot+}$ – 2F – 2, 2), 207 (1), 189 (2), 164 (10), 149 (10), 136 (10), 124 (10), 109 (15), 96 (75), 81 (30), 74 (100), 69 (20), 55 (50); anal. calcd. for $C_{14}H_{26}F_2O_2$: C, 63.61; H, 9.91; F, 14.37. Found: C, 63.52; H, 9.78; F, 14.18.

*(vii) 9,9-Difluorotetradecanoic acid (**2c**).* This acid was isolated (18 mg, 68% yield) from 25 mg (0.1 mmol) of the starting alcohol **13c**. IR 2930, 2850, 1700 (CO), 1465, 1295, 935 cm^{-1}; ^1H NMR δ 3.64 (*t, J* = 6.5 Hz, 2H, CH_2CO), 1.90–1.68 (4H, CH_2CF_2), 1.66–1.51 (4H, CH_2CH_2CO and $CH_2CH_2CF_2$), 1.52–1.40 (2H, $CH_2CH_2CF_2$), 1.50–1.20 (12H, CH_2), 0.90 (*t, J* = 7 Hz, 3H, CH_3); ^{13}C NMR δ 180.0 (CO), 125.4 (*t, J* = 240 Hz, CF_2), 36.3 (*t, J* = 25.5 Hz, CH_2CF_2), 36.2 (*t, J* = 25.5 Hz, CH_2CF_2), 34.0 (CH_2), 31.5 (CH_2), 29.2 (CH_2), 29.0 (CH_2), 28.8 (CH_2), 24.6 (CH_2), 22.4 (CH_2), 22.2 (*t, J* = 4.5 Hz, $CH_2CH_2CF_2$), 22.0 (*t, J* = 4.5 Hz, $CH_2CH_2CF_2$), 13.9 (CH_3); ^{19}F NMR δ −98.17 (*quint, J* = 16.5 Hz); MS *m/z* (–OMe ester) 238 ($M^{\cdot+}$ – 2F – 2, 2), 207 (5), 196

(5), 164 (15), 150 (10), 135 (10), 110 (45), 95 (25), 87 (20), 81 (30), 74 (100), 69 (25), 55 (45); anal. calcd. for $C_{14}H_{26}F_2O_2$: C, 63.61; H, 9.91; F, 14.37. Found: C, 63.59; H, 9.83; F, 14.18.

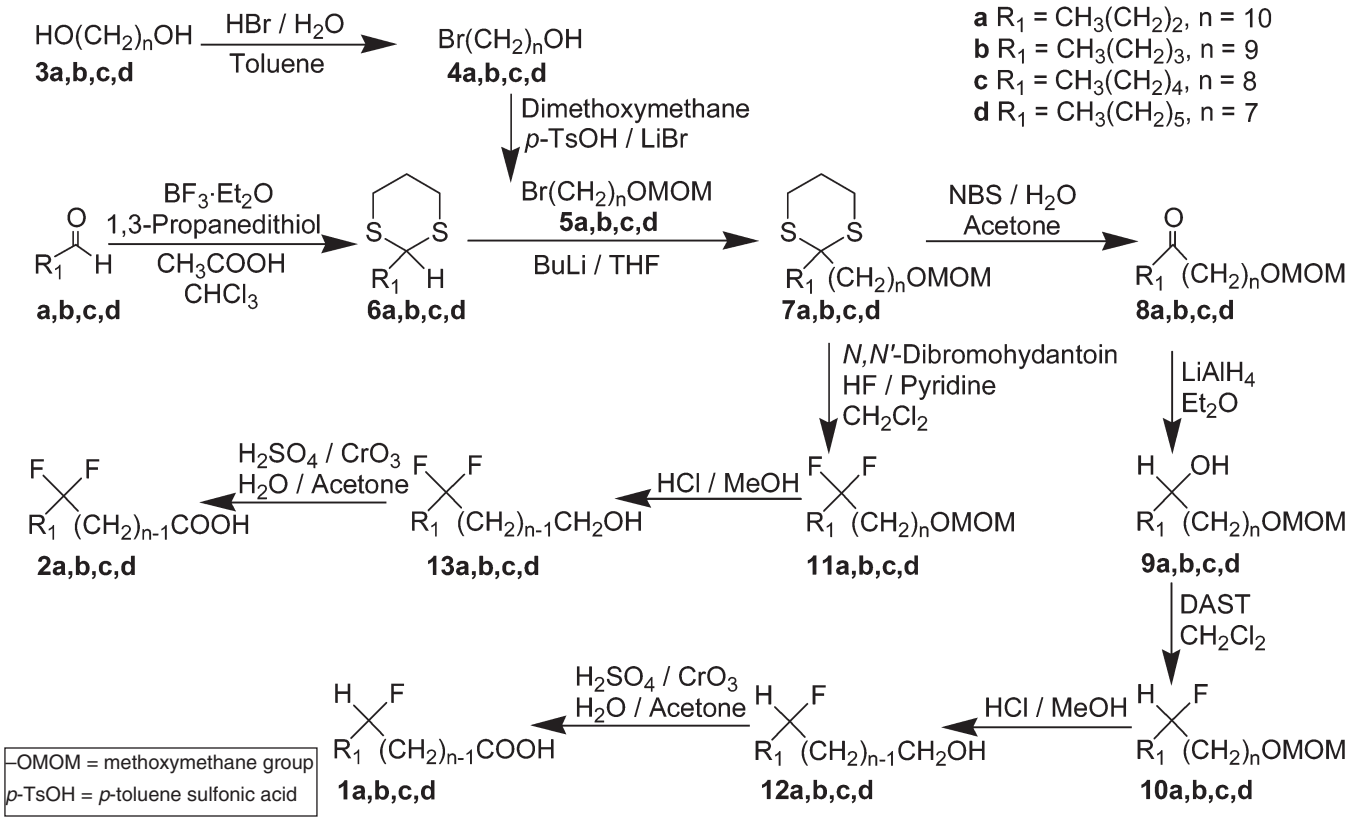
*(viii) 8,8-Difluorotetradecanoic acid (**2d**).* This acid was isolated (10 mg, 63% yield) from 15 mg (0.06 mmol) of the starting alcohol **13d**. IR 2930, 2850, 1700 (CO), 1465, 1295, 935 cm^{-1}; ^1H NMR δ 3.64 (*t, J* = 6.5 Hz, 2H, CH_2CO), 1.90–1.68 (4H, CH_2CF_2), 1.68–1.51 (2H, CH_2CH_2CO), 1.50–1.20 (16H, CH_2), 0.89 (*t, J* = 6.5 Hz, 3H, CH_3); ^{13}C NMR δ 180.0 (CO), 125.4 (*t, J* = 240 Hz, CF_2), 36.3 (*t, J* = 25.5 Hz, CH_2CF_2), 36.2 (*t, J* = 25.5 Hz, CH_2CF_2), 33.9 (CH_2), 31.6 (CH_2), 29.0 (CH_2), 29.0 (CH_2), 28.8 (CH_2), 24.4 (CH_2), 22.5 (CH_2), 22.2 (*t, J* = 4.5 Hz, $CH_2CH_2CF_2$), 22.1 (*t, J* = 4.5 Hz, $CH_2CH_2CF_2$), 14.0 (CH_3); ^{19}F NMR δ –98.13 (*quint, J* = 16.5 Hz); MS *m/z* (–OMe ester) 238 ($M^{\cdot+}$ – 2F – 2, 2), 226 (5), 207 (5), 182 (10), 164 (15), 150 (10), 136 (10), 128 (15), 109 (15), 95 (25), 87 (15), 81 (25), 74 (100), 69 (25), 55 (35); anal. calcd. for $C_{14}H_{26}F_2O_2$: C, 63.61; H, 9.91; F, 14.37. Found: C, 63.57; H, 9.75; F, 14.21.

RESULTS AND DISCUSSION

Preparation and characterization of mono- and gem*-difluoro myristic acids.* The synthesis of myristic acids selectively substituted as mono- and *gem*-difluoro compounds at positions C8, C9, C10, and C11 was carried out as described in Scheme 1.

In this chemical pathway, properly functionalized dithianes **6** were used as myristic acid chain precursors. Thus, coupling of dithianes **6** with methoxymethane-protected bromo-derivatives **5** in presence of *n*-butyllithium afforded the corresponding dithianes **7** with the complete aliphatic chain. Recently, we used this kind of versatile intermediate for the preparation of mono- and di-deuterated tridecanoic acids (18). In the same way, reaction of dithianes **7** with *N*-bromo-succinimide in the presence of water afforded ketones **8**, which gave rise to the corresponding alcohols **9** upon reduction with $LiAlH_4$; these eventually could be resolved to each one of the corresponding enantiomers (18). Because our main goal was to synthesize the different monofluoroderivatives as racemic mixtures, no procedures for their stereoselective formation were contemplated at this stage. Thus, introduction of a fluorine atom was achieved by displacement of the alcohol funtionality of **9** with DAST (21) using CH_2Cl_2 as solvent. Although the yields of fluorination were acceptable, olefinic side products were also detected, as reported elsewhere (21,22). In the worst case, purification was achieved by flash chromatography on silica gel.

On the other hand, ketones are reported to be good substrates for the preparation of the *gem*-difluoro derivatives. But it was not possible to obtain the corresponding *gem*-difluoro derivatives when ketone **8** was treated with DAST or the mixture DAST/HF/pyridine using CH_2Cl_2 as solvent. Furthermore, we detected no difluorinated product when the reaction was carried out with CF_2Br_2 in presence of zinc (23). However, *gem*-difluoro compounds were prepared straightfor-



SCHEME 1

wardly by reaction of the previously obtained dithianes **7** with 1,3-dibromo-5,5-dimethylhydantoin in the presence of HF/pyridine according to the procedure reported by Sondej and Katzenellenbogen (20). Methoxymethane deprotection of mono- and *gem*-difluoro derivatives **10** and **11** to afford the alcohol intermediates **12** and **13**, respectively, was accomplished by acid treatment with HCl/MeOH (0.5 M). Final Jones oxidation gave rise to the corresponding fluorinated FA **1** and **2** with good yields. Characterization of the mono- and difluorinated products was carried out by ^{1}H, ^{13}C, and ^{19}F NMR.

Biochemical experiments. None of the compounds synthesized was converted by the Δ^{11} desaturase of the *S. littoralis* sex pheromone gland, as concluded from the careful examination of the GC–MS chromatograms corresponding to the methanolyzed lipidic extracts of the treated glands, which were identical to those of control tissues, to which no fluoro-FA was administered. These results are in agreement with previously reported data using thia-FA (24). In that case, among 8-, 9-, 10-, 11-, 12-, and 13-thiatetradecanoic acids, only the 13-thiaderivative was transformed into both (*Z*)- and (*E*)-11-thiatetradecenoic acids, and (*E*)-11-thiatetradecenoic acid was further converted into (*Z*,*E*)-13-thia-9,11-tetradecadienoic acid. These overall results suggest that the substrate binding to moth Δ^{11} desaturases is very sensitive to heteroatom substitution, at least at positions C8, C9, C10, C11, and C12, since replacement of methylene by the CH_2 bioisosteric sulfur or that of hydrogen by fluorine produces FA analogs that are not desaturated at C11. In contrast, transformation of both thia-FA and fluoro-FA by the yeast Δ^{9} desaturase has been reported by Behrouzian and Buist (and references cited therein) (25).

As summarized in Figure 1, only the 11-monofluoroderivative **1a** produced a moderate inhibition (50% at 1:1 substrate/inhibitor ratio). Since the assays were performed with racemic **1a**, it is reasonable to expect a higher inhibitory potency of the pure active enantiomer. This aspect is now under investigation in our laboratory.

The present work represents another example of the potential use of fluorinated compounds in biochemical studies of enzyme inhibition. The results obtained here confirm that there is no general rule that makes possible an easy prediction and that there is a dependence on the type and source of the enzyme studied.

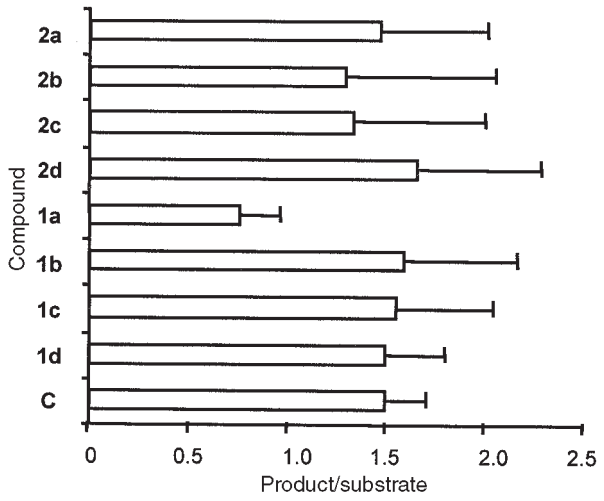


FIG. 1. Effect of the fluorotetradecanoic acids **1a–d** and **2a–d** (for chemical structures see Scheme 1) on the Δ^{11} desaturase of *Spodoptera littoralis*. Inhibition was determined on pheromone glands *in vitro* as described in the Experimental Procedures section. The FAME were analyzed by GC–MS and the ratios between the ions 243 (methyl d_3(*E*)-11-tetradecenoate, product) and 245 (methyl tetradecanoate, substrate) were calculated from their corresponding areas. Since the $M^{\cdot+}$of methyl tetradecanoate is more abundant than that of methyl tetradecenoate, the values in Figure 1 do not represent actual mass ratios between both compounds. Data correspond to the mean ± SD of five replicates. C, control.

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Synthesis and Characterization of Deoxycholyl 2-Deoxyglucuronide: A Water-Soluble Affinity Labeling Reagent

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ABSTRACT: Acyl glucuronides, which are biosynthesized by the action of glucuronosyltransferases to material for detoxification, are water-soluble and chemically active; they produce irreversible protein adducts *via* both the transacylation mechanism and the imine mechanism. The acyl group at the C-1 position migrates from the anomeric carbon to the C-2 position of the glucuronic acid moiety, producing the aldehyde group at the C-1 position, where the protein easily condenses through a Schiff's base, in the open-chain aldose form. The elimination of the hydroxyl group at the C-2 position therefore may prevent a protein-bound adduct *via* the imine mechanism. In this paper, we describe the synthesis and characterization of an acyl 2-deoxyglucuronide of deoxycholic acid as a model compound to investigate its possible utility as a water-soluble affinity labeling reagent for lipophilic carboxylic acids. The solubility of deoxycholyl 2-deoxyglucuronide in an aqueous solution was sufficient under physiological conditions, and the desired material reacted with model peptides to produce covalently bound adducts only *via* the transacylation mechanism.

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Glucuronidation is one of the major metabolic pathways for drugs and other xenobiotics (1). The principal purpose of this metabolism is the transformation of lipophilic biological materials into strongly dissociated compounds that are more water soluble; it also serves as an important detoxification mechanism. It takes place through not only the hydroxyl and amino groups but also the carboxyl group of the lipophilic materials (2), and the acyl glucuronides formed through the carboxyl group are rapidly excreted into blood and urine (3). The acyl glucuronides are chemically very active and react with free amino groups of proteins to produce irreversible protein adducts (4–7), which may be related to liver injuries caused by hypersensitive reactions (8,9). The mechanism of protein adduct formation involves the displacement of the glucuronic acid moiety with nucleophiles, such as the amino groups of the protein molecules. Alternatively, the acyl group migrates away from the anomeric carbon at C-1, promoting the possibility of the aldehyde group at the C-1 position in the open-chain aldose form, which undergoes a condensation reaction (4,5). The aglycone is therefore bound at the migrated center by an ester bond (10) and is linked with the protein molecule through the glucuronic acid moiety.

Bile acids are the major metabolites of cholesterol and undergo glucuronidation and sulfation. The conjugation of bile acids with glucuronic acid involves not only the hydroxyl group at the C-3 position on the steroid nucleus but also the carboxyl group at the C-24 position of the bile acid (11–14). We reported the formation of bile acid 24-glucuronides in an incubation mixture of rat liver microsomes (15) and the occurrence of the acyl glucuronides in human urine (16). In addition, we reported the formation of a protein-bound bile acid by the incubation of lithocholic acid 24-glucuronide with peptide and protein (17). The analytical results obtained by matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOFMS) suggested not only that the acyl group was linked directly to protein by nucleophilic substitution reaction, in which the sugar moiety functioned as a leaving group, but also that the open-chain aldose form at the C-1 position of the sugar moiety produced by the acyl migration reacted to produce a Schiff's base with a free amino group of the protein. Because the acyl group migrates away from the anomeric carbon to the neighboring C-2 position, the elimination of the hydroxyl group at the C-2 position may admit no acyl migration, resulting in the formation of a transacylation product as the sole protein-bound adduct.

Affinity labeling produces a stable covalent bond between the low-M.W. ligand and the interactive site of protein, making it possible to probe the structure around the ligand binding site by using MS (18–20). Two common reactions, one photochemical and the other a chemically active reaction, have been used for this purpose. Haloketones (19,21), epoxides (18), and active esters (22,23) are activated carboxylic acid derivatives that enable the formation of a covalent bond with nucleophiles such as the amino groups on proteins. Among these, an active ester-type reagent is preferred for labeling a target ligand because the reaction can be controlled easily by adjusting the pH. However, the active esters used to date, which include *p*-nitrophenyl ester and *N*-hydroxy-

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Abbreviations: CHCA, α-cyano-4-hydroxycinnamic acid; DCA, 3α,12α-dihydroxy-5β-cholan-24-oic acid, deoxycholic acid; DCA-24dG, 1-*O*-(24-deoxycholyl)-2-deoxy-β-D-glucopyranuronic acid, deoxycholyl 2-deoxyglucuronide; HR-MS, high-resolution MS; MALDI-TOFMS, matrix-assisted laser desorption ionization time-of-flight MS; TFA, trifluoroacetic acid.

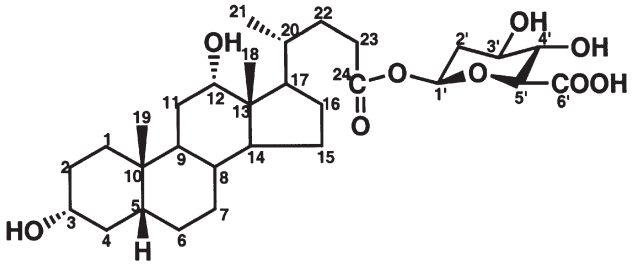

FIG. 1. Structure of 1-*O*-(24-deoxycholyl)-2-deoxy-β-D-glucopyranuronic acid, deoxycholyl 2-deoxyglucuronide (DCA-24dG).

succinimidyl ester, are slightly soluble in water under physiologic conditions. The addition of organic solvent to dissolve the reagents often causes conformational changes in the target protein.

The present paper describes the synthesis and characterization of acyl 2-deoxyglucuronide (Fig. 1). It was synthesized by selective coupling of the carboxyl group on 3α,12α-dihydroxy-5β-cholan-24-oic acid (deoxycholic acid, DCA) as a model carboxylic acid with a D-glucuronic acid benzyl ester-benzyl ether derivative (24), employing the Mitsunobu reaction (25). The properties of acyl 2-deoxyglucuronide were also investigated, and its potential use as an affinity labeling reagent was verified.

EXPERIMENTAL PROCEDURES

Materials and methods. Substance P, α-neo-endorphin and the 6-17 fragment of dynorphin A were purchased from American Peptide (Sunnyvale, CA). 2-Deoxy-D-glucose was obtained from Sigma Chemical (St. Louis, MO), and α-cyano-4-hydroxycinnamic acid (CHCA), as a matrix for MALDI-TOFMS and palladium hydroxide, 20 wt% Pd on carbon (Pearlman's catalyst) were supplied by Aldrich Chemical (Milwaukee, WI). A ZipTip C_{18} cartridge was purchased from Millipore (Milford, MA). *p*-Nitrophenyl deoxycholate and *N*-hydroxysuccinimidyl deoxycholate were synthesized in our laboratory. ^{1}H NMR analysis (300 MHz) was performed using a Hitachi FT-NMR R-3000 (Hitachi, Tokyo, Japan). Chemical shifts are given as the δ value with tetramethylsilane as an internal standard (*s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *dd*, double of doublets; *m*, multiplet). MALDI-TOFMS analysis was performed using Voyager RP (Applied Biosystems, Framingham, MA) and AXIMA-CFR (Shimadzu, Kyoto, Japan) equipped with an N_2 laser (337 nm) under the linear mode. Electrospray ionization mass analysis was performed using an LCmate double focusing magnetic mass spectrometer (JEOL, Tokyo). An LC-10Ai system (Shimadzu) equipped with an SCL-10A_{VP} system controller, LC-10Ai pumps, and a detector (SPD-M10A_{VP}) were controlled with CLASS-VP software. Preparative HPLC for the separation of the α- and β-anomers of a 2-deoxyglucuronic acid benzyl derivative was performed using an L-6000 pump, L-4200 UV-vis detector (Hitachi) equipped with μBondasphere 15 μm C18-100 Å (7.8 mm i.d. × 300 mm; Waters, Milford).

*Synthesis of 1-*O*-(24-Deoxycholyl)-2-deoxy-D-glucopyranuronic acid (DCA-24dG). (i) 1-*O*-Methyl-6-*O*-trityl-2-deoxy-D-glucopyranose (**2**)*. 2-Deoxy-D-glucose (**1**, 5.0 g) in 13 mL of methanol was added to 1.5 mL acetyl chloride/methanol (1:5, vol/vol), and stirred for 2 h at room temperature. The reaction was terminated by the addition of silver carbonate. After concentration *in vacuo*, the residue was dissolved in 100 mL pyridine and added to 8.0 g trityl chloride, and the whole mixture was stirred overnight. Extraction and purification of the product were carried out as described previously (26). A white powder was obtained (**2**, 9.3 g, 79% yield). ^{1}H NMR ($CDCl_3$) δ: 1.62–1.71 (1H, *m*, 2′-H_{ax}), 2.08–2.19 (1H, *m*, 2′-H_{eq}), 3.31 (2.4H, *s*, –OCH_3 α-anomer), 3.36–3.62 (4.6H, *m*, –OCH_3, β-anomer, and 4′-, 5′-, 6′-H), 3.88–3.96 (1H, *m*, 3′-H), 4.43 (0.2H, *dd*, *J* = 2.9 Hz and 10.1 Hz, 1′-H, α-anomer), 4.78 (0.8H, *d*, *J* = 3.3Hz, 1′-H, β-anomer), 7.23–7.48 (15H, *m*, –$OC(C_6H_5)_3$). Analysis calculated for $C_{26}H_{28}O_5$: C, 74.26; H, 6.71. Found: C, 74.09; H, 6.68.

*(ii) 3,4-Di-*O*-benzyl-1-*O*-methyl-6-*O*-trityl-2-deoxy-D-glucopyranose (**3**)*. In 21 mL THF, 9.3 g of **2** was mixed with 181 mg tetra-*n*-butyl ammonium iodide, 8.7 mL benzyl bromide, and 1.7 g 60% sodium hydride. The mixture was stirred at room temperature overnight. After extraction and purification (26), a colorless oil was obtained (**3**, 12.0 g, 90.2% yield). ^{1}H NMR ($CDCl_3$) δ: 1.72–1.81 (1H, *m*, 2′-H_{ax}), 2.29–2.35 (1H, *m*, 2′-H_{eq}), 3.38 (3H, *s*), 3.21–3.63 (7H, *m*, –OCH_3, α-anomer, 4′-, 5′-, 6′-H), 3.90–3.99 (1H, *m*, 3′-H), 4.31–4.76 (4H, *m*, –$OC\underline{H}_2C_6H_5 \times 2$), 4.92 (1H, *d*, *J* = 2.5 Hz, 1′-H, α-anomer), 6.89–7.52 (25H, *m*, –$OC(C_6H_5)_3$ and –$OCH_2C_6\underline{H}_5 \times 2$). Analysis calculated for $C_{40}H_{40}O_5 \cdot \frac{1}{4}H_2O$: C, 79.38; H, 6.75. Found: C, 79.47; H, 6.68.

*(iii) 3,4-Di-*O*-benzyl-1-*O*-methyl-2-deoxy-D-glucopyranose (**4**)*. Sulfuric acid/ethanol (8.0 mL 1:3, vol/vol) was added to an ethanolic solution of **3** (7.8 g). The mixture was stirred at –10°C for 30 min. After extraction and purification (26), a colorless oil was obtained (**4**, 3.8 g, 91% yield). ^{1}H NMR ($CDCl_3$) δ: 1.60–1.70 (1H, *m*, 2′-H_{ax}), 2.26–2.32 (1H, *m*, 2′-H_{eq}), 3.30 (2.4H, *s*, –OCH_3, α-anomer), 3.47–3.53 (1.6H, *m*, 4′-H and –OCH_3, β-anomer), 3.61–3.79 (3H, *m*, 5′-H and 6′-H), 3.95–4.03 (1H, *m*, 3′-H), 4.40 (0.2H, *dd*, *J* = 2.4 Hz and 10.4 Hz, 1′-H, β-anomer), 4.60–4.97 (4.8H, *m*, 1′-H, α-anomer and –$OC\underline{H}_2C_6H_5 \times 2$), 7.26–7.34 (10H, *m*, –$OCH_2C_6\underline{H}_5 \times 2$). Analysis calculated for $C_{21}H_{26}O_5$: C, 70.37; H, 7.31. Found: C, 69.32; H, 7.40.

*(iv) Benzyl 1-*O*-methyl-3,4-di-*O*-benzyl-2-deoxy-D-glucopyranuronate (**5**)*. Compound **4** (3.8 g) in 15 mL acetone was added to 15 mL anhydrous chromic acid (5.0 g) in 17% sulfuric acid (vol/vol, 15 mL) and stirred at room temperature for 1 h. The residue, obtained by extraction (26) in 5 mL of DMSO, was stirred overnight at room temperature together with 1.0 g of sodium hydrogen carbonate and 3.0 mL of benzyl bromide. After extraction and purification (26), a colorless oil was obtained (**5**, 1.8 g, 39% yield). ^{1}H NMR ($CDCl_3$) δ: 1.72–1.81 (1H, *m*, 2′-H_{ax}), 2.21–2.28 (1H, *m*, 2′-H_{eq}), 3.35 (3H, *s*, –OCH_3, α-anomer), 3.74–3.79 (1H, *t*, *J* = 8.7 Hz, 4′-H), 3.92–3.99 (1H, *m*, 3′-H), 4.26 (1H, *d*, *J* = 8.6 Hz, 5′-H),

4.49–4.81 (4H, *m*, –OC$\underline{H}_2C_6H_5 \times 2$), 4.91 (1H, *t*, *J* = 2.7 Hz, 1′-H, α-anomer), 5.12–5.20 (2H, *m*, –COOC$\underline{H}_2C_6H_5$), 7.16–7.38 (15H, *m*, –OCH$_2$C$_6\underline{H}_5 \times 3$). Analysis calculated for $C_{27}H_{30}O_6$: C, 71.98; H, 6.71. Found: C, 71.59; H, 6.44.

*(v) Benzyl 1-*O*-acetyl-3,4-di-*O*-benzyl-2-deoxy-*D*-glucopyranuronate (**6**).* Compound **5** (1.8 g) in 9.0 mL of acetic acid/acetic anhydrous (21:3, vol/vol) was mixed with 1.8 mL ice-cold sulfuric acid/acetic acid (1:10, vol/vol). The mixture was stirred at room temperature for 15 min, and then it underwent extraction and purification (26) to give **6** (1.0 g, 54% yield) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ: 1.84–1.93 (1H, *m*, 2′-H$_{ax}$), 2.06 (2.7H, *s*, –OCOCH$_3$, α-anomer), 2.14 (0.3H, *s*, –OCOCH$_3$, β-anomer), 2.21–2.27 (1H, *m*, 2′H$_{eq}$), 3.79–3.84 (1H, *dd*, *J* = 8.6 Hz and 8.6 Hz, 4′-H), 3.90–3.98 (1H, *m*, 3′-H), 4.35 (1H, *d*, *J* = 8.8 Hz, 5′-H), 4.47–4.81 (4H, *m*, –OCH$_2$C$_6$H$_5$ × 2), 5.15 (2H, *s*, –COOC$\underline{H}_2C_6H_5$), 5.74–5.78 (0.1H, *m*, 1′-H, β-anomer), 6.32 (0.9H, *dd*, *J* = 2.7 Hz and 2.7 Hz, 1′-H, α-anomer), 7.08–7.45 (15H, *m*, –OCH$_2$C$_6\underline{H}_5$ × 3). Analysis calculated for $C_{28}H_{30}O_7$: C, 70.28; H, 6.32. Found: C, 70.58; H, 6.23.

*(vi) Benzyl 3,4-di-*O*-benzyl-2-deoxy-*D*-glucopyranuronate (**7**).* Compound **6** (1.0 g) in 7.0 mL ethyl acetate/DMSO (10:1, vol/vol) was added to 0.5 mL of 2-aminoethanol. The reaction mixture was stirred at room temperature for 10 h. After extraction and purification (26), a colorless oil was obtained (**7**, 530 mg, 58% yield). ^{1}H NMR ($CDCl_3$) δ: 1.74–1.83 (1H, *m*, 2′-H$_{ax}$), 2.16–2.22 (1H, *m*, 2′-H$_{eq}$), 3.81 (1H, *dd*, *J* = 8.7 Hz and 8.7 Hz, 4′-H), 3.95–4.08 (1H, *m*, 3′-H), 4.52–4.78 (4H, *m*, –OC$\underline{H}_2C_6H_5 \times 2$), 4.53 (1H, *d*, *J* = 7.7 Hz, 5′-H), 5.15 (2H, *s*, –COOC$\underline{H}_2C_6H_5$), 5.49 (1H, *dd*, *J* = 3.2 Hz and 6.3 Hz, 1′-H, β-anomer), 7.19–7.34 (15H, *m*, –OCH$_2$C$_6\underline{H}_5$ × 3). Analysis calculated for $C_{26}H_{28}O_6$: C, 71.54; H, 6.47. Found: C, 71.32; H, 6.13.

*(vii) Benzyl 1-*O*-(24-deoxycholyl)-3,4-di-*O*-benzyl-2-deoxy-β-*D*-glucopyranuronate (**8**).* A solution of DCA (315 mg) and azodicarboxylic acid diethyl ester (580 µL) in anhydrous THF (1 mL) was added dropwise into a mixture of **7** (275 mg) and triphenylphosphine (210 mg) in THF (2 mL). The whole solution was stirred at room temperature overnight under an argon atmosphere. The reaction was terminated by the addition of hydrogen chloride, and the mixture was evaporated *in vacuo* and extracted with ethyl acetate. The organic layer was washed successively with 5% hydrogen chloride and saturated sodium chloride. The residue obtained was subjected to column LC on a silica gel. The fraction containing α- and β-anomers was further purified by preparative HPLC on a µBondasphere 15 µm C18-100 Å column with water/methanol (1:10, vol/vol) as a mobile phase, to give the β-anomer **8** (retention time of 24 min, 120 mg, 24% yield) as a colorless powder. ^{1}H NMR ($CDCl_3$) δ: 0.67 (3H, *s*, 18-H), 0.91 (3H, *s*, 19-H), 0.95 (3H, *d*, *J* = 6.3 Hz, 21-H), 3.58–3.68 (1H, *m*, 3′-H), 3.70–3.77 (1H, *m*, 3β-H), 3.82 (1H, *dd*, *J* = 8.5 Hz and 8.5 Hz, 4′-H), 3.97 (1H, *m*, 12β-H), 4.05 (1H, *d*, *J* = 6.6 Hz, 5′-H), 4.47–4.79 (4H, *m*, –OC$\underline{H}_2C_6H_5 \times 2$), 5.15 (2H, *s*, –COOC$\underline{H}_2C_6H_5$), 5.77 (1H, *dd*, *J* = 2.4 Hz and 9.2Hz, 1′-H, β-anomer), 7.14–7.36 (15H, *m*, –OCH$_2$C$_6\underline{H}_5 \times 3$).

*(viii) 1-*O*-(24-Deoxycholyl)-2-deoxy-β-*D*-glucopyranuronic acid (**9**).* Compound **8** (65 mg) in 1.2 mL of ethyl acetate containing 1% acetic acid was mixed with 240 mg of Pearlman's catalyst. The mixture was stirred at room temperature for 30 min under a hydrogen gas atmosphere (1 atm), evaporated under a nitrogen gas stream, and then redissolved in an ammonium phosphate buffer (pH 6.0). The mixture was purified by column LC on a Cosmosil 140C$_{18}$-OPN column and desalted on a Sep-Pak C$_{18}$ cartridge to give **9** as a colorless powder (31 mg, 71.1%). ^{1}H NMR (CD_3OD) δ: 0.70 (3H, *s*, 18-H), 0.93 (3H, *s*, 19-H), 1.00 (3H, *d*, *J* = 6.2 Hz, 21-H), 3.40–3.79 (4H, *m*, 3′-, 4′-, 5′- and 3β-H), 3.95 (1H, *m*, 12β-H), 5.77 (1H, *dd*, *J* = 2.6 Hz and 8.9 Hz, 1′-H, β-anomer). HR-MS calculated for $C_{30}H_{47}O_9$ [M − H]$^-$ theoretical: 551.3220. Found: 551.3201.

Formation of DCA-bound peptide. DCA-24dG (1 µmol) in 150 µL of 50 mM potassium phosphate buffer (pH 7.4) was incubated at 37°C with 20 nmol of the 6-17 fragment of dynorphin A, substance P, or α-neo-endorphin. For each reaction mixture, 6 µL was withdrawn and added to 3 µL of acetic acid to terminate the reaction. After desalting with a ZipTip C$_{18}$ cartridge, a 5-µL aliquot of the reaction mixture was subjected to HPLC analysis. At the same time, a 1-µL aliquot of the mixture was added to 9 µL of CHCA in acetonitrile/water (1:1) containing 0.1% trifluoroacetic acid (TFA), and a 1-µL aliquot of the mixture was subjected to MALDI-TOFMS analysis.

Amino acid sequences of DCA-bound peptides were analyzed by the following procedure. The reaction mixture was separated by HPLC on a linear gradient using a Jupiter C$_{18}$ column (5 µm, 2.0 mm i.d. × 150 mm; Phenomenex, Torrance, CA). Mobile phases A [water/acetonitrile (9:1, vol/vol) containing 0.1% TFA] and B [water/acetonitrile (1:9, vol/vol) containing 0.1% TFA] were delivered at a flow rate of 0.2 mL/min for 50 min along a gradient of 5–45% solvent B, and the analytes were monitored by UV detection at 215 nm. The fraction containing adducts was concentrated, dissolved in water/acetonitrile (1:1) containing 0.1% TFA, and then mixed with saturated CHCA solution. A 1-µL aliquot of the solution was subjected to MALDI-TOFMS analysis.

Effect of pH on formation of DCA-bound peptide and hydrolysis of DCA-24dG. DCA-24dG (1 µmol) was incubated with the 6-17 fragment of dynorphin A (20 nmol) at 37°C in 200 µL of 50 mM potassium phosphate buffer under various pH conditions (pH 5.0, 6.0, 7.4, 8.0, 8.5, 9.0). Aliquots (10 µL) of the reaction mixture were withdrawn at 2, 4, 8, 16, 24, 48, 72, and 96 h. To these aliquots were added first 6 µL of 50% acetic acid for reaction termination and then 1 µg of luteinizing hormone-releasing hormone as an internal standard. A 5-µL aliquot of the solution was analyzed by HPLC along a linear gradient for measurement of DCA-bound peptides formed by the above condition. Another 3 µL of the reaction mixture was withdrawn at the same time. To this aliquot were added first 3 µL of acetic acid for reaction termination and then 2 µg of chenodeoxycholate as an internal standard. The remaining DCA-24dG with hydrolyzed DCA was quantified by HPLC on a Capcell Pak C$_{18}$ UG120 column (5 µm, 4.6

mm i.d. × 150 mm; Shiseido, Tokyo, Japan) with 20 mM ammonium phosphate buffer (pH 6.0)/acetonitrile (2:1, vol/vol) as the mobile phase, at a flow rate of 1 mL/min. The analytes were monitored by UV detection at 205 nm.

RESULTS AND DISCUSSION

Because the glycosyl linkage on an acyl glucuronide is easily hydrolyzed under alkaline conditions, the protecting groups for hydroxyl and carboxyl groups on a 2-deoxyglucuronic acid moiety must be removed under neutral or weakly acidic conditions at the final step of synthesis. The authors synthesized bile acid 24-glucuronides (26) employing a Mitsunobu reaction (25) using a benzyl derivative of D-glucopyranuronate (24), from which the protecting groups can be easily removed by catalytic hydrogenation. Therefore, a benzyl-protected sugar was initially synthesized from 2-deoxy-D-glucose according to the procedure depicted in Figure 2.

2-Deoxy-D-glucose (**1**) was converted to 1-methyl ether, followed by selective protection of the primary hydroxyl group at the C-6 position with a trityl ether group (**2**). The secondary hydroxyl groups at the C-3 and -4 positions were then converted into benzyl ethers (**3**) by a condensation reaction with benzyl bromide in the presence of sodium hydride and tetrabutyl ammonium iodide. After removing the trityl group under acidic conditions to produce **4**, the selective oxidation of the primary hydroxyl group at the C-6 position was carried out, followed by esterification with benzyl bromide in the presence of sodium bicarbonate in DMSO (**5**). The acetolysis of the methyl group at an anomeric position with an equimolar amount of sulfuric acid in a mixed solvent of acetic acid and acetic anhydride gave the 1-*O*-acetyl derivative (**6**). Hydrolysis with 2-aminoethanol in a mixed solution of ethyl acetate and DMSO (10:1, vol/vol) yielded benzyl 3,4-di-*O*-benzyl-2-deoxy-D-glucopyranuronate (**7**) as a mixture of α- and β-anomers. The Mitsunobu reaction (25) using triphenylphosphine and diethylazodicarboxylate in THF effected the condensation of **7** with DCA as a model ligand to produce **8**. After purification by preparative HPLC using a μBondasphere column with water/methanol (1:10, vol/vol) as the mobile phase, all of the benzyl protecting groups of the β-anomer of **8** were removed by hydrogenation with Pearlman's catalyst in ethyl acetate containing 1% acetic acid to give the desired DCA-24dG (**9**).

As mentioned above, acyl glucuronide produces irreversible protein adducts. However, DCA-24dG does not possess the hydroxyl group at the C-2 position of the glucuronic acid moiety, and the formation of the protein adducts may therefore not take place by the imine mechanism. The active ester type of affinity labeling reagents, e.g., *p*-nitrophenyl ester and *N*-hydroxysuccinimidyl ester, can form a covalent bond by nucleophilic substitution. However, these are only slightly soluble in water under physiological conditions (22,23). For the selective cross-linking of a ligand to a target protein without any conformational changes to a protein caused by adding organic solvent to dissolve active esters in the incubation medium, an affinity labeling reagent should be highly water soluble under physiological conditions. The solubility of the desired acyl 2-deoxyglucuronide in 50 mM potassium phosphate buffer (pH 6.0 and 7.4) was compared with that of DCA *p*-nitrophenyl and *N*-hydroxysuccinimidyl esters, the two most common ester forms. DCA-24dG (500 g) was easily dissolved in 25 μL of each phosphate buffer, whereas the same amount of either the *p*-nitrophenyl or *N*-hydroxysuccinimidyl ester of DCA remained insoluble even in 5 mL of the same buffer. Acyl 2-deoxyglucuronide is a highly water-soluble and reactive compound as well as acyl glucuronide; therefore, it may be useful as an affinity labeling reagent.

To investigate the reactivity under physiological conditions, 100 nmol/mL each of model peptides containing a lysine residue, i.e., the 6-17 fragment of dynorphin A (RRIRPKLKWDNQ), substance P (RPKPQQFFGLM-NH_2), and α-neo-endorphin (YGGFLRKYPK), were incubated individually with 50 equiv. of DCA-24dG at 37°C in 50 mM potassium phosphate buffer (pH 7.4). Aliquots of the reaction mixture were analyzed by MALDI-TOFMS every hour. Even after an 8-h incubation, every peptide provided only two mass signals, corresponding exactly to the original and single DCA-

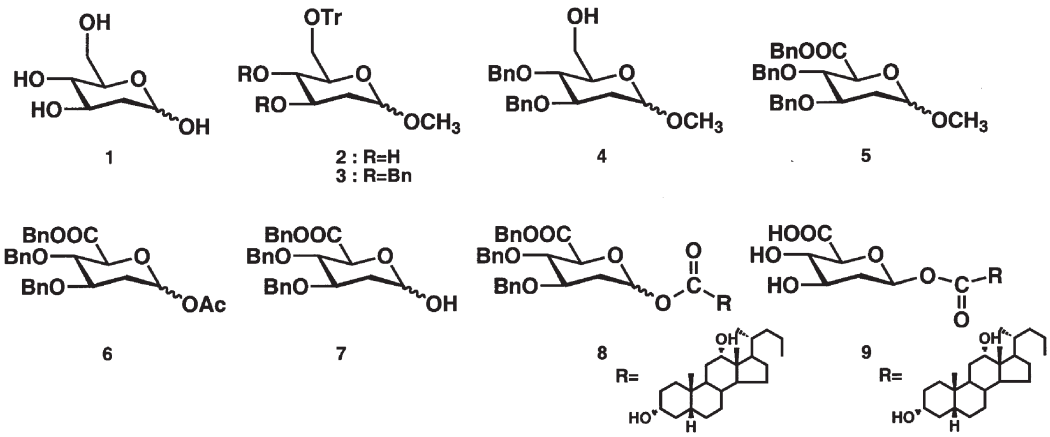

FIG. 2. Synthetic route of 1-*O*-(24-deoxycholyl)-2-deoxy-β-D-glucopyranuronic acid, deoxycholyl 2-deoxyglucuronide (DCA-24dG). Bn = $–CH_2C_6H_5$.

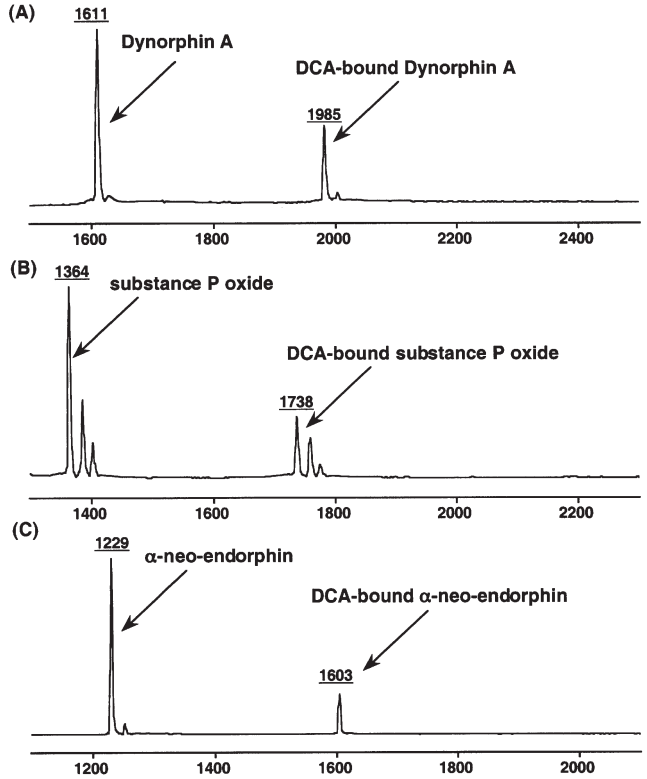

FIG. 3. Matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOFMS) spectra of 3α,12α-dihydroxy-5β-cholan-24-oic acid, deoxycholic acid (DCA)-bound peptides. Peptide: (A) dynorphin A (6-17); (B) substance P; (C) α-neo-endorphin. MS conditions: matrix, α-cyano-4-hydroxycinnamic acid (CHCA); accelerating voltage, 25 kV; grid voltage, 18 kV; guide wire voltage, 50 V.

bound peptides (Figs. 3A–C). There was no signal corresponding to Amadori products formed by condensation through the sugar moiety. These results substantiate our hypothesis that the elimination of the 2-hydroxyl group of the glucuronic acid moiety results in a reduction in the frequency of acyl rearrangement, which would produce these Amadori products.

Although the 6-17 fragment of dynorphin A is a basic peptide, containing a free N-terminal, two lysine residues, and three arginine residues, it was supposed that some product was present. Therefore, a mixed solution of the adducts formed by the incubation with DCA-24dG was subjected to HPLC separation, and three peaks on the chromatogram were collected. All peaks showed one signal at *m/z* 1985 corresponding to the M.W. of dynorphin A with one molecule of DCA bound on the MALDI-TOFMS spectra. The amino acid sequences of those three adducts were therefore analyzed by the post-source decay mode (Table 1). The peptide fragment ion nomenclature used is based on that proposed by Roepstorff and Fohlman (27) as modified by Bieman (28). All of the b-ions of adduct A indicated the addition of one molecule of DCA, demonstrating that DCA was covalently bound to the N-terminal amino group. In the case of adduct B, no mass shift for the b_1, b_2-NH_3, b_3, b_4, b_5, y_4, or y_5 ions was observed, whereas a mass shift corresponding to the addition of one DCA molecule appeared for the c_6, b_7, b_9, b_{10}, b_{11}, y_9, y_{10}, and y_{11} ions. These results suggest that the DCA molecule was bound through the amino group located on the side chain of the lys-6 residue of the peptide molecule. In a similar manner, the binding site of the DCA molecule in adduct C was determined to be the amino group on the side chain of the lys-8 residue because of the lack of mass shift for ions b_1-NH_3, b_2, b_3, b_4, b_5, b_6, a_7-NH_3, and z_4 and the presence of a mass shift corresponding to one DCA molecule for ions b_9, b_{10}, b_{11}, y_9, y_{10}, and y_{11}. These results clearly indicate that the active ester-type affinity labeling reagent, DCA-24dG, reacts with free amino groups located at the N-terminus and the side

TABLE 1
Fragment Ion Mass Values Obtained from MALDI-PSD Spectra of Peptide-Bound DCA Purified by HPLC from the Incubation Mixture of DCA-24dG with the 6-17 Fragment of Dynorphin A[a,b]

Adduct A			Adduct B			Adduct C		
Sequence	Calcd.	Obsd.	Sequence	Calcd.	Obsd.	Sequence	Calcd.	Obsd.
R	**531.8** (b_1)	530.1	R	157.2 (b_1)	157.9	R	157.2 (b_1)	158.0
RR	**688.0** (b_2)	687.2	RR	296.4 (b_2-NH_3)	295.8	RR	296.4 (b_2-NH_3)	296.6
RRI	**801.1** (b_3)	800.0	RRI	426.6 (b_3)	426.6	RRI	426.6 (b_3)	426.3
RRIR	**940.3** (b_4-NH_3)	940.7	RRIR	582.7 (b_4)	581.4	RRIR	582.7 (b_4)	581.7
RRIRP	**1037.4** (b_5-NH_3)	1035.9	RRIRP	680.0 (b_5)	678.3	RRIRP	680.0 (b_5)	677.9
RRIRPK	**1182.6** (b_6)	1183.5	RRIRPK	**1182.6** (b_6)	1181.0	RRIRPK	808.0 (b_6)	806.7
RRIRPKL	**1295.8** (b_7)	1297.9	RRIRPKL	**1295.8** (b_7)	1294.9	RRIRPKL	876.2 (a_7-NH_3)	875.4
RRIRPKLKW	**1593.1** (b_9-NH_3)	1590.7	RRIRPKLKW	**1610.2** (b_9)	1608.8	RRIRPKLK	**1378.9** (a_8-NH_3)	1377.7
RRIRPKLKWD	**1680.2** (a_{10}-NH_3)	1680.5	RRIRPKLKWD	**1725.3** (b_{10})	1723.2	RRIRPKLKW	**1610.2** (b_9)	1607.2
RRIRPKLKWDN	**1839.4** (b_{11})	1838.6	RRIRPKLKWDN	**1839.4** (b_{11})	1838.9	RRIRPKLKWD	**1725.3** (b_{10})	1722.6
RRIRPKLKWDNQ	**1985.4** $[M + H]^+$	1985.4	RRIRPKLKWDNQ	**1985.4** $[M + H]^+$	1985.5	RRIRPKLKWDN	**1839.4** (b_{11})	1838.4
			RIRPKLKWDNQ	**1829.3** (y_{11})	1828.3	RRIRPKLKWDNQ	**1985.4** $[M + H]^+$	1985.1
			IRPKLKWDNQ	**1673.1** (y_{10})	1670.6	RIRPKLKWDNQ	**1812.3** (y_{11}-NH_3)	1812.2
			RPKLKWDNQ	**1560.0** (y_9)	1557.9	IRPKLKWDNQ	**1673.1** (y_{10})	1670.9
			LKWDNQ	803.9 (y_6)	805.7	RPKLKWDNQ	**1560.0** (y_9)	1557.8
			KWDNQ	690.7 (y_5)	692.3	WDNQ	546.5 (z_4)	547.7
R*RIRPKLKWDNQ			RRIRPK*LKWDNQ			RRIRPKLK*WDNQ		

[a]Bold: peptide fragment ion modified with DCA. *Amino acid residue modified with DCA.
[b]Abbreviations: Calcd., calculated; Obsd., observed; MALDI-PSD, matrix-assisted laser desorption ionization-post-source decay; DCA, 3α-12α-dihydroxy-5β-cholan-24-oic acid, deoxycholic acid; DCA-24dG, 1-*O*-(24-deoxycholyl)-2-deoxy-β-D-glucopyranuronic acid, deoxycholyl 2-deoxyglucuronide.

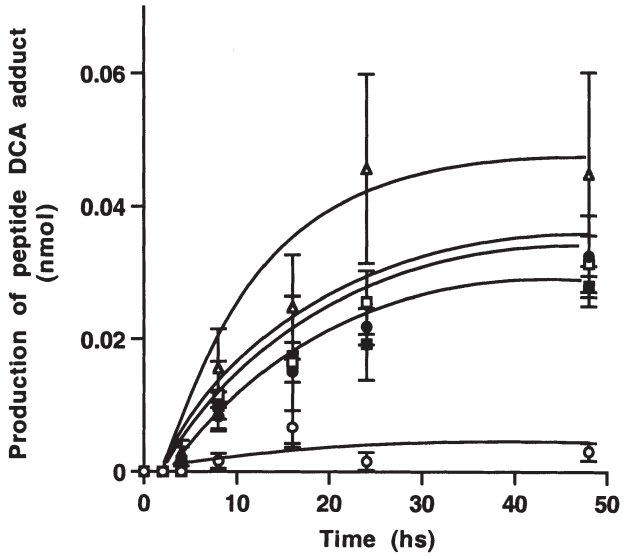

FIG. 4. Effect of pH on the formation of 3α,12α-dihydroxy-5β-cholan-24-oic acid, deoxycholic acid (DCA)-bound dynorphin A (6-17). pH 5.0 (▲), pH 6.0 (○), pH 7.4 (●), pH 8.0 (□), pH 8.5 (■), pH 9.0 (△).

chains of lysine residues. According to the results described above and in the previous paper (17), the binding site of DCA is identified as the N-terminal amino group and/or the side chain of the lys-3 residue for substance P (RPKPQQFFGLM-NH_2), and the side chain of the lys-7 or -10 residue for α-neoendorphin (YGGFLRKYPK).

Affinity labeling occurs preferentially at specific binding sites on the target protein. To reduce any nonspecific labeling, the reaction should take place after the ligand binds to its specific site on the target protein. An activated carboxyl group such as an active ester is commonly more reactive toward nucleophiles under neutral and weakly alkaline conditions; thus, this reaction can be controlled by adjusting the pH of the reaction medium. Therefore, and by way of elimination, the stability of DCA-24dG on its own under various pH conditions was investigated. The rate of DCA-24dG hydrolysis was not strongly influenced by pH, and the residual DCA-24dG decreased with time up to 48 h (data not shown), indicating a hydrolysis rate of 80%. The reactivity of DCA-24dG was then investigated under various pH conditions employing the 6-17 fragment of dynorphin A as a model peptide. Using 50 equivalents of DCA-24dG, the formation of DCA-bound dynorphin A was influenced by the pH of the reaction medium as depicted in Figure 4. Under weakly acidic conditions (pH 6.0), little adduct was formed, whereas under neutral and weakly alkaline conditions, DCA-24dG was more highly reactive with nucleophiles of the peptide.

In conclusion, the possibility of acyl 2-deoxyglucuronide as an affinity labeling reagent, possessing a high aqueous solubility and a high pH-sensitive reactivity toward nucleophiles located around the binding site of the target protein, was investigated. Recent publications have demonstrated that bile acids are natural ligands for an orphan nuclear receptor (29,30). It has also been found that the nature of the structure of the steroid nucleus of bile acids is very important for binding with a FXR/BAR (29,30) and hepatic bile acid acyl glucuronosyltransferase (31) The observations obtained in this report indicate that DCA-24dG is useful as a new water-soluble affinity labeling reagent for clarifying ligand-protein interactions related to protein function.

ACKNOWLEDGMENTS

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Lipid Synthesis and Acyl-CoA Synthetase in Developing Rice Seeds

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ABSTRACT: Developing rice seeds rapidly accumulated storage lipids between 5 and 12 d after flowering. The contents of palmitic, oleic, and linoleic acids increased throughout seed development, while the α-linolenic acid content remained low. The activity of acyl-CoA synthetase varied coincidentally during the period of lipid accumulation, and rice seeds had a sufficient capacity to supply acyl-CoA substrates for TAG synthesis. Acyl-CoA synthetase showed a broad specificity for native FA of rice seeds except for stearic acid, and π electrons of a Δ9–Δ11 double bond in the C_{16}–C_{18} acyl chains were required for its maximal activity.

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Acyl-CoA synthetase or long-chain-FA–CoA ligase (EC 6.2.1.3) catalyzes the activation of FFA to acyl-CoA thioesters and provides acyl substrates for glycerolipid synthesis, acyl chain elongation, β-oxidation, and protein acylation (1):

$$\text{FA} + \text{CoA} + \text{ATP} \xrightarrow{Mg^{2+}} \text{acyl-CoA} + \text{AMP} + \text{PP}_i \quad [1]$$

In developing oilseeds, TAG is synthesized from glycerol 3-phosphate and acyl-CoA by acyltransferases, which sequentially acylate a glycerol moiety in a phospholipid bilayer of the endoplasmic reticulum membrane while *de novo* synthesis of fatty acyl components occurs in plastids (2,3). Acyl-CoA synthetase therefore mediates between *de novo* FA synthesis and glycerolipid assembly. Rice bran oil is among the most important vegetable oil resources in Asia. However, the oil is difficult to process because of its high levels of FFA and other concomitants such as wax and pigments (4). Rice seeds originally have higher FFA contents than other oilseeds, and FFA increase during oil processing by the action of lipase on TAG. The development of processing techniques has enabled us to produce an oil of reasonable quality, but because rice bran contains FFA, it is not utilized efficiently as an oil resource. The high level of FFA in rice seeds is probably a consequence of hydrolysis of TAG by lipase (5). However, whether developing rice seeds have sufficient capacity to produce acyl-CoA thioesters from FFA is not clear. Many reports on acyl-CoA synthetase are related to β-oxidation during germination (6–9), acyl chain elongation (10), or FA exported from the chloroplast (11), and the cDNA encoding acyl-CoA synthetase that participates in TAG synthesis have been isolated from Brassicaceae plants (12,13). In the present paper, we report on the rate of TAG accumulation and the potential for acyl-CoA formation in developing rice seeds. Discussion is also focused on substrate specificity and FA structural requirements of the acyl-CoA synthetase enzyme.

MATERIALS AND METHODS

Reagents. Oleic and linoleic acids were kindly provided by Nippon Oil & Fats Co. (Amagasaki, Japan). Other FA were purchased from Avanti Polar Lipids (Alabaster, AL), Doosan Serdary Research Laboratories (Toronto, Canada), Nu-Chek-Prep (Elysian, MN), and Sigma (St. Louis, MO). Acyl-CoA oxidase (EC 1.3.3.6) of *Candida* sp. was obtained from Toyobo (Osaka, Japan). Catalase of bovine liver was purchased from Sigma.

Plant material and determination of lipid and FA contents. A cultivar of japonica rice, *Oryza sativa* cv. Nipponbare, was grown in the university field. Thirty grains of seed at each developing stage were pulverized in liquid N_2 with a mortar and a pestle. The rice powder was immersed in 5 mL chloroform containing methyl heptadecanoate as an internal standard at room temperature overnight. After filtration of the rice powder *in vacuo,* and after washing the vessel with 10 mL chloroform, the combined chloroform solution was washed with Na_2SO_4-saturated water. The chloroform was evaporated to dryness *in vacuo*, and the lipids obtained were determined gravimetrically. The FA composition of glycerolipids was determined by GC (14).

Assay for acyl-CoA synthetase activity. A membranous particulate fraction was prepared from developing seeds 12 days after flowering (DAF) by centrifugation at 25,000 × *g* for 30 min (15), and protein was determined by a Coomassie Brilliant Blue method (16). Acyl-CoA synthetase activity was measured by using a modification (15) of an enzyme-coupled colorimetric method (17). A typical incubation medium contained 0.15 M MOPS-NaOH (pH 7.7), 1 mM DTT, 0.25 mM CoA Na_3, 4.5 mM ATP Na_2, 10 mM $MgCl_2$, 1% methanol, 1.5 U acyl-CoA oxidase, 2 kU catalase, 0.2 mM potassium salt of FA, 0.55 mM Triton X-100, and 10 μg protein of the 25,000 × *g* membrane preparation in a total volume of 0.2 mL. In the incubation at 30°C for 30 min, acyl-CoA that was synthesized was converted into *trans*-enoyl-CoA and H_2O_2 by the action of acyl-CoA oxidase *in situ*, and catalase then produced formaldehyde from the H_2O_2 and methanol. These sequential reactions were terminated by the addition of 0.2

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Abbreviations: DAF, days after flowering. For comparison of chemical structures, FA in the figures are designated by number of carbon atoms:number of double bonds, followed by the position of double bonds and their geometrical configuration, *cis* or *trans,* such as 18:2 9*c*,12*c* for linoleic acid.

mL 2 M KOH at 0°C. To the chilled alkaline solution was added 0.2 mL of 0.6% 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole, a color-producing reagent for aldehydes, in 0.5 M HCl. The mixture was incubated at 37°C for 10 min, and then 0.5 mL 1% $NaIO_4$ was added. The molar absorption coefficient of the resultant purple dye was 29,200/M/cm at 550 nm. Data are reported as means of triplicate assays.

RESULTS AND DISCUSSION

Lipid synthesis and FA composition. Both seed weight and lipid content varied little at 5 DAF, after which an increase in seed weight was accompanied by an accumulation of total lipids (Fig. 1). The rapid changes that occurred between 5 and 12 DAF with this cultivar were similar to the results reported for an indica cultivar (5), although TAG contents were not determined in the present study. Three-fourths of the total lipid content contained in mature seeds was accumulated at this stage. Palmitic, oleic, and linoleic acids were formed continuously during development, and they became the major FA components of mature seeds. In contrast, the contents of stearic and α-linolenic acids did not increase during seed development and remained low.

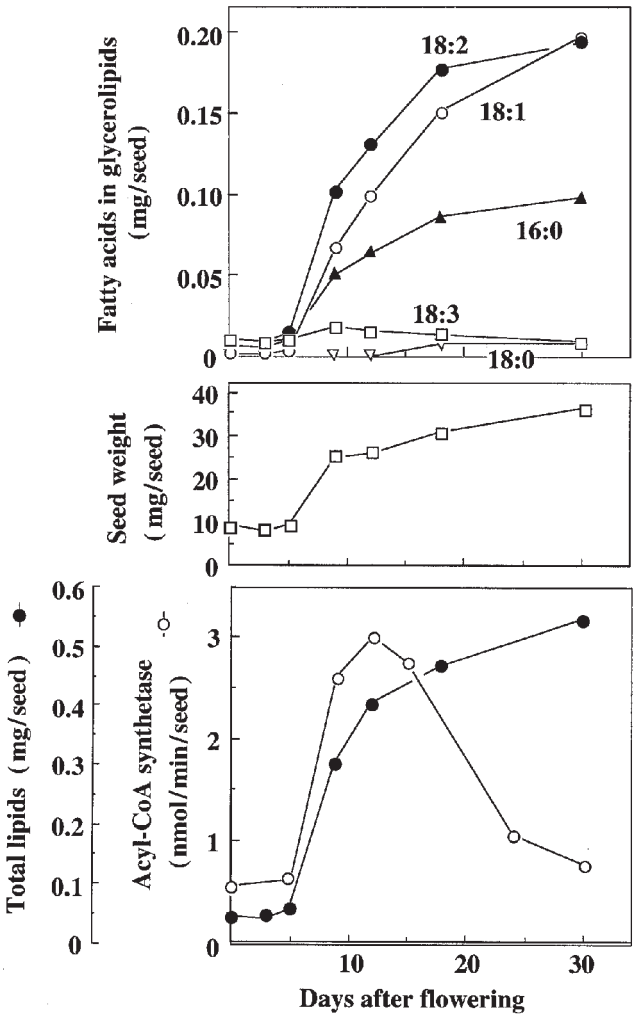

FIG. 1. Changes in FA content, seed weight, total lipid content, and acyl-CoA synthetase activity during seed development. Acyl-CoA synthetase activity is expressed as a membrane-bound activity for oleic acid per seed, and activities in other fractions are neglected. The membrane-bound fraction was prepared from a 1,500 × *g*-10 min supernatant of each homogenate by centrifugation at 25,000 × *g* for 30 min.

Changes in membrane-bound acyl-CoA synthetase activity. A homogenate prepared from developing seeds 12 DAF was fractionated by differential centrifugation (Table 1). Acyl-CoA synthetase activity was mainly associated with the 25,000 × *g* particulate fraction, but this membrane fraction was not identified further. Although the enzyme activity observed might be derived from the endoplasmic reticulum membrane (18,19), it is likely that this active fraction was a mixture of membrane fragments of both the endoplasmic reticulum and plastids. Because acyl-CoA synthetase in leaves is localized on the outer envelope membrane of chloroplasts (20,21), the possibility remains that the plastid envelope is the sole site or one of the sites of acyl-CoA formation for *de novo* TAG synthesis in oilseeds on the analogy between chloroplasts in leaf cells and plastids in seed cells. Recently, Shockey *et al.* (13) found that the *Arabidopsis thaliana* gene of a plastidial acyl-CoA synthetase was strongly expressed in developing seeds.

Membrane-bound acyl-CoA synthetase activity was followed during seed development (Fig. 1). An increase in activity was accompanied by lipid accumulation, and the activity reached a maximum at 12 DAF. At 30 DAF the activity fell to one-fourth of the maximum. This profile of changes in activity is consistent with the current concept that acyl-CoA synthetase plays a key role in TAG formation by supplying fatty acyl components. At 5 to 9 DAF, when TAG was synthesized rapidly, the rate of synthesis was 0.07 μmol/d/seed on average, and the maximal rate of TAG accumulation was estimated at 0.1 μmol/d/seed. Because synthesis of one molecule of TAG requires three molecules of acyl-CoA, the rate of acyl-CoA synthesis must be higher than 0.3 μmol/d/seed. As estimated from the data in Figure 1, the activities of acyl-CoA synthetase at 5 and 9 DAF were at least 0.86 and 3.6 μmol/d/seed, respectively, for oleic acid. The rate of FA activation was thus much faster than that of TAG synthesis, and the developing rice seeds had a sufficient capacity to supply

TABLE 1
Distribution of Acyl-CoA Synthetase Activity in Developing Rice Seeds[a]

Fraction	Protein (mg/seed)	Acyl-CoA synthetase activity (nmol/min/mg protein)	Acyl-CoA synthetase activity (nmol/min/seed)
Homogenate	2.13	10.1	21.5
500 × *g*, 10 min, ppt	1.06	2.5	2.7
5000 × *g*, 20 min, ppt	0.14	19.1	2.8
25,000 × *g*, 30 min, ppt	0.15	40.9	6.3
100,000 × *g*, 90 min, ppt	0.08	9.7	0.8
100,000 × *g*, 90 min, sup	0.38	2.2	0.8

[a]Activity was also detected in a fat layer with poor reproducibility. ppt, precipitate; sup, supernatant.

acyl-CoA substrates to the glycerol phosphate pathway responsible for TAG synthesis. In addition, acyl-CoA synthetase activity was associated with the embryo (23%), the aleurone layer (38%), and the endosperm (39%) in developing seeds at 10 DAF. Because seed TAG is accumulated primarily in the bran, which consists mainly of the embryo and aleurone cells, this activity distribution supports the idea that the acyl-CoA formation from FA is not rate-limiting for TAG synthesis in rice bran. The present data thus suggest that, in developing rice seed cells, FA that have been synthesized *de novo* in plastids and then released from the site of synthesis can all be converted into CoA thioesters by the potent, membrane-bound acyl-CoA synthetase.

General properties and substrate specificity of acyl-CoA synthetase. The optimal pH for the reaction was 7.5–7.8, both in MOPS-NaOH and in Tris-HCl, but the activity was 20% higher in MOPS than in Tris. K_m values were 9.5 μM for CoA and 0.12 mM for ATP. The FA that are synthesized *de novo* in plastids, i.e., palmitic, stearic, and oleic acids, are utilized for the synthesis of glycerolipids in plastids in the form of acyl–acyl carrier proteins. They are also transported to the endoplasmic reticulum, where seed TAG is formed from glycerol 3-phosphate and acyl-CoA. Consequently, the FA substrates of acyl-CoA synthetase in the cell are palmitic, stearic, and oleic acids. Figure 2 shows the dependence of acyl-CoA synthetase activity on the concentrations of these three FA. When simple micelles of each FA salt were supplied as the acyl donor of the enzyme reaction, the physical properties of the FA could affect the interaction between the substrate and the enzyme, depending on the hydrophobic chain. In the experiments reported here, each FA potassium salt was dissolved in a Triton X-100 solution prior to addition. Palmitic and oleic acids were utilized efficiently over the concentrations tested, whereas stearic acid was a poor substrate. This may be related to the fact that stearic acid is a minor component of rice TAG, and low activities for stearic acid have been observed in other plant species (13,15,18).

Activities for different FA species were determined at a fixed concentration of 0.2 mM to reveal the FA structures that acyl-CoA synthetase recognized. Since it was difficult to measure the K_m values of amphipathic molecules that formed micelles, the values of V_{max} but not V_{max}/K_m for various FA potassium salts were compared (Fig. 3). Palmitoleic acid was the most preferred substrate among the FA tested, and palmitic acid was the best substrate among the saturated acids. Oleic and elaidic acids, which are geometrical isomers of each other, showed similar reaction rates. The apparent structure of the *trans* isomer, elaidic acid, resembles the C_{18} saturated FA stearic acid, rather than oleic acid, and the two *cis-trans* isomers are substantially different in physical properties such as phase transition temperature. This means that for enzyme activity, the presence of π electrons in double bonds is critical, but not the geometrical structures or physical properties depending on *cis* or *trans* double bonds. *cis*-Vaccenic acid, C_{18} *cis*-11 monounsaturated, was also effective. Petroselinic acid is an isomer of oleic acid and has a *cis* double bond at position 6. The effectiveness of the *cis*-6 monounsaturated acid as substrate was considerably lower than that of the *cis*-9 isomer, oleic acid. Introduction of *cis* double bonds into positions 12 to 15 of oleic acid did not significantly affect the efficiency, and the reaction rates for linoleic and α-linolenic acids were close to that for oleic acid. A monounsaturated C_{20} FA, *cis*-11 eicosenoic acid, was a very poor substrate, and a short-chain FA, octanoic acid, was completely ineffective. These findings suggest that rice acyl-CoA synthetase recognizes both the length of the fatty acyl

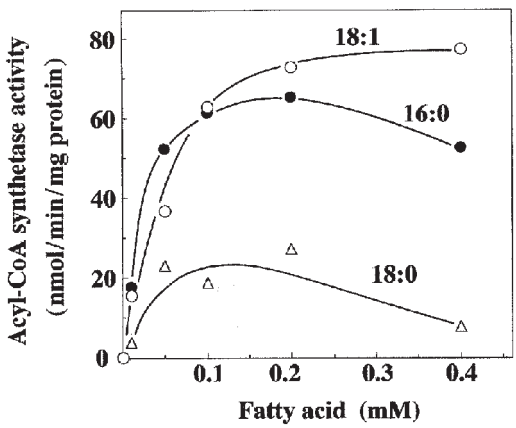

FIG. 2. Dependence of acyl-CoA synthetase activity on FA concentration.

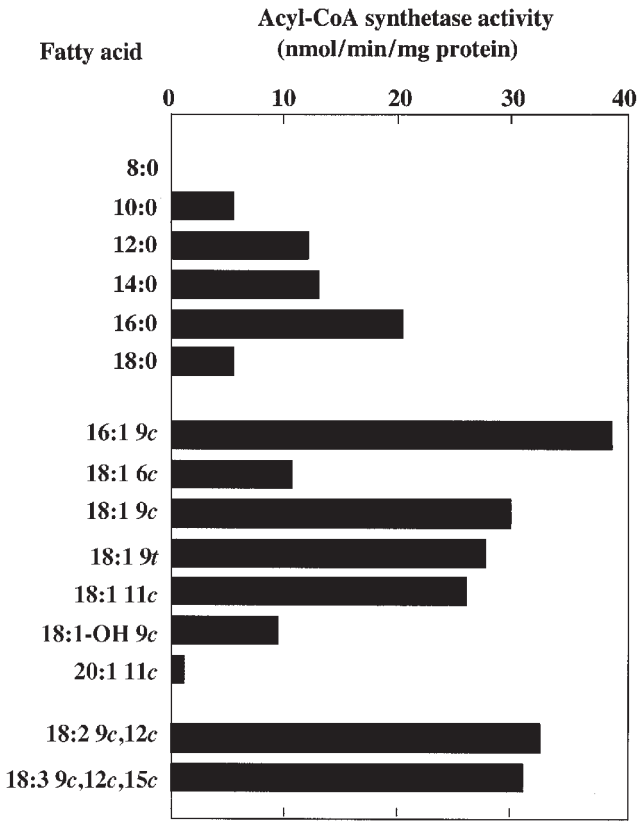

FIG. 3. Substrate specificity of acyl-CoA synthetase. The symbol 18:1-OH 9*c* designates ricinoleic acid.

chain and the position of π electrons in a carbon–carbon double bond from the carboxyl end. In addition, the enzyme requires a double bond at least at positions 9 to 11 for maximal activity. It should be noted that the presence of a hydroxyl group in the hydrophobic chain of ricinoleic acid markedly reduced the stimulatory effect of the double bond at position 9. A similar specificity was observed with an isozyme of rapeseed acyl-CoA synthetases, although the data were shown in V_{max}/K_m (22). The specificity for FA species observed here might be a consequence of the mixed specificities of isozymes, but it was similar to those of other plant species (13,15,16). Thus, one can see that the acyl-CoA synthetases responsible for TAG synthesis in oilseeds have broad specificities for limited FA species.

To reduce the FFA content of rice bran in the future, it may be useful to develop a rice variety with both a controlled lipase activity and a high acyl-CoA synthetase activity in cellular compartments where FFA are released by lipase.

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Phospholipid FA of Piezophilic Bacteria from the Deep Sea

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ABSTRACT: Phospholipid FA (PLFA) profiles were determined on four piezophilic bacteria from the deep sea: *Moritella japonica* DSK1, *Shewanella violacea* DSS12, *S. benthica* DB6705, and *S. benthica* DB21MT-2. The total concentrations of PLFA were higher in strains grown at low pressure (DSK1, 10 MPa, 27.0 mg/g dry wt cells; DSS12, 50 MPa, 24.0 mg/g), and lower in strains grown at high pressure (DB6705, 85 MPa, 1.9 mg/g; DB21MT-2, 100 MPa, 3.0 mg/g). The piezophilic bacteria were characterized by a high abundance of unsaturated FA (62–73% of total FA). In particular, PUFA were detected in all piezophiles examined. *Moritella japonica* DSK1 produced 22:6n-3 (DHA), whereas the three *Shewanella* strains produced 20:5n-3 (EPA) with trace amounts of DHA. The detection of low levels of the medium-chain-length PUFA 18:2n-6 and 18:3 (DSK1) and 20:2 (DB6705 and DB21MT-2) suggests that the biosynthesis of EPA and DHA may be regulated by the formation and desaturation of di- and tri-unsaturated FA.

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Piezophilic bacteria are microorganisms that grow optimally or preferentially at pressures greater than 1 atm (1). Numerous deep-sea piezophilic bacterial strains have been isolated from the water column, sediments, intestinal tracts, and decaying parts of invertebrates in the deep sea and characterized physiologically, genetically, and biochemically (2). It is concluded that piezophily is a general feature of deep-sea bacteria (1). Phylogenetic analyses of the 16S rDNA sequences of sediment samples suggest that most piezophilic bacteria belong to the α-, γ-, δ-, and ε-subdivisions of the Proteobacteria (3). DeLong *et al.* (3) reported that 11 cultivated psychrophilic and piezophilic deep-sea bacteria are affiliated with one of five genera within the γ-subgroup: *Shewanella*, *Photobacterium*, *Colwellia*, *Moritella*, and an unidentified genus.

In biological systems, unsaturated FA and phospholipids (PL) containing these FA play an important role in maintaining membrane fluidity (4). DeLong and Yayanos (4) were among the first to test the responses to pressures from 30 to 50 MPa (at 2°C) of the gram-negative and facultative anaerobic bacterium CNPT3. The concentration of saturated FA decreased from 34 to 25% with pressure, whereas the concentration of unsaturated FA increased from 45 to 75%. There is a striking correlation between growth at high pressure and FA unsaturation index (4). A later study on 11 piezophilic bacteria (from 1,200 to 10,476 m of depth) (5) revealed that these piezophiles produced EPA (20:5) and DHA (22:6), and the amount of these two PUFA increased as a proportion of total FA at higher growth pressures. The compositional change of membrane FA in piezophilic bacteria entails microbial cellular response to the deep-sea low temperature and high-pressure environments (4).

Despite significant advances in the past two decades, the piezophilic bacterial cellular lipid biochemistry remains to be fully characterized. A pertinent question is whether microbial synthesis of PUFA is a widely occurring phenomenon in the marine environment. To this end, the purpose of this study was to characterize the PL FA (PLFA) profiles of a number of piezophilic bacteria: piezo-tolerant, moderately piezophilic, piezophilic, and hyperpiezophilic bacteria isolated from different areas in the deep sea.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Moritella japonica DSK1 and *S. benthica* DB6705 were isolated from the Japan Trench sediment at 6356 m depth (6). *Shewanella violacea* DSS12 and *S. benthica* strain DB21MT-2 were isolated, respectively, from the Ryukyu Trench at 5110 m depth (6) and the Mariana Trench, Challenger Deep (11°22.10′ N, 142°25.85′ E), at a depth of 10,898 m (7). Piezophilic bacteria were cultured according to previously reported procedures (6). Briefly, the bacterial isolates were grown in pressure vessels at 10°C and various pressure (*M. japonica* DSK1, 10 MPa; *S. violacea* DSS12, 50 MPa; *S. benthica* DB6705, 85 MPa; and *S. benthica* DB21MT-2, 100 MPa) in Marine Broth 2216 (Difco Laboratories, Detroit, MI) for 3–5 d. Prior to use, marine broth 2216 was first autoclaved and then filtered through 0.22-μm membrane filters. Cells were harvested in early stationary phase by centrifugation at 8,000 × *g* for 15 min, washed twice with 3% NaCl solution at 4°C, and freeze-dried for lipid analysis.

Lipid extraction and separation. Freeze-dried cells were extracted at room temperature in test tubes containing a mixture of methanol/methylene chloride/phosphate buffer (potassium phosphate, dibasic, 50 mM, pH 7.4) (2:1:0.8, by vol) (8). Crude lipids were collected after phase partitioning by adding

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Abbreviations: PLFA, phospholipid FA.

methylene chloride and deionized water to the test tube to the final ratio of methanol/methylene chloride/water of 1:1:0.9. Total lipids were separated into different lipid classes using miniature columns (Supelco, Inc., Bellefonte, PA) containing 100 mg silicic acid. Neutral lipids, glycolipids, and PL were obtained by sequential elution with 4-mL aliquots of chloroform, acetone, and methanol, respectively (8).

Analysis of FAME by GC–MS. Ester-linked PLFA were subject to a mild alkaline transmethylation procedure to produce FAME (8). The FAME were analyzed on an Agilent 6890 gas chromatograph interfaced with an Agilent 5973 Mass Selective Detector. Analytical separation of the compounds was accomplished using a 30 m × 0.25 mm i.d. DB-5 MS fused-silica capillary column (J&W Scientific, Folsom, CA). Individual compounds were identified from their mass spectra by comparison with standard or published spectra and from their relative retention times. Response factors were obtained for each compound using duplicate injections of quantitative standards at five different concentration levels. Concentrations of individual compounds were obtained based on the GC–MS response relative to that of an internal standard (18:0 FA ethyl ester). Double-bond position and geometry of monounsaturated FA were determined by using methods described by Dunkleblum *et al.* (9).

RESULTS AND DISCUSSION

The PLFA composition of the piezophilic bacteria is summarized in Table 1. The total concentration of FA ranged from 1.9 to 27.0 mg/g dry weight of cells. The FA content may be related to the growth pressure of piezophilic bacteria. The concentrations of FA in piezo-tolerant (DSK1, 10 MPa) and moderately piezophilic strains (DSS12, 50 MPa) (average 27.6 mg/g) were significantly higher than those in piezophilic (DB6705, 85 MPa, 1.9 mg/g) and hyperpiezophilic (DB21MT-2, 100 MPa, 3.0 mg/g). Biosynthesis of FA is a pressure-regulated process (4). It is possible that high hydrostatic pressure may have inactivated some of the enzymes in lipid biosynthesis (10).

TABLE 1
FA Composition[a] of Piezophilic Bacteria[b]

FA	Concentration (% of total FA)			
	DSK1	DSS12	DB6705	DB21MT-2
Total FA (mg/g dry wt cell)	27.0	23.9	1.9	3.0
i-13:0	—	3.5	—	—
3-OH-12:0	—	0.6	0.5	—
c7 14:1	0.8	1.0	—	ND
t7 14:1	6.9	0.9	ND	—
14:1n-5	1.7	—	ND	ND
14:0	13.2	9.5	4.8	2.8
3-OH-13:0	ND	1.8	1.3	—
i-15:0	—	9.0	7.5	3.8
a-15:0	1.8	3.0	—	ND
15:1n-8	0.8	—	ND	ND
15:0	1.1	1.3	2.1	1.4
c7 16:1	7.3	2.0	0.6	37.5
t7 16:1	34.6	23.2	37.5	—
16:1n-5	0.6	—	—	ND
16:0	10.5	10.8	19.9	16.8
17:1n-8	0.6	—	1.1	2.1
17:1n-6	—	—	—	0.9
17:0	—	—	0.5	0.7
18:3	ND	ND	5.6	ND
18:2	1.4	1.0	—	ND
18:1n-9	10.3	3.5	2.0	3.2
18:1n-7	1.6	3.9	10.3	19.7
18:0	ND	ND	0.8	1.7
20:2	ND	ND	0.6	1.4
20:5 (EPA)	ND	27.4	4.9	8.1
22:6 (DHA)	6.4	—	—	—
SFA	25	22	28	23
MUFA	65	34	52	63
PUFA	7.8	28	11	9.5
BFA	1.8	16	7.5	3.8
TUFA/SFA	2.9	2.9	2.2	3.1

[a]ND, not detected; —, <0.5%. SFA, saturated FA; MUFA, monounsaturated FA; BFA, branched FA; TUFA, total unsaturated FA.
[b]DSK1 *(Moritella japonica)* and DB6705 *(Shewanella benthica)* were isolated from the Japan Trench sediment at 6356 m depth (6). DSS12 and DB21MT-2 are *S. violacea* and *S. benthica* strains isolated, respectively, from the Rkukyu Trench at 5110 m (6) and the Mariana Trench, Challenger Deep (11°22.10′ N, 142°25.85′ E) at a depth of 10,898 m (7).

The *Shewanella* group is physiologically diverse and contains strains that are both piezophilic and psychrophilic (11). Strains DSS12, DB6705, and DB21MT-2 belong to the *Shewanella* group 1 as defined by Kato and Nogi (11) and are pressure tolerant (piezophilic) and cold-adapted (psychrophilic). Our results clearly show a biochemical difference between these two genera. For example, DSK1 contained more abundant *anteiso* isomers of the terminal branched FA (Table 1, FA <0.5% of the total FA are not listed). In contrast, piezophiles in the *Shewanella* group (DSS12, DB6705, and DB21MT-2) contained predominantly the *iso* isomers. In addition, the *iso* branched C_{13} and C_{15} FA were consistently present in higher amounts than the corresponding straight-chain FA (*n*-13:0 and *n*-15:0). All strains except DSK1 contained hydroxyl FA, 3-OH-12:0 and -13:0.

The FA were dominated by unsaturated FA in piezophilic bacteria; the ratio of unsaturated to saturated FA ranged from 2.2 to 3.1 (Table 1). Among the unsaturated FA, hexadecenoic acid 16:1n-7 (*cis* and *trans* isomers) was most abundant, accounting for 25.2 (DSS12), 37.5 (DB21MT-2), 38.1 (DB6705), and 41.9% (DSK1) of the total FA. All four piezophilic strains produced PUFA, either DHA (*M. japonica* DSK1) or EPA with trace amounts of DHA (DSS12, DB6705, and DB21MT-2). PUFA also have been observed in *Shewanella* spp. from Antarctic ice cores (e.g., 12). These microflora share the same type of biochemical characteristics by producing EPA or DHA (12). The production of EPA and DHA was characterized as a biochemical trait of piezophilic (4) and psychrophilic/halophilic bacteria, and as a physiological strategy for coping with the combined effects of low temperature and marine salinity (4,12). It is interesting to note that DSK1 produced small amounts of the medium-chain-length PUFA 18:2n-6 and 18:4 (<0.5%), whereas DB6705 and DB21MT-2 contained low levels of 20:2 and 20:3 FA,

suggesting that the biosynthesis of EPA and DHA may be regulated by the formation and desaturation of di- and tri-unsaturated FA (e.g., 12).

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LETTER TO THE EDITOR

Which of the n-3 FA Should Be Called Essential?

Sir:

For most nutritionists the term essential FA (EFA, formerly vitamin F) seems to cover only the PUFA linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) and not the other members of the n-6 and n-3 FA families. Textbooks on biochemistry and nutrition support this view, as they often mention only the two parent FA, e.g., "Linoleate and linolenate are essential fatty acids for mammals" (1). Furthermore, the recent American Dietary Reference Intakes on n-6 and n-3 FA state that a "lack of α-linolenic acid in the diet can result in clinical symptoms of a deficiency" (2), thus focusing on 18:3n-3 as the only essential n-3 FA. The definition of essentiality used in different textbooks is not always clearly stated.

The term EFA was originally coined by Burr and Burr in 1929 (3) for those FA that could reverse deficiency symptoms in young growing rodents caused by a fat-free diet. Some modern nutrition textbooks define EFA as, e.g., FA that perform vital body functions but cannot be made in the body and so are essential in the diet. The classical EFA deficiency symptoms are scaly skin, growth retardation, and impaired reproduction (4). However, severe EFA deficiencies almost never occur in humans, and modern nutritionists are concerned with determining optimal nutritional intake. Therefore, other aspects have been used in defining EFA sufficiency/deficiency, e.g., tissue levels of PUFA. Since 1929 considerable research has been undertaken to clarify the exact biological roles for these essential nutrients.

That the n-6 FA play important biological functions in the water barrier of the skin, as precursors of eicosanoids, and possibly also as second messengers in the process of signal transduction across cell membranes has long been known (5). Our understanding of the essentiality of the n-3 FA has lagged behind, but they too are now recognized as having their own distinct functions, which appear to be primarily related to the incorporation of DHA (22:6n-3) into cellular membranes, especially in neuronal tissue (6). A recent comparison of FA composition among different animal species showed that the percentage of 22:6n-3 in nonneuronal tissues decreases with increasing body size, whereas the percentage of 22:6n-3 in the brain is the same among species (7). Deficiency symptoms resulting from n-3 FA deprivation include impaired visual function and learning ability (6). Thus, the essentiality of n-3 FA seems to be due to a specific function of 22:6n-3 in membranes and neuronal tissue. The specific essential functions of two other common n-3 FA, 20:5n-3 and 18:3n-3, have not been recognized, although recent studies indicate that they have biological activity of their own (8–10), and increased dietary intake of these n-3 PUFA does have beneficial effects on health.

There are two aspects of essentiality: (i) what is essential for survival and optimal functioning (functional essentiality), and (ii) what is required in the diet (dietary essentiality). It can be argued that the long-chain highly unsaturated derivatives (long-chain PUFA: LC-PUFA) of 18:2n-6 and 18:3n-3 are not essential dietary components, since they can be formed at a slow rate in the body from their precursor EFA. Because 18:3n-3 has no known specific function in the body—apart from its excellence as a brain fuel (11) and its role as an LC-PUFA precursor—we do not find it logical to say that 18:3n-3 is the only essential dietary n-3 FA, when the only recognized functionally important n-3 FA appears to be 22:6n-3. 22:6n-3 can be supplied directly in the diet and may in fact be more potent than 18:3n-3 in preventing symptoms of n-3 FA deficiency. The current view assumes that 18:2n-6 and 18:3n-3 are the normal dietary constituents, as they are in the Western diet. However, definitions of essential dietary components should not be related to the dietary habits of specific cultural groups but to the needs of all people. In some cultures the intake of 22:6n-3 and other n-3 LC-PUFA are as dominant as 18:3n-3, e.g., among the Japanese (12), and n-3 LC-PUFA are also major n-3 components in the infant diet. In most Western societies, human breast milk has a low content of n-3 FA; 18:3n-3 constitute 0.5–2% of the FA, and the long-chain derivatives constitute 0.2–0.8%, approximately half of which is 22:6n-3 (6). In populations with a high intake of marine foods, e.g., in Japan and in the Inuit population of Canada, the 22:6n-3 content of milk can be as high as 1–3% of FA (6). Most modern infant formulas contain 18:3n-3 as the only n-3 FA (13). And 18:3n-3 is able to fulfill the n-3 FA requirement of term infants, at least in the sense that it prevents apparent deficiency symptoms. It is currently under debate whether dietary 18:3n-3 optimally fulfills the n-3 FA requirement in infants (6,14) and possibly pregnant and lactating women (15), or whether they might benefit from an additional intake of preformed 22:6n-3.

The situation is somewhat more complex for the n-6 FA, as both 18:2n-6 and arachidonic acid (20:4n-6) seem to have

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Abbreviations: EFA, essential FA; LC-PUFA, long-chain PUFA.

essential biological functions. Classic rodent experiments showed that 18:2n-6 and 20:4n-6 are nearly equipotent in preventing the classical symptoms of EFA deficiency, i.e., skin lesions and growth failure (16). 18:2n-6 is incorporated into ceramides in the water barrier of the skin; these appear to be the components responsible for explaining the importance of n-6 FA in the skin (17). Dietary 20:4n-6 seems to prevent transepidermal water loss *via* retroconversion to 18:2n-6 (18). Rodent knock-out experiments have proved that eicosanoid formation from 20:4n-6 plays an essential role in several processes, e.g., closure of the ductus arteriosus (19).

To our knowledge, no functional studies with animals or humans have investigated the relative ability of 18:3n-3 and 22:6n-3 to cure n-3 deficiency symptoms (dose–response studies). Providing this evidence may be difficult, as n-3 deficiency symptoms (visual function and learning ability) are not as easy to quantify as transepidermal water loss and growth. However, several animal studies have shown that dietary 22:6n-3 is a better source than dietary 18:3n-3 for incorporation of 22:6n-3 into membranes of the central nervous system and other tissues (20–23). It is therefore conceivable that dietary 22:6n-3 also functionally would cover n-3 FA requirements more efficiently. So far, this view is supported by a single study showing that addition of 22:6n-3 and 20:4n-6 prevents a decrease in bioaminergic neurotransmitters in the frontal cortex of EFA-deficient piglets when given in amounts 4 and 40 times smaller than 18:3n-3 and 18:2n-6, respectively (24).

In recent reviews on the n-3 requirement of human infants, 22:6n-3 is called a conditionally essential (25,26) or an optional nutrient (14). This seems awkward when 22:6n-3 is the primary reason for n-3 FA essentiality. We find that it would be reasonable if both aspects of essentiality—that is, functional as well as dietary—were taken into consideration when defining which PUFA are essential. Thus, we suggest that an essential nutrient be defined as follows: "A food component that, directly or *via* conversion, serves an essential biological function and which is not produced endogenously or in amounts large enough to cover the requirements." In the case of vitamin A, retinol is the active component and β-carotenoids are included in the requirement claims according to their retinol-equivalent action. What we propose is that a similar scheme be used for EFA. Thus, because 22:6n-3 may be the *directly* essential n-3 FA, it should be included as an EFA. It would be a practical and simple compromise at present to say that the n-3 and n-6 FA families as such are essential [as originally suggested by Burr and Burr (3)], because all members of the two families seems to cover human needs. Although this is only a matter of terminology, we believe that it plays a role in shaping how we think about PUFA. Currently, pediatric nutritionists debate whether infants have a special dietary need for LC-PUFA. What we need are more studies that define the roles and importance of various PUFA and determine the dietary requirements for PUFA—especially with respect to the n-3 FA family—in different human age and gender groups. Furthermore, more work is needed to determine the bioequivalence of the individual FA of the PUFA families.

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Effect of Conjugated FA on Feed Intake, Body Composition, and Liver FA in Mice

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ABSTRACT: CLA is a generic term describing different isomers of linoleic acid with two conjugated double bonds. Various metabolic effects have been demonstrated following administration of CLA, including a change in body composition in animals. However, the effects of pure CLA isomers are not fully understood. In addition, conjugated octadecatrienoic acids such as calendic acid have not been extensively investigated. In this study, male and female ICR mice were fed pure CLA isomers (*cis*9,*trans*11 or *trans*10,*cis*12) or calendic acid (*trans*8,*trans*10,*cis*12) as their ethyl esters for 6 wk. Body protein content was significantly increased after feeding CLA isomers, either as pure isomers or as a mixture. Calendic acid significantly decreased body fat content in males. CLA (pure isomers or a mixture) significantly decreased body fat in both males and females, with the *trans*10,*cis*12 isomer being the most effective. The effect of the *cis*9,*trans*11 isomer was more pronounced in females than in males. It was concluded that the *trans*10,*cis*12 CLA isomer was mainly responsible for the decrease in fat content in mice, without a significant modification of feed efficiency, and that it was more effective than calendic acid.

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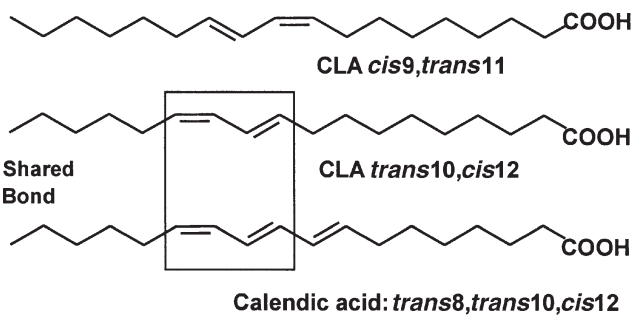
CLA is a generic term that describes different isomers of linoleic acid with two conjugated double bonds. CLA is formed naturally by microorganisms in the rumen and is hence present in meat and dairy products, mainly as the 18:2 *cis*9,*trans*11 isomer. Commercial preparations of CLA contain equal amounts of the *cis*9,*trans*11 and *trans*10,*cis*12 isomers. CLA has been reported to exhibit different potentially beneficial effects on health, including cancer (1), atherosclerosis (2), diabetes (3), and immune function (4). Furthermore, CLA decreases body fat and increases lean body mass in various animal species such as mice, rats, hamsters, chickens, and pigs (5–8). With regard to effects on body composition, the active CLA isomer seems to be the *trans*10,*cis*12 isomer (9,10). Results from human studies are, however, less consistent (11–13).

The mechanism of action is likewise still unclear. Some authors have suggested that the effects of CLA isomers may be mediated through the prostaglandin pathway (14), as possible elongation and desaturation products of the two CLA isomers have been detected in rat livers (14). On the other hand, effects on heparin-releasable lipoprotein lipase, stearoyl-CoA desaturase, leptin levels, peroxisome proliferator-activated receptor, or protein turnover may also explain how CLA affects body composition (15).

In addition, it is unknown whether these effects are specific to CLA or can also be mediated by other FA with a conjugated double bond structure. Park and Pariza (16) demonstrated that a nonadecadienoic acid containing two conjugated double bonds reduced body fat in growing mice by 81%. This nonadecadienoic acid was produced by conjugating the *cis*11,*cis*13 nonadecadienoic acid form, resulting in a mixture of isomers containing *cis*10,*trans*12 (42.3%) and *trans*11,*cis*13 (48.0%), with the remainder being nonconjugated *trans*,*trans* isomers and other minor isomers (16). It was speculated that the *trans*11,*cis*13 isomer of nonadecadienoic acid was an analog corresponding to the *trans*10,*cis*12 CLA isomer and that it might explain the substantial reduction in body fat observed following nonadecadienoic acid feeding in mice (16).

In view of this research, it is of great interest to investigate whether other FA with a similar conjugated double bond structure may also affect body composition in mice. One of these FA is a naturally occurring octadecatrienoic acid named calendic acid. Calendic acid can be derived from oil extracted from the seeds of *Calendula officinalis* (17). Calendic acid has an 18:3 *trans*8,*trans*10,*cis*12 configuration and may be of particular interest because it shares the conjugated *trans*10,*cis*12 double bond with one of the two main CLA isomers. The structures of the two CLA isomers and calendic acid are shown in Scheme 1.

Hence, the objective of the present study was to compare the effects of calendic acid with the effects of CLA isomers on body composition and feed efficiency in mice.



SCHEME 1

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Abbreviation: ECW, empty carcass weight.

TABLE 1
FA Composition (%) of the Lipid Fraction of Each Diet

	Calendic acid	*cis*9,*trans*11	*trans*10,*cis*12	CLA mixture	Control
16:0	4.0	3.6	5.2	4.1	5.1
18:0	3.5	2.8	3.4	3.1	3.4
18:1	53.8	56.5	51.9	53.2	56.1
18:2 n-6	18.1	13.4	15.0	17.0	32.9
*cis*9,*trans*11	—	13.1	1.8	8.4	—
*trans*10,*cis*12	—	3.7	14.1	8.3	—
Calendic acid	11.9	—	—	—	—

MATERIALS AND METHODS

FA. CLA mixed isomers were prepared by transesterification of linoleic acid (TG form to ethyl esters) from sunflower oil followed by conjugation with a base. Calendic acid oil was purchased from BiRo (Sommerschenburg, Germany). The FA composition of the calendic acid oil (TG form) was as follows: 16:0 2.3%, 18:2 (*cis*9,*cis*12) 26.2%, calendic acid 18:3 (*trans*8,*trans*10,*cis*12) 60%, and other FA 11.5%. The content of conjugated FA in the FA preparations of the four treatment groups is summarized in Table 1.

(i) Preparation of linoleic acid ethyl esters. Sunflower oil (2000 g, 2.27 mol) was mixed with 1100 g EtOH (23.9 mol) and 35 g NaOEt (solid, 95%, 0.49 mol). The mixture was refluxed for 1 h. Aqueous H_2SO_4 (5%, 500 mL) was added and the mixture was stirred for 30 min. The aqueous phase was discarded after phase separation, and the organic phase was washed with 500 mL water. The organic phase was dried under reduced pressure (10 mbar) at 100°C for 2 h. Preparation of linoleic acid resulted in a yellow oil (yield: 1950 g, 93%. GC area%: 62.8% linoleic acid, 22.4% oleic acid, 5.7% palmitic acid, 4.1% stearic acid).

(ii) Preparation of CLA ethyl esters. Linoleic acid ethyl esters (62.8% linoleic acid, 1808 g, 3.7 mol), 17 g EtOH (0.37 mol), and 31.1 g (0.37 mol) KOEt were mixed and heated for 8 h at 120°C. The mixture was cooled to 90°C, and 500 mL of aqueous H_2SO_4 (5%) was added under stirring. The mixture was stirred for 30 min. The aqueous phase was discarded after phase separation, and the organic phase was washed with 500 mL water. The organic phase was dried under reduced pressure (10 mbar) at 100°C for 1 h. Preparation of CLA ethyl esters resulted in a yellow oil (yield: 1718 g, 95%. GC area%: 31.1% *cis*9,*trans*11 linoleic acid ethyl esters, 31.0% *trans*10,*cis*12 linoleic acid ethyl esters, 22.3% oleic acid, 5.7% palmitic acid, 4.1% stearic acid). Acid value was 9.6 mg KOH/g oil.

(iii) Preparation of calendic acid ethyl esters. Calendula oil (100 g, 115 mol) was mixed with 46 g EtOH (1 mol) and 8.8 g NaOEt (95%, 0.123 mol). The mixture was stirred at room temperature for 48 h. Aqueous H_2SO_4 (10%, 60 g) was added, and the mixture was stirred for 30 min. The aqueous phase was discarded after phase separation, and the organic phase was washed twice with 100 mL water. The organic solvent was evaporated under reduced pressure (10 mbar) at 40°C for 1 h. Chromatography (hexane/methyl *tert*-butyl ether 88:12) of the crude product yielded pure calendic acid ethyl esters as a yellow oil [yield: 72 g, 68%. GC area%: 16:0 2.7%, 18:2 (*cis*9,*cis*12) 28.7%, calendic acid 18:3 (*trans*8,*trans*10,*cis*12) 59.3%, and other FA 9.3%]. Acid value was 0.5 mg KOH/g oil.

(iv) Preparation and separation of CLA isomers. CLA isomers (*cis*9,*trans*11 and *trans*10,*cis*12) were separated by low-temperature crystallization by a modification of the method described by Berdeaux *et al.* (18). Preparation of CLA ethyl esters from safflower oil was carried out as described above for the production of the CLA isomer mixture from sunflower oil. Purification of ethyl linoleate from safflower oil by urea crystallization was omitted and replaced by a low-temperature crystallization at –35°C to increase the amount of CLA isomers and separate them from the ethyl esters of saturated and monounsaturated FA.

Animals. Six-week-old male (n = 45) and female (n = 45) ICR mice were purchased from Iffa-Credo (L'Arbresle, France). The initial weight of the animals was 33.4 ± 1.3 g for males and 25.5 ± 1.4 g for females. The animal experiment was approved by an internal committee in accordance with French regulations and carried out in our animal house in accordance with Agreement #21100 of the French Ministry of Agriculture.

The mice were housed in groups of three animals in plastic cages in an animal house at a constant temperature (22 ± 1°C) and relative humidity (55–60%) with a 12-h light/dark cycle. They were adapted over 7 d with a control diet (see below) before being allocated to one of five experimental dietary groups (see below). Nine males and nine females were allocated to each group.

Diets. The experimental diets were fed in pelletized form. The diets contained (g/kg): casein 230, cornstarch 360, ethoxyquin 0.1, sucrose 260, high-oleic sunflower/linseed oils (98:2 w/w) 10, ethyl esters 10, mineral mix 10, vitamin mix 50 (19). The control diet contained ethyl esters prepared from safflower oil. Safflower FA ethyl esters were added in sufficient quantities to balance the ethyl ester content to 1% of the diet. The FA content of each diet is summarized in Table 1.

Feed was exchanged three times a week. At that time, the remaining pellets were removed and weighed to determine food intake. The animals were weighed two times a week.

Body composition, feed efficiency, and lipid analyses. After 6 wk on the experimental diets, the animals were anesthetized by isofurane inhalation. Following laparotomy, the liver, heart, and gastrocnemian muscle were removed from the carcass, blotted on filter paper, and weighed. The livers were then frozen in liquid nitrogen and kept at –80°C until further analyses.

After removing the gut, the empty carcasses were weighed,

frozen in liquid nitrogen, and stored at –80°C until body composition determinations were made.

Body composition was determined in 12 animals per group (6 males and 6 females). Briefly, protein content was calculated as nitrogen content multiplied by 6.25. Nitrogen content was measured by the Kjeldahl method. Total lipids were determined by the method of Folch *et al.* (20). Minerals were measured as ash content after incineration at 500°C for 6 h. Feed efficiency was calculated from food consumption and weight gain during the 6-wk experimental period.

Liver lipids were extracted according to the procedure of Folch *et al.* (20) with BHT added in the solvent mixture. The lipid content was determined by gravimetry. Phospholipids were separated from neutral lipids with silica cartridges. The FA from both fractions were methylated using sodium methylate and BF_3 in methanol (14% wt/vol). The resulting FAME were then analyzed by GLC using a Hewlett-Packard Series II gas chromatograph packed with a CPSil88 fused-silica column (100 m × 0.32 mm i.d.). FAME were identified using authentic standards, and quantitative data were obtained using Diamir software (JMBS Developments, Le Fontanil, France).

Statistical analysis. Data are presented as mean ± SD and were compared using Statbox software and a two-way (diet and gender) ANOVA procedure. *Post hoc* analyses were performed using the Newman–Keuls test. *P* values of less than 0.05 were considered significant.

RESULTS

Body weight. Figure 1 shows mean body weight development during the experimental period. Body weight increased from 32 to about 39 g in males and from 24 to about 28 g in females. No significant difference was observed between experimental groups. Weight gain ranged between 4.6 and 7.9 g in males and between 3.3 and 6.1 g in females.

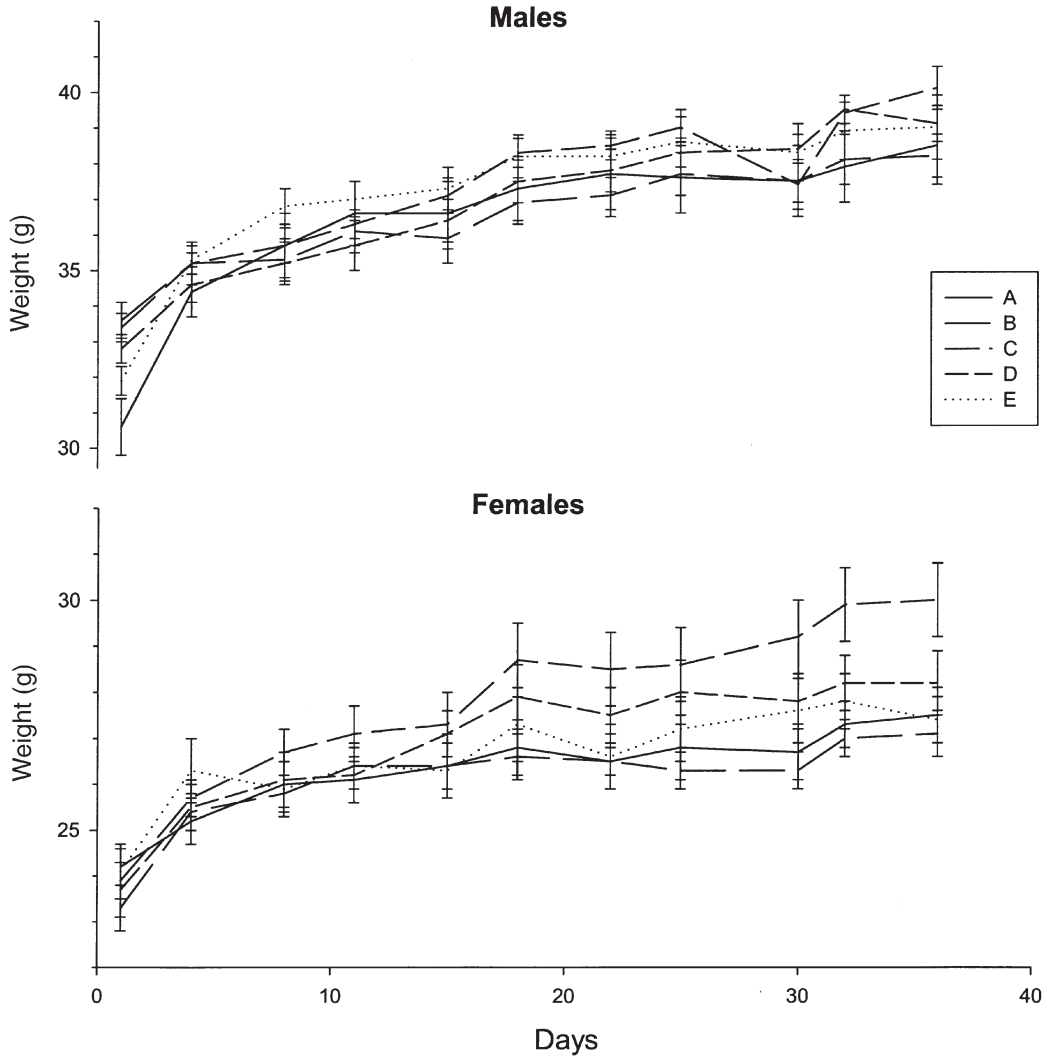

FIG. 1. Body weight development of male and female mice (n = 9 per group and per gender) fed the experimental diets [calendic acid (A), *cis*9,*trans*11 CLA (B), *trans*10,*cis*12 CLA (C), or a CLA mixture (D)] or a control diet (E) for 6 wk. Values are means ± SD of nine independent determinations.

TABLE 2
Feed Efficiency of Male and Female Mice Fed Calendic Acid, *cis*9,*trans*11 CLA, *trans*10,*cis*12 CLA, a CLA Mixture, or a Control Diet

	Calendic acid	*cis*9,*trans*11	*trans*10,*cis*12	CLA mixture	Control
Males	0.046 ± 0.05	0.032 ± 0.04	0.038 ± 0.003	0.040 ± 0.003	0.043 ± 0.007
Females	0.026 ± 0.005	0.029 ± 0.002	0.042 ± 0.006	0.033 ± 0.003	0.027 ± 0.001

[a]Results are expressed as means ± SD of three independent determinations.

TABLE 3
Empty Carcass Weight (ECW) and Organ Weight (in g)

	Calendic acid	*cis*9,*trans*11	*trans*10,*cis*12	CLA mixture	Control	SD
ECW males[a]	25.3	26.4	24.9	25.3	26.2	1.6
ECW females[a]	18.1	18.2	17.7	17.9	18.4	1.0
Liver males[a]	1.48[a]	1.48[a]	2.08[b]	1.76[b]	1.40[a]	0.22
Liver females[a]	1.08[a]	1.08[a]	1.51[b]	1.14[a]	0.94[a]	0.22
Heart	0.160	0.154	0.151	0.164	0.152	0.029
Gastrocnemian muscle	0.333	0.315	0.314	0.329	0.325	0.062

[a]Owing to a gender effect, males and females were compared separately. Values in rows having different superscripts are significantly different ($P < 0.05$).

Feed efficiency. Feed efficiency (expressed as gain/feed) is presented in Table 2. The differences were not significant.

Empty carcass weight (ECW) and organ weight. Table 3 summarizes the weight of the empty carcasses and the different organs studied. The ECW of males varied between 24.9 and 26.4 g, whereas the ECW of females ranged between 17.7 and 18.4 g, without any significant differences between groups.

Liver weight was significantly increased in mice (males and females) fed the *trans*10,*cis*12 isomer (+49% in males and +61% in females, respectively). In males, the CLA

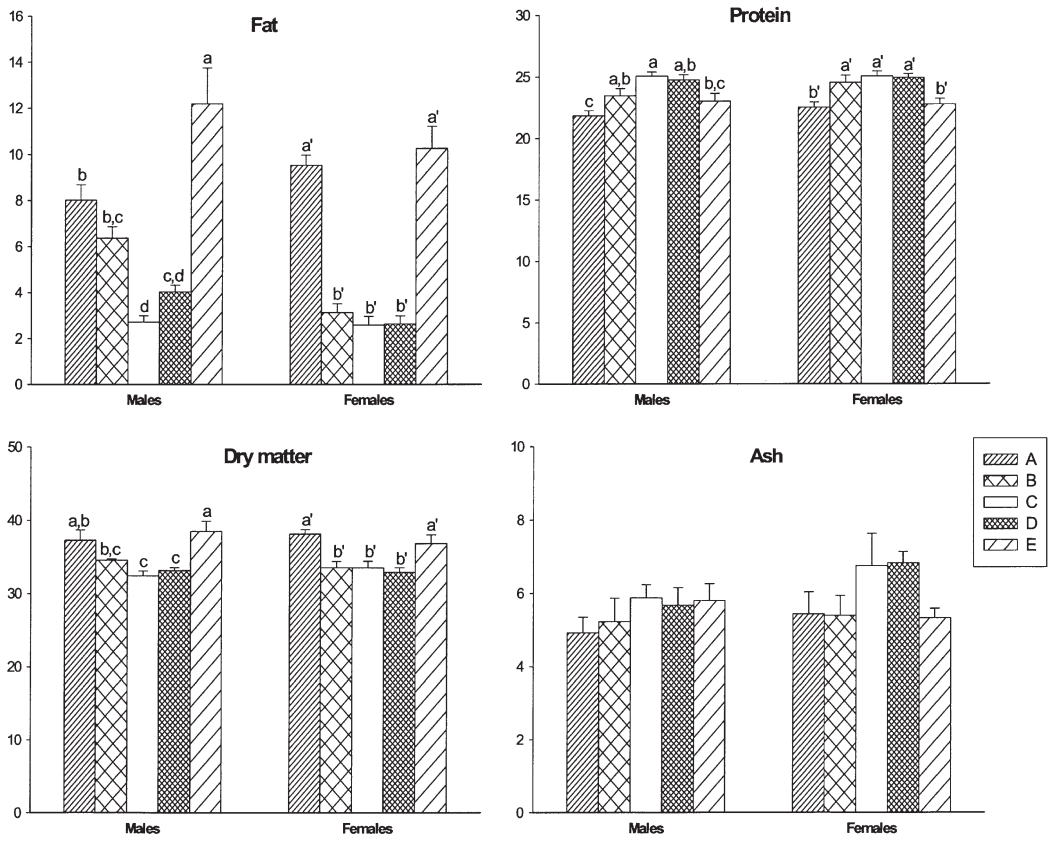

FIG. 2. Body composition (%) of male and female mice ($n = 6$ per group and per gender) fed the experimental diets [calendic acid (A), *cis*9,*trans*11 CLA (B), *trans*10,*cis*12 CLA (C), or a CLA mixture (D)] or a control diet (E) for 6 wk. Values are means ± SD of six independent determinations. For males or females, values having different superscripts are statistically different.

TABLE 4
FA Composition (%) of Liver Phospholipids of Male and Female Mice Fed Calendic Acid (A), *cis*9,*trans*11 CLA (B), *trans*10,*cis*12 CLA (C), a CLA Mixture (D), or a Control Diet (E)

	A	B	C	D	E
			Males		
16:0	19.7 (0.66)	20.6 (2.05)	18.0 (1.37)	19.0 (1.95)	18.8 (1.14)
18:0	14.6 (1.13)	14.8 (0.91)	14.6 (1.05)	14.9 (1.85)	15.7 (1.07)
Σ SAT	34.9 (0.89)[a,b]	35.9 (2.72)[a]	33.1 (0.60)[b]	34.2 (0.31)[a,b]	35.2 (0.86)[a,b]
16:1n-7	1.1 (0.24)[a]	0.8 (0.08)[a]	0.5 (0.10)[b]	0.5 (0.05)[b]	0.8 (0.30)[a]
18:1n-9	12.1 (0.56)[a]	13.7 (0.41)[b]	15.3 (1.68)[b]	14.7 (1.20)[b]	11.0 (0.57)[a]
Σ MONO	16.7 (0.78)[a]	19.2 (1.19)[b]	21.2 (2.23)[b]	19.6 (1.50)[b]	15.0 (0.67)[a]
18:2n-6	12.7 (0.91)[a]	9.8 (1.08)[b]	8.3 (1.22)[b]	8.2 (1.40)[b]	14.4 (1.24)[c]
20:4n-6	22.6 (0.74)[a,c]	20.8 (0.66)[b]	21.1 (1.77)[b,c]	22.6 (0.65)[a,c]	23.9 (0.95)[a]
Σ n-6	39.9 (1.23)[b]	35.5 (1.52)[c]	34.9 (2.00)[c]	35.8 (1.09)[c]	42.7 (0.94)[a]
22:6n-3	7.4 (0.60)[a,b]	6.9 (0.97)[a,b]	7.8 (0.49)[a,b]	8.3 (1.38)[a]	6.3 (0.66)[b]
Σ n-3	7.7 (0.63)[a,b]	7.2 (0.99)[a,b]	8.2 (0.48)[b]	8.6 (1.3)[b]	6.6 (0.68)[a]
18:2 9*c*,11*t*	ND[a]	0.5 (0.38)[b]	ND[a]	0.2 (0.01)[a]	ND[a]
18:2 10*t*,12*c*	ND[a]	0.1 (0.02)[b]	0.1 (0.04)[c]	0.1 (0.08)[b]	ND[a]
Conj 18:3	ND[a]	0.1 (0.01)[b]	0.1 (0.03)[b]	0.1 (0.01)[a,b]	ND[a]
Conj 20:3	ND[a]	0.1 (0.01)[b]	0.1 (0.01)[b]	0.1 (0.00)[a,b]	ND[a]
			Females		
16:0	19.6 (0.90)	19.2 (1.52)	17.7 (1.66)	18.4 (1.77)	18.9 (1.18)
18:0	16.5 (1.89)	16.3 (2.52)	15.7 (1.14)	16.1 (0.93)	17.2 (0.54)
Σ SAT	36.6 (1.23)[a]	35.9 (1.22)[a]	33.8 (0.94)[b]	35.0 (1.05)[a,b]	36.6 (1.40)[a]
16:1n-7	0.9 (0.19)[a]	0.7 (0.15)[a,b]	0.5 (0.12)[b]	0.4 (0.13)[b]	0.6 (0.23)[a,b]
18:1n-9	11.6 (1.15)[a,d]	13.3 (0.45)[b]	16.5 (0.95)[c]	12.7 (1.02)[b,d]	11.1 (0.74)[a]
Σ MONO	15.3 (1.51)[a,b]	17.3 (0.94)[b]	22.1 (1.40)[c]	16.7 (1.39)[b]	14.4 (1.59)[a]
18:2n-6	11.2 (1.48)[a]	9.1 (1.57)[b]	6.9 (0.94)[c]	8.8 (1.61)[b]	12.4 (0.74)[a]
20:4n-6	21.2 (1.67)	20.8 (1.59)	20.4 (1.43)	20.3 (0.97)	22.0 (1.69)
Σ n-6	35.3 (1.04)[b]	33.3 (1.00)[c,d]	32.4 (0.59)[d]	34.5 (0.97)[b,c]	38.3 (1.57)[a]
22:6n-3	11.8 (0.84)[a]	11.2 (1.78)[a,b]	9.2 (1.07)[b]	11.8 (1.05)[a]	10.0 (1.69)[a,b]
Σ n-3	12.2 (0.86)[a]	11.6 (1.87)[a,b]	9.4 (1.04)[b]	12.1 (1.06)[a]	10.2 (1.72)[a,b]
9*c*,11*t* 18:2	ND[a]	0.6 (0.31)[b]	0.1 (0.00)[a]	0.3 (0.06)[c]	ND[a]
10*t*,12*c* 18:2	ND[a]	0.1 (0.00)[b]	0.1 (0.00)[c]	0.2 (0.06)[c]	ND[a]
Conj 18:3	ND[a]	0.1 (0.00)[b,c]	0.1 (0.00)[b]	0.1 (0.00)[c]	ND[a]
Conj 20:3	ND[a]	0.1 (0.02)[b]	0.1 (0.01)[b]	ND[a]	ND[a]

[a]Results are expressed as means (SD) of five independent determinations. SAT, saturated FA; MONO, monounsaturated FA; Conj, conjugated FA; ND, not detected. Values having different superscripts are statistically different ($P < 0.05$).

mixture also induced a significant increase in liver weight (+26%).

No differences in heart and gastrocnemian muscle weights were observed.

Body composition. Data on body composition after the 6-wk experimental period are presented in Figure 2. Calendic acid significantly decreased body fat in males (–34%, $P < 0.05$) but had little effect in females (–7%, not significant). CLA treatment (pure isomers or a mixture) significantly decreased fat content in males as well as in females. The magnitude of reduction in body fat was dependent on the amount of *trans*10,*cis*12 isomer present in the experimental diets. Hence, the largest decrease in body fat was observed in the group fed the pure *trans*10,*cis*12 isomer (–78% in males and –75% in females) and the smallest effect in the group on the *cis*9,*trans*11 isomer-containing diet, in which small amounts of the *trans*10,*cis*12 isomer were still present (–48% in males and –70% in females). Intermediate effects were seen in the group receiving the mixture of CLA isomers.

The protein content was significantly increased after feeding CLA either as pure isomers or as a mixture. These increases ranged from +4.8 to +9.5% in animals fed the *cis*9,*trans*11 and *trans*10,*cis*12 isomers, respectively. The mixture increased protein content by 8.5%. Calendic acid treatment had no significant effect on body protein content.

No significant difference in ash content was observed between the different experimental groups.

FA profiles of liver lipids. Tables 4 and 5 present the FA composition of liver phospholipids and nonpolar lipids, respectively. No calendic acid could be detected in phospholipids of animals fed calendic acid. Small quantities of 18:3 and 20:3 conjugated metabolites were detected in the liver phospholipid fraction of animals fed CLA. Similar trends were observed in neutral lipids. However, small amounts of calendic acid were detected in the liver from animals fed this FA, and higher relative amounts of 20:3 conjugated metabolites in the neutral lipids of livers from animals fed CLA.

The major changes in the liver lipid composition induced by the diets were decreases in n-6 FA in the liver phospholipids and in neutral lipids induced by CLA feeding (pure isomers or a mixture). These effects were greatest in the group fed the *trans*10,*cis*12 CLA isomer.

TABLE 5
FA Composition (%) of Liver Neutral Lipids of Male and Female Mice Fed Calendic Acid (A), *cis*9,*trans*11 CLA (B), *trans*10,*cis*12, CLA (C), a CLA Mixture (D), or a Control Diet (E)[a]

	A	B	C	D	E
			Males		
16:0	22.1 (1.80)	23.4 (2.14)	22.3 (1.34)	22.2 (1.75)	21.8 (1.59)
18:0	1.7 (0.57)	1.8 (0.7)	1.9 (0.82)	1.8 (0.85)	2.2 (0.25)
Σ SAT	25.0 (2.43)	26.3 (2.90)	25.1 (2.15)	24.9 (2.42)	25.2 (4.89)
16:1n-7	4.6 (1.51)^{a}	2.6 (0.71)^{b}	2.0 (0.36)^{b}	2.0 (0.27)^{b}	3.3 (1.74)^{a,b}
18:1n-9	46.8 (1.25)^{b,c}	50.1 (5.20)^{b}	57.6 (2.88)^{a}	58.2 (4.74)^{a}	43.1 (1.50)^{c}
Σ MONO	57.2 (2.07)^{b,c}	60.9 (7.5)^{b}	69.8 (3.77)^{a}	68.9 (4.75)^{a}	52.4 (2.50)^{c}
18:2n-6	12.8 (1.74)^{b}	6.7 (1.80)^{c}	2.7 (0.63)^{d}	3.5 (1.11)^{d}	16.7 (1.89)^{a}
20:4n-6	1.5 (0.37)	1.4 (0.01)	0.1 (0.01)	0.1 (0.01)	2.1 (0.57)
Σ n-6	16.1 (1.81)^{a}	9.3 (3.69)^{b}	3.1 (0.82)^{c}	3.9 (1.44)^{c}	19.2 (3.71)^{a}
18:1n-3	0.2 (0.10)^{a,b}	0.1 (0.02)^{b}	0.2 (0.11)^{b}	0.1 (0.06)^{b}	0.3 (0.15)^{a}
22:6n-3	0.4 (0.08)	0.4 (0.22)	0.1 (0.14)	0.2 (0.09)	0.4 (0.16)
Σ n-3	0.8 (0.09)	0.6 (0.43)	0.5 (0.24)	0.4 (0.18)	0.8 (0.18)
18:2 9*c*11*t*	ND^{c}	1.8 (0.13)^{a}	0.2 (0.03)^{c}	0.6 (0.43)^{b}	ND^{c}
18:2 10t12c	ND^{c}	0.1 (0.55)^{b}	0.3 (0.12)^{a}	0.1 (0.05)^{b}	ND^{c}
Conj 18:3	ND^{c}	0.1 (0.01)^{a}	0.1 (0.04)^{a}	0.1 (0.02)^{b}	ND^{c}
Conj 20:3	ND	0.4 (0.17)	0.7 (0.67)	0.7 (0.70)	ND
Calendic	0.1 (0.01)^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
			Females		
16:0	21.2 (1.37)	21.1 (2.83)	19.6 (3.27)	24.6 (3.99)	23.7 (2.06)
18:0	1.4 (0.20)	1.5 (0.40)	2.3 (0.80)	2.35 (0.35)	2.0 (0.13)
Σ SAT	23.4 (1.41)	23.4 (2.95)	22.6 (3.04)	30.8 (6.74)	27.6 (7.19)
16:1n-7	4.1 (0.81)^{a}	2.5 (0.68)^{b}	1.9 (0.58)^{b}	1.4 (0.76)^{b}	2.6 (1.19)^{b}
18:1n-9	54.3 (2.48)^{a,b}	58.4 (3.44)^{a,b}	61.8 (5.07)^{a}	46.9 (4.90)^{b}	47.7 (4.65)^{b}
Σ MONO	63.8 (2.15)^{a,b}	65.8 (1.87)^{a,b}	71.9 (4.87)^{a}	54.2 (13.51)^{b}	55.8 (9.79)^{b}
18:2n-6	10.9 (1.78)^{a}	6.6 (0.40)^{b}	3.2 (0.68)^{b}	5.4 (0.99)^{b}	12.4 (2.72)^{a}
20:4n-6	0.4 (0.18)	0.4 (0.21)	0.8 (0.88)	1.2 (0.25)	1.5 (0.38)
Σ n-6	12.1 (2.26)^{a,b}	7.6 (3.57)^{b}	4.4 (3.30)^{b}	11.1 (7.31)^{a,b}	15.3 (3.57)^{a}
18:3n-3	0.2 (0.04)	0.1 (0.04)	0.1 (0.03)	0.1 (0.03)	0.2 (0.05)
22:6n-3	0.2 (0.10)	0.2 (0.08)	0.3 (0.07)	0.6 (0.15)	0.7 (0.83)
Σ n-3	0.4 (0.16)	0.3 (0.12)	0.4 (0.34)	1.9 (2.17)	1.0 (1.08
18:2 9*c*,11*t*	ND^{c}	2.3 (0.72)^{a}	0.1 (0.02)^{c}	0.9 (0.26)^{b}	ND^{c}
18:2 10*t*,12*c*	ND^{c}	0.2 (0.06)^{b,c}	0.2 (0.05)^{a,b}	0.4 (0.26)^{a}	ND^{c}
Conj 18:3	ND^{c}	ND^{b,c}	0.1 (0.02)^{a}	0.1 (0.05)^{a,b}	ND^{c}
Conj 20:3	ND^{c}	0.2 (0.09)^{b,c}	0.1 (0.02)^{a}	0.2 (0.11)^{a,b}	ND^{c}
Calendic	0.1 (0.02)^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}

[a]Results are expressed as means (SD) of five independent determinations. Values having different superscripts are statistically different ($P < 0.05$). For abbreviations see Table 4.

DISCUSSION

Previous studies used naturally occurring octadecatrienoic acid-containing preparations based on tung oil (rich in α-eleostearic acid; 18:3 *cis*9,*trans*11,*trans*13) in feeding experiments in rats (21,22) and laying hens (23) to investigate the FA metabolism of trienoic acids. This, however, is the first study comparing the effects of calendic acid, an octadecatrienoic acid with an 18:3 *trans*8,*trans*10,*cis*12 configuration, and CLA on feed intake, feed efficiencies, and body composition in mice. No significant effect of CLA isomers or calendic acid on body weight, ECW, or heart and gastrocnemian muscle weights was observed. Liver weight was significantly increased in animals fed the *trans*10, *cis*12-CLA isomer, which is in accordance with previous findings (24). The increase in liver weight was, to a lesser extent, also observed in mice fed the CLA mixture (significant only in male animals). These results indicate that the increase in liver weight is attributable to the *trans*10,*cis*12 CLA isomer.

Conjugated FA had major effects on body composition. All of the conjugated FA tested induced a significant reduction in fat content. For calendic acid, however, these effects were significant only in male animals, whereas minor effects on body fat were observed in female mice. In accordance with previous publications (5–8), CLA decreased body fat content when fed as an isomeric mixture or as the pure *trans*10,*cis*12 isomer. However, the present data indicate that the *cis*9,*trans*11 CLA isomer (also named rumenic acid), which is the natural isomer present in food items derived from ruminants, may also be active in regard to body fat mass. Its efficiency in females was equal to that of the *trans*10,*cis*12 isomer. Nevertheless, it is possible that the reduction in body fat observed in the group receiving the *cis*9,*trans*11 isomer was due to small amounts of *trans*10,*cis*12 isomer present in the *cis*9,*trans*11 FA preparation (Table 1). These minor quantities may have been sufficient to reduce body fat in female animals, which may be more sensitive to changes in body fat in response to CLA.

The modifications in body fat by conjugated FA were partly balanced by an increase in body protein content as well as an increase in dry matter.

Calendic acid, which has a conjugated structure (18:3 *trans*8,*trans*10,*cis*12) and shares the *trans*10,*cis*12 double bond with one of the CLA isomers, seems to be less effective than CLA. Hence, the effects of octadecadienoic and octadecatrienoic acids with conjugated double bond motifs differ significantly even when parts of the double bond structure are matched. In contrast, a 19:2 FA with two conjugated double bonds was more effective in reducing body fat in growing mice than CLA (16). The nonadecadienoic FA composition used in that experiment was a mixture of mainly two isomers, namely, *cis*10,*trans*12 (42.3%) and *trans*11,*cis*13 (48.0%), with the remainder being minor quantities of other isomers (16). It was speculated that the *trans*11,*cis*13 isomer of nonadecadienoic acid was a corresponding analog of the *trans*10,*cis*12 CLA isomer and may explain the substantial reduction in body fat observed following nonadecadienoic acid feeding in mice (16). It is conceivable that the *trans*11,*cis*13 nonadecadienoic FA and the *trans*10,*cis*12 CLA isomer are metabolized *via* desaturation and elongation and that these metabolites are biologically active. Thus, small quantities of conjugated 18:3 and 20:3 metabolites were detected in liver lipids (particularly conjugated 20:3 metabolites in neutral lipids) of CLA-fed animals, which corresponds to previous findings (14). Owing to its double bond structure, it is unlikely that calendic acid can undergo desaturation. Furthermore, the lack of conjugated 20:3 metabolites in liver lipids of animals fed calendic acid suggests that calendic acid is also not elongated. Hence, calendic acid does not share any common downstream metabolites with the *trans*10,*cis*12 CLA isomer and may therefore be less effective than the *trans*10,*cis*12 CLA isomer. Nevertheless, calendic acid significantly reduced body fat in male animals, with smaller effects in females. This suggests that the effects of calendic acid on body composition are mediated by a different mechanism.

In addition, our data demonstrate that CLA feeding decreased n-6 FA concentrations in liver lipids. This result was not found in animals fed calendic acid. The physiological significance of this difference remains unclear, but it should be taken into account to explain the effects obtained with CLA compared to calendic acid.

This study confirmed previous findings that the *trans*10,*cis*12 CLA isomer accounts for the decrease in body fat content in mice. Calendic acid, the naturally occurring octadecatrienoic acid with a conjugated double bond motif, was less effective in reducing body fat. Nevertheless, it would be intriguing to investigate whether calendic acid shares some of the other reported effects of CLA, such as anticarcinogenic or antiatherogenic effects.

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Recoveries of Rat Lymph FA After Administration of Specific Structured ^{13}C-TAG

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ABSTRACT: The potential of the specific structured TAG MLM [where M = caprylic acid (8:0) and L = linoleic acid (18:2n-6)] is the simultaneous delivery of energy and EFA. Compared with long-chain TAG (LLL), they may be more rapidly hydrolyzed and absorbed. This study examined the lymphatic recoveries of intragastrically administered L*L*L*, M*M*M*, ML*M, and ML*L* (where * = ^{13}C-labeled FA) in rats. Lymph lipids were separated into lipid classes and analyzed by GC combustion isotope ratio MS. The recoveries of lymph TAG 18:2n-6 8 h after administration of L*L*L*, ML*M, and ML*L* were 38.6, 48.4, and 49.1%, respectively, whereas after 24 h the recoveries were approximately 50% in all experimental groups. The exogenous contribution to lymph TAG 18:2n-6 was approximately 80 and 60% at maximum absorption of the specific structured TAG and L*L*L*, respectively, 3–6 h after administration. The tendency toward more rapid recovery of exogenous long-chain FA following administration of specific structured TAG compared with long-chain TAG was probably due to fast hydrolysis. The lymphatic recovery of 8:0 was 2.2% 24 h after administration of M*M*M*. This minor lymphatic recovery of exogenous 8:0 was probably due to low stimulation of chylomicron formation. These results demonstrate tendencies toward faster lymphatic recovery of long-chain FA after administration of specific structured TAG compared with long-chain TAG.

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The major fat component of food is TAG, which contain mainly long-chain FA. Short- and medium-chain FA are components of milk and milk products (1) as well as coconut oil, but they are rare in other dietary fats. However, medium-chain TAG have gained attention because of their properties of rapid hydrolysis, absorption, and metabolism in the organism (2–5).

Dietary TAG are hydrolyzed to FFA and *sn*-2 MAG (2-MAG) in the small intestine. The efficiency of intestinal hydrolysis and absorption of TAG varies in relation to FA chain length and degree of unsaturation. Short- and medium-chain TAG are hydrolyzed and absorbed faster than long-chain TAG (6,7), whereas the intestinal absorption of 2-MAG is a passive process (8), not influenced by FA chain length or degree of unsaturation.

Short- and medium-chain FA are primarily transported across the enterocyte to the portal vein (9,10) and then directed to oxidation in the liver (11), whereas only a minor fraction is utilized in reacylation of 2-MAG to TAG in the enterocyte (12,13). Long-chain FA are, however, resynthesized to TAG, which are assembled in chylomicrons, secreted from the enterocyte, and transported by the lymphatic system (14,15). The chain length and the position of FA in the TAG structure thus determine subsequent metabolism.

Long- and medium-chain FA can be incorporated into the same TAG (a structured TAG) with the use of enzymatic interesterification (16,17). MLM is a specific structured TAG with medium-chain FA located in the *sn*-1,3 positions and a long-chain FA in the *sn*-2 position of the glycerol backbone. MLL and LLM are specific structured TAG with one medium-chain FA located in the *sn*-1 or *sn*-3 position and two long-chain FA at the other positions, here collectively referred to as MLL.

Lymphatic transport of structured TAG has been examined in several studies. Christensen *et al.* (18) observed a significantly higher transport of PUFA in the mesenteric lymph the first hours after administration of MLM (where L = salmon oil FA and M = capric acid, 10:0) compared to chemically randomized TAG. The randomized TAG had the same FA composition as MLM but a similar distribution of the different FA in all three positions of the glycerol backbone. After 24 h, however, the accumulated lymphatic transport of PUFA in the experimental groups was similar. Jensen *et al.* (19) observed a higher mesenteric lymphatic transport of linoleic acid (18:2n-6) after administration of MLM compared with randomized TAG in rats pre-fed fish oil for 4 wk. Straarup and Høy (20) observed a significantly higher mesenteric lymphatic transport of oleic acid (18:1n-9) and linolenic acid (18:3n-3) in rats after administration of MLM (where L = rapeseed oil FA and M = 10:0) compared with randomized TAG, a physical mixture of MMM and LLL and rapeseed oil. Ikeda *et al.* (12) observed a tendency toward higher thoracic lymphatic transport of 18:2n-6 after administering MLM, compared with LLL and LML (where L = 18:2n-6 and M = caprylic acid, 8:0). When comparing MLM with a physical mixture of MMM and LLL, a significantly higher accumulated lymphatic transport of 18:2n-6 was observed 24 h after MLM administration.

Nevertheless, a mesenteric lymphatic study of the absorption of continuously infused ^{14}C-labeled MLM, MML, and

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Abbreviations: AP, atom percentage; APE atom percentage enrichment; *, ^{13}C-labeled FA; LLL, long-chain triacylglycerol; MLM and MLL, specific structured triacylglycerols; MMM, medium-chain triacylglycerol; *sn*-2 MAG, 2-monoacylglycerol.

LLL (where L = 18:2n-6 and M = 8:0) demonstrated that LLL was absorbed better than the two structured TAG (21). Some of the differences in the resulting lymphatic transport of FA in rats after administration of structured TAG compared with randomized TAG or long-chain TAG probably were due to experimental differences (mesenteric vs. thoracic duct lymph collection) and differences in FA species (8:0 or 10:0) and structure besides the experimental design (single meal or constant infusion). Furthermore, only the last-mentioned study could distinguish between dietary and endogenous lymphatic FA. Labeling dietary oils with stable isotopes enables both differentiation and calculation of the amounts of exogenous and endogenous FA in the lymph samples (22) and thus provides new possibilities for understanding and quantifying the time-related lymphatic transport of dietary TAG.

To calculate the lymphatic recovery of dietary specific structured TAG and separate the exogenous and endogenous lymphatic FA, this study compared the lymphatic transport of FA after administration of ML*M (where L = 18:2n-6, M = 8:0, * = ^{13}C-labeled FA), ML*L*, L*L*L*, and M*M*M*.

MATERIALS AND METHODS

Animal experiments. Male Wistar rats (200–250 g; Møllegården Breeding and Research Centre, Lille Skensved, Denmark) were kept 10 d in our animal housing unit before the experiment. They were fed chow pellets (Altromin no. 1324; Chr. Petersen A/S, Ringsted, Denmark), had free access to water, and were housed with two or three individuals in each plastic cage with wood litter. The temperature was 21 ± 1°C, humidity was 50 ± 5%, and the light period lasted from 6 AM to 65 PM.

The experiments were approved by the Danish Committee for Animal Experiments. On the day of the surgery, rats were anesthetized with Hypnorm (0.315 mg/mL fentanyl citrate and 10 mg/mL fluanisone; Janssen Pharmaceutica, Beerse, Belgium) and Dormicum (5 mg/mL; Roche, Basel, Switzerland) mixed in the ratio 1:1:2 (Hypnorm/Dormicum/sterile water). A clear vinyl tube (o.d. 0.8 mm, i.d. 0.5 mm; Critchley Electrical Products Pty. Ltd., Castle Hill, NW South Wales, Australia) was inserted into the mesenteric lymph duct. A silicone tube (o.d. 3.0 mm, i.d. 1.0 mm; Polystan, Værløse, Denmark) was inserted into the fundus region of the stomach and fixed with a purse-string suture.

After surgery, rats were placed in Bollman cages (24) with access to tap water and an infusion of saline solution with glucose (8.474 g/L NaCl, 0.298 g/L KCl, 55.49 g/L glucose; all chemicals were purchased from Sigma Aldrich, Steinheim, Germany) through the gastrostomy tube (2 mL/h). Two to 4 h after surgery, rats received 0.2 mL analgesic (Torbugesic, 1:10 dilution; Fort Dodge Laboratories, Fort Dodge, IA) subcutaneously. The next morning, lymph was collected for an hour (0-sample). Rats were then administered an oil bolus (see below) through the gastrostomy tubing. Lymph was collected in 1-h fractions for the next 8 h, in a night fraction the following 15 h, and in a 1-h fraction the next morning, collected 23 h after administration of the lipid bolus. To prevent coagulation, 0.1 mL EDTA (10% wt/vol Na_2-EDTA/2H_2O; Merck, Darmstadt, Germany) was added to the lymph collection tubes. After the experiment, rats were killed with an overdose of pentobarbital. Lymph samples were kept at –20°C until analysis.

Production of specific structured TAG. Specific structured TAG were produced by batch interesterification of caprylic acid (8:0) and LLL (where L = linoleic acid, 18:2n-6; both from Sigma Aldrich). The two components were mixed at a substrate ratio of 8:1 (mol/mol) and incubated with immobilized *sn*-1,3-specific lipase (10 wt% of the solution, Lipozyme IM; Novozymes A/S, Bagsvaerd, Denmark) at 50°C for 5 h with constant stirring. After incubation the solution was filtered to remove the lipase. MLM and MLL (M = 8:0) were purified by preparative HPLC (25).

Analysis of oils. LLL, MMM (Sigma Aldrich), MLM, and MLL were transmethylated by KOH in methanol for determination of FA compositions. FA compositions of the *sn*-2 position of MLM and MLL were determined by Grignard degradation (27), followed by TLC (silica gel 60; Merck) separation, recovery of the 2-MAG, and KOH methylation. FAME were analyzed by a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard GmbH, Waldbronn, Germany) with a fused-silica capillary column (SP-2380, 60 m, i.d. 0.25 mm, 0.20 µm film; Supelco Inc., Bellefonte, PA). The injector was at 260°C and was used in the split mode with a split ratio of 1:20. Initial oven temperature was 70°C, which was maintained for 0.5 min, raised to 160°C at 15°C/min, then raised 1.5°C/min to 200°C, held for 15 min, and finally raised 30°C/min to 225°C. This temperature was maintained for 10 min. The temperature of the detector (flame ionization) was 300°C. FA were identified by comparison with retention times of authentic FAME standards (Nu-Chek-Prep Inc., Elysian, MN). FA compositions of the TAG and of the *sn*-2 position of the structured TAG are presented in Table 1. The 18:2n-6 content of LLL was 99.5 wt% and the 8:0 content of MMM was 99.2 wt%.

The oil bolus. The lymph-cannulated rats received one of four different oils. The oil bolus consisted of 0.3 mL TAG and 0.3 mL taurocholate solution (20 mM taurocholate and 10 mg/mL choline). The solution was mixed two times for 10 s on a Whirly mixer and sonicated for 10 s (M.S.E. 150-watt Ultrasonic Disintegrator; M.S.E. Inc., Crawley, England; settings: power = medium and amplitude = 3). The TAG were LLL, MMM, and two specific structured TAG, MLM and MLL. The LLL was added L*L*L* (where * = ^{13}C-labeled FA; Larodan, Malmö, Sweden) and had an atom percentage (AP) of 1.13091%; the MMM solution added was M*M*M (Cambridge Isotope Laboratories, Andover, MA), which had an AP of 1.37509%.

TABLE 1
FA Composition (mol%) of the TAG and the *sn*-2 MAG (2-MAG) of the Specific Structured TAG[a]

	MLM		MLL/LLM	
FA	TAG	2-MAG	TAG	2-MAG
8:0	63.4	5.9	31.2	6.7
18:2n-6	36.6	94.1	68.8	93.4

[a]Data represent an average of three to six determinations. M = caprylic acid (8:0); L = linoleic acid (18:2n-6).

The specific structured TAG added were ML*M or ML*L* and had AP of 1.10803 and 1.11609%, respectively.

Analysis of lymph. Internal standards [TAG (15:0), phospholipid (15:0), and FFA (15:0); Sigma Aldrich] were added to lymph samples before extraction (28). For total FA composition, the extract was methylated by KOH-catalyzed methylation. In addition, the extract was separated into lipid classes by TLC in a system of heptane/isopropanol/acetic acid (95:5:1, by vol). The individual bands were identified under UV light by use of standards following spraying with 2,7-dichlorofluorescein. TAG, phospholipids, and FFA spots were scraped off and methylated by BF_3-catalyzed methylation (29,30).

All lymph and experimental oil methyl esters were analyzed by GC combustion isotope ratio MS (Delta^{PLUS}; Thermo-Finnigan, Bremen, Germany) for quantification of FA and determination of the $^{13}C/^{12}C$ ratio of the FA carbon atoms. Samples were injected at 250°C with a split ratio of 1:15 and separated on a fused-silica capillary column (same as above) with the following temperature program: The initial temperature (70°C) was maintained for 3 min and then raised to 150°C at 15°C/min, to 169°C at 1.5°C/min, to 173°C at 0.5°C/min, to 188°C at 3°C/min, and finally to 200°C at 20°C/min, where it was maintained for 14 min.

The stability of the measurements was checked every day by a standard on–off test (18 injections of CO_2) and a B-scan (control of all mass values, especially water, nitrogen, and argon). CO_2 injections at the beginning and end of each run ensured correct determinations of sample δ values. The individual FA were identified by comparing retention times with retention times of authentic and quantitative standards (Nu-Chek-Prep Inc.). The standards were also used to calculate response factors of the individual FA.

Calculations. The AP is a quantitative description of the ^{13}C-enrichment of a sample. The AP of a sample (AP_S) is calculated from the ratio of $^{13}C/^{12}C$ of the sample (δ_S) and a reference δ of a specific limestone, Pee Dee Belemnite (where δ = 0.0112372) (31). Calculation of AP_S is given in Equation 1 (32):

$$AP_S = \frac{100 * 0.0112372 * (0.001 * \delta_S + 1)}{1 + 0.0112372 * (0.001 * \delta_S + 1)} \quad [1]$$

From Equation 1 and the AP of the 0-sample (AP_0), the atom percent enrichment (APE_S) of the sample is calculated:

$$APE_S = AP_S - AP_0 \quad [2]$$

With the APE_S, the APE of the oil (APE_{oil}), and the amount of FA in the sample (W_S), the exogenous content (ExC_S) of the sample is calculated:

$$ExC_S\,(mg) = W_S\,(mg) * \frac{APE_S}{APE_{oil}} \quad [3]$$

The % recovery of the administered dose is then:

$$\%\ \text{recovery} = \frac{ExC_S\ (mg)}{\text{dose administered (mg)}} * 100 \quad [4]$$

The equations were derived and modified from Tissot *et al.* (33).

Statistics. Unless mentioned, amounts and ratios are stated as mean ± SEM. Differences in recoveries after administration of different oils were compared with one-way ANOVA, and differences between groups were evaluated with Tukey's test. The exogenous contribution of 18:2n-6 and the total lymph transport of FA following administration of different oils was compared with Kruskal–Wallis one-way ANOVA on ranks. The level of significance was $P < 0.05$. All statistical calculations were performed using SigmaStat (version 2.03; Jandel Corporation, Erkrath, Germany).

RESULTS

The total lymphatic volume after 24 h was 50.4 ± 10.7 mL (mean ± SD). No significant differences in lymph flow were observed between experimental groups.

Recoveries of ^{13}C-labeled 18:2n-6 and ^{13}C-labeled 8:0. The recoveries of ^{13}C-labeled lymphatic TAG 18:2n-6 tended to be higher 5–8 h after administration of ML*M and ML*L* compared with that of L*L*L*, although not statistically significant (Fig. 1A). The recoveries of ^{13}C-labeled 18:2n-6 in lymph TAG 8 h after administration of L*L*L*, ML*M, and ML*L* were 38.6 ± 5.1, 48.4 ± 4.1, and 49.1 ± 5.5%, respectively. After 24 h, however, similar total recoveries of ^{13}C-labeled 18:2n-6 in the three experimental groups were observed (51.0 ± 4.9, 51.1 ± 4.1, and 54.7 ± 5.0% after administration of L*L*L*, ML*M, and ML*L*, respectively).

The recovered amount of exogenous 18:2n-6 (mg) in lymphatic TAG was significantly higher 24 h after administration of L*L*L* and ML*L* (94.8 ± 9.3 and 84.0 ± 8.7 mg, respectively) compared with that of ML*M (54.7 ± 5.7 mg) (Fig. 1B).

The recoveries of ^{13}C-labeled 18:2n-6 in lymph phospholipids were significantly higher 4–8 h after ML*M administration compared with L*L*L* administration (Fig. 2A). The recoveries of ^{13}C-labeled 18:2n-6 in the phospholipid fraction 24 h after feeding rats L*L*L*, ML*M, or ML*L* were 2.2 ± 0.2, 3.1 ± 0.4, and 2.5 ± 0.3%, respectively.

The recovery of ^{13}C-labeled 18:2n-6 in lymph FFA was significantly higher 6–7 h after administration of ML*L* compared with L*L*L* and ML*M administration. After 8, 23, and 24 h, the recovery of 18:2n-6 was significantly higher following ML*L* administration compared with L*L*L* administration (Fig. 2B). The recoveries of ^{13}C-labeled 18:2n-6 in lymph FFA were 0.5 ± 0.0, 0.9 ± 0.1, and 0.6 ± 0.1% 24 h after administration of L*L*L*, ML*L*, and ML*M, respectively.

The ^{13}C-labeled 18:2n-6 was not metabolized into other FA, since no ^{13}C-enriched FA other than 18:2n-6 was identified in lymph samples in any of the lipid classes analyzed.

The lymphatic recovery of ^{13}C-labeled 8:0 8 h after administration of M*M*M* was 2.2 ± 0.4%. Furthermore, 0.10 ± 0.06% of the administered ^{13}C-labeled 8:0 was recovered in 16:0 and 0.04 ± 0.01% was recovered in 18:0, but no ^{13}C-labeled 10:0, 12:0, or 14:0 was detected.

The exogenous contribution of 18:2n-6 to the lymph. The contributions of exogenous 18:2n-6 to the total pool of lymphatic TAG 18:2n-6 were not significantly higher after

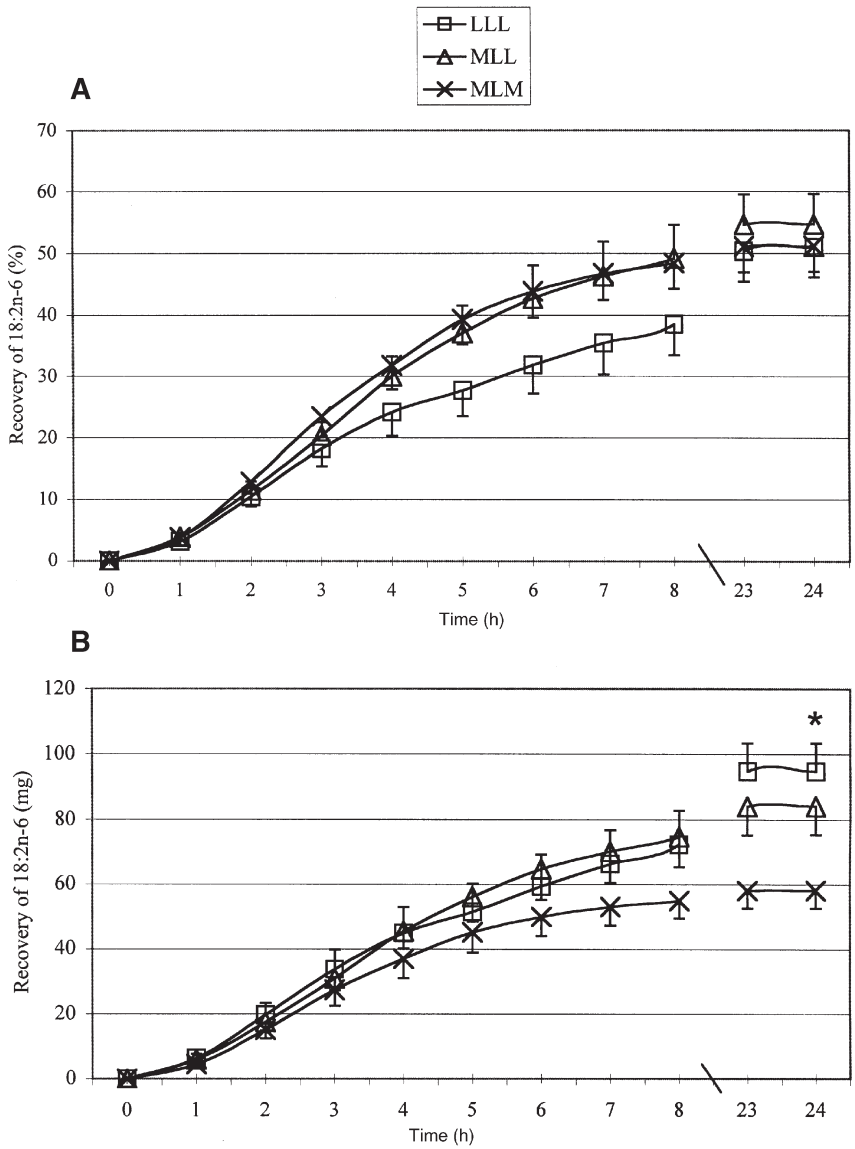

FIG. 1. Recovery of exogenous 18:2n-6 in lymphatic TAG (A) in percentage of administered dose and (B) in milligrams after administration of LLL, MLM, or MLL (n = 5–10; mean ± SEM). Asterisk (*) indicates significantly higher (P = 0.002) recovery (in mg) of ^{13}C-labeled 18:2n-6 24 h after feeding L*L*L* and ML*L* compared with ML*M (where * indicates ^{13}C-labeled FA). M = caprylic acid (8:0; L = linoleic acid (18:2n-6).

administration of ML*M and ML*L* (approximately 80%) compared with L*L*L* (approximately 60%) during the first hours of absorption (Fig. 3A).

Likewise, although not significant, higher exogenous contributions to lymph phospholipid 18:2n-6 were observed during the first hours following administration of ML*M or ML*L* (approximately 55–60%) compared with L*L*L* administration (approximately 40%) (Fig. 3B).

A tendency toward a higher exogenous contribution to lymph FFA 18:2n-6 was observed after administration of ML*M or ML*L* compared with L*L*L* during maximal absorption, with contributions of approximately 70–75% and 55%, respectively (Fig. 3C).

Lymphatic transport of FA. The lymphatic transport (mg/h) of 18:2n-6 and 8:0 (Figs. 4A and 4B) illustrates the sum of exogenous and endogenous FA. The lymphatic transport of 18:2n-6 peaked 2–3 h after LLL administration (at approximately 22 mg/h) and tended to be higher than that following MLL and MLM administration (approximately 17 and 14 mg/h, respectively) (Fig. 4A). The lymphatic transport of 18:2n-6 was approximately 1.5 mg/h after MMM administration. The lymphatic transport of 8:0 peaked 2 h after MLM or MLL administration at 0.7–1.7 mg/h, with a tendency to reach the highest level in the group fed MLM (Fig. 4B). The maximal transport of 8:0, observed 3 h after MMM administration, was approximately 1 mg/h.

The concentrations of the endogenous FA were constant and similar in all experimental groups during the 24 h of

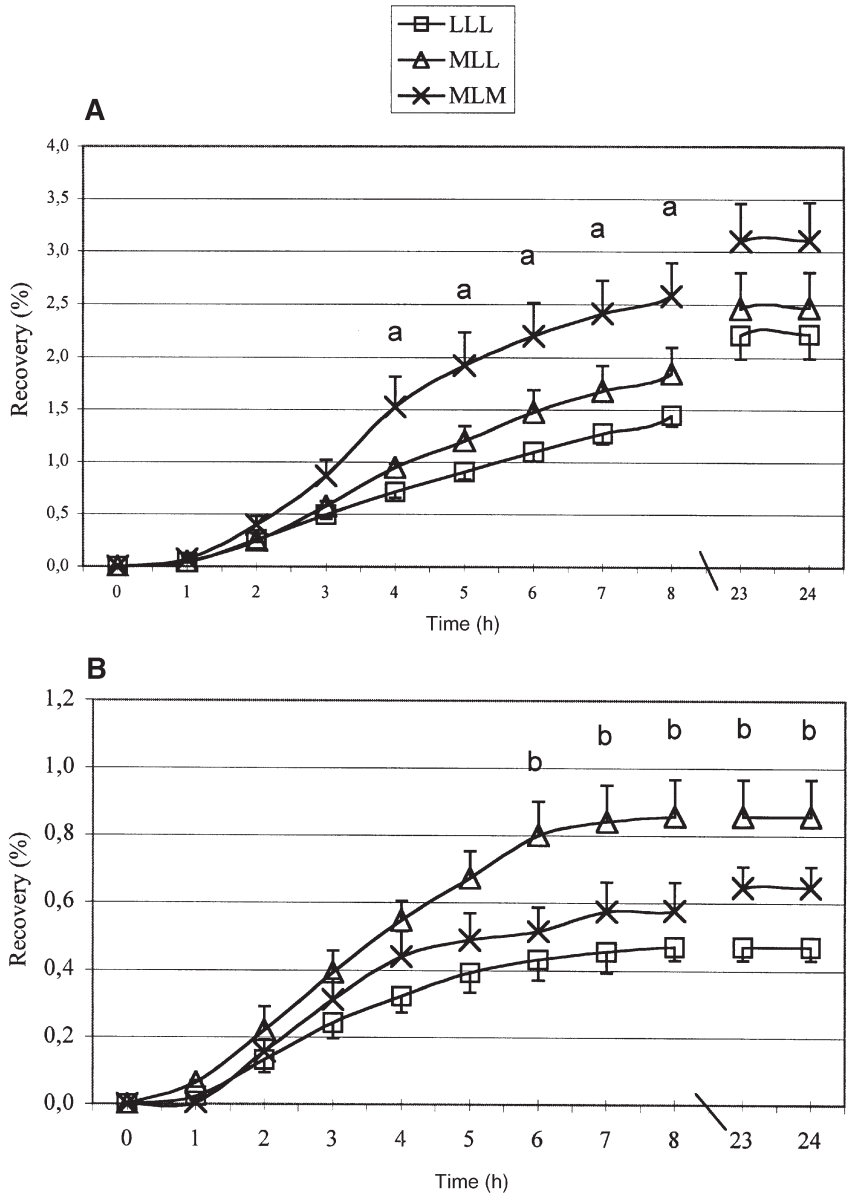

FIG. 2. Recovery of ^{13}C-labeled 18:2n-6 in (A) lymph phospholipids and (B) lymph FFA after administration of LLL, MLM, or MLL (n = 5–10; mean ± SEM). [a]Significantly higher (P = 0.012–0.018) recovery of ^{13}C-labeled 18:2n-6 in lymph phospholipids following ML*M compared with L*L*L* administration. [b]Significantly higher (P = 0.019–0.044) recovery of ^{13}C-labeled 18:2n-6 in lymph FFA 6–7 h after ML*L* administration compared with ML*M and L*L*L* administration, and 8, 23, and 24 h after ML*L* administration compared with L*L*L* administration (where * indicates ^{13}C-labeled FA). For other abbreviations see Figure 1.

lymph collection. Lymphatic transport of 16:0, 18:0, 18:1n-9, and 20:4n-6 was approximately 1–1.5, 0.4–0.6, 0.2–0.4, and 0.4–0.7 mg/h, respectively.

DISCUSSION

The present study indicated that the lymphatic absorption pattern of exogenous 18:2n-6 following administration of specific structured TAG (ML*M and ML*L*) tended to be different from that of long-chain TAG (L*L*L*). A higher lymphatic recovery of exogenous 18:2n-6 during the first 8 h following administration of ML*M and ML*L* (Fig. 1A) might be due to the rapid hydrolysis of medium-chain FA from the *sn*-1,3 positions of the TAG (34,35). However, 24 h after administration of oils the resulting recoveries of exogenous 18:2n-6 in lymph TAG were similar. Christensen *et al.* (18) observed a significantly higher lymphatic transport of PUFA in rats the first 8 h after administration of specific structured TAG compared with randomized TAG. Nevertheless, 24 h after administration of

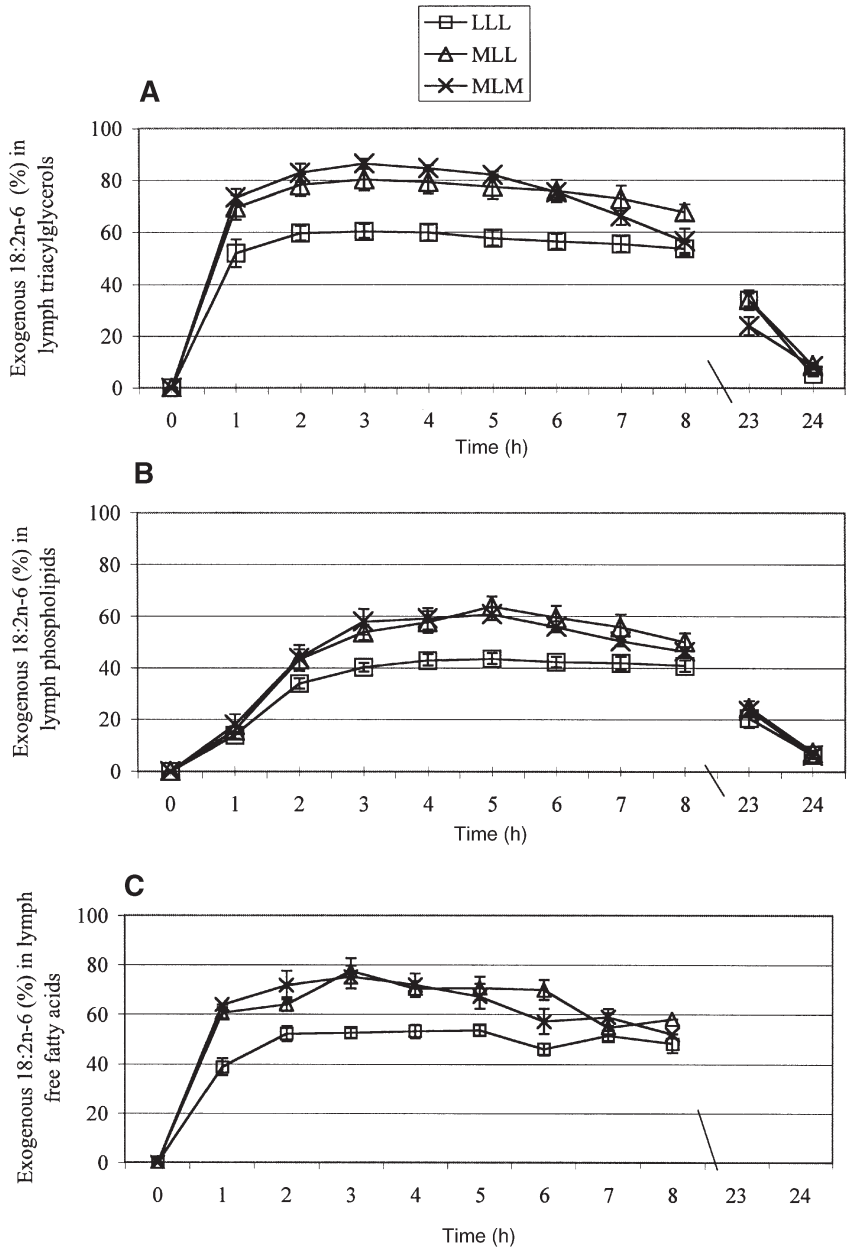

FIG. 3. Exogenous 18:2n-6 in (A) lymphatic TAG, (B) phospholipids, and (C) FFA after LLL, MLM, or MLL administration (n = 5–10; mean ± SEM). For abbreviations see Figure 1.

oils, the accumulated FA transport was similar, which was in agreement with the present study.

The recovery of exogenous 18:2n-6 did not exceed 50–55% after oil administration. This result agreed with the transport of an entirely exogenous FA in the study of Straarup and Høy (36) (20:5n-3: 41%). Furthermore, in a study with constant infusion of ^{14}C-labeled LLL and MLM, the lymphatic recoveries of long-chain FA were 62 and 48%, respectively (21).

Part of the ^{13}C-labeled 18:2n-6, which was not recovered in the present study, probably entered the portal vein (37–39), was oxidized, remained in the enterocyte, or drained into other intestinal lymphatics (40). ^{13}C-Labeled 18:2n-6 may have stayed in the intestinal lumen, but it seems unlikely that the pancreatic lipase would not have hydrolyzed the oil bolus, and the flow of saline–glucose solution (2 mL/h) through the gastrostomy tube also secured the gastric emptying. However, during a feeding study of 4 wk, approximately 15% of the dietary structured TAG were detected in feces (41).

All lymph phospholipids containing ^{13}C-labeled 18:2n-6 had been resynthesized in the enterocyte during absorption since no phospholipids were administered in the oil bolus. The recovery of 2–3% of the administered ^{13}C-labeled 18:2n-6 in the phospholipid fraction in the present study agrees well with the results of Whyte *et al.* (42), who observed approximately 4% of administered ^{14}C-labeled FA (16:0, 18:1n-9, 18:2n-6, and 18:0) in lymph PC, whereas 95% of the detected ^{14}C-labeled FA were recovered in lymph TAG.

The recovery of exogenous 18:2n-6 in lymph FFA was only

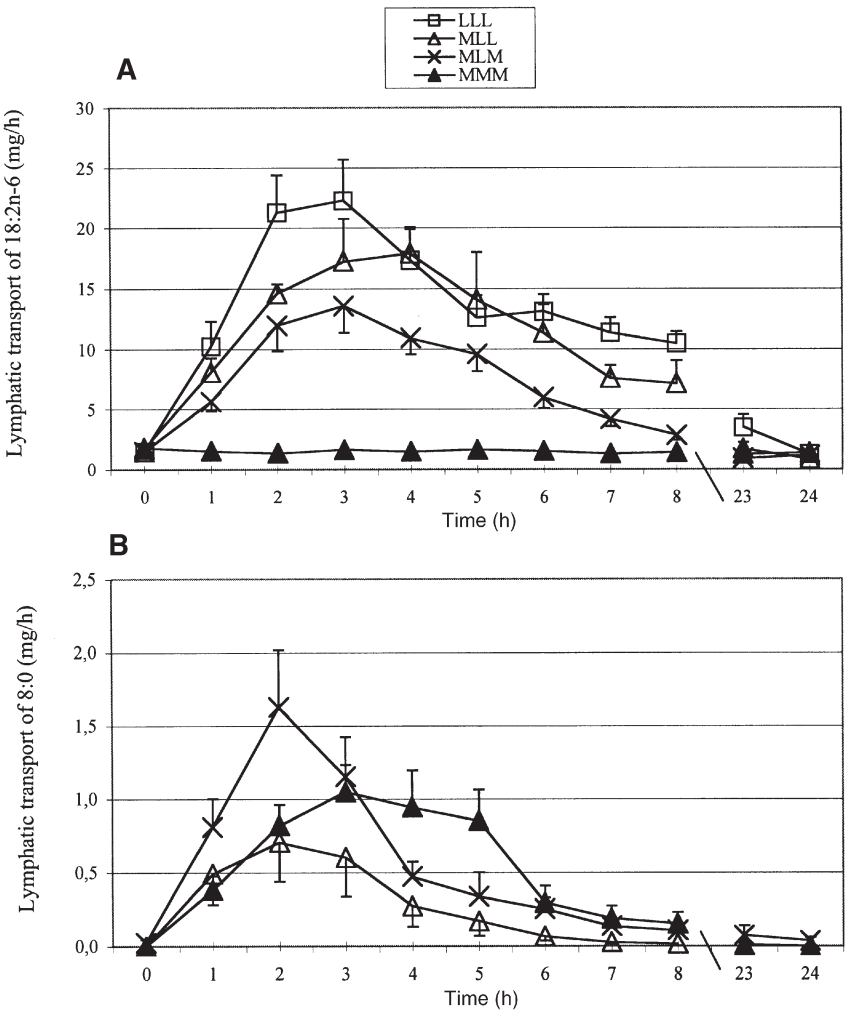

FIG. 4. Lymphatic transport (mg/h) of (A) 18:2n-6 and (B) 8:0 following LLL, MLM, MLL, or MMM administration (n = 7–10; mean ± SEM). For abbreviations see Figure 1.

0.1–0.5%. 18:2n-6 in the form of FFA would probably be transported *via* the portal vein (37,43).

The lower exogenous contribution of 18:2n-6 to lymph phospholipids in all experimental groups compared with lymph TAG and FFA might be due to supplies from several endogenous sources, e.g., the bile and membranes from old intestinal cells. Whyte *et al.* (42) and Arvidson and Nilsson (44) demonstrated with administration of ^{14}C-labeled FA or ^{3}H-choline that the FA composition of the lymph phospholipids was relatively constant and independent of the exogenous FA composition, which implied that lymph phospholipids consisted of a large endogenous fraction of FA.

The low lymphatic recovery of ^{13}C-labeled 8:0 following M*M*M* administration (2.2%) was probably due to a minor stimulation of chylomicron formation by the medium-chain TAG (45). This was further emphasized by the low endogenous lymphatic transport of 18:2n-6 after M*M*M* administration (Fig. 4A).

Mu and Høy (25) demonstrated a recovery of 7.3% of 8:0 after administration of MLM, and Tso *et al.* (21) observed a lymphatic 8:0 recovery of 8.6% by constant infusion of MML. The lymphatic transport of 8:0 after ML*M administration in the present study was 7.6% of the administered dose, which agreed well with these studies. In the present study ML*M was composed of approximately 50% long-chain FA and 50% medium-chain FA (wt%), and the transport was approximately four times as high as that after M*M*M* administration. This agreed well with the results of a lymphatic study following administration of 50% tripelargonin (TAG 9:0) and 50% safflower oil, which resulted in five times as high lymphatic transport compared with the transport after administration of pure tripelargonin (46).

The small amounts of lymphatic ^{13}C-labeled 16:0 and 18:0 observed after M*M*M* administration might have been formed by elongation or by oxidation and *de novo* synthesis in the enterocyte or by hepatic chain-lengthening and recycling through bile phospholipids. Transformation of 8:0 to long-chain FA in the intestinal cell has been indicated before (47), but the pathway has not yet been elucidated.

This study reveals a tendency toward faster lymphatic

recovery of exogenous 18:2n-6 when administered as MLM compared with LLL. A higher but insignificant endogenous lymphatic contribution of 18:2n-6 observed in rats fed L*L*L* compared with rats fed ML*M, ML*L*, or M*M*M* indicates that lipid structures affect the stimulation of the endogenous 18:2n-6 lymphatic contribution.

The results have implications for enteral feeding regimes using structured TAG to obtain the positive effects of long- and medium-chain FA (3). The present study supports the argument for using specific structured TAG for the rapid provision of PUFA and energy instead of using either long-chain TAG or medium-chain TAG.

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TG Containing Stearic Acid, Synthesized from Coconut Oil, Exhibit Lipidemic Effects in Rats Similar to Those of Cocoa Butter

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ABSTRACT: Lipase-catalyzed interesterification was used to prepare structured TG from coconut oil TG by partially replacing some of the atherogenic saturated FA with stearic acid, which is known to have a neutral effect on lipid levels in the body. The level of stearic acid was increased from 4% in the native coconut oil to 40% in the structured lipids, with most of the stearic acid being incorporated into the *sn*-1 and *sn*-3 positions of TG. When structured lipids were fed to rats at a 10% level for a period of 60 d, a 15% decrease in total cholesterol and a 23% decrease in LDL cholesterol levels in the serum were observed when compared to those fed coconut oil. Similarly, the total and free cholesterol levels in the livers of the rats fed structured lipids were lowered by 31 and 36%, respectively, when compared to those fed coconut oil. The TG levels in the serum and in the liver showed decreases of 14 and 30%, respectively, in animals fed structured lipids. Rats fed cocoa butter and structured lipids having a similar amount of stearic acid had similar lipid levels in the serum and liver. These studies indicated that the atherogenic potential of coconut oil lipids can be reduced significantly by enriching them with stearic acid. This also changed the physical properties of coconut oil closer to those of cocoa butter as determined by DSC.

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Dietary lipids influence lipid homeostasis in the body, the disturbance of which contributes to the etiology of diseases such as atherosclerosis and cholesterol cholelithiasis (1). Oils and fats from natural sources are mixtures of various types of TG that contain different combinations of FA moieties. Chain lengths of the FA residues, their degree of unsaturation, and their position on the glycerol backbone influence the physical and chemical properties of oils and fats. Fats and oils high in mixtures of saturated FA have been known to raise the concentration of plasma cholesterol, although the individual saturated FA do not contribute equally to this effect (2).

Coconut oil (CO), a widely consumed edible oil in the southern parts of India, contains medium-chain TG. It also has a very high saturated FA content, with the major FA being lauric (12:0), myristic (14:0), and palmitic (16:0) acids. These FA have varying degrees of hypercholesterolemic effects (3). Hence, this oil is referred to as being atherogenic, resulting in a very limited market as an edible oil. This necessitates the modification of the FA composition of CO so as to reduce its atherogenicity and enhance its nutraceutical value without changing its basic physical and organoleptic properties.

Nutritionists are of the opinion that no edible oil available in nature has the ideal combination and distribution of FA on their TG moieties. Enzymatic interesterification reactions using specific lipases have been used as a tool for the modification of vegetable oils and fats to correct the imbalances in FA compositon (4,5). The lipids thus structurally modified, in which the physical and chemical properties have been altered, are termed structured lipids (SL). They have been found to possess superior metabolic and health benefits when compared to oil or fat blends of a similar FA composition (6).

By using acidolysis reactions, we previously substituted some of the saturated FA in CO with stearic acid (18:0) (7). Research carried out on humans (8,9) and animals (10) has shown that stearic acid has a neutral effect on the cholesterol and TG levels in the body in contrast to the hypercholesterolemic effect of other long-chain saturated FA. The incorporation of stearic acid causes a physical change in CO from a semisolid into a hard fat at ambient temperature (7).

Stearic acid-rich fats such as cocoa butter (CB) are known to exhibit the unique property of being solid fats at ambient temperature but melting sharply in the temperature range of 25–35°C. This imparts a brittle solid form to the fat at room temperature, but it melts completely just below body temperature, which makes it an ideal fat for use in the manufacture of chocolates. The high cost of CB has prompted many investigators to search for more economical CB substitutes or CB extenders. CB has unique TG structures represented by POP, POSt, and StOSt (where P is palmitic acid, O is oleic acid, and St is stearic acid) on the glycerol backbone. Earlier, we were able to incorporate graded amounts of stearic acid into CO lipids by a lipase-catalyzed acidolysis reaction, which altered its physical properties as assessed by DSC (7). In the present investigation we incorporated stearic acid into CO TG under controlled conditions so as to obtain a fat whose levels of stearic acid were similar to those of CB. We then evaluated the solid fat content at various temperatures using a differential scanning calorimeter. The effect of this SL as a dietary source of fat on serum and liver lipids of rats was evaluated in comparison with that found in rats fed an equivalent amount of either CO or CB.

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Abbreviations: CB, cocoa butter; CO, coconut oil; SL, structured lipids.

MATERIALS AND METHODS

Materials. Refined CO was obtained from a local supermarket. CB was obtained from Campco India Ltd. (Puttur, India). Lipozyme IM60 from *Rhizomucor miehei* was obtained from Novo Nordisk Bioindustrials, Inc. (Danbury, CT), Cholesterol, triolein, dipalmitoyl-PC, and thiobarbituric acid were purchased from Sigma (St. Louis, MO). Stearic acid, heparin, and $MnCl_2$ were obtained from Sisco Research Laboratory (Mumbai, India). Digitonin was purchased from BDH Chemicals (Mumbai, India). All solvents used were of analytical grade and distilled before use.

Synthesis of SL. SL enriched in 18:0 were synthesized from CO by using enzymatic interesterification as described previously (7). Large-scale preparation of SL was carried out in a stirred batch reaction (11). Immobilized lipase, Lipozyme IM60, was used at 5% (w/w) of substrates to catalyze the enzymatic acidolysis. CO and stearic acid were used at a molar ratio of 1:0.5. Each batch consisted of 100 g of CO and 22 g of stearic acid with 6 g of immobilized lipase charged to a 1-L stoppered conical flask. Incubation was carried out at 37°C for 4 h with constant agitation. SL prepared in different batches were pooled. The TG obtained from the pooled reaction mixture after scale-up were purified by column chromatography. The reaction mixture was placed in a round-bottomed flask, and hexane was removed with a Büchi rotary evaporator (Postfach, Switzerland). A mixture of silica gel (100–200 mesh size) and alumina (20 g each) was activated at 200°C for 2 h and cooled in a desiccator. A slurry of this mixture was made in hexane and then packed in 4 × 35 cm glass columns. The sample (30 g) was loaded on the column and eluted with 350 mL of hexane/diethyl ether (95:5, vol/vol). The fractions containing TG, as detected by TLC (in a solvent system of petroleum ether/diethyl ether/acetic acid 80:20:1, by vol) were pooled and the solvent was removed in a vacuum rotary evaporator. The purity of TG was checked by rechromatography on TLC, and the repurified TG were pooled and stored in a dark bottle at −20°C under nitrogen.

Analysis of FA in the sn*-2 position of TG.* FA in the *sn*-2 position of TG were determined following the method of Luddy *et al.* (12). Briefly, 1 mg of TG was mixed with 1 mL of 1 M Tris-HCl buffer (pH 7.6), 0.25 mL of 0.05% sodium cholate, 0.1 mL of 2.2% $CaCl_2$, and 1 mg of pancreatic lipase. The mixture was incubated in a water bath at 37°C for 3 min, mixed vigorously for 1 min, centrifuged at 980 × *g*, extracted with 3 mL diethyl ether, and dried over anhydrous sodium sulfate. Aliquots were spotted on TLC plates along with the reference standard and developed in hexane/diethyl ether/acetic acid (50:50:1, by vol). The band corresponding to the 2-MAG reference standard was eluted with chloroform/methanol (2:1, vol/vol), saponified, methylated using BF_3 in methanol (13), and analyzed by GC (Shimadzu 14B, fitted with an FID) using a fused-silica capillary column (25 m × 0.25 mm, Parmabond FFAP-DF-0.25; Macherey-Nagel GmBH Co., Düren, Germany). The operating conditions were: column temperature, 160°C; injector temperature, 210°C; and detector temperature 250°C. Column temperature was programmed to rise at 6°C/min, and the final temperature was 240°C. Nitrogen gas (30 mL/min) was used as the carrier. Individual FA were identified by comparing with the retention times of authentic standards from Nu-Chek-Prep (Elysian, MN) and were quantified by an on-line Chromatopac CR-6A integrator. Individual FA were quantified using pentadecanoic acid as the internal standard.

Experimental animals. Animal experiments were conducted after receiving clearance from the Institutional Animal Ethical Committee according to norms laid down by the Government of India. Male Wistar rats [OUTB-Wistar, IND-cft (2c)], 50.0 ± 3.0 g, were grouped by random distribution. Three groups were formed consisting of seven rats each. Rats were placed in individual cages in an approved animal facility. The animals had free access to food and water throughout the study. Fresh diets were fed daily, and food intake and growth were monitored regularly.

Diet composition. The ingredients used in the basal diets were (g/100 g): casein 20, cellulose 5, sucrose 60, AIN-76 mineral mix 3.5, AIN-76 vitamin mix 1, methionine 0.3, choline chloride 0.2, and fat 10 (14). CO, CB, or SL was fed to rats at a 10% level in the diet. After 60 d, rats were fasted overnight and sacrificed under ether anesthesia. Blood was drawn by cardiac puncture and serum was separated by centrifugation. Livers were removed and rinsed in ice-cold saline, blotted, weighed, and stored at −20°C until analyzed.

Analysis of lipids. Lipids were extracted from serum and tissues by the method of Folch *et al.* (15). Serum lipid peroxide and TBARS in the liver were analyzed as described by Yagi (16) and Buege and Aust (17), respectively.

Serum and liver cholesterol levels were quantified by the method of Searcy and Bergquist (18). HDL cholesterol was measured after precipitating apolipoprotein B-containing lipoproteins with a heparin–$MnCl_2$ reagent (19). Free cholesterol in serum and liver was estimated after precipitating it with 0.5% digitonin (20).

Phospholipids were analyzed by the method of Stewart (21) using dipalmitoyl-PC as the reference standard. TG were estimated by the method described by Fletcher (22).

Fecal samples were collected for five consecutive days, dried, weighed, and the lipids extracted by the method of Folch *et al.* (15) after acidifying the samples.

The FA composition of dietary lipids, serum, tissue, and fecal lipids were analyzed by GC as methyl esters.

Analysis of the thermal profile. A differential scanning calorimeter [model DSC(+); Rheometric Scientific, Epson, United Kingdom] was used to determine the thermal profile and solid fat content of lipids as described previously (7).

Protein estimation. Protein was estimated in the serum and liver homogenates by the method of Lowry *et al.* (23) using BSA as the reference standard.

Statistical analysis. Data were expressed as mean ± SD. ANOVA was used to evaluate the differences between groups (24).

RESULTS

FA composition of dietary lipids. FA analysis of the dietary lipids showed that 12:0 was the predominant FA (49%), followed by 14:0 (24%) in CO (Table 1). The major FA detected at the *sn*-2 position of CO were 14:0, 12:0, and 16:0.

CB contained 23% 16:0, 37% 18:0, and 38% 18:1. The major FA at the *sn*-2 position of CB was 18:1 (72%), followed by 16:0 (13%). The SL synthesized from CO had 40% 18:0, 27% 12:0, and 11% 14:0. At the *sn*-2 position of SL, the major FA observed were 12:0 and 14:0, followed by 16:0 and 18:1. A similar level of 18:0 was observed in total lipids as well as at the *sn*-2 positions of CB and SL.

Effect of dietary lipids on growth parameters. The gain in body weight of rats fed different dietary lipids was not significantly different, although the SL- and CB-fed groups consumed slightly higher amounts of food than the CO-fed group (Table 2). The food efficiency ratio, calculated as the ratio of weight gained by the animal to the food consumed, was marginally lower in the rats fed SL and CB. There were no significant differences in the liver weights of rats fed different dietary lipids.

Serum lipid profile. The total cholesterol levels in the serum of CB- and SL-fed groups were similar but significantly lower by 11 and 15%, respectively, when compared to the CO-fed group (Table 3). The levels of free cholesterol in animals fed CB and SL were significantly lower by 12 and 20%, respectively, as compared to those fed CO. Cholesterol ester levels also decreased in the CB- and SL-fed groups. The levels of HDL cholesterol did not vary significantly among the different dietary groups. However, the levels of VLDL + LDL cholesterol were lowered by 20 and 23%, respectively, in the CB- and SL-fed groups as compared to the CO group. TG levels in the CB-fed group were lower by 22%, whereas the SL-fed group showed a decrease of 14% when compared to the CO-fed group. The phospholipid and lipid peroxidation levels were not significantly different among the rats fed different dietary lipids.

Table 4 shows the FA profile of serum lipids in rats fed different dietary lipids. Small amounts of 12:0 and 14:0 were detected in animals fed CO. 18:1 and 16:0 were the other major FA observed in this group. The levels of 18:0 were increased by 4.22- and 4.25-fold in animals fed SL and CB as compared to those given CO. However, the level of 18:1 was comparable in all groups. The levels of 18:2 and 20:4 were similar in animals fed CO and SL but showed significantly lower levels in animals fed CB.

Liver lipid profile. The levels of total cholesterol in the livers of animals fed CB and SL were lower by 44 and 31%, respectively, than in the livers of animals fed CO (Table 5). The levels of free cholesterol in the CB- and SL-fed groups showed decreases of 30 and 36%, respectively, when compared to the CO-fed group. Similarly, the levels of cholesterol esters in the CB and SL groups showed decreases of 47 and 27%, respectively, when compared to the CO-fed group. The TG levels in the liver were similar in the CB- and SL-fed groups but showed a 30% decrease when compared to the CO-fed group. The lipid peroxide levels in the CO-, CB-, and SL-fed groups were similar.

Table 6 shows the liver FA profile of animals given different dietary lipids. 12:0 and 14:0 were observed in small

TABLE 1
FA Composition (%) of Dietary Lipids[a]

	CO		CB		SL	
FA	Total	*sn*-2	Total	*sn*-2	Total	*sn*-2
8:0	1.9	—	—	—	—	—
10:0	2.7	1.8	—	—	1.5	1.8
12:0	48.8	29.8	—	—	27.1	27.8
14:0	23.6	30.1	—	—	11.0	28.9
16:0	8.5	15.6	22.5	13.3	6.8	15.2
18:0	3.6	3.0	37.4	6.6	40.4	5.9
18:1	9.0	12.5	38.4	71.9	10.0	12.7
18:2	1.8	6.6	1.5	8.1	2.0	8.9
SFA	89.1	80.3	60.0	19.9	79.9	79.6
MUFA	9.0	12.5	38.4	71.9	7.9	12.7
PUFA	1.8	6.6	1.5	8.1	1.0	8.9
P/S	0.02	0.08	0.025	0.40	0.012	0.11

[a]CO, coconut oil; CB, cocoa butter; SL, structured lipids enriched in 18:0; SFA, saturated FA; MUFA, monounsaturated FA; P/S, ratio of PUFA/saturated FA; —, below the detection limit of 0.5%.

TABLE 2
Growth Parameters of Rats Fed CO, CB, and SL[a]

Parameters analyzed	CO	CB	SL
Gain in body weight (g/60 d)	197 ± 14	206 ± 17	195 ± 12
Total food consumed (g)	410 ± 12[a]	479 ± 25[b]	485 ± 19[b]
Food efficiency ratio	0.48 ± 0.05	0.43 ± 0.06	0.40 ± 0.03
Liver weight (g/100 g body weight)	4.65 ± 0.4	4.83 ± 0.5	4.75 ± 0.5
Fecal lipids (mg/g)	6.88 ± 2.6	5.19 ± 1.2	7.21 ± 2.8

[a]Values show the mean ± SD of seven rats. Values in the same row with common superscript letters are not significantly different ($P < 0.001$). For abbreviations see Table 1.

TABLE 3
Effect of SL on the Serum Lipid Profile of Rats in Comparison with Those Fed CO and CB[a]

Parameters analyzed	CO	CB	SL
Total cholesterol (mg%)	74.5 ± 1.3[b]	65.8 ± 3.0[b]	63.0 ± 2.4[b]
Free cholesterol (mg%)	25.9 ± 0.8[a]	22.9 ± 0.7[b]	20.6 ± 0.8[b]
Cholesterol esters (mg%)	48.7 ± 1.5	43.0 ± 2.8	42.4 ± 2.4
HDL cholesterol (mg%)	22.7 ± 1.0	24.2 ± 1.2	23.3 ± 0.9
VLDL + LDL cholesterol (mg%)	51.9 ± 2.1[a]	41.5 ± 2.4[b]	39.7 ± 3.0[b]
TG (mg%)	169.9 ± 6.9[a]	132.4 ± 6.8[b]	145.6 ± 6.2[b]
Phospholipids (mg%)	100.1 ± 2.0	107.9 ± 2.8	97.7 ± 2.0
Lipid peroxidation (nmol/mg protein)	7.6 ± 0.4	7.4 ± 0.16	7.2 ± 0.2

[a]Values are the mean ± SD of seven rats. Values in the same row with common superscript letters are not significantly different ($P < 0.001$). For abbreviations see Table 1.

TABLE 4
Effect of SL on the Serum FA (%) Profile of Rats in Comparison with Those Fed CO and CB[a]

FA	CO	CB	SL
12:0	2.6 ± 0.5	ND	ND
14:0	3.0 ± 1.2	ND	ND
16:0	32.7 ± 1.3	33.5 ± 0.8	25.1 ± 0.5
18:0	3.1 ± 1.3[a]	41.6 ± 1.1[b]	38.0 ± 1.7[b]
18:1	39.8 ± 1.3	41.6 ± 1.1	38.0 ± 1.7
18:2	7.8 ± 1.8[a]	5.3 ± 2.3[b]	9.5 ± 0.6[a]
20:4	10.5 ± 0.4[a]	4.0 ± 1.1[b]	11.3 ± 1.8[a]

[a]Values are the mean ± SD of seven rats. Values in the same row with common superscript letters are not significantly different ($P < 0.001$). ND, not detected; for other abbreviations see Table 1.

amounts in both the CO- and SL-fed groups, whereas the levels of 18:0 in animals fed CB and SL were higher by 47 and 78%, respectively, when compared to that found in the CO-fed group. Ten to 20% higher levels of 18:1 were observed in the CB- and SL-fed groups, although the levels of 18:2 and 20:4 were comparable in all three groups.

Fecal lipids. The amount of 18:0 excreted in fecal matter was significantly higher in animals fed CB and SL than in those fed CO (Table 7). Similarly, high amounts of 12:0 and 14:0 were excreted by animals fed CO and SL. These studies indicated that the FA usually found in large quantities in dietary lipids were found at higher levels in the fecal lipids. The amounts of fecal lipids excreted were also comparable in all three groups of animals (6.43 ± 0.88 mg/g fecal matter, combined mean ± SD of 7 animals/group).

Thermal profile of lipids. Figure 1 shows the solid fat content of SL containing 40% stearic acid over solid fat content of CB containing 37% stearic acid and CO containing only 3.6% stearic acid. The solid fat content of CO was 86% at 10°C, 62% at 15°C, and 24% at 20°C. On the other hand, CB had a solid fat content of 100% at 25°C, which was reduced to 76% at 35°C and to 5% at 40°C. The solid fat content of

TABLE 5
Effect of SL on the Liver Lipid Profile of Rats in Comparison with Those Fed CO and CB[a]

Parameters analyzed	CO	CB	SL
Total cholesterol (mg/g)	7.21 ± 0.2[a]	4.01 ± 0.2[b]	4.96 ± 0.6[b]
Free cholesterol (mg/g)	3.48 ± 0.6[a]	2.42 ± 0.2[b]	2.24 ± 0.23[b]
Cholesterol esters (mg/g)	3.73 ± 0.6[a]	1.99 ± 0.1[b]	2.72 ± 0.6[c]
TG (mg/g)	12.84 ± 2.2[a]	8.97 ± 1.2[b]	8.89 ± 0.7[b]
Phospholipids (mg/g)	11.54 ± 1.8[a]	9.82 ± 1.3[b]	14.16 ± 1.1[c]
Lipid peroxidation (nmol/mg protein)			
Basal	1.67 ± 0.17[a]	2.33 ± 0.3[a]	21.58 ± 0.13[a]
Induced	2.19 ± 2.3[a]	3.16 ± 0.3[a]	2.49 ± 0.2[a]

[a]Values are the mean ± SD of seven rats. Values in the same row with common superscripts are not significantly different ($P < 0.001$). For abbreviations see Table 1.

TABLE 6
Effect of SL on the Liver FA (%) Profile of Rats in Comparison with Those Fed CO and CB[a]

FA	CO	CB	SL
12:0	1.8 ± 0.3	ND	0.8 ± 0.4
14:0	2.7 ± 0.5	0.82 ± 0.1	2.8 ± 0.1
16:0	28.9 ± 0.3	30.9 ± 1.3	25.4 ± 0.9
16:1	1.8 ± 0.3	0.7 ± 0.1	0.8 ± 0.1
18:0	4.9 ± 0.8[a]	7.2 ± 0.7[b]	8.7 ± 1.2[c]
18:1	34.0 ± 2.3[a]	37.4 ± 1.5[b]	40.7 ± 1.8[a]
18:2	8.8 ± 1.2	9.68 ± 0.08	10.8 ± 1.4
20:4	13.8 ± 2.1	12.86 ± 1.1	14.0 ± 2.5

[a]Values are the mean ± SD of seven rats. Values in the same row with common superscripts are not significantly different ($P < 0.001$). For abbreviations see Tables 1 and 4.

SL containing stearic acid ranged between that of CB and that of CO: It had a solid fat content of 64% at 25°C, which decreased to 18% at 35°C, 5% at 40°C, and 0% at 45°C.

DISCUSSION

In the present study, SL enriched in stearic acid were synthesized from CO TG by using enzymatic acidolysis. CO is by nature rich in 12:0, 14:0, and 16:0, all of which are considered to have atherogenic effects. These FA were partially replaced by 18:0, which is known to have a neutral effect on serum lipids. Under controlled conditions, the level of 18:0 in CO TG was increased from 4 to 40% to obtain a lipid having a stearic acid content similar to that of CB. The SL containing 18:0 had a melting profile and a solid fat content between those of CO and CB.

The positional distribution of FA in TG indicated that whereas the *sn*-2 position of CB is predominantly occupied by oleic acid, that of SL contained a mixture of 12:0, 14:0, and 16:0. The distribution of FA in the *sn*-2 position of SL was similar to that of CO, indicating that the glycerol backbone of the original oil was retained in the SL. However, unlike CO, the SL contained a level of 18:0 similar to that of CB. Like CB, 18:0 in the SL was found mostly in the *sn*-1,3 positions.

When CB or SL was given as a source of dietary fat, the gain in body weight was similar to that observed in CO-fed animals. However, the amount of food consumed by animals fed CB or SL was higher than that of the CO-fed group. Sim-

TABLE 7
Effect of SL on the Fecal FA Profile of Rats in Comparison with Those Fed CO and CB[a]

FA	CO	CB	SL
12:0	12.2 ± 2.5	ND	10.31 ± 2.1
14:0	2.2 ± 3.1	ND	16.1 ± 2.8
16:0	26.1 ± 2.2	36.8 ± 3.8	28.52 ± 2.5
18:0	10.7 ± 1.3	38.4 ± 1.8	33.76 ± 2.5
18:1	23.4 ± 0.8	14.3 ± 2.1	7.50 ± 0.7
18:2	5.8 ± 0.6	10.5 ± 1.1	3.1 ± 0.8

[a]Values are the mean ± SD of seven rats. For abbreviations see Tables 1 and 4.

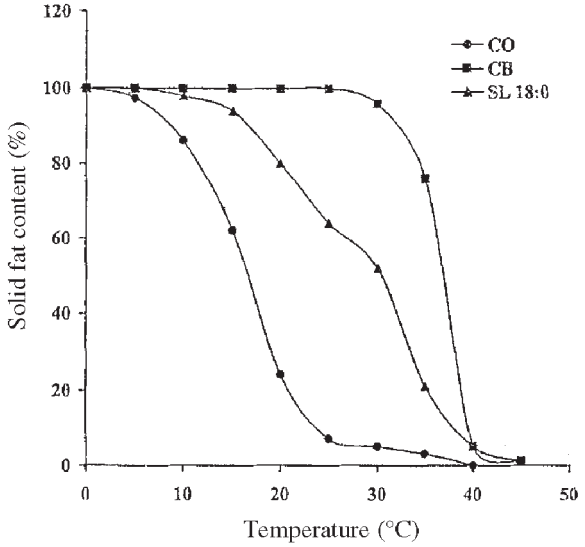

FIG. 1. Percent solid fat content of coconut oil (CO), cocoa butter (CB), and structured lipid enriched with 18:0 (SL).

ilar results were reported earlier, in which rats consumed more of a stearate-rich diet but showed the same growth rate as control animals (25). Stearate is thought to be absorbed poorly in humans (26); however, in our studies, even though higher levels of stearate were excreted in the fecal matter of animals given CB- or SL-containing diets, there was no significant difference in the amount of total lipids excreted among the different dietary groups. This indicates that stearate-containing lipids were absorbed as efficiently as CO lipids. The higher levels of stearic acid found in fecal lipids indicate the abundant nature of FA present in the diets fed to animals.

Rats fed either CB or SL enriched in 18:0 had lower levels of cholesterol and TG in serum as compared to animals fed CO. Most of the decrease in cholesterol levels was reflected in the LDL fraction but did not alter the HDL cholesterol fractions. These studies are in agreement with those reported previously (9,27,28). There is general agreement that, unlike palmitic and myristic acids, stearic acid does not raise serum cholesterol levels. Several mechanisms have been postulated for the neutral effects of stearic acid-containing lipids on lipid profiles in humans and in experimental animals (29). Stearic acid-containing diets are, in general, poorly digested and absorbed. However, more recent studies have indicated that as a part of tristearin, stearic acid has impaired absorption, but as mono- and distearin glycerides or when present in a mixture of TG, stearic acid is almost completely absorbed by rats and human beings (8,28). In our study, the amount of lipids excreted in the fecal matter of rats was comparable in animals given CO, CB, or SL enriched in 18:0, indicating that the absorption mechanism for these lipids was not impaired. Further, the increase in stearic acid levels in serum and liver lipids indicates that stearic acid is well absorbed from the diet and is utilized efficiently for metabolic processes.

Stearic acid is also considered to be desaturated rapidly to oleic acid. Because of this, even though stearic acid is a saturated FA, it does not suppress the rate of receptor-dependent LDL removal from plasma and also may not increase the rate of LDL cholesterol production (26,30). Further, conversion of stearic to oleic acid would increase the unsaturation index of tissue phospholipids and the fluidity of cell membranes, which in turn may enhance the receptor-mediated clearance of LDL (31). In our studies, the level of 18:1 in serum lipids was not affected and was marginally increased by 20 and 10%, respectively, in liver lipids after feeding diets containing CB or SL. However, the levels of cholesterol esters in serum as well as in hepatic tissues were significantly reduced after feeding 18:0-enriched diets. Stearic acid is a poor substrate for esterifying enzymes such as LCAT and ACAT.

When included in the diet as the sole source of fat, CB or SL enriched in 18:0 lowered the cholesterol and TG levels in serum as well as in hepatic tissues compared to animals given CO. This study also indicated that even though a fat might be enriched in a saturated FA such as stearic acid, the serum lipid levels might not increase. Further, incorporation of stearic acid into CO TG can significantly reduce its atherogenic potential and render it a neutral fat. Therefore, a lipase-catalyzed acidolysis reaction can be used as a tool to modulate the atherogenic potential of CO without significantly affecting the physical nature of the lipid.

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Enzyme-Resistant Fractions of Beans Lowered Serum Cholesterol and Increased Sterol Excretions and Hepatic mRNA Levels in Rats

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ABSTRACT: Feeding rats beans with resistant starch reduces their serum cholesterol concentration; however, the mechanism by which this occurs is not fully understood. We examined the effects of enzyme-resistant fractions of adzuki (*Vigna angularis*) and tebou (*Phaseolus vulgaris*, var.) beans on serum cholesterol and hepatic mRNA in rats. Rats were fed a cholesterol-free diet with 50 g of cellulose powder (CP)/kg, 50 g of an enzyme-resistant fraction of adzuki starch (AS)/kg, or 50 g of an enzyme-resistant fraction of tebou starch (TS)/kg diet for 4 wk. There were no significant differences in body weight, liver weight, and cecum contents among the groups, nor was there a significant difference in food intake among the groups. The levels of serum total cholesterol, VLDL + intermediate density lipoprotein + LDL-cholesterol, and HDL cholesterol in the AS and TS groups were significantly ($P < 0.05$) lower than in the CP group throughout the feeding period. Total hepatic cholesterol in the CP group was significantly ($P < 0.05$) lower than in the AS and TS groups, fecal cholesterol excretion in the TS group was significantly ($P < 0.05$) greater than in the CP and AS groups, and the fecal total bile acid concentrations in the AS and TS groups were significantly ($P < 0.05$) higher than in the CP group. Cecal acetate, propionate, and *n*-butyrate concentrations in the AS and TS groups were significantly ($P < 0.05$) higher than in the CP group. The level of hepatic scavenger receptor class B1 (SR-B1) mRNA in the TS group was significantly ($P < 0.05$) higher than in the CP group, and the levels of hepatic cholesterol 7α-hydroxylase mRNA in the AS and TS groups were significantly ($P < 0.05$) higher than in the CP group. These results suggest that AS and TS have a serum cholesterol-lowering function due to the enhanced levels of hepatic SR-B1 and cholesterol 7α-hydroxylase mRNA.

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Some starch in the normal diet escapes digestion and assimilation in the human small intestine. This starch was termed resistant starch (RS) by EURESTA (1). Like dietary fiber, RS is also known to ferment in the large intestine to produce short-chain FA (SCFA), which may be involved in lowering serum cholesterol concentrations (2). Another mechanism suggested for this hypocholesterolemic effect has focused on enhanced fecal steroid excretion leading to an up-regulation of bile acid biosynthesis (3); however, there are no reports on the relationship between feeding RS and gene expression or activity of cholesterol 7α-hydroxylase.

Beans are unique foods, rich in complex carbohydrates, protein, dietary fiber, and starch. Relatively few studies have investigated the digestibility of RS from beans in the small intestine of humans (4) and the content of SCFA in the hindgut of rats fed processed bean flours (5). Our previous report also demonstrated the hypocholesterolemic effect of adzuki (*Vigna angularis*) or tebou (*Phaseolus vulgaris*, var.) starch compared with a cornstarch diet (6). However, neither retrograded bean starches increased the excretion of fecal sterols (6). On the other hand, antihyperlipidemic activities of RS might be due to the acceleration of bile acid excretion (3,7). Recently, physiological approaches for *in vitro* determination of the indigestible fraction in foods have been reported (8–11). These methods include incubation with pepsin followed by further incubation with a mixture of porcine pancreatic enzyme and amylglucosidase. However, there is very little published information on the physiological properties of the above-mentioned indigestible fraction (e.g., RS or protein). Therefore, to understand the potential mechanism of the hypocholesterolemic action of bean RS, an *in vivo* physiological investigation of the enzyme-resistant fraction was necessary.

In this study, we investigated the effect on serum lipids, liver lipids, fecal lipids, and hepatic mRNA of the enzyme-resistant fractions of adzuki and tebou beans made under the same physiological conditions by incubation with pepsin and pancreatic enzymes.

MATERIALS AND METHODS

Animals. Male F344/DuCrj rats (8 wk old) were purchased from Charles River Japan Inc. (Yokohama, Japan). Rats were housed individually in cages with *ad libitum* access to food and water. The animal facility was maintained on a 12-h light/dark cycle, and the temperature was maintained at 23 ± 1°C with 60 ± 5% RH. Animals were randomly assigned to three groups of

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Abbreviations: AS, enzyme-resistant fraction of adzuki starch; CP, cellulose powder; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IDL, intermediate density lipoprotein; RS, resistant starch; RT-PCR, reverse transcription-polymerase chain reaction; SCFA, short-chain FA; SR-B1, scavenger receptor class type B1; TS, enzyme-resistant fraction of tebou starch.

five each. There were no significant differences among groups in body weight and serum total cholesterol concentrations at the start of the experiment. Body weight was recorded weekly and feed consumption was recorded daily. This experimental design was approved by the Animal Experiment Committee of Obihiro University of Agriculture and Veterinary Medicine. All animal procedures described conformed to NIH guidelines (12).

Preparing the enzyme-resistant fractions of beans. Enzyme-resistant fractions of beans were prepared by following the method of Åkerberg *et al.* (8), as modified in our laboratory. Sample preparation and analysis were carried out using the same process. First, raw beans were washed and boiled at 93°C for 2 h. The boiled beans were allowed to reach room temperature by adding cool water. The product was then filtered to separate the bean hulls. The yield of dried filtrate was approximately 79% based on weight. Next, the filtrate fraction was incubated at 37°C for 2 h 30 min with 0.1 N HCl buffer containing 0.5% pepsin. After incubation, 1 N NaOH solution was added to stop the reaction. After being centrifuged at 13,000 × *g* for 15 min, the residue was incubated at 37°C for 20 h with 0.1 M phosphate buffer containing 0.8% pancreatin and 2.1% potassium sorbate. After incubation, the enzyme reaction was stopped by adding HCl, and the pH was adjusted to 4.0. The sample was centrifuged at 13,000 × *g* for 15 min and the supernatant was removed. The indigestible residue in the crucible was washed with ethanol and acetone to remove residual water. The residue remaining in the crucible was dried in an oven at 50°C for 15 h and then cooled in a desiccator. Thereafter, the RS was analyzed by the modified method of Prosky *et al.* (13), and protein, lipid, carbohydrate, moisture, and ash contents were determined by AOAC procedures (14). Compositions of the enzyme-resistant fractions of adzuki and tebou (g/100 g) were as follows: moisture, 9.9 and 7.5; protein (N × 6.25), 20.0 and 9.1; lipid, 0.6 and 1.2; ash, 2.8 and 2.8; RS, 51.2 (insoluble RS, 39.7; water-soluble RS, 11.5) and 62.7 (insoluble RS, 44.9; water-soluble RS, 17.8); carbohydrate, 66.7 and 79.4.

The composition of each diet is shown in Table 1. All diets were based on the AIN-76 purified rodent diet (15) in which carbohydrates account for 603 g/kg, protein accounts for 250 g/kg, and fat accounts for 50 g/kg. The experimental groups were fed a diet that contained either 50 g/kg of enzyme-resistant adzuki starch (AS) or tebou starch (TS) for 4 wk, and a control group of rats was fed 50 g/kg of cellulose powder (CP). The Hokkaido Tokachi Area Regional Food Processing Technology Center (Obihiro, Hokkaido, Japan) kindly provided the adzuki and tebou beans.

Analytical procedures. Blood samples (1 mL) were collected from the jugular veins of fasting rats between 0800 and 1000 h. Samples were taken into tubes without an anticoagulant. After the samples stood at room temperature for 2 h, serum was prepared by centrifugation at 1500 × *g* for 20 min. At the end of the experimental period of 4 wk, all fecal excretions were collected for 2 d. Fecal weights did not differ significantly among groups (CP, AS, and TS: 2.74 ± 0.33, 2.82 ± 0.37, and 3.62 ± 1.11 wet g/d, respectively). The rats were sacrificed by ether inhalation, and the liver and cecum were quickly removed, washed with cold saline, blotted dry on filter paper, and weighed before freezing for storage.

Chemical analysis. Total cholesterol and HDL cholesterol concentrations in the serum were determined enzymatically using commercially available reagent kits (assay kits for the TDx system; Abbott Laboratory Co., Irving, TX). The VLDL + intermediate density lipoprotein (IDL) + LDL cholesterol concentration was calculated as follows: [VLDL + IDL + LDL cholesterol] = [total cholesterol] – [HDL cholesterol].

Total lipids were extracted from liver and feces by using a mixture of chloroform/methanol (2:1, vol/vol) (16). The neutral sterol portion in each total lipid sample obtained by saponification was acetylated (17) and analyzed by GLC using a Shimadzu 14A chromatograph (Kyoto, Japan) with a DB17 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA) with nitrogen as the carrier gas. Acidic sterols in feces were measured by GLC following the method of Grundy *et al.* (18). Individual SCFA in the cecum were measured by GLC with a

TABLE 1
Composition of the Control and Experimental Diets

Ingredients	Dietary group		
	CP	AS	TS
	(g/kg diet)		
Casein	250	250	250
Corn oil	50	50	50
Minerals (AIN-76)[a]	35	35	35
Vitamins (AIN-76)[b]	10	10	10
Cellulose powder (CP)	50	—	—
Adzuki starch (AS)[c]	—	50	—
Tebou starch (TS)[d]	—	—	50
Choline chloride	2	2	2
Sucrose	603	603	603

[a]AIN-76 mineral mixture (15).
[b]AIN-76 vitamin mixture (15).
[c]As, enzyme-resistant fraction of adzuki starch.
[d]TS, enzyme-resistant fraction of tebou starch.

glass column (2000 × 3 mm) packed with 80–100 mesh Chromosorb W-AW DMCS with H_3PO_4 (100 mL/L) as the liquid phase after adding H_3PO_4 by the procedure of Hara *et al.* (19).

RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR), and Southern blot analysis. Total RNA was isolated by the acid guanidium/phenol/chloroform method, using Isogen (Nippon Gene, Tokyo, Japan) from liver (20). mRNA encoding HMG-CoA reductase, LDL receptors, cholesterol 7α-hydroxylase, scavenger receptor class B type 1 (SR-B1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as an invariant control) were analyzed by semiquantitative RT-PCR and subsequent Southern hybridization of PCR products with each inner oligonucleotide probe as described previously (21). The primers used for PCR were as follows: for SR-B1, the upstream primer was 5′-GTAGGGCCCAGAAGACACCAC-3′ and the downstream primer was 5′-CCTGCCACCGCTGCCACTTAC-3′. Blots were hybridized with an SR-B1 probe of a 54-base oligonucleotide (5′-TGCCGTGTGGACAGTGTGACATCTGGGGGG-CTCAGGACGTGGCACTGGCGGGTTG-3′). The relative quantity of mRNA was estimated by densitometry scanning with X-ray film.

Statistical analysis. Data are presented as means and SD. The mean and SD for serum total cholesterol, HDL cholesterol, and VLDL + IDL + LDL cholesterol for each time point were calculated. The significance of differences among treatment groups was determined by ANOVA with Duncan's multiple-range test (SAS Institute, Cary, NC). Differences were considered significant at $P < 0.05$.

RESULTS

Feed intake, rat growth, and liver weight. No differences were observed in the body weight, feed intake, liver weight, and cecum contents among the groups (Table 2). The cecum pH in all groups also showed no significant difference.

Tissue lipid concentration. Figure 1 shows the serum total cholesterol, VLDL + IDL + LDL cholesterol, and HDL cholesterol concentrations in rats fed each enzyme-resistant fraction. The serum total cholesterol, VLDL + IDL + LDL cholesterol, and HDL cholesterol concentrations in the AS and TS groups were significantly ($P < 0.05$) lower than in the CP group throughout the feeding period. The liver cholesterol concentration in the CP group was significantly ($P < 0.05$) lower than that in the AS and TS groups (Table 2).

Hepatic mRNA. The relative quantities of mRNA were determined by Southern hybridization of PCR-amplified HMG-CoA reductase cDNA, LDL receptor cDNA, cholesterol 7α-hydroxylase cDNA, and SR-B1 cDNA in the rat liver. The values of HMG-CoA reductase, LDL receptors, cholesterol 7α-hydroxylase, and SR-B1 mRNA were normalized to the value of GAPDH mRNA. The values of the enzyme-resistant fractions of bean-fed rats were expressed relative to the range values of the CP diet group, which were normalized to 100. The relative quantities of HMG-CoA reductase mRNA (CP, AS, and TS: 100 ± 19, 96 ± 25, and 90 ± 24, respectively) and LDL receptor mRNA (CP, AS, and TS: 100 ± 48, 138 ± 57, and 151 ± 38, respectively) were unaffected by diet. However, LDL receptor mRNA level in the AS and TS groups tended to be elevated compared to the CP group. The relative levels of hepatic cholesterol 7α-hydroxylase mRNA (Fig. 2) in the AS and TS groups were significantly ($P < 0.05$) higher than in the CP group. In addition, the level of hepatic SR-B1 mRNA (Fig. 2) in the TS group was significantly ($P < 0.05$) higher than in the CP group, and the level of hepatic SR-B1 mRNA in the AS group tended to be elevated compared with the CP group.

Cecal SCFA and fecal lipid concentrations. Table 2 shows the SCFA concentrations in rat cecum. Cecal acetate, propionate, and *n*-butyrate concentrations in the AS and TS groups were significantly ($P < 0.05$) higher than in the CP group, and the cecal total SCFA concentrations in the AS and TS groups were also significantly ($P < 0.05$) higher than in the CP group.

Table 3 shows the cholesterol and bile acid concentrations in the feces of rats. Fecal cholesterol excretion in the TS group was significantly ($P < 0.05$) higher than in the CP and AS groups. Furthermore, the fecal cholic, chenodeoxycholic, deoxycholic,

TABLE 2
Body Weight, Food Intake, Liver Weight, Cholesterol Concentration, Cecum Contents, Short-Chain FA (SCFA) Concentrations, and pH in Rats Fed CP, AS, or TS for 4 wk[a]

	Dietary group		
Component	CP	AS	TS
Initial body weight (g)	150 ± 3[a]	148 ± 3[a]	149 ± 9[a]
Body weight gain (g/4 wk)	74 ± 6[a]	78 ± 9[a]	81 ± 9[a]
Food intake (g/4 wk)	367 ± 23[a]	358 ± 26[a]	389 ± 20[a]
Liver weight (g/100 g body weight)	10.0 ± 0.4[a]	8.8 ± 0.9[a]	9.0 ± 1.1[a]
Liver cholesterol (μmol/g)	1.85 ± 0.25[a]	3.27 ± 0.39[b]	3.11 ± 0.62[b]
Cecal content (g/100 g body weight)	3.6 ± 0.5[a]	3.1 ± 0.4[a]	3.2 ± 0.5[a]
Cecal total SCFA (μmol/g content)	26.0 ± 8.9[a]	116.7 ± 32.4[b]	99.4 ± 44.3[b]
Cecal acetic acid (μmol/g content)	16.6 ± 5.3[a]	79.5 ± 36.1[b]	59.1 ± 33.0[b]
Cecal propionic acid (μmol/g content)	5.3 ± 4.9[a]	15.0 ± 6.9[b]	15.4 ± 6.0[b]
Cecal butyric acid (μmol/g content)	4.1 ± 1.8[a]	22.2 ± 5.4[b]	24.9 ± 6.9[b]
Cecal pH	7.16 ± 0.11[a]	6.95 ± 0.13[a]	6.94 ± 0.17[a]

[a]Values are expressed as means ± SD for five rats. Means within the same rows bearing different superscript roman letters are significantly different ($P < 0.05$). For other abbreviations see Table 1.

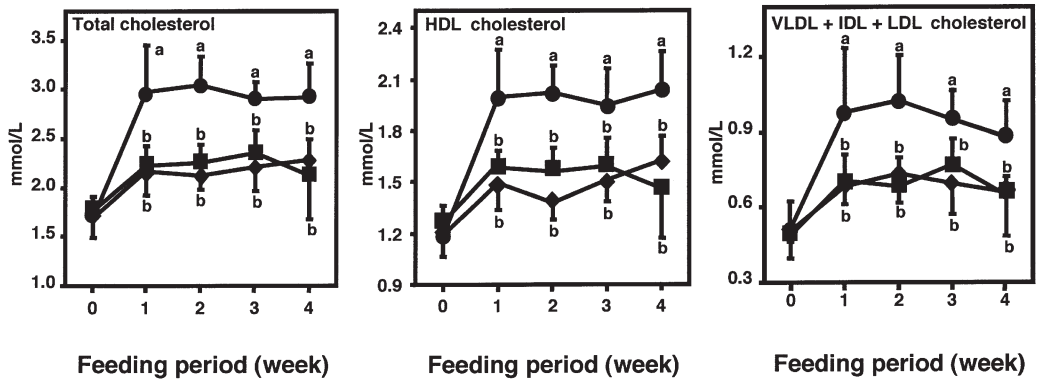

FIG. 1. Serum total cholesterol, VLDL + intermediate density lipoprotein (IDL) + LDL cholesterol, and HDL cholesterol concentrations in rats fed bean CP (●), AS (■), or TS (◆) for 4 wk. Each value represents the mean and SD for data obtained from five animals. Mean values (a, b) were significantly different ($P < 0.05$), as determined by ANOVA with Duncan's multiple-range test. CP, cellulose powder; AS, enzyme-resistant fraction of adzuki starch; TS, enzyme-resistant fraction of tebou starch.

and lithocholic acid concentrations in the AS and TS groups were significantly ($P < 0.05$) greater than in the CP group, and the total bile acid contents in the AS and TS groups were significantly ($P < 0.05$) higher than in the CP group.

DISCUSSION

In the present study, we examined the effects of feeding enzyme-resistant fractions of beans on serum cholesterol and hepatic mRNA levels in rats. Serum total cholesterol concentrations in the AS and TS groups were significantly lower than in the CP group. Most of the serum cholesterol in rats is associated with the HDL fraction (22), and it has been suggested that lowering the HDL cholesterol concentration may be an important factor in lowering the serum total cholesterol concentration. In fact, the serum HDL cholesterol concentrations in the AS and TS groups were significantly lower than in the CP group. The lower HDL cholesterol concentrations in the AS and TS groups may be related to the accelerated removal of HDL cholesterol *via* the hepatic SR-B1, because substituting an enzyme-resistant fraction for cellulose in the diet promoted SR-B1 mRNA expression. Dietary fiber and RS have been reported to decrease or have no effect on liver cholesterol concentration (3,21,23). However, the liver cholesterol concentration in the groups fed the enzyme-resistant fractions of beans was significantly higher than in the CP group, and the expression of hepatic HMG-CoA reductase mRNA among the groups in the present experiment was unaffected, consistent with our previous study (6). Although the reason for this result remains unclear, it may be speculated that the increase in liver cholesterol concentration was due to an enhanced level of hepatic SR-B1 mRNA.

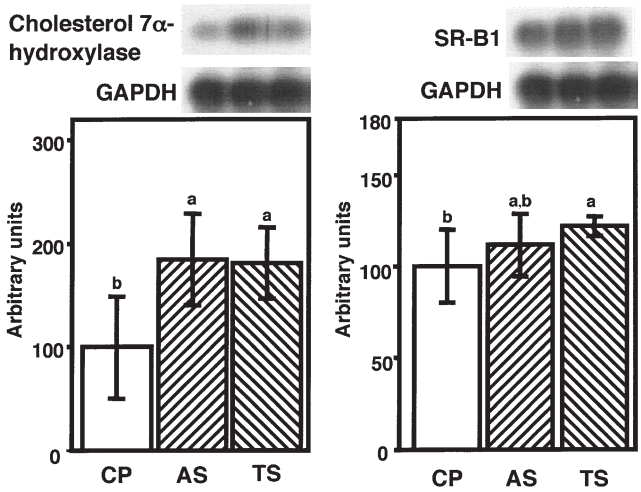

FIG. 2. Hepatic cholesterol 7α-hydroxylase mRNA and scavenger receptor class type B1 (SR-B1) mRNA expressions in rats fed various enzyme-resistant fractions of beans for 4 wk. Each value represents the mean and SD for data obtained from five animals. Mean values were significantly different ($P < 0.05$), as determined by ANOVA with Duncan's multiple-range test. The values of 7α-hydroxylase and SR-B1 mRNA were normalized to the value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and values for the rats fed AS and TS diets are expressed relative to the average values for rats fed the CP diet, which was set to 100. Inset illustrates the representative Southern hybridization of polymerase chain reaction-amplified 7α-hydroxylase and SR-B1 cDNA of hepatic RNA. For abbreviations see Figure 1.

The hypocholesterolemic effect of dietary fiber has been attributed to its ability to inhibit intestinal absorption of bile acids and neutral steroids, resulting in greater fecal bile acid and total steroid excretions. Moundras *et al.* (24) reported that the plasma cholesterol-lowering effect of dietary fiber was derived from the increased fecal loss of steroids, and others (3,25) have reported that the antihyperlipidemia activities of RS were due to the acceleration of bile acid excretion in animals. Buhman *et al.* (26) also demonstrated that feeding psyllium to rats enhanced the level of hepatic cholesterol 7α-hydroxylase mRNA as well as fecal bile acid and total steroid excretions. In this study, feeding enzyme-resistant starch fractions significantly increased fecal bile acid and hepatic cholesterol 7α-hydroxylase mRNA levels

TABLE 3
Fecal Steroid Concentrations in Rats Fed CP, AS, or TS for 4 wk[a]

Component	Dietary group		
	CP	AS	TS
	(μmol/100 g body weight/d)		
Cholesterol	2.54 ± 0.51[a]	2.65 ± 0.81[a]	6.66 ± 1.57[b]
Cholic acid	<0.01	0.01	<0.01
Chenodeoxycholic acid	<0.01	0.04 ± 0.01	0.03 ± 0.02
Deoxycholic acid	0.02 ± 0.01[b]	0.10 ± 0.03[a]	0.11 ± 0.05[a]
Lithocholic acid	0.03 ± 0.01[b]	0.14 ± 0.04[a]	0.17 ± 0.09[a]
Total bile acid	0.05 ± 0.03[b]	0.28 ± 0.07[a]	0.31 ± 0.14[a]

[a]Values are expressed as means ±SD for five rats. Means within the same rows bearing different superscript roman letters are significantly different ($P < 0.05$). For abbreviations see Table 1.

compared with the CP group. An increase in cecal SCFA concentration was observed in response to the elevated excretion of bile acids in rats fed AS and TS. Some investigators (3,23) have reported that feeding RS to rats lowered the plasma cholesterol concentration and increased the cecal SCFA concentration and fecal bile acid excretion. Although it is not clear whether SCFA potentiates an enhanced fecal steroid excretion, Illman *et al*. (27) reported that SCFA was positively correlated with the cecal neutral steroids and bile acids. Furthermore, a putative role of SCFA in mediating the cholesterol-lowering effect of fiber has been proposed (2), probably in relation to an inhibition of the metabolism of the major lipogenic precursors such as acetate or lactate (28,29). In this study, the cecal total SCFA level was positively correlated with the fecal total bile acid concentration, the correlation coefficient (r) being 0.744 ($P < 0.01$) (Fig. 3). Therefore, the effect of RS on serum cholesterol concentrations may reflect an accelerated fecal excretion of bile acid due to the enhanced cecal SCFA concentration.

In conclusion, in rats fed the enzyme-resistant fractions of adzuki and tebou beans, the hypocholesterolemic effects were evident compared with rats fed a cellulose diet. The enzyme-resistant fractions elevated fecal acidic steroid excretions and reduced the concentrations of serum total cholesterol, HDL cholesterol, and VLDL + IDL + LDL cholesterol. The cholesterol-lowering effect of enzyme-resistant fractions appears to be due to the enhanced level of cholesterol 7α-hydroxylase mRNA in the AS and TS groups, the level of SR-B1 mRNA in the AS group, and fecal steroid excretion accelerated by SCFA in the AS and TS groups.

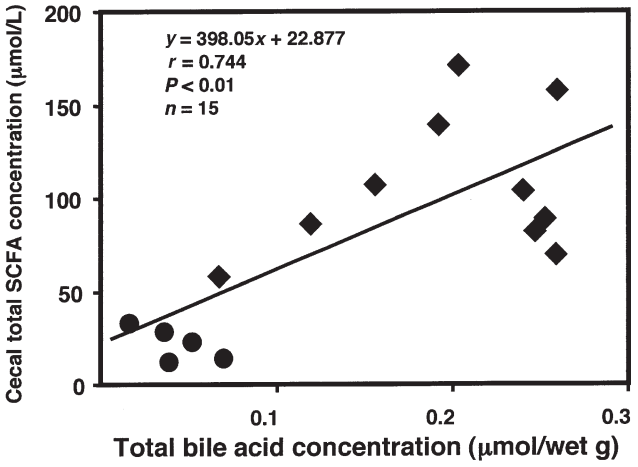

FIG. 3. Correlation between fecal total bile acid concentration and cecal total short-chain FA (SCFA) concentration in the control group (●) and two experimental groups (◆) for 4 wk. The values were significantly different ($P < 0.01$), as determined by simple correlations.

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Tissue Deposition of n-3 FA Pathway Intermediates in the Synthesis of DHA in Rainbow Trout *(Oncorhynchus mykiss)*

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ABSTRACT: The tissue distribution of newly synthesized 22:6n-3 and intermediate PUFA was examined in rainbow trout to further our understanding of the metabolism of this EFA in fish. Rainbow trout were fed a pulse of deuterated linolenic acid (D_5-17,17,18,18,18-18:3n-3), and the tissue distribution of deuterated anabolites was determined at intervals up to 35 d post-dose by GC-negative chemical ionization MS of the pentafluorobenzyl derivatives. D_5-22:6n-3 was the major deuterated FA in liver and cecal mucosa 2 and 5 d post-dose. All the n-3 FA pathway intermediates were found in liver, cecal mucosa, and blood including D_5-24:5n-3 and D_5-24:6n-3. Brain and eyes also contained the full suite of intermediate deuterated FA, but with a different profile from liver when analyzed over a longer time course up to 35 d. D_5-20:5n-3 was the major component in brain up to 7 d, after which D_5-22:6n-3 became predominant, but D_5-22:5n-3 constituted *ca.* 20% of FA throughout the time period. The pattern in eyes was similar but less pronounced. In visceral adipose tissue there was a much greater accumulation of the initial substrate, D_5-18:3n-3, with D_5-18:4n-3 and D_5-22:6n-3 the predominant deuterated FA at all time points. There was a similar though less pronounced trend in eye socket adipose tissue. The C_{24} PUFA were not detected in visceral fat and barely detected in eye socket fat. The results show that the kinetics of accumulation and depletion of the various n-3 PUFA differ between tissues. The presence of pathway intermediate FA provides evidence that liver and ceca possess the full metabolic pathway for synthesis of 22:6n-3, whereas brain and eyes are less active, with an accumulation of pentaene intermediate FA, and adipose tissue is inactive.

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The importance of 22:6n-3 in the development and function of the brain and retina in vertebrates is well recognized (1). However, the chemistry underpinning the unique biological roles of 22:6n-3 is still not understood despite much recent work on the physicochemical properties of PUFA-containing phospholipids (2). In spite of its importance, there is little information on the rate of synthesis of 22:6n-3 from its 18-carbon precursor α-linolenic acid. Regarding many species, there is continuing debate on the quantitative requirement for 22:6n-3 and the bioequivalence of 18:3n-3 and 22:6n-3, i.e., the amounts of 18:3n-3 or 22:6n-3 that are required in the diet to satisfy the requirement for 22:6n-3.

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Abbreviations: DFA, deuterated fatty acid; FAEE, fatty acid ethyl ester.

We have recently measured the rate of 22:6n-3 synthesis from 18:3n-3 in rainbow trout using a deuterated tracer with quantitation by GC-negative chemical ionization-MS (3). The accumulation of newly synthesized 22:6n-3 in the whole fish peaked at about 14 d, giving a rate of 0.54 μg/g fish/mg 18:3 eaten/d over the first 7 d with peaks in liver and brain achieved at days 7 and 24 post-dose, respectively (3). By using the same technique, we found the pyloric ceca to accumulate newly synthesized 22:6n-3 faster and at a higher concentration than liver, indicating that pyloric ceca were also an important site of 22:6n-3 synthesis (4).

The synthesis of 22:6n-3 in vertebrates is now known to occur *via* C_{24} PUFA intermediates with elongation of 22:5n-3 to 24:5n-3, Δ6-desaturation to 24:6n-3, and finally peroxisomal β-oxidation to give 22:6n-3 (5). In the present study the n-3 PUFA pathway intermediates 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3, 24:5n-3, and 24:6n-3 were measured in liver and ceca, tissues active in the synthesis of 22:6n-3; in brain and eyes, tissues that accumulate 22:6n-3 and may be able to synthesize PUFA; and in adipose tissue, which is not thought to synthesize 22:6n-3. Eye socket adipose tissue was examined since this is a significant adipose deposit in salmonids and tuna orbital oil contains a high concentration of 22:6n-3 (6). This could be a source of 22:6n-3 for the retina. The tissues chosen for analysis were therefore expected to show different patterns of PUFA accumulation and depletion consistent with their differing functions and requirements for 22:6n-3.

MATERIALS AND METHODS

Chemicals. Chloroform, methanol, ethanol, isohexane, and diethyl ether were HPLC grade from Fisher (Loughborough, Leicestershire, United Kingdom). Diisopropylamine, anhydrous acetonitrile, and pentafluorobenzyl bromide were obtained from Aldrich (Gillingham, Dorset, United Kingdom). D_5-17,17,18,18,18-linolenic acid was purchased from Cambridge Isotope Laboratories (Andover, MA) as the FA ethyl ester (FAEE). Linseed oil was from ICN (Basingstoke, Hampshire, United Kingdom) and refined olive oil from Tesco supermarkets. High-oleic acid sunflower oil was a gift from Croda Chemicals (Goole, United Kingdom). Fish meal was from Biomar (Grangemouth, United Kingdom). All other chemicals were from Sigma (Poole, Dorset, United Kingdom).

Synthesis of 21:4n-6 and 24:6n-3. Heneicosatetraenoic acid (Δ6,9,12,15-21:4) ethyl ester was prepared by a one-carbon

addition to 20:4n-6 FA (7). The product was obtained in 51.2% yield and was 98.9% pure by GC and GC–MS of the FAME. Tetracosahexaenoic acid (Δ-6,9,12,15,18,21-24:6) ethyl ester was similarly prepared by two sequential one-carbon additions to 22:6n-3 and was obtained in 30.2% yield, 94.0% pure.

Fish and diets. Rainbow trout approximately 2 g size were obtained from a commercial hatchery and kept in a running freshwater aquarium at ambient temperature (3.5 to 16.5°C) on a 14 h/10 h light cycle. Fish were fed a diet based on casein and a blend of vegetable oil containing predominantly oleic acid with 18:2n-6 and 18:3n-3 at approximately 1% each, this to maximize 22:6n-3 synthesis and satisfy the fish's EFA requirements. The full composition of the diet was described previously (3). The final diet provided 50% crude protein and 11% oil blended to give, as a percentage of the final diet, 0.99% 18:2n-6, 1.02% 18:3n-3, and 0.12% highly unsaturated FA (20:5n-3 and 22:6n-3) from the fish meal, which was added to make the diet palatable and readily accepted by the fish. The remaining FA were predominantly 16:0 (1.02%) and 18:1n-9 (7.18%). The fish were fed this diet for at least 25 wk before starting the experiments.

Preparation of labeled diet. A small portion of diet containing D_5-18:3n-3 FAEE and 21:4n-6 FAEE was prepared as follows. An oil sample containing 10 mg D_5-18:3n-3 FAEE, 2.5 mg 21:4n-6 FAEE, 153 mg of high-oleic acid sunflower oil, and 61 μg antioxidant was dissolved in 0.82 mL isohexane and 1.335 g of dry diet mix was added. The isohexane was then removed at 37°C under nitrogen and the diet desiccated *in vacuo* for 18 h. The diet was mixed thoroughly and 0.95 mL water was added and mixed to a stiff paste. This was extruded through a 1-mL disposable syringe, dried at room temperature for 2–3 h, and cut into 3–4 mm lengths. The diet was stored under argon at −20°C and was used within 3 d. 21:4n-6 FAEE was added to diets as a marker since it is much less readily catabolized than 18:3n-3 and therefore gives a higher recovery, enabling more precise determination of the amount of labeled diet eaten by individual fish (3).

Experimental protocol. Groups of 10 to 21 fish were acclimated in a 100-L circular tank with running water for at least 4 d before starting an experiment. They were then fed the labeled diet, all of which was observed to be eaten. Next, the fish were fed the normal unlabeled diet daily and sampled at intervals thereafter. The temperature ranged from 9.0 to 16.0°C during the experiments.

Fish were anesthetized with MS 222 (ethyl 3-aminobenzoate methane sulfonate) and bled from the tail vein into a weighed vial containing heparinized saline (100 U/mL of 0.9% NaCl). The fish were weighed and individual tissues dissected for analysis as described earlier (3,4). Portions of liver, cecal mucosa, and blood were taken for protein determination by the method of Lowry *et al.* (8). Samples were homogenized in chloroform/methanol 2:1 (by vol) by using a Potter or UltraTurrax homogenizer, and an extract was prepared by the method of Folch *et al.* (9). Tritricosanoyl glycerol (tri23:0) standard was added to each tissue sample before homogenization. Samples were kept on ice under nitrogen during workup and were stored at −20°C under argon.

Quantification of FA. One milligram of total lipid was saponified with 2 mL of 0.1 M KOH in 95% (by vol) ethanol under nitrogen for 1 h at 78°C. Nonsaponifiable material was removed by extracting with isohexane/diethyl ether (2:1, vol/vol), the aqueous phase was acidified, and FFA were extracted with diethyl ether. Pentafluorobenzyl esters were then prepared from 100 μg FFA using acetonitrile/diisopropylamine/pentafluorobenzyl bromide (1000:10:1, by vol) at 60°C for 30 min under nitrogen (10). Excess reagent and solvent were removed under nitrogen, and samples were dissolved in isohexane and stored at −20°C under argon until analyzed.

Calibration standards of individual FA (18:3n-3, 18:4n-3, 20:4n-6, 21:4n-6, 20:5n-3, 22:5n-3, 22:6n-3, and 24:6n-3) with 23:0 were prepared by varying the amount of each FA while keeping the 23:0 constant and plotting the peak area ratio against the mass ratio of the different FA. Sample volumes for analysis were adjusted such that the amount of 23:0 injected onto the GC–MS was constant. Pentafluorobenzyl esters were chromatographed and quantified on a Fisons MD 800 GC–MS fitted with an on-column injector and a ZB wax column (60 m × 0.32 mm i.d., 0.25 μm film thickness) (Phenomenex, Macclesfield, Cheshire, United Kingdom), with helium as carrier gas (column head pressure 20 psi) and by running in negative chemical ionization mode with methane as reagent gas (pressure 7 psi). The temperature program was 80–240°C at 40°/min, then 240°C for 50 min. FA were identified by selective ion scanning for the required masses using a dwell time of 80 ms and cycle time of 20 ms, and quantified by reference to the appropriate FA calibration curve. Arachidonic acid (20:4n-6) was used as the standard for 20:4n-3, and 24:6n-3 as the standard for 24:5n-3.

RESULTS

At 2 and 5 d after feeding tracer, all the intermediate FA on the n-3 FA biosynthetic pathway were identified and quantified in liver, cecal mucosa, and blood. The total amounts of deuterated FA (DFA) recovered were highest in cecal mucosa (370 ± 190 ng/mg protein/mg D_5-18:3n-3 eaten) at day 2 ($n = 4$) compared with liver (97 ± 44 ng/mg protein/mg D_5-18:3n-3 eaten) and blood (5.8 ± 2.3 ng/mg protein/mg D_5-18:3n-3 eaten). The values in liver and blood rose to 151 ± 141 ng/mg protein/mg D_5-18:3n-3 eaten and 8.4 ± 5.3 ng/mg protein/mg D_5-18:3n-3 eaten, respectively, at day 5 ($n = 6$), but fell in cecal mucosa to 214 ± 110 ng/mg protein/mg D_5-18:3n-3 eaten.

In liver at day 2 (Fig. 1A) D_5-22:6n-3 was the most abundant labeled n-3 PUFA (34.7%), followed by D_5-20:5n-3 (16.1%) and D_5-22:5n-3 (13.9%), with D_5-18:3n-3, D_5-18:4n-3, D_5-20:4n-3, and D_5-24:6n-3 all contributing between 7.5 and 9.5% of DFA. By day 5 the proportion of D_5-22:6n-3 had risen to 73.6%, with all other FA decreasing; D_5-20:5n-3 and D_5-22:5n-3 were the only other FA present at more than 5% (Fig. 1A). In cecal mucosa (Fig. 1B) D_5-22:6n-3 was again the most abundant labeled FA constituting 36.7% at day 2 and 57.0% at day 5. D_5-18:3n-3 and D_5-18:4n-3 were more abundant in cecal mucosa than in liver at

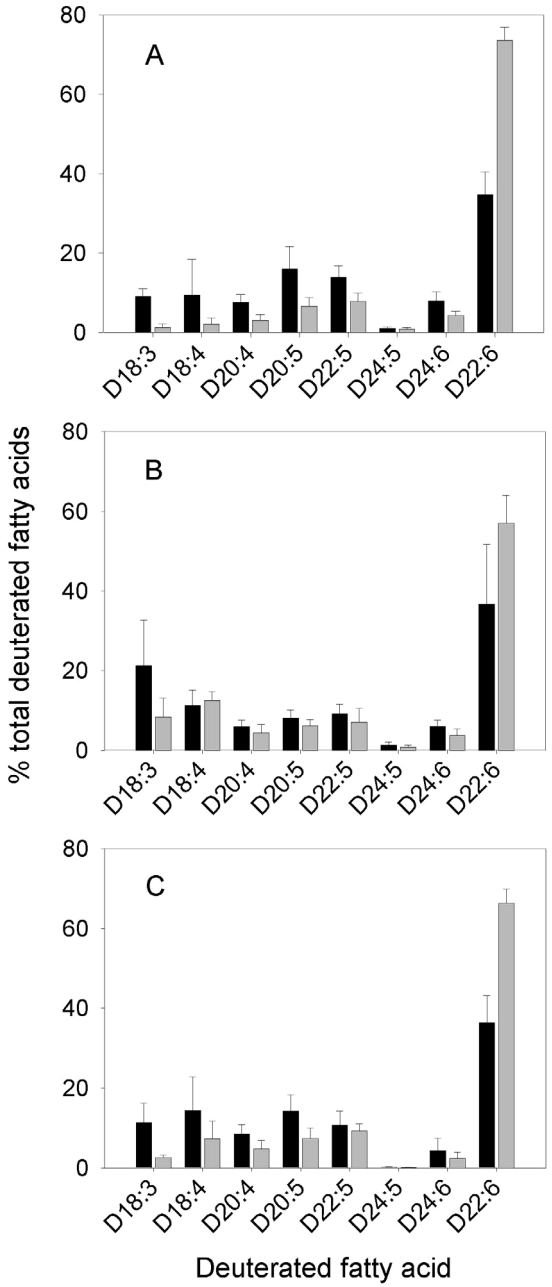

FIG. 1. The percentage distribution of D_5-18:3n-3 and anabolites in liver (A), cecal mucosa (B), and blood (C) 2 and 5 d post-dose of D_5-18:3n-3. Mean ± SD of $n = 4$ at day 2 and $n = 6$ at day 5. Day 2 = black bars, day 5 = gray bars. The weight of the fish was 60.1 ±11.3 g.

both times, whereas D_5-20:5n-3 and D_5-22:5n-3 were less important than in liver, contributing 8.1 and 9.2%, respectively, at day 2 (Fig. 1B). The two C_{24} PUFA intermediates were present in cecal mucosa in amounts very similar to liver at days 2 and 5 (Fig. 1B). The profile of DFA in blood (Fig. 1C) was very similar to that in liver and cecal mucosa, with D_5-22:6n-3 constituting 36.4% at day 2 and 66.3% at day 5 and no other FA contributing more than 10% by day 5. D_5-18:3n-3 contributed only 2.6% by day 5 in blood, and D_5-24:5n-3 was barely detectable at both times (Fig. 1C).

The tissue distribution of DFA was determined in liver, brain, eye, visceral adipose tissue, and eye socket adipose tissue over a longer time course, from 3 to 35 d post-feeding of tracer (Figs. 2–5). Total DFA in liver fell over the 5 wk from 0.91 to 0.12 µg DFA/g fish/mg D_5-18:3n-3 eaten (Fig. 2). In brain, total DFA increased from 0.14 to 0.30 µg DFA/g fish/mg D_5-18:3n-3 eaten, peaking at day 24, and in eyes increased from 0.16 to 0.53 µg DFA/g fish/mg D_5-18:3n-3 eaten over the same time (Fig. 2). Visceral adipose tissue contained the largest amount of DFA, and the levels were more constant than in liver, brain, and eyes, between 0.75 and 1.34 µg DFA/g fish/mg D_5-18:3n-3 eaten from day 7 to day 35 (Fig. 2). The amounts of DFA in eye socket adipose tissue remained between 0.28 and 0.33 µg DFA/g fish/mg D_5-18:3n-3 eaten between 7 and 35 d (Fig. 2).

In liver (Fig. 3) D_5-20:5n-3 was the largest component at day 3 (41.2%) but declined sharply, and D_5-22:6n-3 was the predominant DFA from day 7, constituting over 65% of label from day 14 onward. D_5-22:5n-3 was the second-most abundant DFA at days 14 and 24. The amount of D_5-18:3n-3 in liver was very low (<2.1%) at all times, whereas D_5-18:4n-3 was slightly more abundant and increased at the later time points. D_5-24:6n-3 was present at all times in a low but relatively constant amount (4.1 to 5.1%), but D_5-24:5n-3 was present in much lower amounts (0.09 to 0.36% of DFA) (Fig. 3).

In brain (Fig. 4A) D_5-20:5n-3 was the major DFA at day 3 (64.3%) and, although falling rapidly, still constituted 22.5 to 28.1% of DFA from day 14 onward. In contrast, D_5-22:6n-3 increased sharply from day 3 (4.9%) to day 7 (28.7%) and much more slowly from day 7 to day 35 (39.3%). D_5-22:5n-3 also was important in brain, constituting 16.6 to 20.9% of DFA from day 7 (Fig. 4A). The other DFA (D_5-18:4n-3, D_5-20:4n-3, D_5-24:5n-3, and D_5-24:6n-3) were not detected in brain at day 3 and were present at a low but relatively constant

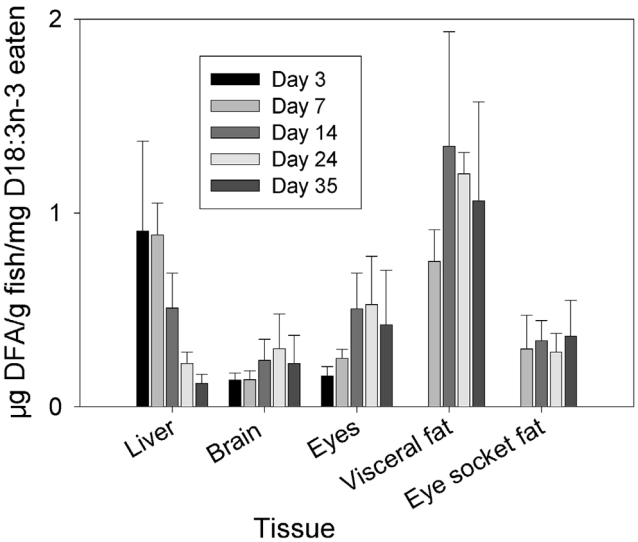

FIG. 2. The distribution of total deuterated FA (DFA) in different tissues at intervals after ingesting a pulse of D_5-18:3n-3. Values are mean ± SD of $n = 5$ for day 3, $n = 4$ for day 7, $n = 5$ for day 14, $n = 3$ for day 24, and $n = 4$ for day 35. The weight of the fish was 5.58 ± 1.03 g at day 3, increasing to 10.41 ± 4.41 g at day 35.

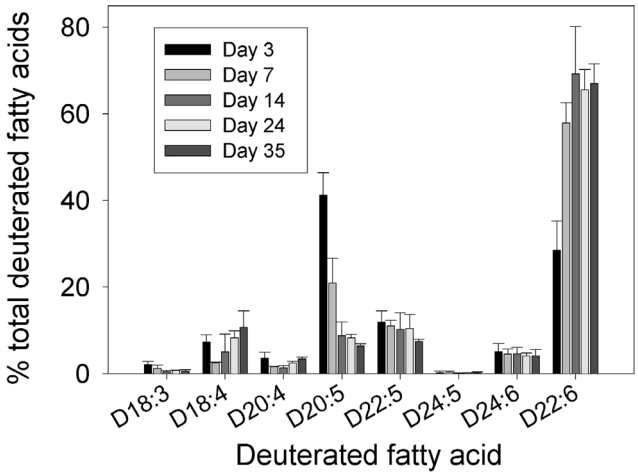

FIG. 3. The percentage distribution of D_5-18:3n-3 and anabolites in liver at 3, 7, 14, 24, and 35 d post-dose of D_5-18:3n-3. Other details are as in Figure 2.

level (<6.6%) from day 7 to day 35 (Fig. 4A), with D_5-24:5n-3 making up 1.5 to 4.2% and D_5-24:6n-3 3.7 to 5.8% of DFA. The initial substrate, D_5-18:3n-3, constituted <3.1%

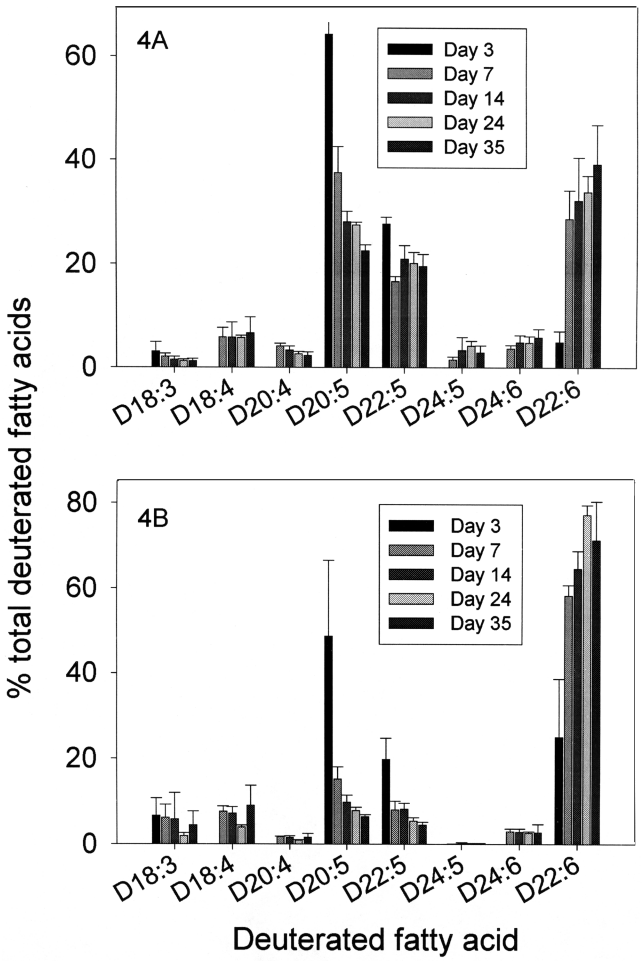

FIG. 4. The percentage distribution of D_5-18:3n-3 and anabolites in brain (A) and eyes (B) at 3, 7, 14, 24, and 35 d post-dose of D_5-18:3n-3. Other details are as in Figure 2.

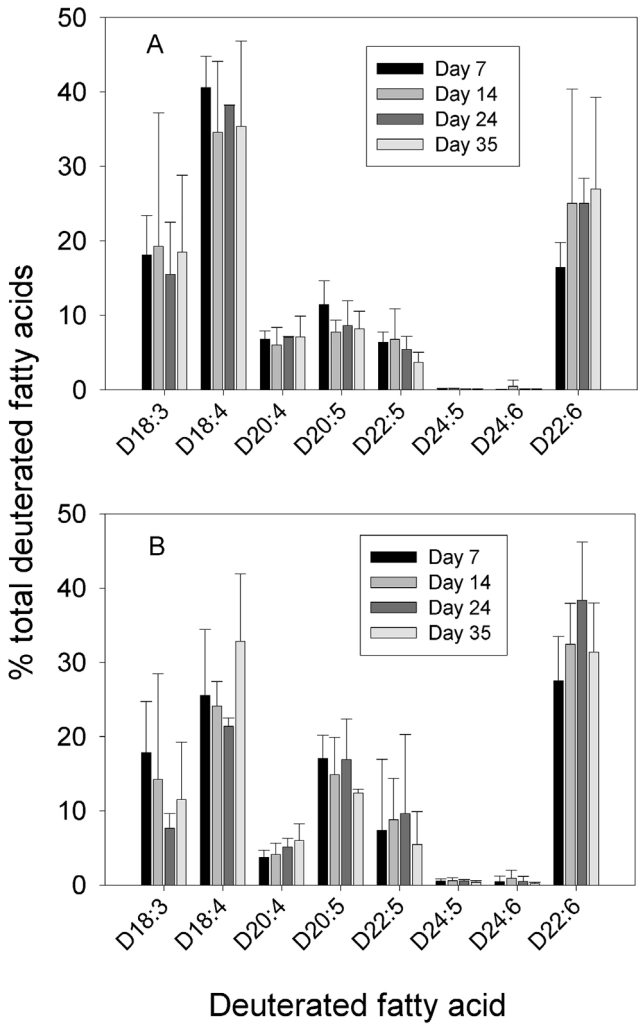

FIG. 5. The percentage distribution of D_5-18:3n-3 and anabolites in visceral (A) and eye socket (B) adipose tissue at 7, 14, 24, and 35 d post-dose of D_5-18:3n-3. Other details are as in Figure 2.

of DFA at all times. Eyes (Fig. 4B) showed a similar pattern to brain (Fig. 4A), with a large peak of D_5-20:5n-3 at day 3 (48.7%), falling sharply by day 7 (15.2%), whereas D_5-22:6n-3 increased from 24.9 to 58.4% over the same time and constituted over 70% of DFA on days 24 and 35. D_5-22:5n-3 made a smaller contribution than in brain, mirroring the trend in D_5-20:5n-3. D_5-24:6n-3 and D_5-20:4n-3 were present at very low levels in eyes, and D_5-24:5n-3 was barely detectable (Fig. 4B). Eyes also had no detectable D_5-18:4n-3, D_5-20:4n-3, or D_5-24:6n-3 at day 3.

In visceral adipose tissue (Fig. 5A) D_5-18:4n-3 was the predominant DFA at all times (34.5 to 40.6%), followed by D_5-22:6n-3 (16.5 to 27.0%) and D_5-18:3n-3 (15.5 to 19.3%). D_5-20:4n-3, D_5-20:5n-3, and D_5-22:5n-3 each constituted 5–10% in visceral adipose tissue; D_5-24:5n-3 and D_5-24:6n-3 could not be detected in most samples (Fig. 5A). Eye socket adipose tissue (Fig. 5B) contained proportionately less D_5-18:3n-3 and D_5-18:4n-3 and more D_5-20:5n-3, D_5-22:5n-3, and D_5-22:6n-3 than visceral adipose tissue. The two C_{24}

PUFA intermediates were barely detectable in eye socket adipose tissue.

DISCUSSION

All the intermediate FA of the n-3 pathway were identified in liver and cecal mucosa, consistent with the pathway proposed by Voss *et al.* (5). D_5-24:6n-3 was more abundant than D_5-24:5n-3 in all tissues at all times. However, relatively more D_5-24:5n-3 was found in brain than in other tissues. This DFA was barely detected in blood, eyes, and adipose tissue. D_5-24:6n-3 was also present at very low levels in adipose tissue. The presence of newly synthesized C_{24} PUFA is consistent with liver, ceca, brain, and eyes being active in 22:6n-3 synthesis, whereas the absence of these DFA in adipose tissue suggests that tissue is inactive.

D_5-22:6n-3 was the major labeled product in all tissues except visceral fat, which accumulated D_5-18:3n-3 and D_5-18:4n-3 to a much greater extent than other tissues. The substrate, D_5-18:3n-3, was present in very low concentrations in all tissues except adipose tissue. This was expected, since in fish C_{18} PUFA are usually esterified as TAG and are not found in polar lipid to any great extent. There was little accumulation of intermediate DFA in liver or in cecal mucosa even at 2 d post-dose. However, in the livers of the smaller fish from the longer time course, D_5-20:5n-3 was the major DFA at day 3. This may reflect the amount of food already in the gut when tracer was eaten and consequently slower digestion and absorption in fish of this size. The pattern of DFA in blood mimicked that of liver and cecal mucosa closely, although D_5-24:5n-3 was barely detectable in blood. All other intermediate FA were present in blood, indicating that FA participating in the biosynthetic pathway are not tightly retained at the site of biosynthesis within cells.

The patterns of deposition of newly synthesized 22:6n-3 and the n-3 pathway intermediates in liver differed from those found in earlier *in vitro* studies. Experiments carried out with isolated hepatocytes or liver microsomes from trout over a few hours showed much higher recoveries of tracer in the original substrate and intermediates (11,12) than in this study. For example, in hepatocytes incubated with [1-^{14}C]18:3n-3, 31.6% of radioactivity in polar lipid was recovered in the substrate after 3 h, with 10.5% in 20:4n-3, 17.4% in 20:5n-3, and 18.4% in 22:6n-3 (11). The proportions of these FA in neutral lipid were lower, with more substrate, elongation products, and 18:4n-3 in neutral lipid. In liver microsomes, 18:4n-3 (67.4%) and 18:3n-3 (21.8%) were the major labeled products, with much smaller amounts of 20:4n-3 and 20:5n-3. In the absence of peroxisomes 22:6n-3 was not formed (12). These differences are a function of the slow rate of synthesis of 22:6n-3 and the necessarily short incubation times used in *in vitro* studies. Accretion of 22:6n-3 in trout liver peaked at 14 d post-feeding (3). It is not surprising therefore that *in vitro* incubations show much higher proportions of starting material and labeled intermediates and less final product than feeding experiments carried out over much longer times.

The two neural tissues showed a different pattern of DFA accumulation. Accretion into brain and eyes was very slow, with the maximal deposition of DFA at 24 d. D_5-20:5n-3 was the major DFA in brain up to day 7 and D_5-22:6n-3, the predominant FA thereafter. However, D_5-22:5n-3 constituted *ca.* 20% of DFA at later time points. Thus, in brain the C_{20} to C_{22} and C_{22} to C_{24} elongase steps appear to be slow, resulting in a buildup of 20:5n-3 and 22:5n-3. It is not clear from this type of experiment whether brain has appreciable Δ6- or Δ5-desaturase activity. The very low levels of D_5-18:3n-3, D_5-18:4n-3, and D_5-20:4n-3 could be due to a failure of these FA to cross the blood–brain barrier, or to very active Δ6- and Δ5-desaturases, leading to depletion of these substrates and a buildup of D_5-20:5n-3. However, the buildup of D_5-24:5n-3 compared to liver and cecal mucosa suggests low Δ6-desaturase activity. The ratio of the final Δ6-desaturase products D_5-24:6n-3 + D_5-22:6n-3 to the immediate substrate D_5-24:5n-3 in brain was much lower than in liver, cecal mucosa, and eyes at all times, and the ratio of D_5-22:6n-3 to D_5-24:6n-3 was also lower in brain than in the other tissues. The usefulness of this type of analysis in an open system is limited, but the results do suggest that the C_{24} PUFA Δ6-desaturase and final peroxisomal β-oxidation steps are relatively inactive in trout brain. It is possible that Δ6-desaturase isoenzymes with different substrate specificities are involved in the conversion of 18:3n-3 to 18:4n-3 and 24:5n-3 to 24:6n-3. However, recent cloning experiments in rats and humans showed that the same Δ6-desaturase acts on C_{18} and C_{24} PUFA (13,14). In zebrafish a cDNA with a high similarity to mammalian Δ6-desaturase had both Δ6- and Δ5-desaturase activity when expressed in yeast (15).

Trout astrocytes incubated with [1-^{14}C]linolenate for 7 d gave the majority of recovered tracer as substrate (64%) with 18:4n-3, 20:4n-3, and 20:5n-3 in decreasing order accumulating 12.6 to 5.9%, and only 0.7% was recovered as 22:6n-3 (16). However, in rat brain astrocytes incubated with 18:3n-3 there was a marked accumulation of 22:5n-3, showing that the synthesis of 22:6n-3 was limited by the elongation of 22:5n-3 (17). This is consistent with the findings of our *in vivo* study. In felines, liver and brain were found to act in tandem to synthesize 22:6n-3 from 18:3n-3 (18). PUFA with chain lengths up to 22:5n-3 were found in liver and plasma, whereas subsequent products (24:5n-3, 24:6n-3, and 22:6n-3) were found only in brain (18). In mice it was found after intraperitoneal injection of [1-^{14}C]18:3n-3 that newly synthesized 22:6n-3 accumulated in liver within 2 h, but brain was still accumulating 22:6n-3 at 72 h post-dose, while hardly any was found in retina after this time (19). The mechanism for providing 22:6n-3 to the brain may therefore involve several different cell types and differ between species. Such differences would account for the different results from *in vitro* and *in vivo* experiments.

The eyes showed a similar pattern of deposition of intermediate DFA to brain, with an early accumulation of D_5-20:5n-3 and D_5-22:5n-3, but levels then dropped further than in brain. The D_5-22:6n-3 to D_5-24:6n-3 ratio was higher in

eyes than in brain. Again, it is not possible to conclude whether the retina, or more probably the retinal pigment epithelium, has biosynthetic activity or whether FA are selectively sequestered from the blood. In frogs, retina accumulated 18:4n-3 from [1-^{14}C]18:3n-3 and 20:5n-3 from [3-^{14}C]22:5n-3, whereas retinal pigment epithelium was able to convert all n-3 PUFA intermediates to 22:6n-3, suggesting that retinal pigment epithelium is a source of 22:6n-3 for the retina (20). Bovine retina was, however, able to convert [1-^{14}C]22:5n-3 to 22:6n-3 (21).

The present study did not suggest that eye socket adipose tissue was a depository of newly synthesized 22:6n-3 for the retina. The role of eye socket adipose tissue in fish is unclear, but it represents a sizable deposit. In the fish in this study, it made up 43% by weight of tissue and 14% by weight of lipid of visceral adipose tissue (data not shown). In mammals, eye socket adipose tissue has been suggested to have a purely structural role to cushion the eye from damage from the bony socket (22), and as such might therefore be expected to be relatively inert. Pressure changes during underwater high-speed swimming in fish would make cushioning of the eyes even more important. The amount of eye socket fat in tuna and its high content of 22:6n-3 make it a valuable source of 22:6n-3-rich oil (6). We are unaware of work that would suggest a special structural role for 22:6n-3-rich TAG, in contrast to the numerous studies that have shown the unique roles of phosphoglycerides containing 22:6n-3 (e.g., Ref. 23). A possible reason for the high content of 22:6n-3 in eye socket adipose tissue could be as a source of 22:6n-3 for the retina. However, the results here did not clarify this issue since eye socket fat did not selectively accumulate newly synthesized 22:6n-3. We are not aware of studies that have examined the uptake and mobilization of different FA in eye socket adipose tissue from fish.

The tissue distribution and kinetics of deposition of newly synthesized n-3 PUFA were largely consistent with our present understanding of their metabolism and function in different tissues. Liver and ceca, tissues active in 22:6n-3 synthesis (3,4), quickly converted ingested D_5-18:3n-3 to D_5-22:6n-3 with low levels of intermediate PUFA at early time points. The presence of low levels of D_5-24:5n-3 and D_5-24:6n-3 were consistent with the pathway of 22:6n-3 synthesis proposed by Sprecher and coworkers (5). Brain and eyes have a large requirement for 22:6n-3, especially during early development, and were therefore expected to accumulate 22:6n-3 over a longer time. This was the case, but the accumulation of D_5-20:5n-3 and D_5-22:5n-3 in brain and eyes suggested that neural tissue has relatively inactive C_{20}–C_{22} and C_{22}–C_{24} elongases and/or C_{24} PUFA Δ6-desaturase activity, leading to an accumulation of the C_{20} and C_{22} pentaene intermediates. Visceral adipose tissue, with no functional requirement for 22:6n-3, showed the lowest percentage accumulation of this FA. Eye socket adipose tissue, which might be able to supply the retina with 22:6n-3, showed a slightly greater accumulation of D_5-22:6n-3. Adipose tissue contained barely measurable amounts of deuterated C_{24} PUFA, consistent with an inability to synthesize PUFA.

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Solubilization of Carotenoids from Carrot Juice and Spinach in Lipid Phases: I. Modeling the Gastric Lumen

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ABSTRACT: Our understanding of the factors determining the bioavailability of carotenoids from fruits and vegetables is poor. The apolar nature of carotenoids precludes their simple diffusion from the food structure to the absorption site at the enterocyte. Therefore, there is interest in the potential pathways for solubilization in the gut before absorption. We have studied the transfer of carotenoids from carrot juice and homogenized spinach into lipid phases that mimic the intestinal lumen at the start of digestion. In this paper we report on their transfer into olive oil under conditions pertaining to the gastric environment. A comparison between preparations of raw spinach and of carrot, in which the intact cells have been largely broken, suggests that the membrane-bound carotenoids of spinach are more resistant to transfer than the crystalline carotenoids of carrot. Lowering the pH and pepsin treatment enhance the transfer from raw vegetables. The process of blanching and freezing spinach destroys the chloroplast ultrastructure and leads to (i) a substantial increase in transfer of the carotenoids to oil and (ii) an attenuation or reversal of the enhancement of transfer seen with reduced pH or with pepsin treatment. Similar effects are seen after blanching carrot juice. Our results show that removal of soluble protein and denaturation of membrane proteins enhances the partition of carotenoids into oil. For both vegetables there is no evidence of preference in the extent of transfer of one carotenoid over another. This suggests that partitioning into oil under gastric conditions is not the stage of digestion that could lead to differences in carotenoid bioavailability.

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Dietary carotenoids from fruits and vegetables have been implicated as contributing to antioxidant (1), anticarcinogenic (2), immunogenic (3,4), and cardiovascular protection (5) activities in humans. Therefore, there is interest in understanding the factors determining the bioavailability of carotenoids from plant materials. The apolar nature of carotenoids precludes their simple diffusion from the plant structures to the absorption site at the enterocyte. Current thinking (6–8) proposes that carotenoids are solubilized into a gastric oil phase before being transferred to mixed micelles in the duodenum. To understand the factors determining bioavailability and the relative importance of different potential solubilization pathways before absorption, we have been studying lipid systems that model the lumen of the gut during the digestion of vegetables containing carotenoids. In this paper, our previous observations (6) on transfer of carotene from raw carrot juice into olive oil are extended to include another vegetable, spinach. The effects of blanching and pepsin treatment are also reported. By studying how the amount of carotenoid transfer is correlated with protein concentrations, we have gained insight into how changes in pH and in the state of the carotenoid-containing organelles can potentially affect carotenoid bioavailability at the gastric stage. The accompanying paper (9) is concerned with the first stages of digestion in the duodenal environment and solubilization in micelles.

The factors that determine the ease of transfer of carotenoids from vegetable material to an oil phase and therefore their bioavailability include the molecular nature of the carotenoids and the structure and stability of the organelles in which they are sequestered. *Trans*-α- and -β-carotene, the major carotenoids in carrot, are completely apolar. In contrast, the xanthophylls, such as lutein, typically found in greatest amounts in green vegetables, have one or more polar groups. For pure carotenoids in oil-in-water emulsions, xanthophylls have been found to accumulate preferentially at the oil–aqueous phase interface, whereas carotenes are found within the bulk oil phase (7). Carrot and spinach were chosen for comparison because the organelles in which the carotenoids are found in these two vegetables are very different. In carrot, the carotenes are in a crystalline state in the chromoplasts. Each crystal is surrounded by a membrane to form a carotene body (10). The results of our previous work (6) are consistent with carotene from carrot juice passing directly from the crystals into the oil phase. Lutein is present in small amounts in carrot. However, its location has not been explicitly studied. In spinach the major carotenoids are lutein and *trans*-β-carotene, which are located in photosystems I and II of the inner chloroplast membranes (11). These carotenoid molecules are therefore directly associated with lipoproteins and lipid.

Other important factors contributing to the bioavailability of the carotenoids relate to the structure of the vegetable tissue containing the organelles. Thus, the decreased particle size of carrot increases both the partition of carotenes into oil *in vitro* (6) and absorption *in vivo* (12,13). Similar results for absorption have been obtained with spinach (14). Cooking also enhances the amount absorbed (14,15) by breaking down cell walls (16). In the work reported here, comparing carrot with spinach, the complications due to differences in gross vegetable structure were avoided by using carrot juice and

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homogenized spinach. In both cases, intact cells and cell walls were largely removed.

MATERIALS

Commercially available washed carrots were purchased and stored at 1–2°C at 98% RH for use within 2 wk. The spinach used for most of the carotenoid transfer experiments was whole-leaf frozen spinach stored at –30°C. It was prepared by washing leaves in cold water, followed by blanching for 80 s at 90°C in a Lubot Wakker Eng. Bv Asperen-Holland blancher and cooling to 10°C in 80 s. The spinach was sealed in foil laminate pouches and frozen rapidly. Experiments were also done using commercially available raw spinach, blanched as above but not frozen. In both of these cases, the leaves were cut into 1 to 2 cm^2 pieces before being processed as described in the Methods section.

Nylon mesh with a nominal 63-μm hole size was purchased from John Staniar & Co. (Manchester, United Kingdom). Agarose (Type VII, low gelling temperature), pepsin, Bradford reagent, bovine albumin (FA-free), BHT, and olive oil (low acidity) were obtained from Sigma-Aldrich (Poole, United Kingdom). Chloroform was Normapur from Merck-BDH (Lutterworth, United Kingdom). Sources of the carotenoids that were routinely incorporated in the HPLC calibrations were as follows: lutein, lycopene, *trans*-α- and -β-carotene from Sigma Chemicals; and zeaxanthin and β-cryptoxanthin from Rotec Scientific (Milton Keynes, United Kingdom). All other reagents were of Analar or HPLC grade.

METHODS

The carrot and spinach preparations were designed to remove as much variability as possible attributable to particle size and to the amount of matrix compartmentalizing the carotenoid-containing organelles. Carrot juice was prepared from the outer cortex of peeled carrots in a Moulinex juicer (Type CF1A). The juice was then filtered through one layer of nylon mesh. Blanched carrot juice was prepared from the filtered juice by heating and cooling as described above for spinach.

Three preparations of spinach suspensions were used: blanched-frozen, blanched, and raw. For the blanched-frozen spinach suspension, the frozen leaves (20 ± 5 g) were suspended in cold 70 mM NaCl (150 mL), stirred until defrosted, and then homogenized in a Waring blender (8011 commercial blender) at maximal speed for four 10-s periods. The homogenate was filtered as described above. For the purpose of studying spinach carotenoid transfer to oil under similar conditions as for carrot juice, it was necessary to concentrate the chloroplasts. The filtrate was centrifuged at 1800 × g_{max} for 10 min at 4°C. The green pellet was suspended in 20 mL of 70 or 150 mM NaCl. The blanched and raw spinach suspensions were prepared in the same way from blanched and raw spinach leaves, respectively, except the raw spinach required only one 10-s period of homogenization and passed easily through the 63-μm filter. The blanched spinach tended to block the filter.

For transmission electron microscopy, samples of spinach suspensions and carrot juice were mixed with an equal volume of 6% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). After centrifugation, the pellet was washed three times in buffer and then mixed with a small amount of 1% molten agarose and cooled over ice. Cubes of this material were post-fixed in 1% osmium tetroxide for 3 h, dehydrated in a graded ethanol series, and transferred to acetone. The samples were then infiltrated and embedded in Spurr resin and sectioned with a diamond knife. Sections showing silver-gray interference colors were stained sequentially in a saturated solution of uranyl acetate in 50% ethanol and in Reynold's lead citrate for examination in a JEOL 1200EX transmission electron microscope (JEOL, Tokyo, Japan) using an accelerating voltage of 60 or 80 kV. Prior to electron microscopy, samples were examined by light microscopy.

The carotene concentration in the filtered carrot juice was measured routinely as described (6) by extracting the juice into a hexane phase after treatment with THF and methanol and measuring the absorbance at 450 nm. This method does not distinguish between α- and β-carotene and does not extract all the lutein, which is present in amounts an order of magnitude less than the carotenes. When information on the detailed composition of the juice used for experiments was required, it was extracted three times by the method of Bligh and Dyer (17) with 0.1% BHT in the chloroform phase. An aliquot of the combined extracts was dried under nitrogen, dissolved in dichloromethane, and diluted in the mobile phase for HPLC analysis.

The carotenoid composition of the spinach suspension (Table 1) was measured by centrifuging a known volume at 10,000 × g_{max} for a few minutes, removing the colorless supernatant, and extracting the pellet four times with acetone containing 0.1% BHT. The extracts were combined and treated as described for the carrot juice for analysis by HPLC.

Transfer of carotenoids to olive oil was measured as described earlier (6). To mimic the gastric condition, 30 mL aqueous phase at the required pH, containing carrot juice or spinach suspension at the required concentration and ionic

TABLE 1
Carotenoid Composition of Blanched-Frozen Whole Leaf Spinach, Blanched-Frozen Spinach Suspension, and Raw Carrot Juice

	Spinach[a]		Spinach suspension[a]	Carrot juice[a]
	Concentration (nmol/g) Mean (SD)	mol%	mol% Mean (SD)	mol% Mean (SD)
Lutein	181.8 (10.7)	56.1	61.3 (2.9)	2.45 (0.74)
Zeaxanthin	4.39 (0.35)	1.4		
Trans-α-carotene				28.8 (1.1)
Trans-β-carotene	117.5 (9.05)	36.3	38.7 (2.9)	68.8 (1.2)
Lutein/*trans*-carotenes	1.55 (0.15)		1.59 (0.18)	0.025 (0.01)

[a]The composition was determined by HPLC analysis of 6 samples of drained whole leaf spinach (52.6% solids), 8 samples of carrot juice extracted by the method of Bligh and Dyer (17), and 15 samples of spinach suspension extracted with acetone containing 0.1% BHT.

strength plus 1 mM sodium azide, was pre-equilibrated at 37°C for 20 min in stoppered 50-mL Quickfit flasks. For pepsin treatment at low pH, pepsin was included at a concentration of 120 U/mL. The pre-equilibration was done in a Gallenkamp Plus II incubator with the shaking platform in orbital mode (setting 5.5). Olive oil (6 mL) was then added, and the flasks were returned to the shaking incubator. Samples of the oil phase (30–100 μL) were taken at the required times and centrifuged briefly at 10,000 × *g* to remove any entrained aqueous phase and vegetable tissue. Routinely, carotene from carrot juice was measured by diluting aliquots of the oil (10 to 50 μL) into 1 mL *n*-hexane and measuring the absorbance at 450 nm using a millimolar extinction coefficient of 137.4 (18). However, spectra were measured to assess whether significant amounts of noncarotene compounds were contributing to the 450 nm absorbance, in which case a more comprehensive HPLC analysis would have been required. For the work reported here, this situation did not arise, and therefore our results are given in terms of total carotene in the oil with no distinction being made between *trans*-α- and -β-carotene. HPLC analysis was required for the spinach suspension carotenoids solubilized in oil because of the presence of chlorophyll obscuring the carotenoid peaks and the comparable β-carotene and lutein concentrations.

As reported previously (6), transfer of carotene to oil from carrot juice was done routinely with juice diluted into 70 mM NaCl. The effect of ionic strength was examined by raising the NaCl concentration in the raw carrot juice to effectively 150 mM NaCl by adding 1.5 M NaCl to the filtered juice until the electrical conductance was the same as that of 150 mM NaCl. This juice was then diluted to the required carotene concentration with either 150 mM NaCl or water plus 70 mM NaCl, to give effective NaCl concentrations of 150 and 70 mM, respectively. Sodium azide was included in the diluents to give a final azide concentration of 1 mM. The juice was then titrated to the required pH by the addition of HCl or NaOH.

HPLC measurements of carotenoids were performed on a Vydac C18 201-TP54 analytical column (250 × 4.6 mm i.d.; Phenomenex, Cheshire, United Kingdom) fitted with biocompatible 2-μm Teflon frits, a Spherisorb ODS 2 metal-free precolumn (5 μm; 100 × 4.6 mm i.d.), and an ODS 2 PEEK guard cartridge (Alltech Associates, Lancashire, United Kingdom). The mobile phase, acetonitrile/methanol/dichloromethane (75:20:5 by vol) containing 50 mM ammonium acetate and 0.05% (wt/vol) triethylamine, was pumped at a flow rate of 1.5 mL/min using an isocratic pump (Kontron Instruments, Milton Keynes, United Kingdom). The column temperature was maintained at 19.3°C by use of a water jacket and circulating water bath. Samples were injected *via* a 50-μL loop using a Rheodyne 7125 valve. Carotenoids were detected at 450 nm using an L-3000 UV/vis detector (Merck, Dorset, United Kingdom), and data were collected using a Chromperfect data station (Justice Innovations, Auchtermuchty, Fife, United Kingdom). Carotenoid concentrations were calculated by comparison of peak areas to those of standard solutions of carotenoids.

For HPLC analysis, olive oil samples were added to dichloromethane and immediately diluted into the mobile phase so the final dichloromethane concentration was 10 to 20% in the complete mobile phase. These solutions were then injected onto the column. It has been reported that carotene may not be very stable in dichloromethane solutions (19). For this reason, the time between diluting the experimental samples into the mobile phase and injection onto the column was kept to less than 15 min, and the diluents were stored on ice. After 1 h β-carotene concentrations in the diluents kept at room temperature fell by 12%. Lutein concentrations were unchanged. Other methods of extracting the carotenoids from experimental media were tried, but the direct method described above gave the most reproducible results and the most efficient extraction.

After dilution of the spinach suspension into experimental media and incubations, the amounts of the minor carotenoids (zeaxanthin and *cis*-β-carotene) transferred to oil were very low, which made their quantification difficult. Furthermore, the *cis*-β-carotene peak contained a contribution from pheophytin *b*, which is the primary light-harvesting molecule in photosystem II. Low pH treatment converts chlorophyll to pheophytin, which virtually obscures the *cis*-β-carotene peak. For these reasons, when measuring spinach carotenoid transfer we focused only on the major carotenoids lutein and *trans*-β-carotene. The limit for detection of each carotenoid was 1 ng/mL. For quantification, with a CV around 10%, 2.5 ng/mL was required.

The amounts of carotenoids solubilized in the oil were expressed as mean (±SD). Significant differences were assessed by Student's *t*-test.

Solutions of what will be referred to later as soluble carrot protein were prepared as described earlier (6), by centrifuging carrot juice and taking the supernatant. (This also contains other soluble components.) Soluble protein was measured in carrot juice, raw and blanched spinach suspensions, and experimental incubations by Bradford's microprotein assay (20) using methods described previously (6). Total protein was measured by the Bradford assay (20) in the presence of 0.1% SDS after solubilizing the membrane proteins in 5% SDS. SDS (0.1%) was also included in the bovine albumin standards. To prevent further hydrolysis in samples taken from low pH incubations, the pH was immediately adjusted to between 7 and 8 by the addition of NaOH. Insoluble protein was calculated as (total protein – soluble protein).

Owing to circumstances beyond our control, the stock of frozen spinach was lost. This happened before it was realized that it would be worthwhile to make measurements of protein in incubations under conditions similar to those in which carotenoid transfer to oil had been measured. However, the frozen spinach had been blanched. As described in the results section, the blanching procedure caused a drastic loss of organelle structure so that the appearance of frozen and blanched spinach suspensions was indistinguishable. Furthermore, most of the soluble protein was removed when the chloroplasts were concentrated. Therefore, it was felt justifiable to use measurements of protein on blanched spinach as

reflecting, in relative terms, what would have happened to the protein in frozen spinach suspensions.

RESULTS

Nature of vegetable material. (i) Raw and blanched carrot juice. The raw carrot juice has been described previously (6). In brief, light microscopy showed it consisted of diffuse material with large numbers of carotene crystals up to 20 μm in length, both floating free and attached to cell debris. Electron microscopy (Fig. 1A) showed that the diffuse material was cell contents with extensive vesiculation. Some intact chromoplasts were present as well as carotene crystals, both free and membrane-bounded (carotene bodies). The carotenoid composition of the juice is shown in Table 1. The composition is given as mole percentage because the analyses were done on different dilutions of juice in experimental media.

After blanching, the material present in the carrot juice tended to aggregate. This is illustrated in the electron micrograph in Figure 1B, which shows that blanching led to the presence of densely staining precipitates that were absent in the raw material. The precipitates are thought to be coagulated lipid and protein. This idea is supported by protein analysis results. Table 2 shows that blanching caused a substantial loss of soluble protein: from 56.3 ± 10.5% for raw juice to 13.7 ± 6.1% for blanched juice. Thus, most of the protein became insoluble. In contrast, blanching had no effect on the total carotene concentration in the juice, and carotenoid crystals were present in both raw and blanched carrot juice.

(ii) Raw and blanched-frozen spinach suspensions. Light microscopy showed that the suspensions made from raw spinach, blanched spinach, and blanched-frozen spinach consisted predominantly of free chloroplasts and other plastids. The blanched and blanched-frozen spinach suspensions also contained a few whole and partially broken cells and some cell-wall fragments. In the raw spinach suspension there were even fewer tissue aggregates and broken cells. Those that were present were partially surrounded by cell walls. Otherwise, cell walls were largely absent.

Electron microscopy showed that the raw spinach suspensions consisted of intact and broken chloroplasts, mitochondria, and a few cell-wall fragments (Fig. 2A). The chloroplast native structure was well maintained; intact grana and thylakoid membranes were clearly visible. In contrast, blanched-frozen spinach suspensions (Fig. 2B) showed no distinct organelle features. The blanching had clearly led to coagulation of lipids and proteins, leaving an amorphous mass of densely stained material. Electron micrographs of blanched and blanched-frozen spinach suspensions were indistinguishable. Therefore, for the blanched-frozen material the most damaging step for organelle ultrastructure occurred during blanching. The precipitates formed during blanching would tend to block the pores in the filter. This explains why the blanched-frozen and blanched suspensions passed through the filter with difficulty.

For raw spinach preparations, soluble protein was low (5.3

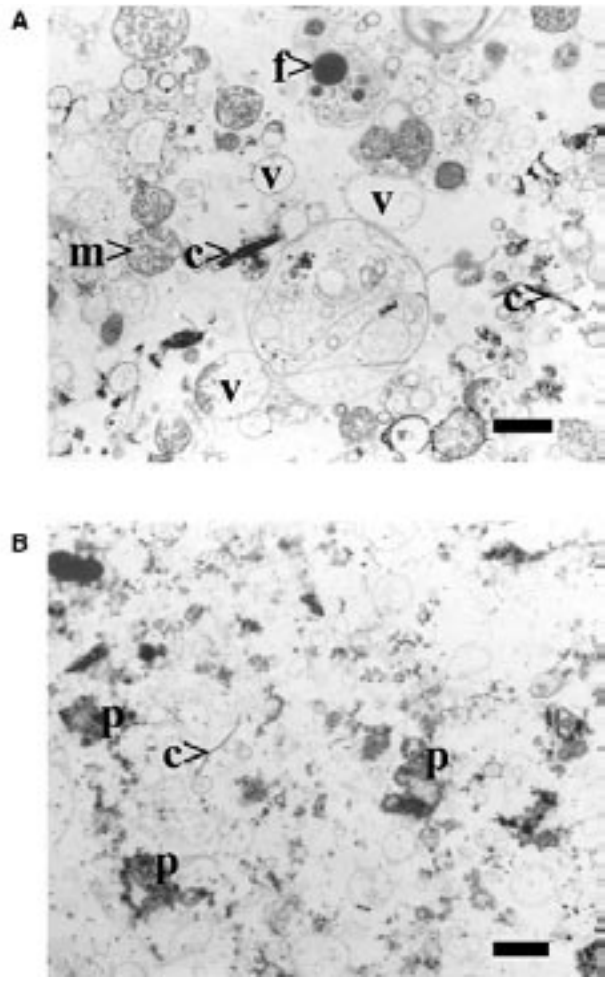

FIG. 1. Transmission electron micrographs of (A) raw carrot juice and (B) blanched carrot juice. In (A), **c** indicates carotene crystals [the one on the right is membrane-bounded (a carotene body)]; **v**, vesicles; **m**, mitochondria; and **f**, fat droplet. In (B), carotene crystals (**c**) and densely stained precipitates (**p**) are visible. The scale bars represent 1 μm.

TABLE 2
Protein Content of Carrot Juice and Spinach Suspensions[a]

	Total protein (μg/mL)	Soluble protein (μg/mL)	% Soluble	Carotene (μM)
Raw carrot juice	1967 ± 599	1460 ± 452	56.3 ± 10.5 (12)	196.3 ± 52.4 (6)
Blanched carrot juice	1461 ± 443	193 ± 72	13.7 ± 6.1 (9)	200.4 ± 48.2 (6)
Raw spinach suspension	6780 ± 98.5	362 ± 48	5.3 ± 0.8 (3)	78.9 ± 31.6 (3)
Blanched spinach suspension	ND	344 ± 52	ND	60.0 ± 25.3 (9)

[a]Results are given as mean ± SD (number of measurements). ND, not done

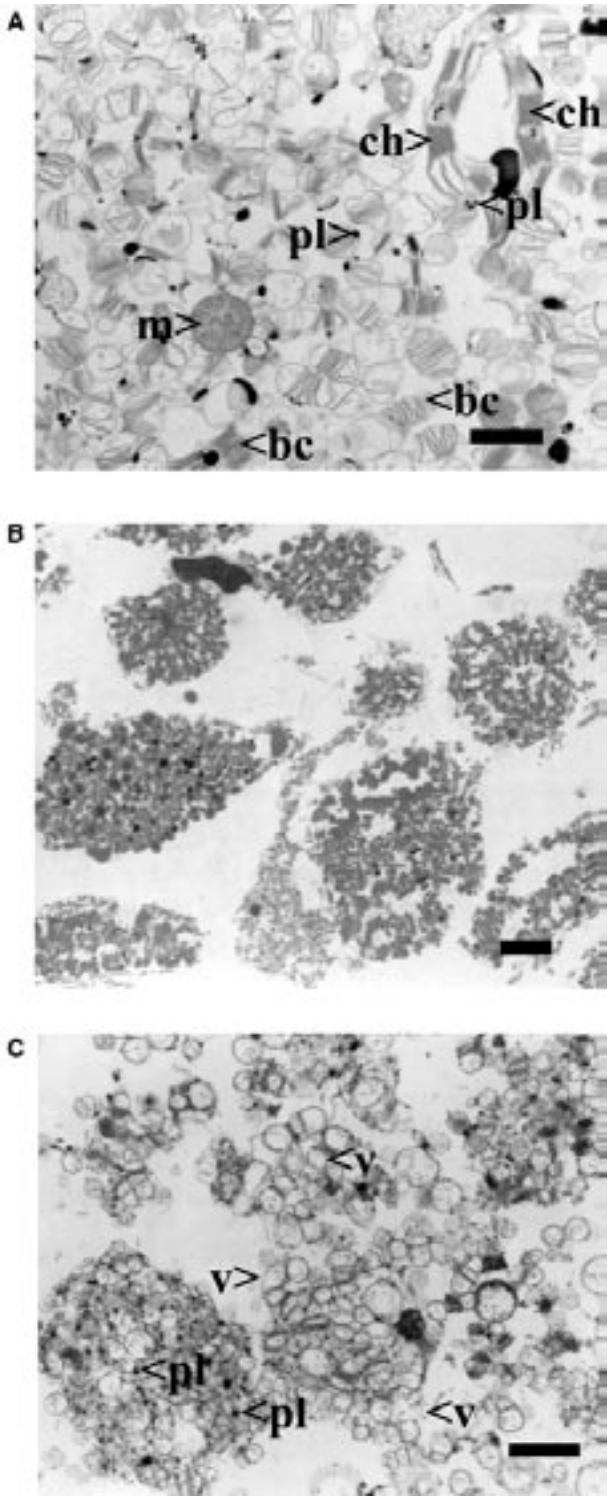

FIG. 2. Transmission electron micrographs of (A) raw spinach suspension, (B) blanched-frozen spinach suspension, and (C) raw spinach suspension after incubation at pH 2.5 for 0.5 h. In (A), **ch** indicates intact chloroplasts with grana and thylakoid membranes; **bc**, broken chloroplasts; **pl**, plastoglobuli; and **m**, a mitochondrion. In (B), these features are lost: The organelles have formed densely stained coagula, which are featureless apart from the presence of droplets, which may be plastoglobuli. Panel C represents an intermediate state where there is some coagulation but vesicles (**v**) and plastoglobuli (**pl**) are visible. The scale bars represent 1 μm.

± 0.8% of the total) because most of the soluble protein had been removed when the chloroplasts were concentrated. Upon blanching, protein precipitation/denaturation was irreversible, and it was impossible to measure accurately total protein in blanched spinach suspensions because of the extreme difficulty of solubilizing the insoluble protein, even in 10% SDS at 100°C. The results from spinach suspension protein analyses are presented in Table 2.

(iii) Effect of low pH. The gastric environment was mimicked by incubating the raw carrot juice and raw spinach suspensions at pH 2.5. The effects of blanching and low pH were very similar. As reported previously for carrot (6), acid treatment made the carrot and spinach material prone to aggregation and led to the appearance of dark coagulated cytoplasm in the electron micrographs (Figs. 7A and 8C in Ref. 6 for carrot and Fig. 2C in this paper for spinach). Chloroplast ultrastructure was largely, but not completely, lost (Fig. 2C). In comparing Figures 2B and 2C, it is clear that blanching has a more destructive effect on spinach organelle ultrastructure than low pH. Many vesicles (perhaps swollen plastids) were present at low pH that were not seen in blanched material. These vesicles were not present in spinach that had been blanched and then subjected to low pH treatment.

The effect of low pH on soluble protein implied by the electron microscopy was supported by protein analysis. Thus, low pH treatment of raw carrot juice led to an increase in the insoluble fraction at the expense of soluble protein. Insoluble protein in blanched juice was less affected by a fall in pH since the soluble protein had already been precipitated. For raw spinach, low pH treatment initially slightly increased the amount of soluble protein, which then fell. Blanched spinach showed no increase in soluble protein with acid treatment, implying an irreversible precipitation. Since these effects are time dependent, are influenced by the presence of pepsin, and are relevant to understanding the results on carotenoid transfer to oil, they are shown below, together with the transfer results.

Stability of carotenoids at low pH. Carotenoids are known to be less stable in acid media (21). To interpret results on transfer of carotenoids to oil, it was important to know whether there was any carotenoid loss due to the low pH. We could detect no loss of carotenoids in carrot juice and blanched-frozen spinach suspension for up to 2 h at 37°C and pH 2.5 even in the presence of pepsin. However, this was not true for the raw spinach suspension, where 5% of lutein and *trans*-β-carotene was lost within 20 min and between 10 and 20% in 2 h. Addition of pepsin increased these losses, with lutein being particularly sensitive. Thus, at pH 2.5 in the presence of pepsin, 76% of lutein and 50% of *trans*-β-carotene had disappeared within 2 h. Although the raw spinach carotenoids were more stable at pH 6.5 (less than 5% lost in 2 h), the losses at low pH were one of the reasons why fewer studies were done with the raw spinach. The reason for the difference may well be that blanching inactivated lipoxygenase enzymes and/or destroyed some of the more vulnerable carotenoids.

Effect of pH on carotenoid transfer to olive oil. (i) Carrot juice. The amount of carotene transferred to olive oil from raw

carrot juice is enhanced by lowering the pH (6). This is illustrated in Figure 3A (solid lines). Both the absolute amounts transferred and the enhancement seen at low pH were variable between different batches of juice (see Fig. 3, Ref. 6). Within one batch of juice, carotene transfer was linearly related to the concentration of carotene in the aqueous phase. In the present work, blanching of juice was found to enhance transfer when the measurements were made at pH 6.5. The effect with blanched juice of lowering the pH was opposite to that seen with raw juice: Low pH decreased carotene transfer (dotted lines in Fig. 3A). As a consequence, the effect of blanching on carotene transfer measured at low pH was usually not significant. (Another example of this is shown in Fig. 6A.)

The different effects of low pH treatment on raw and blanched juice are reflected in what happens to the protein. Thus, for raw juice, Figure 3B(i) shows that low pH caused a time-dependent decrease in soluble protein, which appeared largely as insoluble protein. (There was only a small amount of protein hydrolysis, since the total protein fell by only 11%.) In contrast, for blanched juice, low pH produced a decrease in insoluble protein [Fig. 3B(ii)] attributable to hydrolysis, since soluble protein did not change significantly from its initial low level but the total protein declined by 32%.

(ii) Spinach suspensions. For the blanched-frozen spinach suspension in 150 mM NaCl, the amounts of carotenoid transferred to oil were more reproducible and, bearing in mind the variability of the carrot results, were broadly comparable to the carotene transferred from the carrot juice. Lowering the pH gave a small but significant enhancement of transfer of lutein and *trans*-β-carotene [solid lines in Figs. 4A(i) and 4A(ii) for lutein and *trans*-β-carotene, respectively]. In general, the pH effect was smaller than that seen with carrot juice. For example, after incubation of carrot juice with oil for 1 h, typical experiments showed that the carotene concentrations in the oil were from 1.8 to12.5 times larger at pH 2.5 than at pH 6.5 (Fig. 3, Ref. 6). For spinach, the same pH change and incubation time increased lutein and *trans*-β-carotene concentrations in the oil by a factor of 1.67 ± 0.32 (mean ± SD, $n = 4$).

Figures 4B(i) and 4B(ii) incorporate the results from several experiments showing that, as for carrot juice, the amount of transfer was linearly related to the concentration of carotenoid in the aqueous phase. This meant that the results from different experiments using different concentrations of carotenoids could be combined and the amounts transferred expressed as a percentage of the total initially present in the aqueous phase.

Figures 4C(i) and 4C(ii) show the averaged results from two experiments on the raw spinach suspension. The amounts of carotenoid transferred were an order of magnitude smaller than for the blanched-frozen spinach, with less than 1% of lutein and *trans*-β-carotene moving into the oil after 2 h at pH 6.5. However, in spite of the instability of the raw spinach carotenoids in acid media, lowering the pH to 2.5 gave a relatively large effect, with about 2 and 4% of lutein and *trans*-β-carotene, respectively, reaching the oil phase after 2 h.

In spite of the relatively large effect of acid treatment on carotenoid transfer with raw spinach, unlike raw carrot juice,

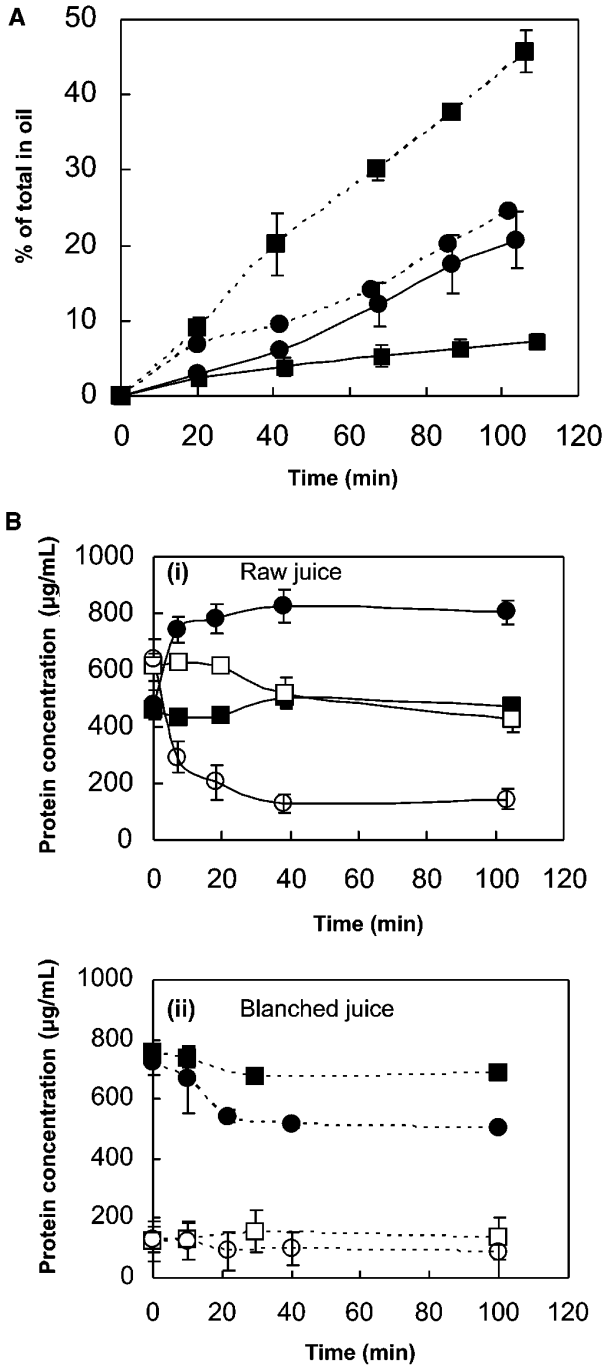

FIG. 3. (A) Transfer of carotene from raw (solid lines) and blanched (dotted lines) carrot juice to olive oil as a function of time measured at pH 6.5 (■) and pH 2.5 (●). Each point shows the mean and SD of three measurements. The initial aqueous-phase carotene concentration was 109 µM. At pH 6.5, in comparing blanched and fresh juice, carotene solubilization was significantly higher after 20 min for the blanched juice ($P < 0.01$). In comparing the results at the different pH values, after 20 or 60 min for blanched and fresh juice, respectively, solubilization was significantly different ($P < 0.01$). (B) The protein concentrations in incubations of (i) raw and (ii) blanched juice. The conditions indicated by the symbols are as in (A) but with solid and open symbols referring to insoluble and soluble protein, respectively. Each point represents the mean and range of values for two experiments. The results refer to the same concentration of carotene as used in (A), but different batches of juice were used.

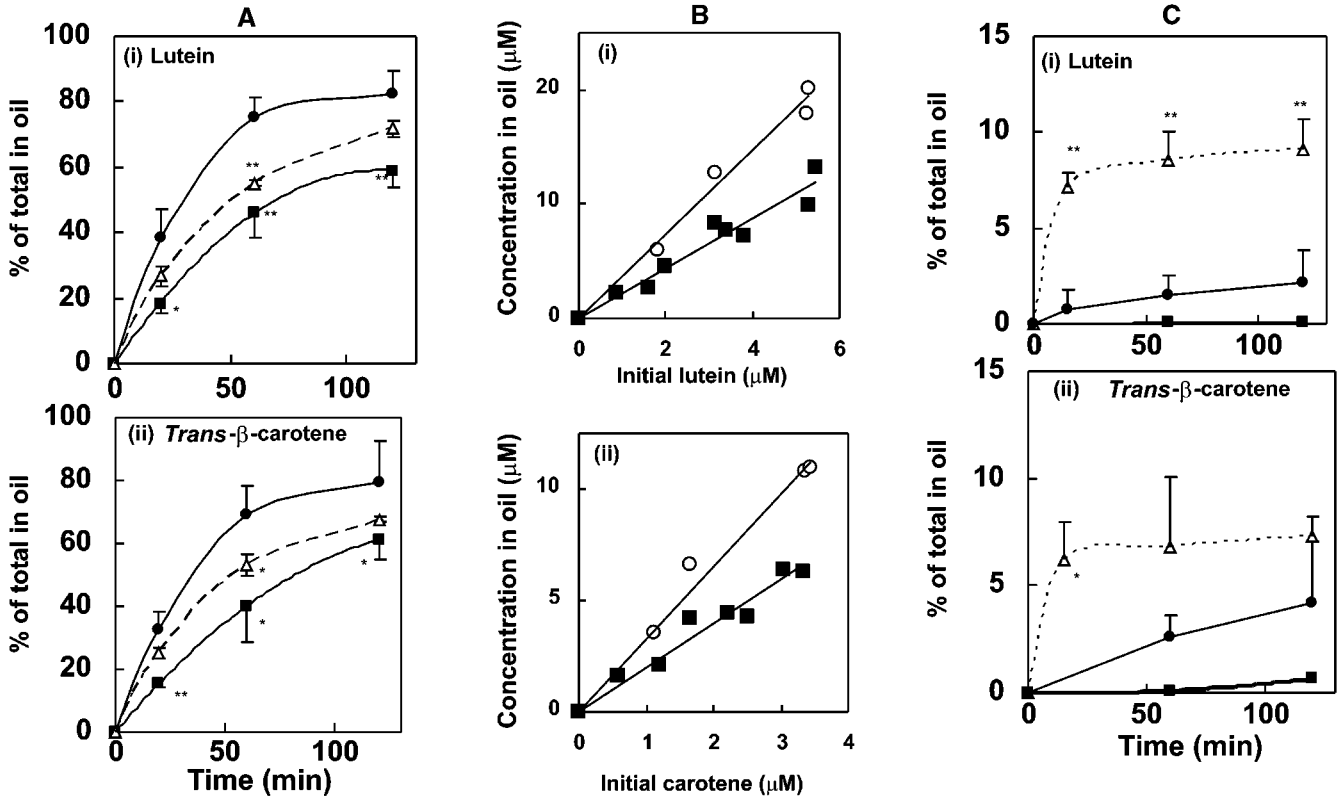

FIG. 4. Transfer of (i) lutein and (ii) *trans*-β-carotene from (A) frozen-blanched and (C) raw spinach suspensions to olive oil measured at pH 2.5 ● (solid line), pH 6.5 ■ (solid line), and pH 2.5 with pepsin △ (dotted line). Each point shows the mean and SD of three experiments. Significantly different from the means measured at pH 2.5 without pepsin: $*P < 0.05$, $**P < 0.01$. The initial aqueous-phase concentrations ranged from 3.15 to 5.57 for lutein and 1.57 to 3.44 μM for *trans*-β-carotene. Justification for combining data from experiments with different starting carotenoid concentrations is shown in B(i) and B(ii), where results from several experiments are combined to show the amount of carotenoid in the oil after 1 h, plotted as a function of the initial concentration in the aqueous phase: (○) pH 2.5, (■) pH 6.5.

this was not reflected in any dramatic change in either insoluble or soluble protein (see solid lines in Fig. 5B). Of course, the difference may be somewhat illusory since the starting soluble protein level was so low. Like raw carrot juice, there was little hydrolysis, with total protein falling by 12%. For the blanched spinach (see solid lines in Fig. 5A), as far as could be determined (since total protein measurements were impossible), the blanched carrot and spinach proteins behaved similarly with soluble protein unaltered by low pH treatment. (The reader is reminded that, for the protein measurements, as mentioned at the end of the Methods section, we used blanched spinach as a model for blanched-frozen spinach.)

Effect of pepsin treatment on transfer of carotenoids to oil. (i) Carrot juice. Figure 6A shows that for raw carrot juice, inclusion of pepsin at pH 2.5 enhanced carotene transfer to oil above the level observed in the absence of pepsin. If, after treatment for 1 h with pepsin in the absence of oil, the pH was raised to 6.5 and carotene transfer to oil measured, the enhancement of transfer persisted (results not shown). If the pH were kept at 2.5 after the pepsin treatment, then one would expect that the amount of carotene transferred to oil would be even higher owing to the continued activity of the pepsin and to the inherent pH sensitivity of the transfer process. This expectation was confirmed (results not shown).

Figure 6A also shows the effect of pepsin on transfer of carotene to oil from blanched carrot juice. The pepsin-induced enhancement shown with raw juice was much reduced for the blanched juice. For raw juice, by comparing the effect of low pH treatment alone with low pH plus pepsin, one can see that pepsin reduced the amount of insoluble protein [Fig. 6B(i)]. For blanched juice [Fig. 6B(ii)] the opposite appeared to be the case. This effect was small, and since pepsin had no significant effect on soluble protein it probably indicates some change in the protein due to hydrolysis affecting the protein assay (20).

(ii) Spinach suspensions. For the raw spinach suspension, Figures 4C(i) and 4C(ii) show that pepsin treatment enhanced carotenoid transfer. (For *trans*-β-carotene, variability in the results means the enhancement is only significant at short times.) As for carrot, the enhanced transfer persisted if pepsin treatment in the absence of oil was followed by measurements at pH 6.5 (results not shown). The blanched-frozen spinach behaved differently. Here, as shown in Figures 4A(i) and 4A(ii), pepsin treatment reduced the amounts of lutein and *trans*-β-carotene in the oil. In the context of soluble protein inhibiting carotenoid transfer to the oil, it is significant that with pepsin treatment of blanched spinach suspensions, soluble protein rose and the elevated level was maintained throughout the incubations (see Fig. 5A). In contrast, as

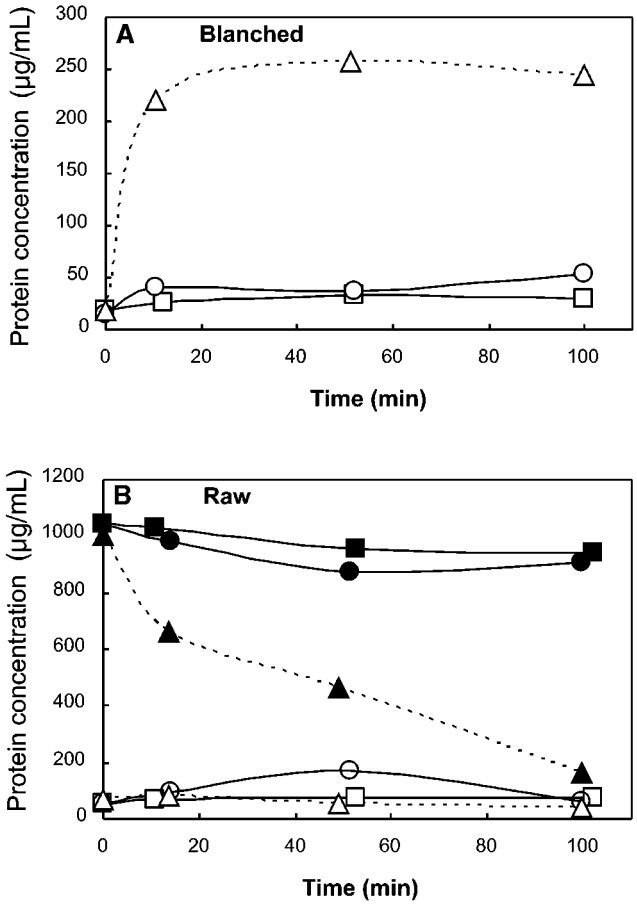

FIG. 5. Protein concentrations in incubations of (A) blanched and (B) raw spinach suspensions. The conditions indicated by the symbols and lines are as in Figures 4A and 4C, with filled and empty symbols referring to insoluble and soluble protein, respectively. Each point represents the mean and range of values for two experiments. The concentration of the carotenoids was approximately four times the highest concentration used in the transfer experiments.

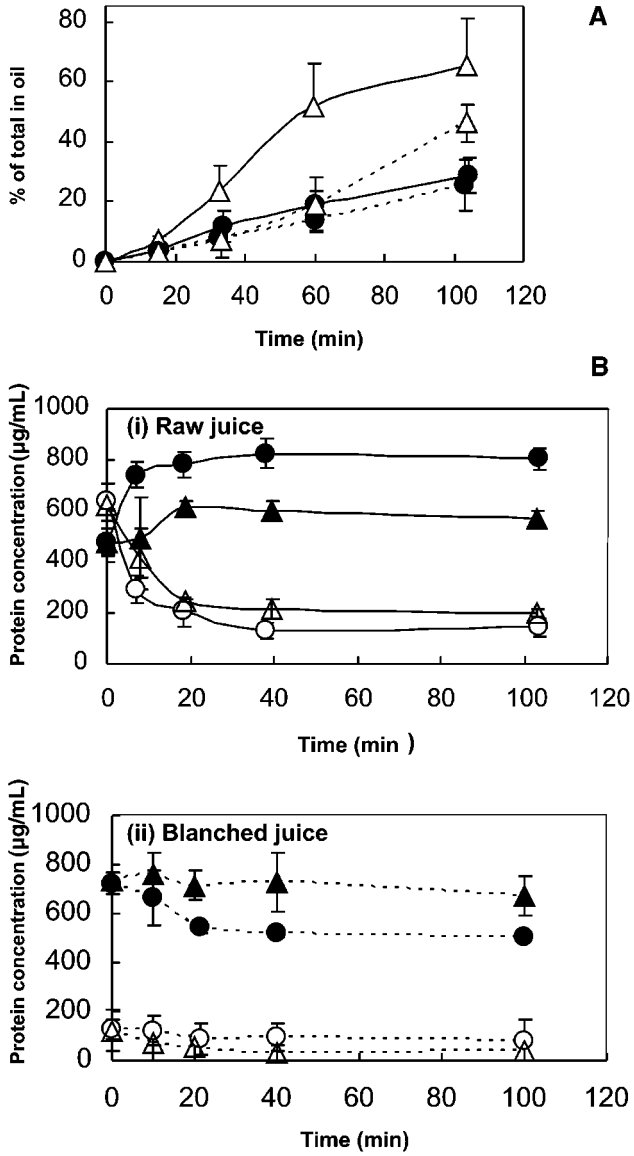

FIG. 6. (A) Comparison of the effect of pepsin on transfer of carotene from raw (solid line) and blanched (dotted line) carrot juice to olive oil at pH 2.5. The initial carotene concentration was 95 µM. Each point represents the mean and range of values for two experiments. (△) With pepsin, (●) without pepsin. (B)The protein concentrations in incubations of (i) raw and (ii) blanched carrot juice. The conditions indicated by the symbols are as in (A), but with filled and empty symbols referring to insoluble and soluble protein, respectively. Each point represents the mean and range of values for two experiments.

shown in Figure 5B, pepsin acting on raw spinach suspensions caused only a small, transitory rise in soluble protein, and insoluble protein was progressively lost.

Influence of soluble protein on carotene transfer to oil from carrot juice. It has previously been suggested (6) that soluble protein acted as an inhibitor of carotene transfer to oil. The present work is consistent with this hypothesis. However, when making comparisons between, for example, raw and blanched carrot juice, or pH 6.5 and 2.5, or the presence and absence of pepsin, it is not only the soluble protein that is changing. Therefore, soluble protein was added to juice to see if this inhibited carotene transfer at pH 6.5. Typical results from such an experiment are shown in Figure 7. From raw juice, preparations were made of soluble protein and blanched juice. Aliquots of raw and blanched juice were diluted three times into 70 mM NaCl + 1 mM azide together with raw soluble protein solutions so that the latter contributed from 0 to 63% of the total volume. Transfer to the oil phase was then measured. Figure 7 shows the results after 2 h incubation with the oil. The addition of soluble protein does indeed inhibit transfer of carotene from both raw and blanched juice. However, to achieve the low level of transfer with blanched juice that was seen with raw juice, it was necessary to add much more soluble protein than that which was present in the raw juice incubations. A similar trend was seen after 1 h incubation with oil (results not shown).

Effect of ionic strength on carotenoid transfer to oil. (i) Carrot juice. Figure 8A shows typical experiments indicating that increasing the ionic strength had no effect at pH 2.5. At pH 6.5 a small but significant enhancement of transfer

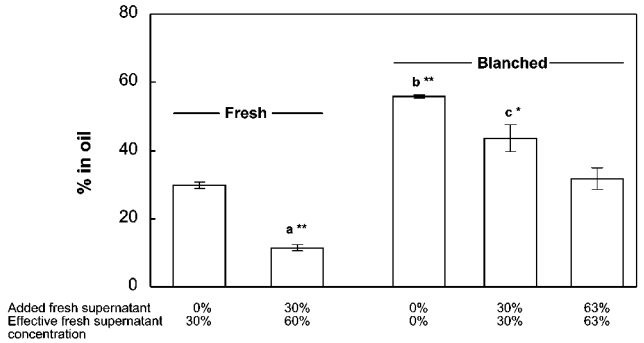

FIG. 7. Typical experiment showing the effect of adding raw carrot juice supernatant to raw and blanched juice on carotene transfer to olive oil measured after 2 h. Results are the mean and SD of values for three experiments on the same batch of juice. The carotene concentration was 86 ± 10 μM. The presence of different letters over the error bars indicates that solubilization was significantly different from the raw juice without any added supernatant. $^{**}P < 0.01$, $^{*}P < 0.05$.

occurred when the NaCl concentration was increased. These observations are consistent with our suggestion (6) that one factor in determining carotene transfer to the oil phase is the surface potential at the oil–aqueous phase interface. The surface potential is negative at pH 6.5 and rises to near zero at pH 2.5. A surface potential near zero facilitates adhesion of the oil to carrot tissue containing the carotene crystals, carotene bodies, and chromoplasts so the carotene can pass through the oil–water interface and dissolve in the oil.

(ii) Frozen spinach suspension. Figures 8B(i) and B(ii) show the results of similar experiments using frozen spinach where the spinach suspension pellet was diluted into either 150 or 70 mM NaCl. Here, in contrast to the situation with carrot juice, raising the ionic strength at pH 6.5 had no effect on either lutein or *trans*-β-carotene transfer to the oil. At pH 2.5 there was a rise in the amounts transferred, but this was significant only for lutein. These observations point to different controlling factors for the two vegetables in partitioning of the carotenoids.

It can also be seen from Figure 8B that the low pH enhancement of transfer seen in 150 mM NaCl is apparently a kinetic effect in 70 mM NaCl. For up to an hour in 70 mM NaCl, both lutein and *trans*-β-carotene concentrations in the oil were higher at the lower pH, but after 2 h the concentrations were the same and indistinguishable from those measured in 150 mM NaCl at pH 6.5.

DISCUSSION

The work described in this paper develops further our initial study modeling digestion within the gastrointestinal tract of carotenoids from vegetable tissue (6). Intestinal absorption of carotenoids is dependent on their solubilization in lipid phases. Digestion in the stomach initiates this process. We consider our results in terms of the physicochemical principles determining solubilization of the carotenoids from the vegetables into the gastric oil phase. Solubilization in the duodenal lumen is considered in Reference 9.

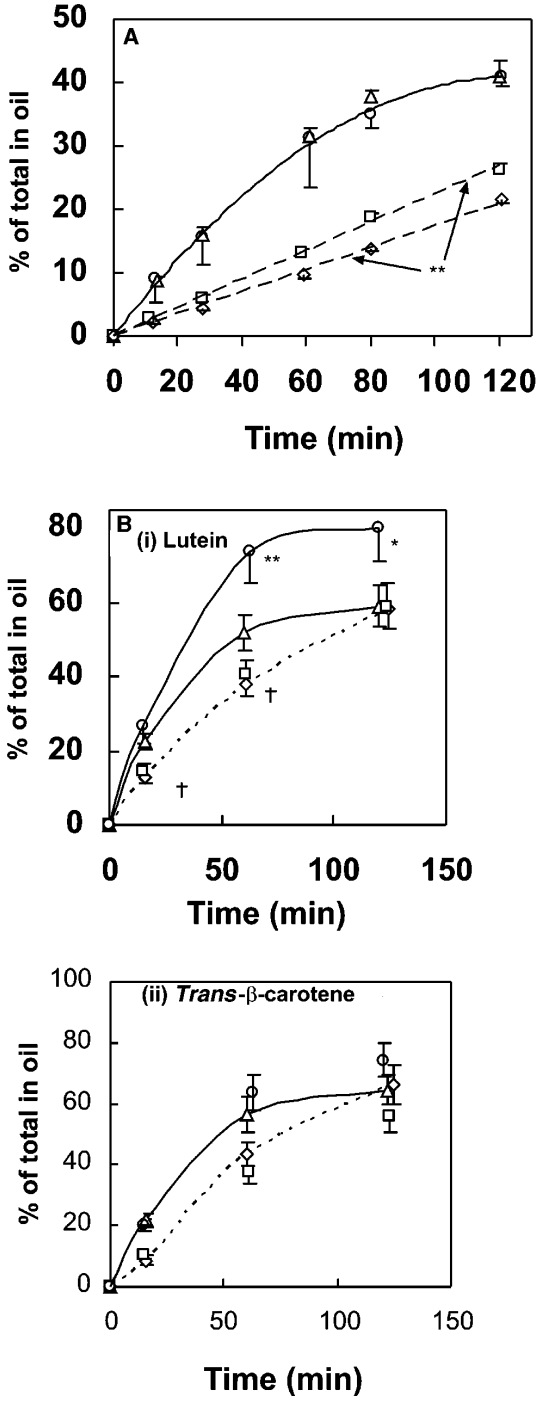

FIG. 8. The effect of ionic strength on transfer of carotenoids to olive oil. (A) Transfer of carotene from raw carrot juice. The carotene concentration was 63 ± 3 μM. (B) Transfer from blanched-frozen spinach suspension, with the concentrations of *trans*-β-carotene and lutein being 2.9 to 3.3 and 4.7 to 5.1 μM, respectively. (○) 150 mM NaCl, pH 2.5; (△) 70 mM NaCl, pH 2.5; (□) 150 mM NaCl, pH 6.5; (◇) 70 mM NaCl, pH 6.5. The data are shown as the mean and SD of three experiments made on the same batches of carrot juice and frozen spinach suspension. In (A), for carrot, ** indicates a significant difference in solubilization ($P < 0.001$) at pH 6.5 as shown by an *F*-test on linear regression of the data. In (B), for spinach, significant differences at the same times are indicated by ** ($P < 0.01$) and * ($P < 0.05$) for the two ionic strengths at pH 2.5; † ($P < 0.05$) for the two pH values at an ionic strength of 70 mM.

The insolubility of carotenoids in water precludes simple diffusion from the vegetable to the site of absorption during passage through the gut. To our knowledge, there is no evidence that carotenoids, either in the crystalline state or in membranes, can move by endocytosis into enterocytes. Therefore, it must be assumed that all absorbable carotenoids are solubilized in lipid phases before they can pass through the mucosal membrane. Within the gastric lumen, the principal lipid phase consists of TAG either as an emulsion or as a bulk phase. Both of these forms have been reported within the gastric lumen of human volunteers (22). In this work, olive oil has been used as the gastric oil phase because it is a fat that is commonly consumed by humans. By reference to solubility data in the literature (7), it is apparent that in our systems carotenoid solubilization was not limited by the solubility of the carotenoids in the oil.

Clearly, our model system is a simplification of the gastric lumen. Lipase has been omitted, so the effect of this enzyme and the products of lipolysis at the interfaces and bulk phases have been ignored. Within the gastric environment, hydrolysis is limited to about 10% of the total FA that potentially can be released from TAG (23–25) and hence its contribution is assumed to be small.

The initial environment of carotenoids in the vegetable preparations. The principal carotenoids within carrot are *trans*-α- and -β-carotene. They are located within the chromoplasts as crystalline bodies (10). In spinach the location of carotenoids within the tissue is very different. They are found in the inner membranes of the chloroplasts, mainly in the lipoprotein complexes of photosystems II and I (11). Furthermore, the relative amounts of the two types of carotenoids are different, with lutein being present at a higher level than *trans*-β-carotene.

Since in carrot juice the cellular structures containing the chromoplasts were disrupted and the cell walls were removed, an analogous system was used for spinach consisting largely of chloroplasts. Thus, for the two vegetable preparations, carotenoid solubilization from the organelles sequestering the carotenoids could be compared without the complications of differences in gross cellular structure. The effect of the blanching procedure had a large effect on the chloroplast structure. The native membrane structure and arrangement were completely lost, leaving a coagulated amorphous mass. The aggregated/denatured proteins became largely detergent-insoluble, and so the native lipoprotein–carotenoid interactions must have been altered in the frozen spinach suspensions. Previous structural studies (26) on the effect of cooking and freezing had concentrated on the breakdown of cells and cell wall structures with scant reference to the effect on organelles. Experimentally, there is some advantage in using blanched spinach because the carotenoids are more stable. With carrot juice, although blanching has a destructive effect on membrane-bounded organelles (Figs. 1A and 1B), carotene crystals are still present. Thus, it is likely that carotene–lipid and carotene–protein interactions were disrupted but the stable carotene–carotene interactions left largely unchanged. For both carrot and spinach, high-resolution electron microscopy and spectroscopic studies could give further information on the effect of blanching on the intermolecular interactions within the organelles.

The gastric environment: transfer of carotenoids to oil. In our previous studies (6) on the pH effect of carotene partitioning from carrot juice to an oil phase, transfer was facilitated by the adherence of oil droplets to the carrot material, thereby juxtaposing carotene crystals, carotene bodies, and intact chromoplasts with the oil–aqueous phase interface. The relative ease of aggregation was enhanced by low pH when the surface charge of the oil droplets was at a minimum. The pH dependence of the surface charge and the transfer to oil of

TABLE 3
Summary of Carotenoid Transfer and Protein Results[a]

	Carotenoid transfer to oil			Protein	
	Carrot juice	Spinach suspension		Carrot juice	Spinach suspension
			Effect of decreased pH		
Raw	↑	↑	Soluble	↓	—
			Insoluble	↑	—
Blanched	↓	↑	Soluble	—	—
			Insoluble	↓	?
			Effect of pepsin at pH 2.5		
Raw	↑	↑	Soluble	—	—
			Insoluble	↓	↓
Blanched	↑	↓	Soluble	—	↑
			Insoluble	↑	?
			Effect of blanching on measurements at pH 6.5		
	↑	↑	Soluble	↓	↓
			Insoluble	↑	?

[a]↑ Indicates a significant increase ($P < 0.05$), ↓ a significant decrease ($P < 0.05$), — no significant change, and ? not measured.

exogenous β-carotene also suggested the presence of some inhibitory factor (possibly protein) from the carrot juice present at the oil droplet interface and/or surrounding the crystallites themselves.

The results obtained for raw spinach are qualitatively similar, but the different environments of the carotenoids in the raw spinach chloroplast compared to that of carotene crystals (free and membrane-bounded in carotene bodies and chromoplasts) in carrot juice are evident from the different proportions of total carotenoid partitioned into the oil phase. The low level of transfer from the raw spinach reflects the stability of the unmodified chloroplast membranes in which the carotenoids are integrated. The hypothesis that soluble protein inhibits carotenoid transfer to oil is supported by the experiments of Figures 3, 5, and 6 (summarized in Table 3), which show that a significant fall in soluble protein was associated with a rise in the transfer of carotenoid to the oil phase. Furthermore, the single case of elevated soluble protein (spinach suspension at pH 2.5 with pepsin, Fig. 5) gave a decrease in transfer. Therefore, it was of interest to see if a system with low soluble protein and relatively high carotenoid transfer could be transformed into a system with low carotenoid transfer by adding soluble protein. Figure 7 shows that, by using blanched carrot juice and adding a preparation of soluble protein from raw juice, we were able to inhibit carotene transfer. The inhibition was not so large as to reduce the transfer to the level seen with the raw juice, indicating the different amount of soluble protein was not the only difference between raw and blanched juice.

One additional effect of blanching carrot juice is to increase the amount of insoluble protein. Since carotene crystals are associated with the insoluble aggregates, it is likely that with low soluble protein content, carotene transfer from the aggregates across the oil interface is facilitated. Another possible factor is destabilization of carotene–lipid/protein interactions (27).

Of course, the solution of soluble protein that was added also contained other components that could inhibit transfer of carotene to the oil. A prime candidate for providing this inhibition is soluble fiber, which is present in carrot (28,29) and has been found to decrease carotenoid bioavailability *in vivo* (30,31). Work is planned to investigate whether soluble carrot fiber does indeed inhibit partition of carotene from carrot chromoplasts into oil.

The importance of carotenoid–protein interactions in controlling the partition process with raw vegetables is shown by the effect of pepsin. For both carrot and spinach, pepsin hydrolyzes insoluble protein and enhances transfer. For raw carrot juice, an additional enhancing factor is the loss of soluble protein.

The factors that determine the partitioning for these raw vegetables are clearly modified for the processed vegetables. The disruption of native structure, as well as enhancing transfer, alters the effect of low pH and pepsin treatment. For blanched carrot, the enhancing effect of acid seen with raw juice becomes an inhibition (Fig. 3A). This may be due to the loss of insoluble protein: first, that which is intercalated between the carotene layers in the carotene bodies, and second, that which is precipitated at the low pH. The latter facilitates the adherence of carrot material to the oil interface. For blanched-frozen spinach, low pH treatment enhances transfer as seen with raw spinach, but the effect is relatively attenuated [compare Figs. 4A(i) and (ii) with Figs. 4C(i) and (ii)]. This is expected, as the blanching treatment alone destroys chloroplast native structure. Therefore, blanching and low pH treatment will have only a relatively small effect. When pepsin is present in the blanched-frozen spinach suspension, there is an inhibition compared to low pH treatment alone. It is likely that this is due to pepsin releasing soluble protein, which is resistant to hydrolysis. For blanched carrot, pepsin still causes an enhancement of carotene transfer to the oil, but the enhancement is relatively small compared to what is seen with raw juice. Overall, these observations indicate that the effect of blanching is greater on the spinach chloroplast than the carrot chromoplast.

For raw carrot, the adherence of carotene-containing tissue to the oil interface is clearly influenced by surface charge/potential effects. Thus, raising the ionic strength at pH 6.5 when the surface potential has a negative value (6) facilitates carotene transfer to oil (Fig. 8A), whereas at low pH, when the surface potential is near zero, increasing the ionic strength has no effect. With the blanched-frozen spinach suspension, a different situation exists, with increasing ionic strength having a significant effect on lutein transfer only at low pH. This may only be a kinetic effect, and our results suggest that with spinach, carotenoid partitioning is largely controlled by the integrity of the chloroplast membranes.

It is of interest that the relative proportions of the carotenoids within the tissues were usually maintained after transfer to the oil. For carrot juice, a limited number of HPLC analyses showed that after 2 h incubation with oil, the *trans*-α- to -β-carotene ratio was 0.36 ± 0.04, which is not significantly different from the ratio in the carrot juice (Table 1). A similar situation, with one exception, was found for the blanched-frozen spinach suspension: At times less than 1 h in the absence of pepsin, the lutein/*trans*-β-carotene ratio tended to be higher: 2.08 ± 0.4 at pH 6.5 and 2.41 ± 0.03 at pH 2.5 compared to 1.59 + 0.18 in the starting spinach suspension. This suggests that, apart from some kinetic enhancement of lutein passing through the oil interface, a degree of organization persists until the point of transfer. It also indicates that partitioning into oil is not the digestive stage that could lead to differences between carotenoid bioavailability if the carotenoids are in equivalent environments in the organelles. Further, it highlights the importance of close proximity of the oil interface to the organelles to facilitate transfer.

Transfer of carotenoids from vegetables to sunflower oil has been studied in the stomachs of human subjects fed intragastrically (8). Equal amounts of lutein in cooked chopped spinach and β-carotene in cooked carrot puree were fed together with the oil. After 20 min, both carotenoids were present in the oil phase at comparable concentrations, with around

20–30% of the total being recovered in the oil phase. Our percentages of transfer for carrot juice and blanched-frozen spinach suspensions to the oil at the same time are similar to these values. At later times in the human studies, carotene from carrot continues to leave the vegetable material and pass into the oil, whereas with spinach, after the initial phase of loss of lutein from the tissue to the oil, the remaining lutein stays in the tissue. The time courses of these results from the human studies are similar to ours, with the exception that in our systems much more lutein in the spinach can eventually be solubilized in the oil. We believe this difference reflects the importance of vegetable matrix effects: In carrot paste the tissue is finely divided so that, like our carrot juice, the chromoplasts can easily reach the oil. However, in chopped spinach the cell matrix would provide a barrier to a large fraction of the chloroplasts.

Studies with humans also have looked at the fate of the carotenoids in the duodenum. Tyssandier *et al.* (8) showed, as has other work (9,32,33), the importance of solubilization in the duodenal micelles in limiting the overall bioavailability of carotenoids from vegetables.

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Solubilization of Carotenoids from Carrot Juice and Spinach in Lipid Phases: II. Modeling the Duodenal Environment

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ABSTRACT: We have been investigating the factors determining the bioavailability of carotenoids from vegetables. The previous paper [Rich, G.T., Bailey, A.L., Faulks, R.M., Parker, M.L., Wickham, M.S.J., and Fillery-Travis, A. (2003) Solubilization of Carotenoids from Carrot Juice and Spinach in Lipid Phases: I. Modeling the Gastric Lumen, *Lipids 38*, 933–945] modeled the gastric lumen and studied the solubilization pathway of carotenes and lutein from carrot juice and homogenized spinach to oil. Using the same vegetable preparations, we have extended our investigations to solubilization pathways potentially available in the duodenum and looked at the ease of solubilization of carotenes and lutein within simplified lipid micellar and oil phases present within the duodenum during digestion. Micellar solubility of raw spinach carotenoids was low and was enhanced by freezing, which involved a blanching step. The efficiency of solubilization of carotenoids in glycodeoxycholate micelles decreased in the order $\text{lutein}_{\text{carrot}} > \text{lutein}_{\text{blanched-frozen spinach}} > \text{carotene}_{\text{blanched-frozen spinach}} > \text{carotene}_{\text{carrot}}$. Frozen spinach carotenoids were less soluble in simple micelles of taurocholate than of glycodeoxycholate. The results comparing the solubility of the carotenoids in mixed micelles (bile salt with lecithin) with simple bile salt micelles are explained by the relative stability of the carotenoid in the organelle compared to that in the micelle. The latter is largely determined by the polarity of the micelle. Below their critical micelle concentration (CMC), bile salts inhibit transfer of carotenoids from tissue to a lipid oil phase. Above their CMC, the bile salts that solubilize a carotenoid can provide an additional route to the oil from the tissue for that carotenoid by virtue of the equilibrium between micellar phases and the interfacial pathway. Mixed micellar phases inhibit transfer of both carotenoids from the tissue to the oil phase, thereby minimizing this futile pathway.

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A substantial body of evidence suggests that ingestion of foods rich in carotenoids is associated with improved health, leading, for example, to a decreased risk of cardiovascular disease (1) and some cancers (2), particularly those associated with aging. The structure of the food and the way it is processed modify the absorption of these micronutrients from the diet (3,4). The origin of this effect is thought to be the hydrophobicity of the carotenoids, which prevents them from diffusing directly from the food structure to the site of absorption. Instead the carotenoids must be solubilized within the mixed micelles formed during digestion. Currently, there is a lack of information on the breakdown of food structure during digestion and the solubilization pathways present within the gastrointestinal tract.

Previous publications from this group have modeled, *in vitro*, the relative ease of release of carotenoids into ingested lipid from disrupted carrot and spinach tissue within the gastric environment (5,6). In spite of disrupting the cells in the vegetable tissue and removing cell walls, we found that in the time scale of gastric emptying, transfer of the carotenoids to the lipid phase (olive oil) was not complete. We have also investigated the effluent of ileostomy patients fed carotenoid-containing fruits and vegetables together with TG (7). One result of this *in vivo* work was that, under all the conditions studied, the tissue retained a significant proportion of the carotenoid. Thus, we suggest that carotenoid release is not complete within the gastric environment and that the tissue emptied into the duodenum still retains a significant proportion of the original carotenoid content.

Once the tissue and gastric emulsion phase enter the duodenal environment, they are bathed in the pancreatic secretions, i.e., the bile. This contains a number of surface-active agents including the bile salts (BS) and phospholipids (PL). Competitive adsorption of these surfactants significantly modifies the interface of the emulsion droplets (8), although the effect of this on carotenoid solubilization is unknown. They also form micellar and vesicular structures within the digesta (9) and the hydrophobic core of these colloidal particles provides a further solubilization site for the carotenoids. The long-chain FA formed by the action of lipase on the lipid emulsion droplets are also solubilized within these aggregated structures. The resulting mixed micellar phase is thought to be the principal diffusion vehicle for the carotenoids to the mucosal membrane.

The structure of the micellar particles is ordered and therefore constrained by packing considerations of its components (10). Solubilization of a hydrophobic molecule within the structure will depend upon its size and polarity. No systematic study of this has been undertaken, and hence the capacity of physiologically relevant micelles for carotenoids is as yet undefined.

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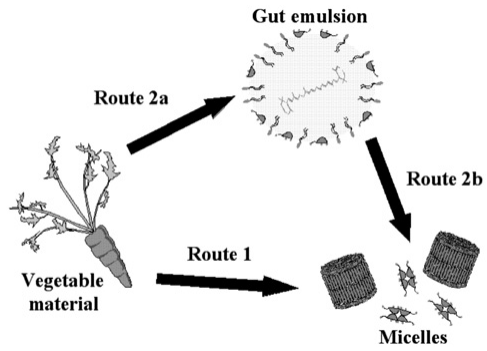
Abbreviations: CMC, critical micelle concentration; BS, bile salts; PL, phospholipid.



SCHEME 1

In summary, upon emptying into the duodenum a number of lipid structures can solubilize the carotenoids—an emulsion phase and micellar phases consisting of BS and PL (Scheme 1). Current thinking requires solubilization of the carotenoids by the emulsion phase prior to transfer to a mixed micellar phase (11). However, this ignores the possibility of direct solubilization of residual carotenoid in the vegetable tissue by the micellar phase.

The possible routes of solubilization from vegetable tissue to micelle are: Route 1, tissue to micelle: The direct solubilization from the tissue occurs without initial solubilization within the emulsion or oil phase. Route 2a, tissue to oil: This is the first part of what is thought to be the dominant route and continues the transfer initiated within the gastric environment. Route 2b, oil to micelle: This is the transfer of carotenoid from the oil phase to the micellar phase and completes route 2.

A study by Borel *et al.* (11) investigated the influence of lipase activity on the oil to micellar transfer for the main carotenoids (route 2b) and found an absolute requirement for lipase action to enable the carotene to transfer between an emulsion and micellar phase. Within this study we do not include the action of pancreatic lipase, as we aim to test the significance of routes 1 and 2a on the solubilization of the carotenoid before an in-depth analysis of the effect of lipase activity on the whole system. As in our previous paper on modeling the gastric solubilization of carotenoids from vegetables (6), we use carrot juice and preparations of spinach consisting of free plastids. This avoids complications due to the tissue architecture impeding access of the lipid-solubilizing agents to the sites of the carotenoids.

MATERIALS

The carrots and blanched-frozen spinach that were processed to make carrot juice and spinach suspensions are described in our previous paper (6). The spinach suspension was made in 150 mM NaCl. The sources of other materials were as before (6), with the additional chemicals sodium glycodeoxycholate (98%) and sodium taurocholate (98%) from Sigma-Aldrich (Poole, United Kingdom), and egg lecithin (L-α-PC), grade I, from Lipid Products (S. Nutfield, United Kingdom). All other reagents were of Analar or HPLC grade. Nylon membrane syringe filters (0.2 μm) were from Nalgene (Rochester, NY).

METHODS

Solubilization methodology. Solubilization of carotenoids in micelles was measured in 150 mM NaCl and 1 mM sodium azide at pH 6.5. To mimic digestion in the stomach, there was a 1-h 37°C preincubation with shaking on the orbital shaker of the carrot juice or spinach suspension where the pH was either 6.50 ± 0.05 or 2.5 ± 0.05. For pepsin treatment the enzyme was included in the low pH incubate at a concentration of 120 U/mL. (As described in the Results section, a preincubation was not always used.) After 1 h the pH was adjusted to 6.5, and samples were added to 150 mM NaCl with and without micelles and incubated at 37°C for 1 or 2 h. At the end of each incubation, samples were centrifuged at 17,000 × g_{max}, 37°C for 2 min and the supernatants filtered through 0.2-μm pore size nylon filters. The filtrates were stored at −30°C under a layer of argon before analysis by HPLC.

The micelles used were either simple BS (sodium glycodeoxycholate or sodium taurocholate) or mixed micelles of egg lecithin PL and BS. These systems were prepared by drying chloroform/methanol solutions of BS ± PL under nitrogen followed by overnight *in vacuo*. The lipids were then hydrated in 150 mM NaCl for 1 h at 37°C.

Transfer of carotenoids to olive oil was measured as described before (6).

Carotenoid analysis. HPLC measurements of carotenoids were performed as described (6). Aqueous samples containing carrot juice had high soluble protein concentrations and gave cloudy solutions when diluted into the mobile phase. They were therefore extracted three times by the Bligh and Dyer method (12). The chloroform extracts were dried under nitrogen and the dry residues dissolved in dichloromethane before dilution into the mobile phase. In experiments studying the effect of surfactants on transfer of carotenoids from carrot juice to olive oil, we found the lutein concentrations in the oil were very low, reflecting the low lutein concentration in the carrot (see Table 1, Ref. 6). In some experiments we measured only total carotene concentrations, as described previously (5), by diluting the olive oil samples into *n*-hexane and measuring the absorbance at 450 nm.

Measurement of critical micelle concentrations (CMC). BS CMC under the same conditions of ionic strength and pH as were used in the solubilization studies were measured by the pyrene fluorescence method (13). This measures the intensity of pyrene fluorescence peaks at 383 and 372.5 nm. F_R, the ratio of these peak intensities, reflects the partition of pyrene into the micelles and hence shows micelle formation. We found our data were best fitted by the equation $F_R = F_R^\circ + (K \cdot \Delta F_{max})/(K + e^{-([BS] \cdot c \cdot \Delta F_{max})})$, where [BS] is the BS concentration (mM) and F_R°, ΔF_{max}, K, and c are constants.

RESULTS

The carrot and spinach preparations we used were designed to provide a standard sample with minimal amount of tissue matrix above the organelle level sequestering the carotenoids. The

appearance of the raw carrot juice and the spinach suspensions as seen by light and electron microscopy has already been described (6). The reader is reminded that blanching the spinach led to complete loss of chloroplast ultrastructure.

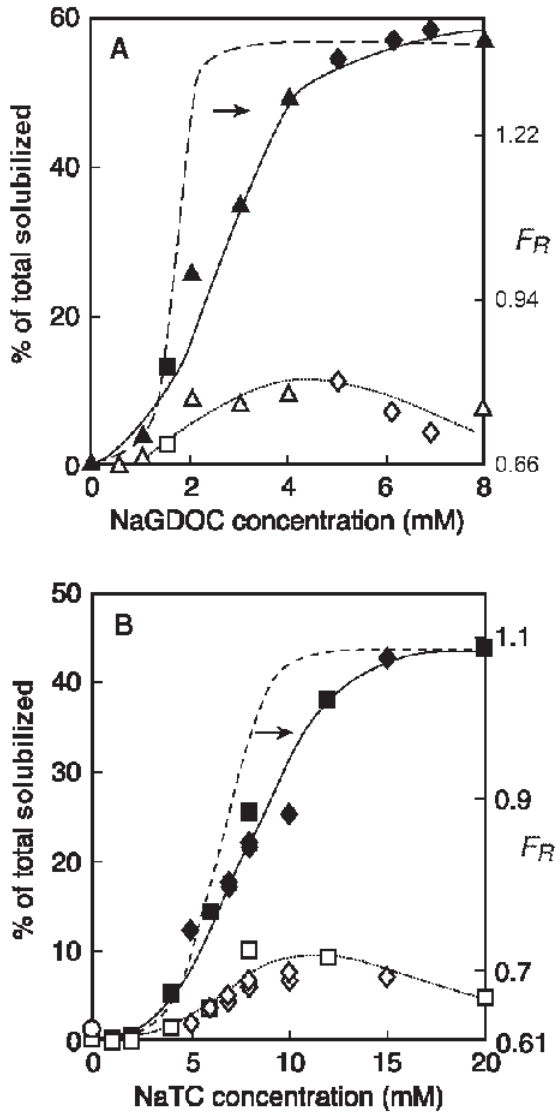


FIG. 1. Solubilization of blanched-frozen spinach carotenoids as a function of concentration of bile salt: (A) with sodium glycodeoxycholate (NaGDOC) and (B) with sodium taurocholate (NaTC). Open symbols show transfer of *trans*-β-carotene, and solid symbols show transfer of lutein. Different symbols represent different experiments. For (A) the total aqueous concentrations were from15.7 to 24.0 μM for lutein and 10.2 to 15.0 μM for *trans*-β-carotene. For (B) the total aqueous concentrations were from 15.1 to 24.0 μM for lutein and 9.7 to 15.0 μM for *trans*-β-carotene. The dashed line refers to the right-hand axis and shows the best fit for F_R, the pyrene fluorescence peak ratio data, which was described by the equation $F_R = F_R^o + (K{\cdot}\Delta F_{max})/(K + e^{-([BS]\cdot c\cdot\Delta F_{max})})$, where [BS] is the millimolar bile salt concentration and F_R^o, ΔF_{max}, K, and c are constants. For (A) [and (B)] $F_R^o = 0.66$ (0.61), $\Delta F_{max} = 0.72$ (0.48), $K = 0.00018$ (0.0028), $c = 6.85$ (1.881) with the maximum deviation of experimental data from the fitted line being 2.4% (1.5%).

Route 1: Direct solubilization of carotenoids from vegetable tissue into duodenal surfactants. PL and BS can form a range of aggregated structures (14) as a function of the BS/PL mole ratio. At low BS/PL ratios, the BS are solubilized within the PL vesicles, whereas at intermediate ratios mixed micelles of both surfactants are found. At high BS/PL ratios, simple BS micelles can coexist with the mixed micelles. We have investigated the direct solubilization of carotenoids from carrot and spinach tissue preparations by each of the phases found at physiological BS/PL ratios, i.e., simple and mixed micelles. The PL was egg lecithin, and the BS were glycodeoxycholate and taurocholate, chosen as representative of relatively nonpolar and polar BS, respectively (15). In the absence of surfactants, no intact carotenoids could be detected in the aqueous phase.

(i) Solubilization of carotenoids in simple BS micelles. Blanched-frozen spinach suspension. We found that with glycodeoxycholate and taurocholate, 1 and 2 h, respectively, were needed to reach the maximal amount of solubilization. After these periods, 87–95% of the carotenoids were recovered in the aqueous phase and vegetable material, which was extracted by the Bligh and Dyer method (12). Preliminary experiments also showed that preincubations at pH 6.5 or pH 2.5 ± pepsin did not significantly alter the concentrations of solubilized carotenoids. Figure 1A shows solubilization of spinach carotenoids by glycodeoxycholate and Figure 2B by taurocholate, as a function of BS concentration. The figures also show (dotted lines) the ratio (F_R) of the intensity of pyrene fluorescence peaks at 383 nm to that at 372.5 nm. (F_R reflects the partition of pyrene into micelles and the dipolar environment surrounding the pyrene monomers in the micelles (14) and hence identifies the formation of the micellar phase at the BS CMC). The solubilized carotenoid concentrations increased in the region of the CMC values of the BS (1–2 mM and 4–7 mM for glycodeoxycholate and taurocholate, respectively). Lutein was solubilized more than *trans*-β-carotene in taurocholate and glycodeoxycholate micelles, and glycodeoxycholate was more efficient than taurocholate at extracting lutein. With *trans*-β-carotene, it was difficult to determine which was the more efficient BS. Certainly, the rise in amount solubilized was steeper and occurred at a lower BS concentration for glycodeoxycholate, correlating with its lower CMC, but at higher concentrations the amounts of *trans*-β-carotene solubilized with both BS appeared to fall. Possible explanations for this are explored in the Discussion section. Figure 2 shows the maximal amounts solubilized (empty bars) and also the solubilization results in mixed micelles (hatched bars), which we present after considering the carrot solubilization results.

(ii) Solubilization of carotenoids in sample BS micelles. Carrot juice. Preliminary experiments showed that the total amount of carotenoids solubilized by the micelles was very low, with maximal solubilization achieved within 2 h. In fact, we made measurements only with glycodeoxycholate because visual inspection of the aqueous phase with taurocholate showed there had been very little carotenoid solubilization. Solubilized lutein increased in the region of the CMC of the BS, so that 70 to 90% of the total lutein was solubilized by 4

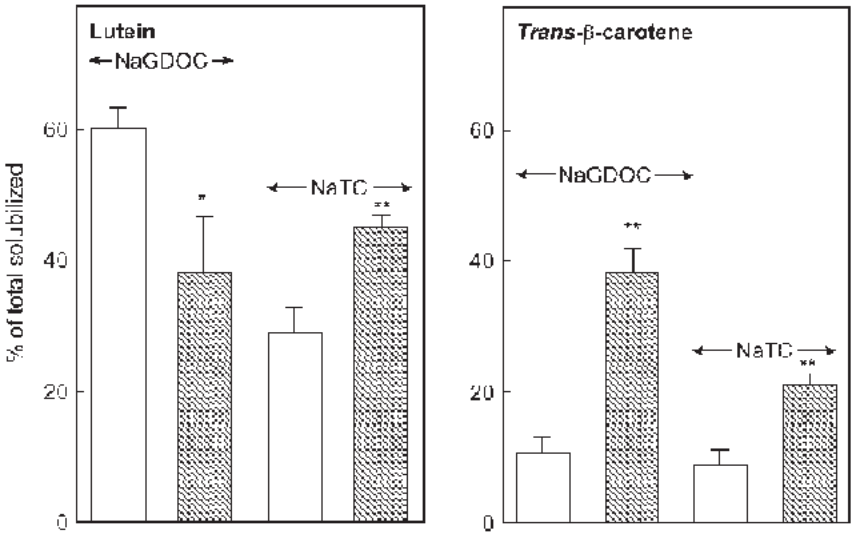


FIG. 2. Solubilization of blanched-frozen spinach suspension carotenoids by 8 mM bile salts (empty bars), 8 mM bile salt + 5 mM phospholipid (hatched bars). The bile salts were NaGDOC (left-hand bars) and NaTC (right-hand bars). Measurements made at 2 h at pH 6.5 after 1 h pretreatment at pH 2.5. Each data point represents the mean and SD of values for three experiments. Asterisks indicate that solubility in the mixed micelles was significantly different from that in the simple micelles (**$P < 0.01$, *$P < 0.05$). The carotenoid concentrations ranged from 5.2 to 25.5 μM for lutein and 4.6 to 15.6 μM for *trans*-β-carotene. For abbreviations see Figure 1.

mM BS. At higher BS concentrations, the solubilized lutein did not increase further. In contrast, less than 2% of the *trans*-α- and -β-carotenes were solubilized. It must be emphasised that in absolute terms the amounts solubilized were very small, that is, in the micromolar range for lutein and less than 1 μM for the carotenes. Furthermore, since the amounts of solubilized carotenes were very small, our assays were accurate to only about 15%. As observed for spinach carotene, when the BS concentration increased above the CMC, there were indications that the amounts of solubilized *trans*-α- and -β-carotene passed through a maximum. Low-pH pretreatment depressed *trans*-α- and -β-carotene solubilization without affecting lutein solubilization. Including pepsin in the low pH pretreatment restored the carotene to the pH 6.5 pretreatment levels. The amounts solubilized in 8 mM glycodeoxycholate are summarized in Figure 3 together with the solubilization results we obtained when PL was added with the BS.

(iii) Solubilization of carotenoids within mixed micelles. The mixed micellar phase of BS and PL would be the dominant phase at physiological concentrations of bile. Indeed, even if BS are added in isolation they may form mixed micelles by solubilizing lipids present in the vegetable material. If this BS extraction of lipids were absent, adding PL with BS in our experiments would provide a rather different micellar environment than that provided by adding BS alone. Therefore, it is of interest to determine in our systems how added mixed micelles (8 mM BS with 5 mM PL) affect carotenoid solubilization, particularly in comparison with solubilization with BS alone. Figures 2 and 3 show the results we obtained with the blanched-frozen spinach suspension and carrot juice, respectively. For comparative purposes, these figures include the maximal amounts solubilized with BS alone. We did experiments with a pretreatment for 1 h at either pH 6.5 or 2.5, and for carrot juice only, at pH 2.5 with pepsin. It is only for the solubilization of carotenes from carrot juice (where the amounts solubilized are very low) that the pretreatments had a significant effect. For this reason, for spinach, in Figure 2 we show only the results from the pH 2.5 pretreatment experiments.

The addition of PL had a large effect on the solubilization of *trans*-β-carotene from the spinach suspension, as shown in Figure 2, with solubilization in the presence of glycodeoxycholate increased from 12.9 to 45.5% by the addition of lecithin. This degree of solubilization was comparable to that obtained for lutein in the same system. This enhancement was also seen in the experiments with carrot juice when the juice was pretreated at pH 2.5 (± pepsin). In contrast, for both carrot and spinach preparations, the solubility of lutein was depressed by the presence of PL, whereas it was enhanced in spinach if the more polar BS, taurocholate, was used. Some of these differences between the two BS may be because taurocholate was used at a concentration only just above the CMC, so the volume fraction of micelles was lower than for glycodeoxycholate.

Comparing carrot juice with spinach, one can see that the percentages of lutein solubilized in the mixed glycodeoxycholate-PL micelles were comparable, but since carrot contains very little lutein the absolute amount of lutein solubilized from carrot juice was very small, giving a micellar concentration of 1–2 μM. In contrast, the percentages of carotene solubilized were an order of magnitude larger for the spinach suspension

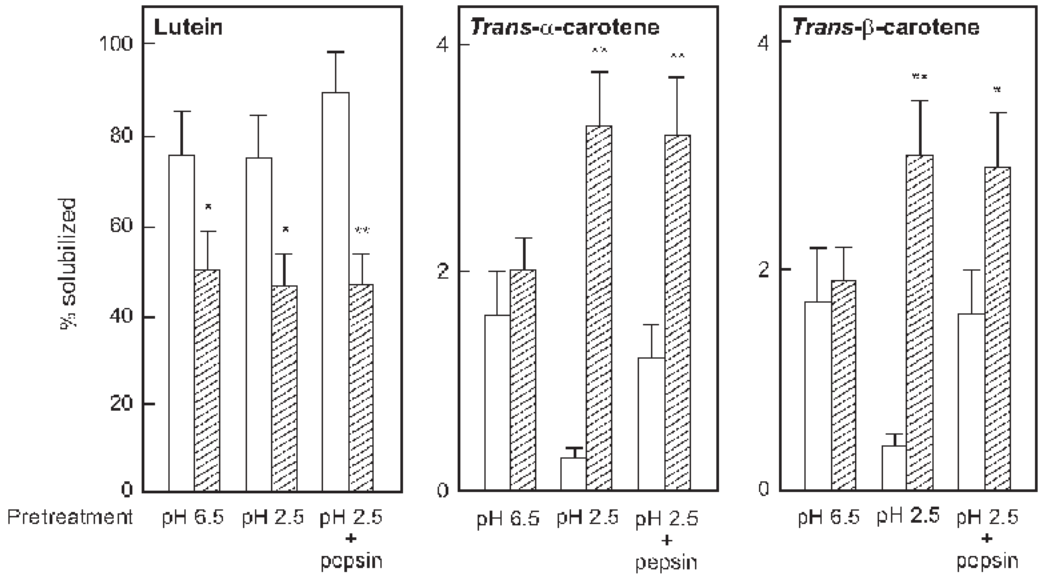


FIG. 3. Solubilization of carotenoids from carrot juice by 8 mM Na GDOC (empty bars) and 8 mM NaGDOC + 5 mM phospholipid (hatched bars). Measurements were made at 2 h at pH 6.5 after 1 h pretreatment at either pH 6.5 or pH 2.5 or pH 2.5 + pepsin. Results are the mean and SD for three experiments. Significant differences between solubilization in mixed and simple micelles are indicated as in Figure 2. The carotenoid concentrations were 2.1 ± 0.1 μM for lutein, 18.9–29.7 μM for *trans*-α-carotene, and 45.5–73.5 μM for *trans*-β-carotene. For abbreviations see Figure 1.

than for the carrot juice. Since the starting concentrations are comparable, larger absolute amounts were extracted into the mixed micelles from the spinach suspension. We believe this difference reflects the stability of the carotene crystalline state in the chromoplast of carrot compared to their situation in the blanched-frozen spinach suspension, where the native chloroplast membrane structure has been destroyed by the blanching.

(iv) Solubilization in micelles. Comparison between raw and blanched-frozen spinach suspensions. Significantly lower amounts of carotenoids were solubilized from the raw spinach suspension compared to the frozen spinach suspension, in both mixed micelles of glycodeoxycholate plus PL and simple micelles of glycodeoxycholate alone. To achieve maximum solubilization of 2 h, an incubation with the micelles was required rather than 1 h with the blanched-frozen spinach suspension. Another observation pointing to the importance of the integrity of the photosystems in controlling solubilization was that, as stated earlier, low-pH pretreatment of the blanched-frozen spinach had no effect on solubilization in mixed micelles, whereas with the raw spinach it enhanced the amounts solubilized. Thus, comparing preincubations at pH 6.5 with those at pH 2.5, there was an enhancement of the amounts of lutein and *trans*-β-carotene solubilized from fresh spinach into glycodeoxycholate plus PL micelles, from 2.9 to 4.8 and from 3.1 to 3.9%, respectively.

Route 2a: Effect of duodenal surfactants on transfer of carotenoids to oil. Within the duodenal environment, the micellar phases will coexist with emulsion droplets supplied by the continuing emptying of the stomach. We investigated the relative ease of solubilization within the lipid droplets in the presence of the micellar phase.

(i) Carrot juice. We studied the effect of the major PL in bile, PC, and the BS (sodium glycodeoxycholate) on the transfer of carotene from carrot juice to olive oil. We mimicked the situation in the gastrointestinal tract by preincubating the juice for 1 h at pH 2.5 with and without pepsin. The pH was then raised to 6.5 and the juice added to flasks containing solutions with and without the surface-active agents followed by the oil. As described below, to obtain reproducible results, we found it necessary to exclude oxygen from the flasks containing PL before adding the juice. Figure 4A shows the results when we measured total carotene in the oil after 1 h. For each treatment there was a control where transfer was measured in the absence of surface-active agents (empty bars). [The variability in control values reflects the inherent difference between batches of carrot juice described previously (5)]. It is apparent that the presence of 5 mM PL slightly enhanced the transfer. One explanation for this is that PL acts as an efficient emulsifier, increasing the oil/aqueous phase surface area for attachment of carotenoid chromoplasts and carotene bodies and crystals. Serendipitously, we found that if the PL was allowed to oxidize, the opposite effect was observed and inhibition of carotene transfer occurred (results not shown).

As shown in Figure 4A, 8 mM glycodeoxycholate inhibited the transfer, and the inhibition persisted in the presence of 5 mM PL. Oxidized PL with glycodeoxycholate gave the greatest inhibition with less than 0.5% of carotene reaching the oil (results not shown).

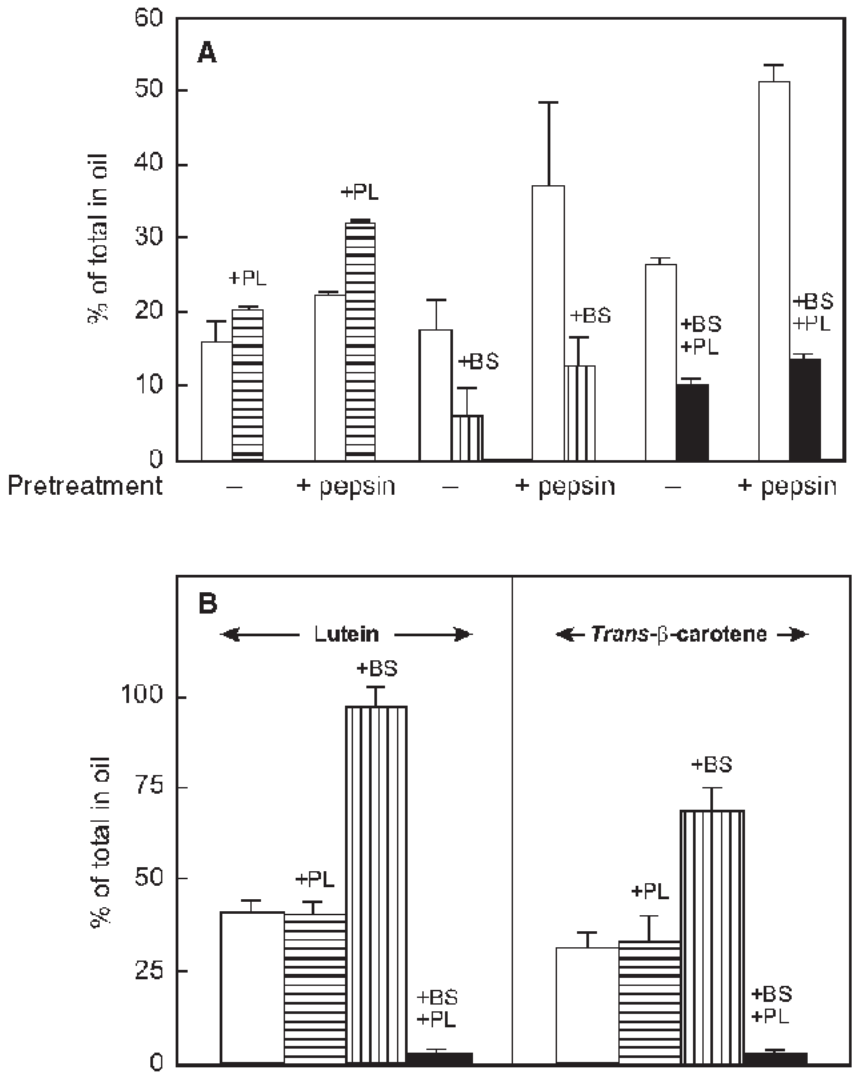


FIG. 4. The effect of duodenal surfactants on transfer of carotenoids to olive oil after 1 h of incubation with oil: (A) total carotene from carrot juice and (B) lutein and *trans*-β-carotene from the blanched-frozen spinach suspension. The carrot juice was pretreated for 1 h at pH 2.5 with and without pepsin (120 U/mL). The transfer to oil was measured at pH 6.5. This pretreatment was omitted for spinach. Open bars represent the controls with no additions, horizontal hatching with 5 mM phospholipid, vertical hatching with 8 mM bile salt (BS, NaGDOC), and solid bars with 5 mM phospholipid and 8 mM bile salt. Each data point represents the mean and range of values for two experiments. For abbreviations see Figure 1.

(ii) Blanched-frozen spinach suspension. Similar experiments were carried out on the spinach suspension. In view of the lack of significant effect of pepsin on carotenoid transfer in this system, pepsin pretreatment was not included. Figure 4B shows that PL alone had no effect on the carotenoid transfer. Whereas glycodeoxycholate inhibited carotene transfer from carrot juice, the opposite effect occurred with spinach, with a significant rise in lutein and *trans*-β-carotene transfer to the oil on adding the BS. As for carrot juice, the presence of PL and BS together inhibited the transfer.

Carotenoid transfer to oil as a function of BS concentration. We have shown previously (5,6) that the surface charge of the emulsion droplets has a greater influence on the ease of solubilization of carotene from carrots than from spinach. Shielding of this charge by pH or ionic strength adjustment allows aggregation of the tissue and droplet and promotes solubilization within the lipid phase. An explanation for the BS inhibition of transfer of the carotene from carrot juice to oil observed in this study is that BS imparts a negative charge to the oil surface, thereby preventing the organelle/oil aggregation. This effect would be less significant for frozen spinach carotenoids, where the surface potential is less important and separate from events in the aqueous phase. Here the BS may extract lipids from the vegetables to form mixed micelles, which can solubilize the

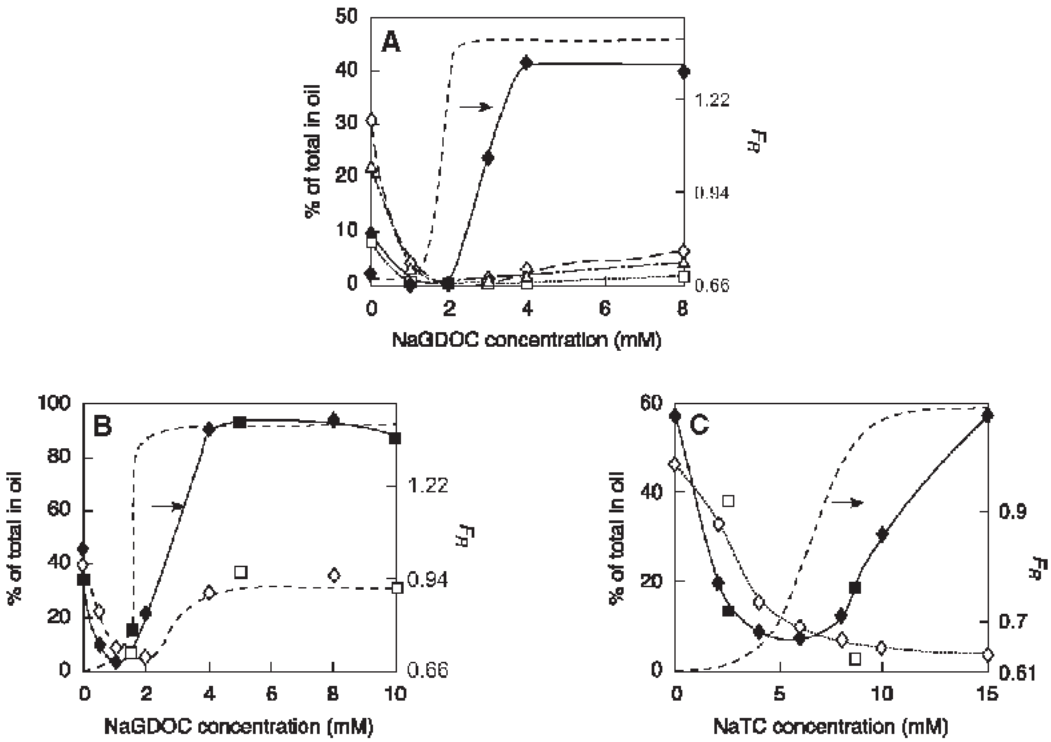


FIG. 5. Transfer of carotenoids to olive oil as a function of BS concentration. (A) From carrot juice after 2 h incubation with NaGDOC: (◆) lutein, (◇), total *trans*-α- + -β-carotene; (△) *trans*-β-carotene; (□) *trans*-α-carotene. The initial concentrations in the aqueous phase were 85.6, 151.3, and 7.50 μM for *trans*-α-carotene, *trans*-β-carotene, and lutein, respectively. The dashed line refers to the right-hand axis showing the pyrene fluorescence peak ratio data. (B) From the blanched-frozen spinach suspension after 1 h incubation with NaGDOC. Different symbols represent different experiments. Solid symbols are for lutein; unfilled symbols are for *trans*-β-carotene. The initial aqueous concentrations were 1.59–3.41 μM for lutein and 1.04–2.21 μM for *trans*-β-carotene. The dashed line refers to the right-hand axis and shows the best fit for F_R, the pyrene fluorescence peak ratio data. (C) From the frozen spinach suspension after 2 h incubation with NaTC. Symbols are as in (B). The initial aqueous concentrations were 1.60 ± 0.02 and 1.11 ± 0.06 μM for lutein and *trans*-β-carotene, respectively. For other abbreviations see Figure 1.

carotenoids so that they are either competitively inhibited from moving into the oil (carrot) or provided with another pathway to reach the oil (spinach). We resolved to gain further understanding of the situation by studying how the transfer to oil depended on bile salt concentration when it was added alone.

(i) Carrot juice. As shown in Figure 5A, for carrot juice low concentrations of glycodeoxycholate did indeed inhibit transfer of the carotenes and lutein to oil. Above the CMC, as the BS concentration was increased the amounts of *trans*-α- and -β-carotene in the oil showed only a slight increase. In contrast, the lutein concentration in the oil showed a much larger percentage rise in the region of the CMC. As a consequence, in spite of the lower amounts of lutein compared to the carotenes in the carrot, the concentration of lutein in the oil at high BS levels was comparable to that of the carotenes.

(ii) Blanched-frozen spinach suspension. For the spinach suspension, Figure 5B shows that as for the carrot, the presence of glycodeoxycholate below the CMC inhibited carotenoid transfer to oil. In the region of the CMC, *trans*-β-carotene and lutein concentrations in the oil rose significantly higher than with carrot. The effect was largest with lutein, so that by 4 mM BS virtually all the lutein was in the oil. In contrast, *trans*-β-carotene concentrations in the oil rose no higher than the level measured in the absence of BS.

To study the effect of polarity of the BS, we compared these results with those obtained using taurocholate. Figure 5C shows that, as for glycodeoxycholate the amounts of lutein transferred fell until the CMC was reached and then rose, but they did not level off above the CMC. Clearly, taurocholate is less efficient than glycodeoxycholate in facilitating the transfer to oil, as the final level reached at 15 mM bile taurocholate was no higher than the value without BS. Interestingly, with the more polar BS the *trans*-β-carotene concentration in the oil fell monotonically as the BS concentration was increased.

DISCUSSION

When the contents of the stomach are ejected into the duodenum, they are immediately exposed to duodenal surfactants. The carotenoids must become solubilized within the micellar phases formed by these surfactants and diffuse to the mucosal membrane to be absorbed by the body. Thus, we hypothesize

that it is at this point in digestion where the differences between micellar solubilization of the carrot and spinach carotenoids will influence the ability of the carotenoids to reach the site of absorption. Within this study we have sought to examine the relative ease by which carotenoids from carrot and spinach can become solubilized in the micellar phase available within the gut lumen during duodenal digestion. To simplify this analysis we will discuss solubilization within simple and mixed micellar phases *via* two alternative routes (1 and 2) as identified in Scheme 1.

Route 1: Direct solubilization from tissue within simple and mixed micelles. For simple micelles, onset of solubilization was achieved around the CMC of the BS (Fig. 1), suggesting that the solubilizing moiety was the micelles, as opposed to a more unstructured, random aggregate. In this respect, the BS are behaving like classical surfactants. They differ from this classical behavior above the CMC where, as the BS concentration was increased, the amount solubilized leveled off for lutein and fell for the carotenes. Thus, although the number of micelles was increased, no further solubilization occurred. These observations could be explained by a change in form of the micelles as the BS concentration is increased above the CMC, either as a change in structure (9) or by solubilizing of additional components that inhibit further solubilization for lutein and actually decrease that for carotene. The additional component(s) inhibiting carotene solubilization could be lutein itself and/or lipids. A neutral PL, such as PC would be an unlikely candidate to fulfill such a role because, when we added it to BS to make mixed micelles, the solubility of carotene increased (Figs. 2 and 3).

The efficiency of solubilization of carotenoids in glycodeoxycholate micelles decreases in the order $\text{lutein}_{\text{carrot}} > \text{lutein}_{\text{blanched-frozen spinach}} > \text{carotene}_{\text{blanched-frozen spinach}} > \text{carotene}_{\text{carrot}}$. Taurocholate micelles also showed a greater affinity for lutein compared to carotene from blanched-frozen spinach preparations (Figs. 1 and 2). We interpret this result as the free energy difference between the *in situ* and solubilized state increasing in the opposite order. Thus, for carotene, the crystalline state found in carrot is the most stable, and its apolar nature precludes an equilibrium toward the more polar environment of a micelle. The membrane-bound carotene within spinach is less stable (especially after blanching) and transfers more easily to a micellar environment. The fact that lutein from carrot partitions well into the micelles argues against it being located in the same crystals as the carotene. In fact, evidence in the literature implies that carotene is present only in the crystalline carotene bodies (16,17). It is possible that lutein is located in a liquid state in the plastoglobuli also found in the chromoplasts (17–19). This would be consistent with the yellow color of these organelles as opposed to the orange of the carotene bodies (18).

For both carrot and spinach, the effect of adding PL/BS mixed micelles correlated with the polarity of the BS and the carotenoid. PL, with its substantial hydrophobic moiety, increased the hydrophobicity of the micellar core and thus decreased the solubility of lutein in the mixed micelles compared to the glycodeoxycholate micelles. This interpretation is in agreement with the finding that the solubility of nonpolar carotenes was increased in the same system. Taurocholate, the more polar BS and a much less efficient solubilizing agent, was studied with only the spinach preparation. Here the presence of PL within the micelle invariably enhanced the micellar solubility of both carotenoids, thus confirming that the simple, more polar micelle was a relatively poor solubilizing entity for both carotenoids. Without a complete analysis of the micellar composition, it is impossible to have a complete picture of the factors determining carotenoid partitioning from the vegetables into the micelles. For example, PL is known to protect biological membranes from disruption by BS (20). Therefore, any increase in carotenoid concentration in the micelle caused by PL is likely to reflect a lower free energy state in the micelle rather than an easier escape route for the carotenoids.

In conclusion, our data for carrot juice (Fig. 3) imply that any carotene still left in the tissue after digestion in the stomach is less likely to be available for absorption by this route than lutein. In contrast, for spinach (Fig. 2), the abilities of carotene and lutein to pass through the mixed micelle route are comparable. Of course, our model systems underestimate absorption by this route because of the effective increase in the micellar pool, which occurs *in vivo* by recycling of BS.

We described in a previous paper (6) how processing of the spinach (blanching) destabilizes the carotenoid environment in the chloroplast. Thus, carotenoids from blanched-frozen spinach suspensions transfer to an oil phase much more readily than those from raw spinach suspensions. We observed an analogous situation here and have shown that raw spinach suspension carotenoids are poorly solubilized in both simple and mixed micelles compared to those in blanched spinach. We conclude that the increased bioavailability of carotenoids in cooked green vegetables (21,22) is due not only to loss of tissue structure and cell walls but also to disruption of subcellular structures, which allows easier access of lipid phases to the sites of the carotenoids.

There is evidence from human studies (23) that the direct route from vegetables to micelles is important in determining carotenoid bioavailability. In that work carotene from carrot was solubilized in the gastric oil phase more than lutein from spinach. Yet at the duodenal stage, micellar concentrations of the two carotenoids were similar. Therefore, it seems likely that the micelles interact with the vegetable matrix and solubilize the lipid-soluble molecules directly. This idea is supported by other modeling work developed by Failla and coworkers (24,25) to assess carotenoid bioavailability from meals and showed that inadequate gastric solubilization of carotenoids could be compensated for by micellar solubilization in the duodenal phase. Even though Failla's model is much more complex and physiological than ours and is closer to the *in vivo* human situation, we are able to reproduce their key results that determine carotenoid bioavailability. Thus, like them, we have shown that vegetable lutein is solubilized in micelles more than carotene. Since the concentration of carotenoids in cells absorbing carotenoids from micelles is proportional to their micellar concentration (25), this provides

an explanation of why the bioavailability of lutein is higher than that of carotene.

Route 2: Transfer of carotenoids to oil within the duodenal environment. The action of lipase enzymes within the duodenal environment means the oil phase, as a pool for solubilizing carotenoids in the duodenum, effectively disappears. Therefore, efficient transfer of carotenoids from undigested vegetables to this pool would be a futile pathway unless the ease of escaping from the oil phase into the micelles exceeds that of escaping from the vegetable. Partition of carotenoids between oil (triolein) droplets and mixed micelles has been studied by Borel *et al.* (11). They showed that the polar carotenoid zeaxanthin was preferentially located at the oil/aqueous phase interface and could spontaneously transfer to micelles. *Trans*-β-carotene distributed to the bulk oil phase, and its transfer to micelles occurred only if lipolysis was present. The authors interpreted this to mean that carotene requires the production of water-soluble lipids in order to be transferred to the micelles. Another interpretation is that, during lipolysis and loss of the oil phase, carotene becomes solubilized within the FA-rich phases formed transiently at the interface and hence is solubilized directly into the emerging micelles. Whatever the explanation, we conclude that for our systems without lipolysis, once *trans*-β-carotene reaches the oil phase it will not transfer out. This is not the case for lutein, which will distribute between micellar and oil phases as determined by the chemical potential of lutein in each environment.

(i) Effect of BS alone (Figs. 4 and 5). BS below the CMC inhibit transfer of lutein and carotene from both carrot and spinach. For carrot, this is to be expected as the BS will adsorb at interfaces, increasing the negative charge and impeding the adhesion of organelles (plastoglobuli, carotene bodies, etc.) to the oil interface. For both carrot and spinach, there may also be stabilization of the structures containing the carotenoids. This has been observed for photosystem II by Gall and Scheer (26).

Above the CMC the BS can provide another pathway to the oil, and their effect correlates with the solubility of the carotenoid in the micelles. In this pathway the micelles are in dynamic equilibrium with the oil interface, and hence exchange of solubilized material can take place. The solubility of the carotenoid in the micelles, as we have seen, depends on the polarity of BS and carotenoid and the ease with which the carotenoid can escape from the vegetable. Thus, for both carrot and spinach, lutein's passage to the oil is facilitated by glycodeoxycholate. The carotenes from carrot are inhibited from transferring to oil, reflecting the extremely small amount of micellar solubilization and therefore the virtual absence of a micellar pathway to the oil. This is also true for spinach carotene moving to oil in the presence of taurocholate micelles.

(ii) Effect of mixed micelles (Fig. 4). In spite of PL increasing the solubility of carotene from carrot in micelles, mixed micelles have a very similar inhibitory effect on carotene transfer to oil as bile salt micelles alone. Therefore, the equilibrium between micelles and oil phase has not been changed by the presence of PL. In contrast, for the spinach carotenoids, adding PL leads to a drastic inhibition of transfer of both lutein and carotene, so either the micellar environment has become more favorable for both lutein and carotene or the oil/aqueous phase interface has become modified so as to impede the movement of carotenoids into the oil. To summarize: For both vegetables and types of carotenoids, in the duodenal environment transfer of carotenoids from the tissue to the oil is inhibited, thereby minimizing any futile pathway.

In summary, the physical state of the carotenoid within the food is a major determinant of the extent of solubilization of the carotenoid within simple micellar phases. The efficiency of solubilization of carotenoids in glycodeoxycholate micelles decreases in the order $\text{lutein}_{\text{carrot}} > \text{lutein}_{\text{blanched-frozen spinach}} > \text{carotene}_{\text{blanched-frozen spinach}} > \text{carotene}_{\text{carrot}}$. Taurocholate micelles also showed a greater affinity for lutein compared to carotene from blanched-frozen spinach preparations. Mixed micellar phases of PL and BS solubilized the nonpolar carotenoid, carotene, to a greater extent then simple micelles of the same bile salt, but the micellar solubility of the more polar carotenoid, lutein, was decreased. We suggest that carotenes present in carrot tissue, when it is emptied into the duodenum, are less likely to be available for absorption than lutein. For spinach the availability of lutein and carotene are likely to be more comparable, but since the solubility of carotene is more dependent on the nature of the BS present in the mixed micelles, further work is needed using micelles of composition closer to that in bile. However, in spite of this reservation and the simplicity of our model systems, our results that relate to understanding carotenoid bioavailability parallel those obtained from *in vivo* human studies and models that have used more physiological conditions.

Below their CMC, BS inhibit the transfer of carotenoids from tissue to an oil phase under simulated duodenal conditions. Above their CMC, the BS can provide an additional route to the oil from the tissue for lutein by virtue of the equilibrium between micellar phases and the interface. This pathway is not available for carotene due to their low micellar solubility. Mixed micellar phases inhibit transfer of both carotenoids from the tissue to the oil phase, thereby minimizing this futile pathway.

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Peroxisome Proliferator-Activated Receptor α Is Not the Exclusive Mediator of the Effects of Dietary Cyclic FA in Mice

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ABSTRACT: Cyclic FA monomers (CFAM) formed during heating of α-linolenic acid have been reported to interfere in hepatic metabolism in a putatively peroxisome proliferator-activated receptor α (PPARα)-dependent manner. In the present work, CFAM (0.5% of the diet) were administered for 3 wk to wild-type and PPARα-null mice of both genders to elucidate the role of PPARα in mediating the effects of CFAM on the activity of acyl-CoA oxidase (ACO) and ω-laurate hydroxylase (CYP4A), the regulation of which is known to be dependent on the PPARα. Dietary CFAM enhanced CYP4A activity threefold in male and female wild-type mice. This effect was abolished in PPARα-null mice. A twofold induction of ACO activity was found in wild-type female mice fed CFAM; however, no effect was seen in males. In wild-type animals, (ω-1)-laurate hydroxylase (CYP2E1) activity, the expression of which has not been shown to be PPARα dependent, was not affected by the CFAM diet. In contrast, stearoyl-CoA desaturase activity was reduced in wild-type mice. CFAM feeding reduced the activities of ACO, CYP2E1, and stearoyl-CoA desaturase and caused accumulation of lipids in the livers of female PPARα-null mice. These data show that CFAM apparently activate gene expression *via* the PPARα and have profound effects on lipid homeostasis, exacerbating the disturbances preexisting in mice lacking functional PPARα. Although the data emphasize the importance of PPARα in the metabolism of the CFAM, these results show that PPARα is not the exclusive mediator of the effects of CFAM in lipid metabolism in mice.

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Dietary oils contain variable amounts of cyclic FA monomers (CFAM), which can account for up to 0.66% of the FA (1). These are formed from linoleic and α-linolenic acids during the heating of vegetable oil that occurs during the refining process and also during home-frying. Sixteen cyclic FA can be formed from α-linolenic acid (2). These are dienoic FA containing a cyclopentenyl (C_5) or a cyclohexenyl (C_6) ring (Fig. 1). No results are available thus far on the effects of CFAM in humans. However, a few studies have been carried out feeding these FA to rats, and some have shown deleterious effects on rat survival during the first days of life (3,4).

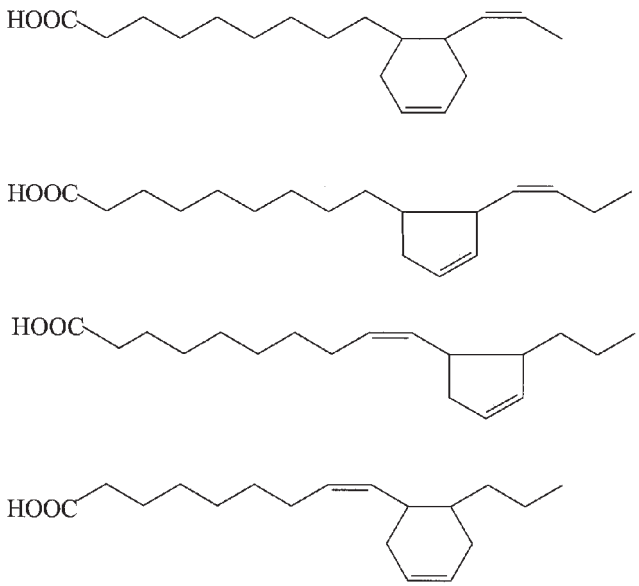

FIG. 1. General chemical structures of the cyclic FA formed from α-linolenic acid. Each structure gives rise to four isomers, accounting for the *Z* and *E* configuration of the allylic double bond and for the *cis* and *trans* configuration of the cycle (from Ref. 2).

Apart from these biological effects, a preferential incorporation of the CFAM having a C5 ring has been reported (5,6), suggesting that C_5- and C_6-CFAM may be metabolized differently. Recently, it was reported that CFAM increase the activities of acyl-CoA oxidase (ACO) and ω- and (ω-1)-laurate hydroxylase (CYP4A and CYP2E1, respectively) in rats and reduce stearoyl-CoA desaturase activity (5). From these studies it has been suggested that CFAM may serve as activators of the peroxisome proliferator-activated receptor α (PPARα). In this context, PPARα may be of importance since it is well established that this ligand-activated nuclear receptor mediates the pleiotropic response to peroxisome proliferators (7,8) and some of the effects due to starvation and diabetes (9,10). A variety of FA have been identified as ligands for PPARα, including saturated and unsaturated long-chain FA and eicosanoids (11–13). CFAM have not been reported to act as ligands for the PPAR, but it is conceivable that these lipid analogs also may act as PPARα activators *in vivo*.

The aim of the present study was to test whether PPARα mediates the induction of CYP4A and ACO activity by feeding CFAM. For this purpose, wild-type and PPARα-null mice of both genders were fed a diet containing CFAM isolated

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Abbreviations: ACO, acyl-CoA oxidase; CFAM, cyclic FA monomer; CYP2E1, (ω-1)-laurate hydroxylase; CYP4A, ω-laurate hydroxylase; PPARα, peroxisome proliferator-activated receptor α; SREBP, sterol regulatory element-binding protein.

from α-linolenic acid. At the end of the feeding period, ACO, CYP4A, CYP2E1, and stearoyl-CoA desaturase activities were measured in liver subcellular fractions. FA composition of the livers was subsequently determined.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Stearic acid was purchased from NEN Products (DuPont NEN, Uppsala, Sweden) and [1-^{14}C]lauric acid from Amersham Pharmacia Biotech (Orsay, France). Cofactors and other chemicals were purchased from Sigma-Aldrich (St. Quentin Fallavier, France), and solvents were obtained from SDS (Peypin, France) and redistilled before use.

Diets and mice. Linseed oil was used as a source of α-linolenic acid and heated (275°C, 12 h under N_2) to form CFAM. The CFAM were isolated and purified, and TAG were prepared as described previously (14). The synthesized TAG contained 50% CFAM (data not shown). A standard chow diet (R36; Lactamin, Vadstena, Sweden) was supplemented with 1% (w/w) of the CFAM-containing TAG as the experimental CFAM-diet or, alternatively, with soybean oil (Sigma) (1% w/w) as the control diet.

Wild-type and PPARα-null mice, on a pure Sv/129 genetic background derived from the original colony of mixed background mice (8), were generously provided by Dr. Frank J. Gonzales and Dr. Jeffrey M. Peters, National Institutes of Health (Bethesda, MD). This study was conducted in compliance with local, legal, and institutional laws regarding the care and use of experimental animals. All animals were housed in a light- and temperature-controlled environment. Forty-nine mice (23 males, 26 females, 88 ± 4 d) were divided into eight groups (of five to seven animals per group). Four groups (two groups of males and two of females) were fed for 3 wk on the experimental CFAM diet while the four other groups were fed on the control diet. At the end of the feeding experiment, mice were killed following euthanasia by carbon dioxide inhalation, and livers were quickly excised.

FA analyses. Total lipids were extracted from the livers using a mixture of chloroform/methanol (2:1, vol/vol) according to Folch *et al.* (15). Methyl nonadecanoate (100 μg) was used as an internal standard. Total FA were derivatized into FAME according to Morrison and Smith (16). Briefly, total lipids were dissolved in 0.3 mL of toluene and 0.7 mL of 7% boron trifluoride in methanol, and heated at 95°C for 90 min. Five milliliters of an aqueous saturated $NaHCO_3$ solution were added, and the methyl esters were extracted twice with 2 mL of hexane. To determine the FA composition of the livers, the methyl esters were analyzed by GLC using a fused-silica column (BPX 70, 50 m length, 0.25 μm film thickness, 0.33 mm i.d.; SGE, Villeneuve Saint Georges, France). The methyl esters in hexane were injected into a split/splitless injector heated at 250°C and detected using an FID set at 280°C. Helium was used as the carrier gas. The column was operated at 60°C for 1 min. The temperature was then increased to 170°C at a rate of 20°C·min^{-1}, kept at 170°C for 10 min, and increased to 220°C at a rate of 3°C·min^{-1} and maintained at 220°C for 20 min.

For increased analytical accuracy, the FAME of these lipid extracts were dissolved in 100 μL of acetone and injected onto a reversed-phase C_{18} column (Nucleosil C18, 5 μm particle size, 250 × 10 mm i.d.; Interchim, Montluçon, France) connected to a Spectra Physics 8810 HPLC system (Spectra Physics, La Verpillière, France). The FAME were detected by using a differential refractometer (Waters 410; Waters, Saint Quentin en Yvelines, France). Acetonitrile was used as the mobile phase at a flow rate of 4 mL·min^{-1}. The methyl esters of the CFAM coeluting with methyl linoleate were collected, dried under reduced pressure, and dissolved in hexane for analysis by GLC. Two polar columns (BPX 70, 50 m length, 0.25 μm film thickness, 0.33 mm i.d., SGE; CPWax 52CB, 30 m length, 0.2 μm film thickness, 0.25 mm i.d.; Chrompack, Les Ulis, France) were used to separate the 16 different CFAM isomers (Fig. 1) under the chromatographic conditions described previously. CFAM identification was performed using a standard mixture of the 16 fully identified isomers, arbitrarily named from a- to h-CFAM for the C5-ring members and from i- to p-CFAM for the C6-ring cyclic FA (2). Linoleic acid was used as standard to calculate the content of CFAM in the collected fraction and finally in total lipids.

Assays of enzyme activities. Subcellular fractionation was performed as reported previously (17). The Δ9 desaturase activity was measured as described previously (17) on 5 mg of freshly prepared microsomal suspensions incubated at 37°C for 15 min with 100 nmol of [1-^{14}C]stearic acid (259 Bq·nmol^{-1} in 3 μL of ethanol). The reaction products were separated by HPLC using a reversed-phase C18 column (YMC-Pack ODS-A, 5 μm particle size, 250 × 4.6 mm i.d.; YMC, Dusseldorf, Germany) and analyzed by a Flo-One β detector (A-280X, Radiomatic).

ACO activity was determined in the peroxisomal-mitochondrial fraction according to Lazarow (18), using palmitoyl-CoA as a substrate.

ω- and (ω-1)-laurate hydroxylation activities were determined as markers of cytochrome P450 4A and P450 2E1, respectively, in the microsomal fraction according to Orton and Parker (19) as modified by Laignelet and co-workers (20) using lauric acid as a substrate. Briefly, 125 μg of microsomal protein was incubated at 37°C for 15 min in the presence of β-NADPH (750 nmol) and lauric acid (2,072 Bq, 64 nmol) in 66 mM Tris buffer, pH 7.4. The reaction was stopped by adding 125 μL of 1 N HCl. Protein was sedimented by centrifugation at 37,620 × *g* for 10 min, and 100 μL of the supernatant was injected onto a HPLC reversed-phase C18 column (5 μm particle size, 150 × 4.6 mm i.d., Spherisorb S5 ODS2; Waters) connected to a Flo-One β detector. The mobile phase used was made up of acetonitrile, 0.1 M ammonium acetate buffer (pH 4.6), and water (32:27:41 by vol) and was run for 4 min at a flow rate of 1.8 mL·min^{-1}. The mobile phase was then changed to 90% acetonitrile and 10% ammonium acetate buffer (0.1 M, pH 4.6, using a 2-min gradient), and held for 4.5 min. In this way, the metabolites formed by ω- and (ω-1)-hydroxylation of lauric acid were separated from the precursor (elution volumes: 12.7, 11.1, and 18.9 mL, respectively) and were quantified by peak integration.

Statistical analyses. Results were analyzed by a two-way ANOVA procedure followed by least squares estimates of marginal means using the SAS Software (SAS Institute, Cary, NC). Interactions between variables (sex, genotype, and diet) and differences were considered significant at $P < 0.05$.

RESULTS

Feeding CFAM significantly increased the ratio of liver to body weight in male wild-type and PPARα-null mice (significant general diet effect at $P < 0.03$, by two-way ANOVA). The greatest effect was observed in male PPARα-null mice (+16%, from 4.22 ± 0.11 to 4.89 ± 0.15% of body weight, $P = 0.03$, Table 1). In contrast, CFAM feeding did not change the relative liver weight in female mice.

Lipid analysis of the livers showed that sex, genotype, and diet interacted on the accumulation of lipids in the liver ($P = 0.03$). These interactions can be explained by the following observations. The lipid content of the livers of female PPARα-null mice was significantly higher than in male mice of both genotypes and compared to wild-type female mice. Feeding the CFAM diet to female mice further increased the lipid content in the PPARα-null mice (from 60 ± 5 to 100 ± 7 μg lipids·mg of liver^{-1}, respectively, $P < 0.0001$), but not in wild-type mice. In addition, one female PPARα-null mouse fed on the CFAM diet showed a steatotic liver.

The CFAM amount and composition of the livers were determined by GLC, combining two polar columns as previously reported by Dobson and co-workers (2) to separate the various isomers. The results, which are summarized in Table 2, show a clear interaction between sex and genotype on the CFAM profile of the livers of mice fed CFAM (P from <0.0001 to 0.02 by two-way ANOVA). The total amount of CFAM was not different in male and female wild-type mice (256 ± 34 and 307 ± 53 ng of CFAM·mg of liver^{-1}, respectively; Table 2), but the amount of CFAM was significantly higher in PPARα-null mice compared to the wild-type mice ($P < 0.001$, Table 2). In addition, the accumulation of CFAM in the PPARα-null mice showed a clear sex difference in that the total amount of CFAM was doubled in the livers of female mice compared to male mice, resulting in the liver CFAM content in the female PPARα-null mice being two- to fivefold higher than in the other animals (Table 2). Interestingly, accumulation of CFAM in the PPARα-null mice was mainly due to an enrichment in the C_5-CFAM as illustrated by the ratio of C_6- to C_5-CFAM, which ranged from 0.35 to 0.41 in the PPARα-null mice and from 0.53 to 0.55 in the wild-type mice ($P < 0.01$, Table 2). The ratio of C_6-CFAM to C_5-CFAM was 1.5 in the diet, whereas it was 0.35–0.55 in the livers of the mice fed on this diet. This difference can be explained either by a reduction in the proportion of the C_6-CFAM (from 61% in the diet to 26–36% in the liver lipids) or by an increase in the C_5-CFAM (from 37% in the diet to 65–74% in the liver lipids).

To elucidate whether the effects of CFAM on gene expression were mediated *via* the PPARα, CFAM were fed to wild-

TABLE 1
Weight and Lipid Content of Livers of Male and Female Wild-Type (+/+) and PPARα-Null Mice (–/–) Administered a Standard Control Diet or a Diet Containing 0.5% of Cyclic FA Derived from α-Linolenic Acid[a,b]

		Liver[c]	
	n	% of body weight	Lipids (μg·mg of liver^{-1})
Males +/+			
Control diet	6	4.35 ± 0.07	42 ± 2[a]
CFAM diet	6	4.71 ± 0.10	39 ± 2[a]
Males –/–			
Control diet	5	4.22 ± 0.11	52 ± 8[a,b]
CFAM diet	6	4.89 ± 0.15*	55 ± 7[a,b]
Females +/+			
Control diet	6	4.48 ± 0.10	46 ± 2[a]
CFAM diet	7	4.48 ± 0.10	53 ± 5[a,b]
Females –/–			
Control diet	7	4.68 ± 0.10	60 ± 5[b]
CFAM diet	6	4.72 ± 0.39	100 ± 7[c]

[a]Given as TAG [cyclic FA monomer (CFAM) diet] for 3 wk.
[b]Results are expressed as means ± SEM.
[c]*$P < 0.05$ vs. PPARα-null males fed on the control diet, by two-way ANOVA followed by least-squares estimates of marginal means. Data not sharing the same superscript roman letter are significantly different at $P < 0.05$ (two-way ANOVA followed by least-squares estimates of marginal means). PPARα, peroxisome proliferator-activated receptor α.

TABLE 2
Cyclic FA Profile of the Liver Lipids of Male and Female Wild-Type (+/+) and PPARα-Null Mice (–/–) Administered a Diet Containing 0.5% of Cyclic FA Derived from α-Linolenic Acid[a,b]

CFAM isomer	Male +/+	Male –/–	Female +/+	Female –/–
	(ng CFAM·mg of liver^{-1})			
a	19 ± 3[a]	58 ± 9[b]	20 ± 3[a]	137 ± 13[c]
b	29 ± 4[a]	72 ± 12[b]	32 ± 5[a]	162 ± 7[c]
c	30 ± 4[a]	82 ± 14[b]	38 ± 7[a]	197 ± 12[c]
d	20 ± 5[a]	56 ± 13[a]	20 ± 10[a]	146 ± 15[c]
e	20 ± 7[a]	70 ± 19[b]	28 ± 11[a,b]	131 ± 22[c]
f	21 ± 2[a]	60 ± 11[b]	30 ± 9[a]	113 ± 11[c]
g	15 ± 3[a]	32 ± 7[b]	19 ± 3[a,b]	75 ± 3[c]
h	14 ± 2[a]	26 ± 6[b]	14 ± 2[a]	71 ± 2[c]
i	10 ± 2[a]	25 ± 4[a]	8 ± 3[a]	38 ± 3[b]
j	19 ± 2[a]	17 ± 2[a]	36 ± 11[b]	57 ± 6[c]
k	32 ± 5[a]	40 ± 5[a]	33 ± 7[a]	76 ± 6[b]
l	2 ± 1[a]	3 ± 2[a]	Traces	Traces
m	9 ± 2[a]	29 ± 7[b]	8 ± 1[a]	66 ± 6[c]
n	17 ± 4[a]	66 ± 13[b]	13 ± 3[a]	125 ± 14[c]
o	Traces	Traces	Traces	Traces
p	Traces	Traces	Traces	Traces
C_5[c]	167 ± 21[a]	456 ± 79[b]	198 ± 34[a]	1032 ± 59[c]
C_6[d]	89 ± 15[a]	184 ± 30[b]	109 ± 19[a]	362 ± 30[c]
Total	256 ± 34[a]	640 ± 109[b]	307 ± 53[a]	1394 ± 85[c]
Ratio C_6/C_5	0.53 ± 0.04[a]	0.41 ± 0.01[b]	0.55 ± 0.02[a]	0.35 ± 0.02[b]

[a]Given as TAG for 3 wk. Structural assignments and GC conditions can be found in the original paper (see Ref. 2).
[b]Results are expressed as means ± SEM. Data not sharing the same superscript roman letter are significantly different at $P < 0.05$ (two-way ANOVA followed by least-squares estimates of marginal means). Traces: under the threshold for GC detection; for other abbreviations see Table 1.
[c]C_5: CFAM showing a cyclopentenyl ring (from a- to h-CFAM) (see Fig. 1).
[d]C_6: CFAM showing a cyclohexenyl ring (from i- to p-CFAM) (see Fig. 1).

type and PPARα-null mice, followed by enzyme activity measurements. The activities of ACO and CYP4A, which are both known to be PPARα-target genes (21,22), were significantly regulated in liver by CFAM feeding, showing interaction between diet, sex, and genotype. There was no significant difference induced by CFAM feeding in males (wild-type or PPARα-null mice), but ACO activity was induced twofold in female wild-type mice (13.9 ± 1.0 nmol·min^{-1}·mg of protein^{-1} in females fed on the CFAM diet vs. 6.4 ± 0.4 nmol·min^{-1}·mg of protein^{-1} in females fed on the control diet, $P < 0.0001$, Table 3). In contrast, ACO activity was reduced fivefold by the CFAM diet in female PPARα-null mice (0.9 ± 0.3 nmol·min^{-1}·mg of protein^{-1} in females fed on the CFAM diet, and 4.7 ± 0.1 nmol·min^{-1}·mg of protein^{-1} in females fed on the control diet, $P < 0.0001$).

The activity of CYP4A in liver was increased (two- to threefold, $P < 0.001$, Table 3) by CFAM feeding of wild-type, but not of PPARα-null, mice, showing a PPARα-dependent regulation by the CFAM feeding. In addition, CYP4A activity was clearly lower in PPARα-null mice compared to wild-type mice. The activity in male PPARα-null mice was about 25% of the activity in male wild-type mice, whereas the activity in female PPARα-null mice was undetectable, demonstrating a PPARα dependence for basal expression.

The CFAM diet did not regulate the CYP2E1 activity in wild-type mice ($P > 0.05$), but the activity was downregulated by the CFAM diet in female PPARα-null mice. In addition, the activity was dependent on both sex and genotype, which interfered individually. Females showed lower CYP2E1 activity than males ($P < 0.0001$), and PPARα-null mice exhibited lower CYP2E1 activity than wild-type animals ($P = 0.03$).

The activity of stearoyl-CoA desaturase has been reported to be regulated *via* peroxisome proliferators and by PUFA (23,24). In the present experiment, feeding the CFAM-containing diet significantly lowered the stearoyl-CoA desaturase activity in both wild-type and PPARα-null mice ($P = 0.0003$, Table 3). In addition, stearoyl-CoA desaturase activity was affected by both genotype and gender ($P < 0.0001$ and 0.014, respectively). The activity was lower in males than in females ($P = 0.014$), and the activity was lower in PPARα-null animals compared to wild type ($P < 0.0001$, Table 3). In addition, the CFAM diet significantly lowered the activity in female PPARα-null mice.

DISCUSSION

PPARα mediates the induction of ACO and CYP4A activities in response to peroxisome proliferators (7,8), and after starvation and during diabetes (9,25). Recently, it was shown that feeding CFAM to rats exerts some peroxisome proliferator-like effects by increasing ACO and CYP4A1 activities (5). To elucidate the role of the PPARα in mediating the effects of CFAM on liver expression of lipid-metabolizing enzymes, we took advantage of the PPARα-null mice model, which has a targeted deletion of the PPARα gene (8). CFAM were fed to wild-type and PPARα-null mice, and their livers

TABLE 3
Enzymatic Activities[a,b] Measured in Subcellular Fractions of Livers of Male and Female Wild-Type (+/+) and PPARα-Null Mice (–/–) Administered a Standard Control Diet or a Diet Containing 0.5% of Cyclic FA Derived from α-Linolenic Acid and Given as TAG (CFAM diet) for 3 wk

		Palmitoyl-CoA oxidase (nmol NADH·min^{-1}·mg protein^{-1})	ω-Laurate hydroxylase (CYP4A) (nmol·min^{-1}·mg protein^{-1})	(ω-1)-Laurate hydroxylase (CYP2E1) (nmol·min^{-1}·mg protein^{-1})	Δ9 Desaturase (pmol·min^{-1}·mg protein^{-1})
Male +/+	Control diet	4.2 ± 0.4^{a}	2.5 ± 0.5^{a}	6.2 ± 0.5^{a}	48.5 ± 7.1
	CFAM diet	5.7 ± 0.9^{a}	6.7 ± 1.0^{c}	5.7 ± 0.6^{a}	27.2 ± 7.0*
Male –/–	Control diet	1.7 ± 0.1^{b}	0.6 ± 0.4^{b}	4.5 ± 2.0^{a}	15.9 ± 1.5
	CFAM diet	2.4 ± 0.2^{b,c}	2.3 ± 0.6^{a,b}	6.0 ± 1.1^{a}	14.2 ± 2.6
Female +/+	Control diet	6.4 ± 0.4^{d}	1.6 ± 0.3^{a,b}	4.1 ± 0.6^{b}	56.3 ± 33.2
	CFAM diet	13.9 ± 1.0^{e}	4.4 ± 0.8^{d}	3.9 ± 0.3^{b}	33.2 ± 1.8*
Female –/–	Control diet	4.7 ± 0.1^{a}	ND	3.0 ± 0.4^{b}	38.6 ± 6.6
	CFAM diet	0.9 ± 0.3^{b,c,f}	ND	1.9 ± 0.3^{c}	19.7 ± 1.8*

[a]Results are expressed as means ± SEM.
[b]Data not sharing the same superscript roman letter are significantly different at $P < 0.05$ (two-way ANOVA followed by least-squares estimates of marginal means).
[c]*$P < 0.05$ vs. animals of the same sex and same strain but fed on the CFAM diet (two-way ANOVA followed by least-squares estimates of marginal means). ND: not detected (under the limit for the detection of the metabolites); for other abbreviations see Table 1.

were analyzed for various enzyme activities and lipid contents. The present results are consistent with our hypothesis, in that dietary CFAM can induce ACO and CYP4A activities in a PPARα-dependent manner in females. Concerning ACO activity, the significant interaction between sex, genotype, and diet on ACO activity cannot discern any clear effect of CFAM: CFAM induced ACO activity by a factor of two in wild-type females, whereas this treatment invoked a decrease in ACO activity in female PPARα-null mice (Table 3). The nonsignificant effect in ACO observed in wild-type males (Table 3) suggested no, or only weak, activation of the PPARα by CFAM. The results on CYP4A, showing induction of the activity in wild-type animals but not in PPARα-null mice (Table 3), demonstrate a dependency on PPARα in mediating the effects of CFAM. Thus, taken togther the data show that other mechanisms besides PPARα are involved in mediating some of the effects of CFAM. One possibility is that other isoforms of PPAR may mediate the effects of CFAM. Indeed, PUFA like linoleic acid, α-linolenic acid, and eicosanoids have been demonstrated to be ligands not only for PPARα (12,26,27) but also for PPARδ (12). Recently, lipid oxidation products in oxidized frying oil were shown to upregulate CYP4A1 and ACO expression and to activate PPARα (28), although in that study the contribution of restricted food intake was not discerned. The ability of CFAM to bind to nuclear receptors remains unknown, and further work with the PPAR isoforms should help to elucidate the effects of CFAM on lipid metabolism.

Livers were moderately enlarged in male PPARα-null mice fed on the CFAM diet when compared to the males fed on the control diet (+16%). A weak, nonsignificant effect was found in wild-type animals (+8%). These findings suggest that dietary CFAM do not cause hepatomegaly *via* PPARα activation, as do peroxisome proliferators. Somewhat unexpectedly, the liver enlargement seen in the male PPARα-null mice was not associated with lipid accumulation, but female PPARα-null mice showed lipid accumulation that was not associated with liver enlargement. The observed accumulation of total lipids and CFAM in female PPARα-null mice is probably due to impaired FA metabolism. Indeed, targeted disruption of the PPARα gene was shown to change the constitutive expression of FA-metabolizing enzymes and FA oxidation capacity (29). Our present findings of lower ACO, CYP4A, CYP2E1, and stearoyl-CoA desaturase activities in the PPARα-null mice, in particular in view of the observed downregulation of ACO activity in female PPARα-null mice fed the CFAM diet, are in line with a generally lower capacity of FA metabolism. This suggests that CFAM may exacerbate the lipid disturbances that have already been reported to occur spontaneously in mice lacking expression of the PPARα. Indeed, we as well as others (8,30) have observed an accumulation of lipids in the livers of untreated female PPARα-null mice compared to females of wild-type mice (+30%, Table 2).

It was also evident that CFAM feeding caused an accumulation of total lipids in the livers of female PPARα-null mice as compared to wild-type females. Also, CFAM accumulated to a much greater extent in the livers of female PPARα-null mice, being two- to fivefold higher than in the other mice. In addition, one female PPARα-null mouse fed the CFAM diet showed a steatotic liver. PPARα plays an important role in regulating expression of lipid-metabolizing enzymes for balancing perturbations in cellular lipid metabolism, and a plausible explanation to our findings is that peroxisomes are of great importance in the metabolism of CFAM. Therefore, a lack of functional PPARα apparently results in lower ACO activity, which may cause the observed accumulation of CFAM isomers shown in Table 2. The accumulation of CFAM in the liver may in turn interfere with general lipid metabolism, leading to a further reduction in ACO, stearoyl-CoA desaturase, and CYP2E1 activities and to the accumulation of lipids in the livers of, in particular, female PPARα-null mice. Indeed, CFAM feeding decreased stearoyl-CoA desaturase activity significantly in all groups except in male PPARα-null mice. However, the stearoyl-CoA desaturase activity in untreated male PPARα-null mice was only one-third, compared to the activity seen in untreated wild-type mice. These results suggest that the effect of CFAM can be attributed to the well-known regulation by PUFA (31,32), and that PPARα is not involved in the regulation of stearoyl-CoA desaturase by CFAM. The mechanism by which lack of PPARα regulates basal stearoyl-CoA desaturase expression differently in female and male mice remains unknown. Nevertheless, recent studies have described the interaction of both estrogens, especially β-estradiol, and PPARα in the regulation of hepatic lipid metabolism (33). Based on the known importance of stearoyl-CoA desaturase in lipoprotein metabolism in the liver (34), a possible involvement of estrogen in the regulation of stearoyl-CoA desaturase in female PPARα-null mice might explain the foregoing results. In addition, sterol regulatory element-binding protein (SREBP)-1c, the main liver isoform of the SREBP family, which is known to play a role in regulating FA synthesis, may also be involved. Indeed, the suppression of hepatic gene expression of lipogenic enzymes, especially of stearoyl-CoA desaturase, by PUFA has been demonstrated to be due to a decrease in mature SREBP-1, and not to be mediated *via* PPARα or -γ . Hence, the SREBP-1c pathway may be of major importance in mediating the effects of CFAM on liver metabolism.

Interestingly, the liver CFAM profiles were significantly different from that of the diet, with an increase in the proportion of the C_5-CFAM and a corresponding decrease in the proportion of the C_6-CFAM (see structures in Fig. 1), as has already been described in rats (5,6). Moreover, the ratio between the amount of the C_6- and C_5-CFAM was significantly lower in the PPARα-null animals than in the wild-type mice. One can surmise that a difference in the metabolism of the C_5-CFAM and the C_6-CFAM would produce the difference observed between the ratio of the C_6- and the C_5-CFAM in the diet and in the livers, leading to the accumulation of the C_5-CFAM. The preferential enrichment of C_5-CFAM in the PPARα-null animals suggests that PPARα may regulate expression of enzymes involved in their metabolism. However,

the biochemical routes of metabolism of CFAM are not clearly identified. Some data suggest that they can be readily β-oxidized in the rat (36), but the kinetic parameters of ACO and carnitine palmitoyltransferase suggest that CFAM would be poorer substrates for these enzymes than their precursor α-linolenic acid (37). It is, however, well known that the combined system of microsomal ω-oxidation and peroxisomal β-oxidation plays a central role in the degradation of a wide variety of endogenous and xenobiotic lipid compounds. For instance, ω-hydroxylation is the first step in the elimination of leukotriene B_4, a PPARα ligand, which is then degraded by peroxisomal β-oxidation (38). It thus may be suggested that CFAM would first be ω-hydroxylated in the endoplasmic reticulum and subsequently β-oxidized in peroxisomes and/or mitochondria. However, ω-hydroxylation of CFAM has not yet been described, and the preferred substrates for oxidation may not be the dicarboxylic cyclic FA, but rather the CFAM. Additional studies are needed to verify this hypothesis.

The results of the present study suggest that PPARα is of importance in the metabolism of cyclic FA in mice but is not the exclusive mediator of the effects of CFAM on the liver. No study has thus far been carried out in humans to determine the effects and the metabolism of these cyclic FA, which occur in the human diet as a result of the consumption of fried vegetable oils. In humans, the expression of PPARα is one order of magnitude lower than in mice (39,40). As a consequence, humans may have a low capacity to metabolize cyclic FA, similar to PPARα-null mice. The metabolism of CFAM and the effects of these molecules in humans remain to be clarified. In addition, further work is warranted to assess the involvement of other nuclear receptors in the mediation of the effects of these cyclic FA.

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Stimulation of Rat Liver Mitochondrial *sn*-Glycerol-3-phosphate Acyltransferase by Polymyxin B *via* Enhanced Extraction of Lysophosphatidic Acid

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ABSTRACT: The purpose of this investigation was to determine how polymyxin B stimulates the activity of mitochondrial glycerophosphate acyltransferase. Polymyxin B did not change the integrity of the mitochondrial outer membrane as judged by testing the latency (>80%) of cytochrome oxidase activity. The stimulation totally disappeared when polymyxin B-treated mitochondria were washed. The FA side chain in polymyxin B was unnecessary for stimulation, as the nonapeptide was as effective as the whole antibiotic. The stimulation by polymyxin B or the nonapeptide was observed only in the presence of BSA. Cytochrome c, when added to the incubation medium instead of albumin, did not stimulate the mitochondrial enzyme, but did produce a stimulatory effect of polymyxin B on the mitochondrial acyltransferase. As reported earlier for the bacterial and microsomal acyltransferase, other polycationic compounds such as spermine and spermidine stimulated mitochondrial glycerophosphate acyltransferase. The stimulation of the mitochondrial acyltransferase by spermine and spermidine also occurred only in the presence of BSA. The analysis of the products of esterification demonstrated the presence of more lysophosphatidic acid (LPA) in the polymyxin B- and polyamine-stimulated assays in comparison to their respective control. Furthermore, in comparison to the albumin-treated control, there was 60% more LPA present in the assay supernatant fractions of polymyxin B-treated samples. Our results suggest that polymyxin B stimulates the mitochondrial glycerophosphate acyltransferase activity by enhancing the extraction of more LPA from the mitochondria to the supernatant fraction.

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Glycerophosphate acyltransferase (EC 2.3.1.15; GAT) catalyzes the first and committed step in the synthesis of glycerophospholipids and TAG from *sn*-glycerol-3-phosphate. In mammalian cells, the enzyme is present in both mitochondrial outer membrane (MOM) and endoplasmic reticulum. The two isoforms of GAT differ in their biochemical properties and regulation. The enzyme acylates glycerophosphate to lysophosphatidic acid (LPA), which is converted to PA by the monoacyl glycerophosphate acyltransferase (EC 2.3.1.51; MGAT) that is also present in both the MOM and endoplasmic reticulum. PA is the key intermediate in the biosynthetic pathway of all glycerolipids.

A substantial amount of knowledge has accumulated on mitochondrial GAT (mtGAT) regarding its properties (1,2), purification (3,4), cloning (5–7), and regulation (8,9). The reason for this thrust of research on mtGAT is because several lines of experiments have provided evidence that the mtGAT regulates the ultimate distribution of FA in major cellular phosphoglycerolipids. The asymmetric distribution of FA is considered to be of major importance in maintaining the functional and structural role of phosphoglycerides in biological membranes (10,11).

Since all molecular species of phosphoglycerides arise through LPA, the specific distribution of FA can be regulated by GAT. The preference of saturated fatty acyl-CoA by the mtGAT suggests (12) that this enzyme may be responsible for the predominance of saturated FA found in position 1 of cellular glycerolipids (13–15). Furthermore, in Ehrlich ascites tumor cells that exhibit a large percentage of unsaturated FA present at position 1 in the major phosphoglycerides (16), the GAT activity is confined to the microsomes only (17). A similar observation was made on the rat ascites hepatocytes (18). There is an age-dependent decline in mtGAT activity when tissue explants are converted to primary cell cultures. The decline in mtGAT activity parallels an increase in the amount of monounsaturated FA at position 1 in choline phosphoglycerides (19).

Indeed, the mitochondrially synthesized LPA can leave the organelles in the presence of FA-binding protein, translocate to the microsomes, and convert to PA (20) and presumably to other glycerophospholipids.

In considering the important role mtGAT plays in the quality control of glycerophospholipids, it is not surprising that both synthesis and activity of GAT are subject to rather complex regulation. The activity of rat mtGAT decreases by starvation and increases by insulin treatment of diabetic animals (21,22). Similarly, the mtGAT mRNA dramatically increases in livers of fasted mice re-fed with a high-carbohydrate diet (23–25). Recent results also indicate that mtGAT activity is controlled by a phosphorylation–dephosphorylation mechanism as well (8,9).

There are selective inhibitors and activators of the mitochondrial and microsomal GAT. For example, microsomal GAT is inhibited by sulfhydryl group reagents (17,26), differ-

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Abbreviations: GAT, glycerophosphate acyltransferase; LPA, lysophosphatidic acid; MGAT, monoacylglycerophosphate acyltransferase; MOM, mitochondrial outer membrane; mtGAT, mitochondrial glycerophosphate acyltransferase.

ent proteases (27), acetone (17), and polymyxin B_1 (28,29). On the other hand, the mitochondrial enzyme is either resistant to or stimulated by the above reagents (17,29,30).

Polymyxin B sulfate has a bactericidal action against almost all gram-negative bacilli except the *Proteus* group. It is also a drug of choice in treating susceptible *Pseudomonas aeruginosa* infections of the urinary tract, meninges, and bloodstream (31). In spite of these antimicrobial effects, polymyxin B is mostly used topically because it exerts effects on eukaryotic metabolism such as lipopolysaccharide-related immune functions (32) and bronchial provocation by mast cell degranulation (33) and enhancement of lipoprotein catabolism (34).

We have used polymyxin B, immobilized by conjugation with agarose, to determine the topography of the catalytic site of the mtGAT. Externally added polymyxin B-agarose can stimulate mtGAT to the same extent as the free antibiotic, suggesting that the catalytic site of the acyltransferase faces the cytosol (30). It is not clear, however, how polymyxin B stimulates mtGAT. In this paper, we have investigated how the antibiotic stimulates the acyltransferase. The results suggest that the antibiotic does not stimulate the enzyme by directly binding with the enzyme or MOM but by helping exogenously added soluble proteins to remove the reaction product from the catalytic site.

EXPERIMENTAL PROCEDURES

Materials. Male Harlan Sprague-Dawley rats were purchased from Taconic Farms (Germantown, NY) and were fed *ad libitum*. *sn*-(2-^{3}H)Glycerol-3-phosphate was purchased from American Radiolabeled Chemicals Inc. BSA (FA-free) was purchased from Boehringer Mannheim (Indianapolis, IN). Polymyxin B, polymyxin B nonapeptide, spermine, and spermidine were obtained from Sigma Chemicals (St. Louis, MO). All other materials were obtained as described previously (27).

Preparation of mitochondria. Rat liver mitochondria from 150–200-g male rats were prepared by differential centrifugation as described previously (12). The purity of the preparations was evaluated by performing GAT assays in the presence or absence of 2 mM *N*-ethylmaleimide, a potent inhibitor of the microsomal GAT (17). The microsomal contamination of the mitochondrial fractions was less than 5%.

Analytical methods. GAT activity was measured by following the incorporation of *sn*-(2-^{3}H)glycerol-3-phosphate into butanol-extractable phospholipids (12,17). Asolectin was omitted from the assay system. The assay of mtGAT was carried out at 37°C for 3 min in a reaction volume of 0.25 mL, containing 0.2 M MES-TES-glycylglycine buffer [morpholine ethane sulfonic acid-*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid-glycylglycine buffer] (pH 7.5), 0.72 mM palmitoyl-CoA, BSA (FA-free) at 4 mg/mL, 0.1 M $MgCl_2$, 0.1 M KCN, and 37.5 mM *sn*-(2-^{3}H)glycerol-3-phosphate (specific activity 1.923×10^{-3} cpm/nmol). The amount of palmitoyl-CoA represents its optimal level for mitochondrial assay. The reaction was initiated by the addition of the subcellular protein that was maintained between 0.2 and 0.4 mg/mL in the incubation medium. For sedimenting mitochondria, the incubated mixture was cooled to 0–4°C and spun at $10,000 \times g$ for 10 min. The pellet was resuspended in 0.3 M sucrose-EDTA solution. The supernatant fluid, the resuspended pellet, or the whole GAT reaction mixture was treated with 1-butanol to extract the radioactive acylation products. Whenever necessary, the products of acylation, chiefly LPA and PA, were separated by TLC (12). Protein concentration was assayed according to the Bradford method (35) using BSA as a standard. FA-free BSA (50 mg/mL) was denatured by heating at 95°C for 10 min (36). Cytochrome c oxidase activity was measured by using the cytochrome c oxidase assay kit (CYTOC-OX1), obtained from Sigma. Cytochrome c was reduced with sodium hydrosulfite (dithionate). The reduced cytochrome c was then reoxidized by the addition of mitochondrial sample. The initial rate of change in absorbance was measured at 550 nm (37).

Statistical analysis. The computer software Stat View 5.12^+ from Brain Power (Calabasas, CA) was used to perform one-way ANOVA. All values are expressed as means ± SEM. The level of significance was set at $P < 0.05$.

RESULTS

Are both the cyclic peptide and FA side chain of polymyxin B necessary for stimulation? Since the permeabilizing property of polymyxin B on gram negative bacterial cells requires both the cyclic nonapeptide and FA side chain (38), it is pertinent to ask whether the same is true for the property of the antibiotic in stimulating mtGAT. We measured the stimulation of mtGAT at different concentrations of polymyxin B and the corresponding nonapeptide. The results are presented in Figure 1. The

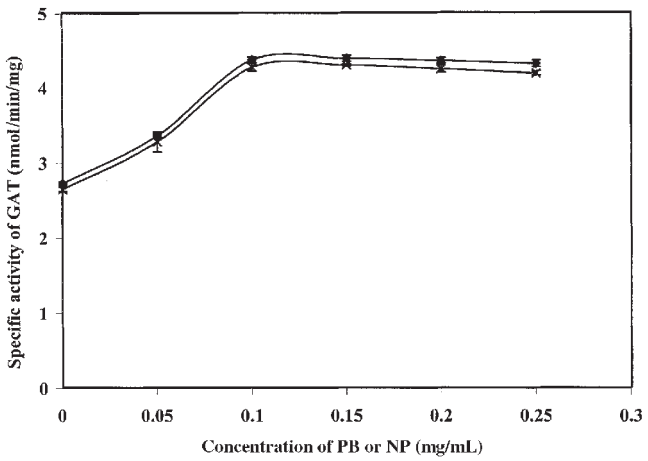

FIG. 1. Effect of polymyxin B (PB) and its nonapeptide (NP) on mitochondrial glycerophosphate acyltransferase (GAT) activity. The reaction was initiated by the addition of the subcellular fraction. The concentration of BSA in the assay system was kept at 4 mg/mL. The assays were performed by using different concentrations of PB (◆) and NP (×). The final protein concentration was adjusted to 0.2 mg/mL. The maximal GAT activities in the presence of PB and NP were 4.36 and 4.26 nmol/min/mg, respectively. The values are the mean ± SEM from four independent experiments.

maximum stimulation by polymyxin B was 60%. This extent of stimulation was achieved at an antibiotic concentration of 0.1 mg/mL and above. These results are in agreement with our previously published data (30). The nonapeptide alone at different concentrations produced stimulation similar to that by polymyxin B. Thus, the acyl group side chain of the antibiotic does not play a role in causing the stimulation of mtGAT.

The influence of polymyxin B on the permeability of mitochondrial outer membrane (MOM). The initial effect of polymyxin B on bacterial cells is to permeabilize the cells, leading to release of internal materials (39,40). Therefore, it is possible that the antibiotic can also damage the MOM by increasing its permeability. The MOM is permeable to molecules up to M.W. of 8 kDa (41,42). This unusual permeability is mainly due to the presence of the VDAC protein (43,44). Although polymyxin B has a M.W. of 1.2 kDa, it is a polycationic molecule. Thus, its passage through the outer membrane is questionable. Accordingly, it is unclear whether the antibiotic should be present on the outer side or both sides of the MOM. We decided to measure any permeability change of the MOM by measuring the latency of cytochrome c oxidase, an inner membrane protein. Reduced cytochrome c (12.5 kDa) cannot penetrate the MOM unless the membrane is damaged. The latency of cytochrome oxidase is used to gauge the damage of the MOM. The results, presented in Figure 2, show that mitochondria, in the presence or the absence of polymyxin B, had very similar latency of cytochrome c oxidase. On the other hand, mitochondria treated with *n*-dodecyl-β-D-maltoside showed full cytochrome c oxidase activity. It is therefore unlikely that the antibiotic causes any damage to mitochondrial membrane leading to an increase in its permeability.

Reversibility of the stimulatory effect of polymyxin B. When polymyxin B interacts with bacterial membrane, the FA side chain interacts with the hydrophobic core of the membrane and the cyclic polycationic peptide interacts with the phosphate groups of the membrane phospholipids. As a result, washing the bacterial cells after polymyxin B treatment does not reverse the interaction of the antibiotic with the membrane (38).

Rat liver mitochondria were incubated with polymyxin B (0.1 mg/mL) and then washed twice with 0.3 M sucrose-EDTA solution by centrifugation. Because polymyxin B is a water-soluble compound, washing of the mitochondria should remove the antibiotic. If the antibiotic is not removed, it must be bound to the mitochondria and should therefore exert its stimulating property after the wash; but it does not. Figure 3 documents the results. The washed mitochondria showed the same GAT activity regardless of whether they were previously treated with polymyxin B. Thus, the possibility of any irreversible binding of polymyxin B with the MOM is ruled out.

Polymyxin B stimulates mtGAT in the presence of a protein. As BSA itself stimulates mtGAT activity (20), the possibility was explored that polymyxin B might show its stimulatory activity better in the absence of BSA. Results presented in Figure 4 demonstrate that in the absence of BSA, the activity of the mtGAT was approximately 60% of that obtained in the presence of BSA (Fig. 4; bars 1 vs. 2). Surprisingly, poly-

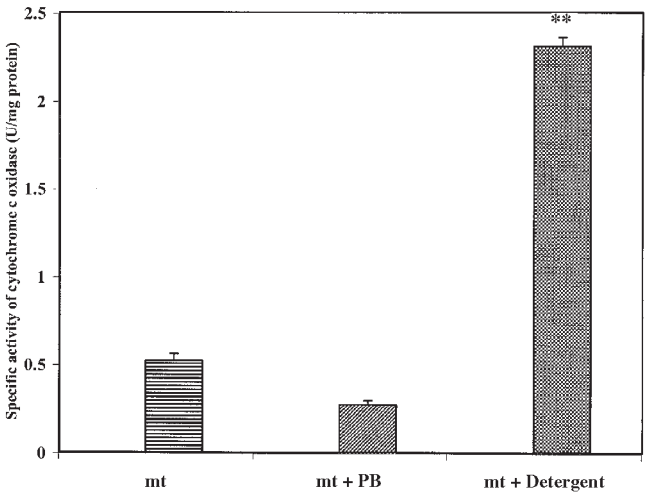

FIG. 2. Cytochrome c oxidase activity for detection of mitochondrial outer membrane integrity in the presence of PB. Cytochrome c oxidase activity was measured using a kit from Sigma Chemicals (St. Louis, MO; see the Experimental Procedures section for details). In the control, the mitochondria were incubated with enzyme dilution buffer (EDB; 10 mM Tris HCl, pH 7.0, containing 250 mM sucrose), and in the other two sets, the mitochondria were incubated with EDB that contained either PB (0.1 mg/mL) or 1 mM *n*-dodecyl-β-D-maltoside (the detergent supplied in the kit). The enzyme was assayed after 10 min of incubation. The values are the mean ± SEM from four independent experiments. **Significantly different from the group containing mitochondria and mitochondria in the presence of detergent, $P < 0.001$. mt, mitochondria; for other abbreviation see Figure 1.

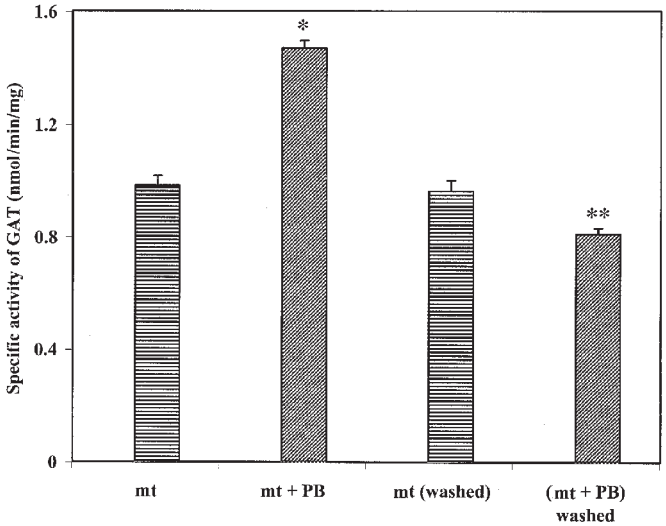

FIG. 3. The reversibility effect of the PB on mitochondrial GAT. Mitochondrial (mt) samples were incubated for 3 min at 37°C in either the absence or the presence of 0.1 mg/mL of PB. The concentration of BSA in the assay system was kept at 4 mg/mL. The mixtures were then centrifuged at 10,000 × *g* for 10 min, and the mt pellets were resuspended in sucrose-EDTA solution. GAT activity was assayed from the resuspended pellets (mt washed and mt + PB washed) and compared with that of the control group (mt and mt + PB). The values are the mean ± SEM from three independent experiments. Statistical differences are denoted by $*P < 0.05$ and $**P < 0.01$. For other abbreviations see Figure 1.

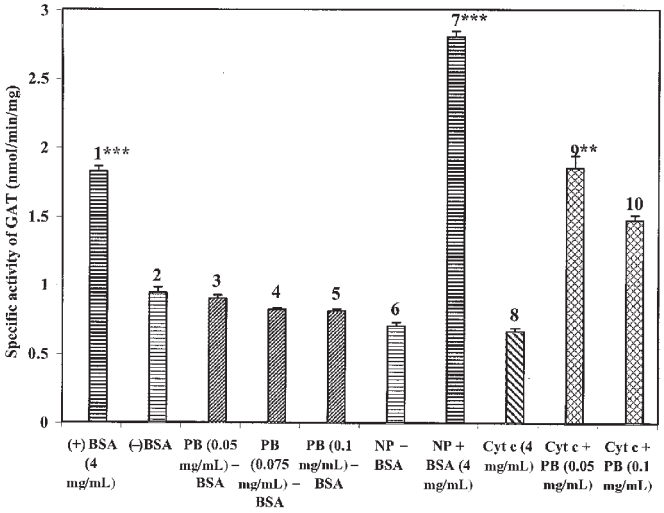

FIG. 4. PB exerts its stimulatory effect in the presence of a protein. The assays were done under the following conditions. Bars 1 and 7: assay includes BSA (4 mg/mL); bars 2–6: assay without BSA; bars 3–5 and 9,10: different concentrations of polymyxin B were used in the assay; bar 6: NP (0.1 mg/mL) was present; and bars 8–10: cytochrome c (4 mg/mL) was present in the assay instead of BSA. The data are the mean ± SEM from four independent experiments. Statistical differences are denoted by $^{**}P < 0.01$ and $^{***}P < 0.001$ relative to bar 8 and bars 2–6, respectively. For abbreviations see Figure 1.

myxin B and the nonapeptide showed no stimulation of mtGAT when the assay was done in the absence of BSA (Fig. 4; bars 2 vs. 3–6). In fact, there was a slight inhibition of the acyltransferase by polymyxin B in the absence of BSA (bars 2–5). The maximum inhibition was observed at a polymyxin B concentration of 0.1 mg/mL.

As the stimulatory effect of polymyxin B on mtGAT takes place only in the presence of BSA, we questioned whether any protein other than BSA could manifest a similar stimulatory effect. Cytochrome c (4 mg/mL) was used in the assay medium in place of BSA. The presence of cytochrome c did not stimulate mtGAT (Fig. 4; bars 2 and 8). This observation was in keeping with the idea that only BSA or liver FA-binding protein could stimulate mtGAT activity when either of these proteins was present in the assay medium (38). The stimulation is caused by specific binding of LPA to these proteins (20). The presence of cytochrome c instead of BSA in the assay medium showed approximately 30% inhibitory effect on mtGAT (bars 2 and 8). The reason for this inhibitory effect is unknown but could be less availability of substrate due to nonspecific binding of palmitoyl-CoA to the protein. However, in the presence of cytochrome c, polymyxin B stimulated mtGAT. The stimulation was maximal (180%) in the presence of the lower dose (0.05 mg/mL) of polymyxin B (Fig. 4; bars 8–10).

Similarly, to determine if BSA in its native form is essential for the stimulation of mtGAT by polymyxin B, denatured BSA was used in the assay medium instead of native BSA. The results (Table 1) show that denatured BSA is more effective in stimulating mtGAT than native BSA (the two control values in Table 1). However, there was still over 50% stimulation of mtGAT due to the addition of polymyxin B (0.1 mg/mL) in the presence of denatured BSA.

TABLE 1
Effect of Denatured BSA on the Stimulation of mtGAT by Polymyxin B[a]

	Enzyme activity (nmol/min/mg)		
Protein in assay	Control	PB (0.05 mg/mL)	PB (0.1 mg/mL)
BSA (4 mg/mL)	2.47 ± 0.58	3.35 ± 0.34	4.28 ± 0.48
Denatured BSA (4 mg/mL)	3.07 ± 0.27	4.26 ± 0.39**	5.20 ± 0.66***

[a]Assays were carried out as described in the Experimental Procedures section. The stimulatory effect of mitochondrial glycerophosphate acyltransferase (mtGAT) by polymyxin B (PB) in the presence of denatured BSA was compared with that in the presence of native BSA. The values are means ± SEM from three independent experiments. $^{**}P < 0.01$ and $^{**}P < 0.001$ relative to the control.

Effect of $MgCl_2$ on the stimulation of mtGAT by polymyxin B. Mg^{2+} ions stimulate mtGAT, presumably by stabilizing the membrane by functioning as cationic bridges between adjacent phosphate groups in the phospholipid bilayer (38). As polymyxin B has four positive charges on the cyclic peptide, the stimulation it exerts on the mtGAT may be influenced by the presence of Mg^{2+}. With this in mind, the effect of polymyxin B on mtGAT was measured in the presence and absence of Mg^{2+}. The results are documented in Figure 5. The mtGAT activity was less in the absence of Mg^{2+}. In the presence of polymyxin B, Mg^{2+} produced an additional stimulatory effect on mtGAT.

Effect of polyamines on mtGAT. Polyamines are polycationic substances and have a structural similarity with poly-

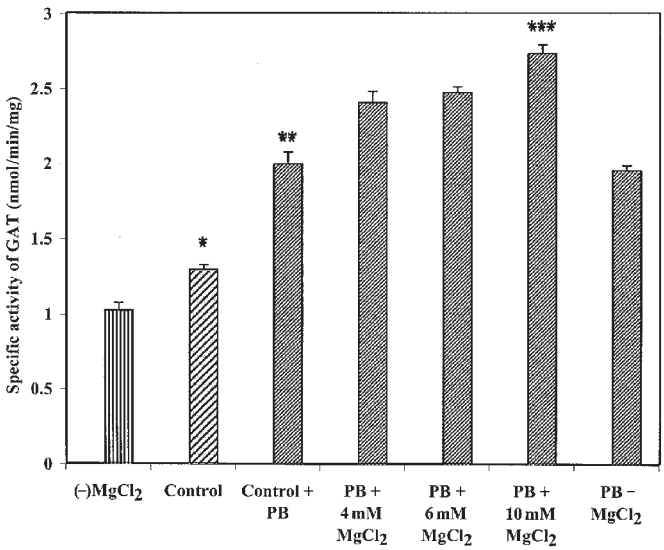

FIG. 5. Comparison of the optimal activity of mitochondrial GAT by PB in the presence of different concentrations of Mg^{2+} ions. Mitochondria (0.6–0.8 mg/mL) were incubated in either the regular GAT assay medium (control) or under different conditions, such as in the absence of magnesium chloride (–$MgCl_2$) or with PB (control + PB), and in the presence of different concentrations of $MgCl_2$. Whenever used, PB was present at 0.1 mg/mL. The values are the mean ± SEM from three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, respectively.

TABLE 2
Effect of Polyamines on mtGAT Activity[a]

Polyamine used	Concentration (mg/mL)	BSA (4 mg/mL) (% activity)	BSA absent (% activity)
Spermine	0	100	56.8 ± 3.80
	0.05	219.4 ± 22.1	42.0 ± 3.16
	0.1	296.9 ±18.15**	48.5 ± 4.02
Spermidine	0.05	256.3 ± 20.64	79.6 ± 5.42
	0.1	348.5 ± 19.36**	65.1 ± 4.58

[a]The assay conditions are described in the Experimental Procedures section. The concentration of polyamines in the entire assay system was as indicated. The reaction was initiated by the addition of the subcellular fraction. The control mtGAT activity (100%) in the presence of BSA was 1.82 nmol/min/mg. The values are means ± SEM from three independent experiments. Statistical differences are denoted by $^{**}P < 0.01$, relative to the group where BSA is absent. For abbreviation see Table 1.

myxin B in terms of positively charged amino acid residues. Table 2 demonstrates the effect of polyamines such as spermine and spermidine on stimulation of mtGAT. In the presence of spermine or spermidine, the activity of mtGAT was also stimulated much more (three- to fourfold) than that of polymyxin B at its optimal dose (0.1 mg/mL). The maximal stimulatory effects of both spermine and spermidine on mtGAT were observed mainly in the presence of BSA in a dose-dependent manner. However, in the absence of BSA, spermidine at its lower concentration (0.05 mg/mL) showed a slight stimulatory effect on mtGAT as compared to the control.

Effect of polymyxin B, spermine, and spermidine on the levels of reaction products of mtGAT. In an effort to determine how polymyxin B increases mtGAT activity, reaction products of mtGAT were analyzed by TLC. More LPA was formed in the presence of BSA (Table 3). When cytochrome c replaced BSA in the assay medium, the LPA/PA ratio remained unchanged from the control samples without BSA. However, when polymyxin B was added to either BSA-, denatured BSA-, or cytochrome c-treated assays, the LPA/PA ratio increased in comparison to the respective controls containing exogenously added protein only. In these samples, the amount of LPA produced in the presence of polymyxin B was two to three times more than that in the respective controls without polymyxin B. The polyamines spermine and spermidine also yielded results similar to, but more pronounced than, those obtained with polymyxin B (Table 3).

Previous results have established that BSA stimulates mtGAT by binding and removing the reaction product LPA from the mitochondria to the supernatant fluid (30). Table 4 documents the results of such an experiment. In the BSA-treated samples, more LPA was produced and most of the LPA was present in the supernatant fraction. On the other hand, when a similar experiment was done in the presence of BSA and polymyxin B, there was 60% more LPA present in the assay supernatant fraction compared to that of the BSA-treated control. Furthermore, the LPA/PA ratio in the polymyxin B-treated samples was approximately double in the supernatant fraction in comparison with that in the presence of BSA only. It is notable that the increase in the phospholipid

TABLE 3
Effect of PB or the Polyamines on the Acylation Products Formed in the Presence of BSA, Denatured BSA, or Cytochrome c[a]

Protein in assay	Control			Polymyxin B (0.1 mg/mL)			Spermine (0.1 mg/mL)			Spermidine (0.1 mg/mL)		
	LPA	PA	Ratio	LPA	PA	Ratio	LPA	PA	Ratio	LPA	PA	Ratio
None	0.32 ± 0.017	0.29 ± 0.021	1.10 ± 0.035	—	—	—	0.55 ± 0.041	0.31 ± 0.012	1.77 ± 0.038	0.48 ± 0.029	0.39 ± 0.018	1.23 ± 0.023
BSA (4 mg/mL)	0.97 ± 0.014	0.22 ± 0.007	4.40 ± 0.32	2.15 ± 0.036	0.36 ± 0.018	5.97** ± 0.27	3.61 ± 0.21	0.54 ± 0.032	6.68** ± 0.32	4.46 ± 0.24	0.53 ± 0.04	8.41 ** ± 0.42
Denatured BSA (4 mg/mL)	0.78 ± 0.024	0.50 ± 0.019	1.56 ± 0.037	1.80 ± 0.041	0.41 ± 0.008	4.39* ± 0.28	—	—	—	—	—	—
Cytochrome c (4 mg/mL)	0.28 ± 0.011	0.31 ± 0.025	0.90 ± 0.09	0.82 ± 0.13	0.21 ± 0.015	3.90* ± 0.37	—	—	—	—	—	—

[a]Mitochondria (0.62 mg/mL) were incubated in the presence of BSA, denatured BSA, or cytochrome c (4 mg/mL) in the GAT assay medium in a total volume of 0.5 mL. The reactions were stopped and the lipids extracted by the addition of 0.5 mL of 1-butanol. Whenever indicated, PB and polyamines were present at 0.1 mg/mL. The formation of lysophosphatidic acid (LPA) and PA was analyzed by TLC. The values of the acylation products (LPA and PA) are expressed as nmol/min/mg. The data shown are the mean ± SEM from three independent experiments; $^{*}P < 0.05$ and $^{**}P < 0.01$ relative to the respective controls, containing only protein. For other abbreviations see Table 1.

TABLE 4
Effect of PB on Acylation Products (LPA and PA) in Mitochondrial Pellet and Supernatant Fraction[a]

Assay condition	Mitochondrial pellet			Supernatant fraction		
	LPA	PA	Ratio	LPA	PA	Ratio
None	0.18 ± 0.015	0.32 ± 0.014	0.56 ± 0.024	0.04 ± 0.003	0.30 ± 0.029	0.13 ± 0.004
BSA	0.24 ± 0.013	0.36 ± 0.02	0.67 ± 0.031	1.07 ± 0.038	0.18 ± 0.01	5.65 ± 0.23
BSA + PB	0.31 ± 0.015	0.39 ± 0.018	0.79 ± 0.013	1.61 ± 0.035	0.14 ± 0.004	11.32 ± 0.27***

[a]Mitochondria were incubated for 3 min in the GAT assay medium in the absence and presence of PB (0.1 mg/mL). In another set of experiments, mitochondria were incubated without BSA for the same time period. The reaction mixtures were centrifuged at 10,000 × *g* for 10 min, and the mitochondrial pellet was resuspended in 0.34 mL of buffer mixture (0.2 M MTG buffer and 0.1 M $MgCl_2$). Both the supernatant fraction and resuspended mitochondrial pellet were extracted with 0.68 mL of 1-butanol. LPA and PA were separated by TLC. Results are presented as LPA and PA produced (nmol/min/mg) in both pellet and supernatant fractions. The values are the mean ± SEM from three independent experiments. Statistical differences are denoted by ***$P < 0.001$ relative to the BSA-treated control. For abbreviations see Tables 1 and 3.

product due to polymyxin B is selective for LPA. The total amount (pellet + supernatant fluid) of PA produced in the presence of polymyxin B and BSA is the same as that produced in the presence of BSA alone. Therefore, it is unlikely that the increase in the LPA in the supernatant fraction is a reflection of an increase in the total pool size of LPA in the presence of polymyxin B.

DISCUSSION

From the polycationic nature of spermine, spermidine, and polymyxin B and their stimulatory effect on glycerophosphate acyltransferase from different sources, it is tempting to propose that the mechanism of stimulation for all these compounds is the same regardless of the source of GAT. However, our results suggest that no such unifying mechanism of action of the polycationic compounds exists for GAT from different sources. First, we will confine our discussion on whether polymyxin B interacts with the MOM. Second, we will examine how polymyxin B can stimulate mtGAT.

The sensitivity of *Escherichia coli* B to polymyxin B is drastically reduced if the acyl side chain of the antibiotic is removed (38). It has been postulated that the acyl side chain of the antibiotic penetrates the hydrophobic domain of the phospholipid bilayer with the cyclic peptide amino groups interacting with phospholipid phosphates. For stimulating mtGAT, the nonapeptide alone is as good as the whole antibiotic (Fig. 1). The nonapeptide moiety has a net positive charge, and it may interact with the negatively charged phosphate groups of the MOM phospholipids. However, in the absence of BSA, neither polymyxin B nor the nonapeptide stimulates mtGAT. Thus, our results suggest no interaction between the antibiotic and the MOM.

It is likely that, as the polymyxin B nonapeptide is much bulkier than Mg^{2+} or Ca^{2+}, it cannot form cationic bridges between adjacent phosphate groups. The results presented in Figure 5 demonstrate that, in the absence or presence of Mg^{2+}, polymyxin B can stimulate mtGAT to the same degree (approximately 62%). Therefore, for stimulating mtGAT, polymyxin B works differently from Mg^{2+}, which is known to prevent the action and binding of polymyxin B to bacterial membrane (38).

Other factors should be added to the preceding considerations. Addition of polymyxin B does not alter the latency of cytochrome oxidase activity of isolated mitochondria (Fig. 2). Moreover, the stimulatory effect of the antibiotic can be totally reversed by washing the mitochondria. Finally, polymyxin B stimulates mtGAT only in the presence of any exogenous protein in the assay medium (Fig. 4). All of these results suggest that polymyxin B does not interact with the MOM to cause stimulation of mtGAT.

The indication of how polymyxin B stimulates mtGAT activity comes from the experiments done in the presence and absence of BSA and other proteins. It is obvious from these experiments that an externally added protein must be present in the assay medium to exhibit the stimulatory effect of polymyxin B. BSA, even when denatured, stimulates mtGAT. On the other hand, cytochrome c does not stimulate the acyltransferase. So not all proteins can stimulate mtGAT. The proteins known to stimulate mtGAT are BSA and FA-binding protein (20). Both these proteins stimulate the mitochondrial enzyme by extracting the LPA formed by the acyltransferase (20). Denatured BSA also has stimulating activity apparently because it still can extract the LPA from the mitochondria. For the stimulatory effect of polymyxin B, the presence of any of these proteins—BSA, denatured BSA, or cytochrome c—in the assay medium is a requirement. So there is lack of specificity as to which protein should be present in the incubation medium. The likely explanation is that polymyxin B (or its nonapeptide) binds to the exogenous protein and modifies the LPA-binding property of the protein so that LPA formed by the mtGAT is removed more efficiently from the reaction center. The presence of more LPA in the supernatant fluid of the polymyxin B-treated samples (Table 4) provides evidence for this suggestion. The removal of LPA at a faster rate, in turn, helps the acyltransferase reaction to go forward at a faster rate. How polymyxin B modifies the LPA-binding capacity of the externally added proteins is not clear from our experiments. But it is possible that polymyxin B binds to any protein and then binds the LPA, through the interaction of its cationic property, with the negatively charged phosphates of the LPA. The affinity of polymyxin B for exogenous protein is more than that for the MOM.

A question remains as to whether polymyxin B, in combination with BSA or other exogenously added protein, increases the binding of palmityl-CoA for presenting to the mtGAT. First, we performed our experiments at the optimal level of the acyl-CoA. Such a facilitated interaction of this set of substrates and the enzyme should lead to the increase of synthesis of both LPA and PA, which is not the case (Table 4). So enhanced interaction of the substrate with the enzyme does not appear to be the mode of action of polymyxin B in stimulating mtGAT.

Although the action of spermine, spermidine, and polymyxin B on bacterial and mitochondrial GAT are all stimulatory, the mode of action of polymyxin B or the polyamines on mtGAT may be different from that on the other systems. The experiments involving the action of the polyamines on bacterial (45) and microsomal GAT were mostly done in the presence of BSA. However, in at least one instance where the experiment was performed both in the presence and absence of BSA. There, the polyamines stimulated microsomal PA and TAG synthesis even in the absence of BSA (46). Therefore, it is possible that the mechanism of stimulation of the microsomal and perhaps of bacterial GAT by the polyamines is different from that of polymyxin B and the polyamines on mtGAT.

The question still remains why polymyxin B stimulates mtGAT but inhibits microsomal GAT. In the presence of BSA, both mtGAT and microsomal GAT are stimulated. However, LPA is the major product with mtGAT, but PA is the chief product with microsomal GAT (30). LPA bound to BSA is a poor substrate for mitochondrial MGAT but a good substrate for microsomal MGAT (20). It is possible that in the presence of both BSA and polymyxin B, their complex with LPA is a poor substrate for microsomal MGAT; the electrostatic interaction between LPA and polymyxin B might make the LPA no longer accessible for microsomal MGAT. Therefore, microsomal GAT, which is assayed by the combined action of GAT and MGAT, exhibits inhibition. The assay of mtGAT, which mainly reflects the conversion of glycerol-3-phosphate to LPA, is actually stimulated.

Previously, we have used polymyxin B/agarose to determine the topography of the catalytic site of mtGAT (30). After realizing how polymyxin B stimulates mtGAT, a question arose whether we took a valid approach to establishing the location of the catalytic site of the mitochondrial enzyme. MOM is impermeable to BSA, which has a M.W. of 67 kDa. The MOM should also be impermeable to polymyxin B/agarose. So if BSA and polymyxin B (attached to agarose) combine together, the combined complex cannot penetrate the MOM. The polymyxin B portion of the complex can, however, bind LPA formed on the outer surface of the MOM. The effect of polymyxin B was exerted from outside the mitochondria. Thus, the approach taken earlier in determining the topographic location of the catalytic site of mtGAT is appropriate.

The stimulation of GAT by the polyamines is well documented in bacteria and microsomes, and by polymyxin B in mtGAT from different animals and organs. So how these polycationic compounds accomplish this stimulation is a legitimate question. The answer, as depicted above, can lead us to a judicious application of these agents in stimulating mtGAT. However, it is unclear at the moment whether the stimulation of mtGAT by polymyxin B stimulates the synthesis of lipids that, in turn, helps in the wound-healing process when the antibiotic is applied topically. Assuming polymyxin B reaches inside the mammalian cell, it can interact with FA-binding protein (20) and thereby stimulate mtGAT and glycerolipid synthesis.

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Electrophile Tocopheryl Quinones in Apoptosis and Mutagenesis: Thermochemolysis of Thiol Adducts with Proteins and in Cells

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ABSTRACT: Electrophile tocopheryl quinones from the phenolic antioxidants γ-tocopherol and δ-tocopherol form Michael adducts with the thiol nucleophile glutathione. These tocopheryl quinones are involved in cytotoxicity, apoptosis, and mutagenesis, and their biologic properties are associated with the depletion of intracellular thiols. We now show that both proteins and tissues treated with the electrophile γ-tocopheryl quinone (γ-TQ) form thiol adducts. The monoglutathion-*S*-yl derivative of γ-TQ was subjected to thermochemolysis with the strong methylating base tetramethylammonium hydroxide. GC/MS showed four signature peaks and a fragmentation pattern characteristic of the thiol adduct. Similarly, pure monoglutathion-*S*-yl and diglutathion-*S*-yl derivatives of δ-TQ were subjected to thermochemolysis, and GC/MS showed characteristic fragmentation patterns for thiol adducts. The four signature peaks were identified when pure proteins with accessible thiol groups (hemoglobin and histone), FBS, and tissue culture medium and cell preparations were treated with γ-TQ. Signature peaks in both complete medium and washed cells showed the presence of both soluble and insoluble thiol adducts. The effective or free arylating electrophile concentration in complete medium should always be evaluated in tissue culture studies. γ-TQ is a mutagen but not a genotoxin; therefore, the histone adduct may be a previously unrecognized histone modification involved in chromatin dynamics leading to mutagenesis.

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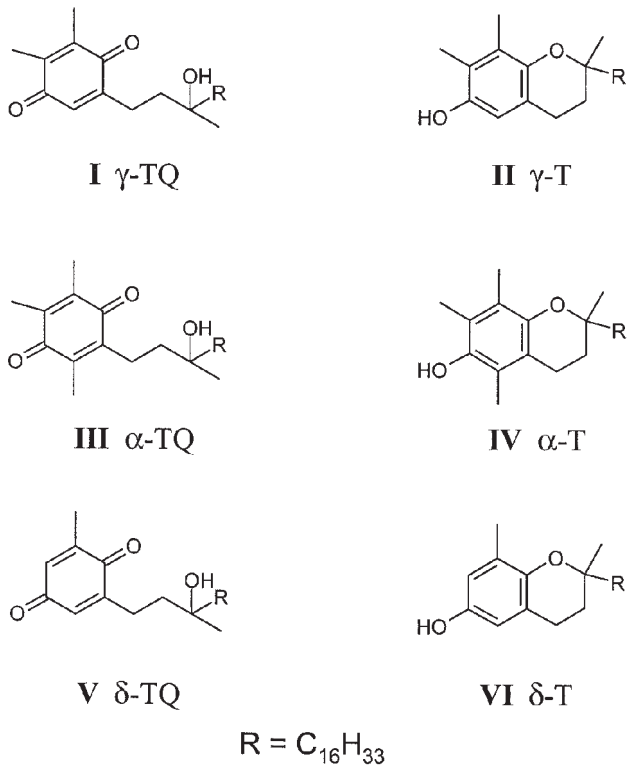

FIG. 1. Structural formulas for tocopherols (T) and tocopheryl quinones (TQ).

γ-Tocopheryl quinone (TQ) **I** (Fig. 1), a *para*-quinone metabolite of the phenolic antioxidant γ-tocopherol (T) **II**, and α-tocopheryl quinone **III**, a *para*-quinone metabolite of the phenolic antioxidant α-tocopherol **IV**, are found in small amounts in animal tissues (1). We have found that, unlike α-TQ **III**, γ-TQ **I** is cytotoxic in tissue cultures of normal cells and both drug-sensitive and multidrug-resistant cancer cells (2–7). γ-TQ **I** stimulates apoptosis (5,6), and although it is not a genotoxin, it enhances transfection and stimulates mutagenesis in cells (7). Thus, specific tocopherols and their quinone metabolites are potential chemotherapeutic agents, possible signaling agents, and putative carcinogens. γ-TQ **I** has a partially methyl-substituted chroman ring that enables it to function as an arylating electrophile in contrast to α-TQ **III**, which has a fully methyl-substituted chroman ring that prevents it from functioning as an arylating electrophile (2–4). The *para*-quinone δ-TQ **V**, a metabolite of δ-T **VI**, is also an arylating electrophile, but very little δ-T **VI** is found in animal tissues, and no attempt has been made to measure the concentration of its quinone in tissues.

Studies with TQ in tissue culture are fully consistent with arylation being the distinguishing difference between α-TQ **III** and γ-TQ **I** that establishes their distinct biologic properties. γ-TQ **I** readily forms adducts with GSH, and the glutathion-*S*-yl adduct, like α-TQ **III**, has no effect on cells in culture (3). The cytotoxicity of γ-TQ **I** is enhanced when cells are preincubated with buthionine-(*S*,*R*)-sulfoximine (BSO) to

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Abbreviations: BSO, buthionine-(*S,R*)-sulfoximine; CEM, drug-sensitive lymphoblastic leukemia cells; DPBS, Dulbecco's phosphate-buffered saline; α-CEHC, 2,5,7,8-tetramethyl-2-(β-carboxyethyl)-6-hydroxychroman; γ-CEHC, 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman; MI, molecular ion; PAH, polycyclic aromatic hydrocarbons; SIC, selective ion chromatogram; T, tocopherol; TQ, tocopheryl quinone; TIC, total ion current; TMAH, tetramethylammonium hydroxide.

reduce their GSH content, and the cytotoxicity of γ-TQ **I** is diminished when cells are treated with *N*-acetylcysteine (NAC) to enhance their intracellular thiol content (4). Intracellular GSH is diminished when cells are incubated with γ-TQ **I** but not with α-TQ **III** (5). Although it is logical to suspect formation of thiol adducts, no direct evidence has been reported to establish the formation of thiol adducts between γ-TQ **I** and naturally occurring nucleophiles in cells and tissues.

A number of studies attribute the cytotoxicity of electrophilic quinones produced from benzene, estrogens, and polycyclic aromatic hydrocarbons (PAH) to the arylation of cellular nucleophiles, including GSH (8). In all instances, the isolation and quantitative estimation of soluble glutathion-*S*-yl adducts were performed by HPLC and MS techniques. Protein adducts are more difficult to isolate and identify, but small peptide adducts have been identified by electrospray MS (9). An alternative approach to identifying adducts derives from a base-cleavage reaction for naturally occurring adducts that was first described in 1933 (10). From this early work, a multistep procedure was developed (base-cleavage followed by permethylation with methyl iodide) to show adduct formation with benzoquinone and substituted benzoquinones, although this method requires from 20- to 200-mg quantities of protein (11–13). We recently described a method to identify adducts between arylating quinone derivatives of PAH and thiol and amine nucleophiles (Briggs, M.K., Desavis, E.D., Mazzer, P.A., and Hatcher, P.G., unpublished observations).

In the present investigation, we used thermochemolysis to cleave adducts between arylating quinones, derived from tocopherol phenolic antioxidants, and accessible thiol groups in GSH, specific proteins, tissue culture medium, and cultured cells. Tetramethylammonium hydroxide (TMAH) is a strong alkaline methylating agent used in highly sensitive thermochemolysis of structural linkages in very small (μg) quantities of large biopolymers through cleavage followed by monomer methylation and the identification of these less polar derivatives by GC/MS (14,15). Thermochemolysis cleaves the tocopheryl hydroquinone adduct to its methyl thioether, converts its hydroquinone groups to methyl ethers, and eliminates its aliphatic hydroxyl group. This relatively nonpolar cleavage product is readily separated and identified by GC/MS.

MATERIALS AND METHODS

Materials. (d)-α-T **IV** (99.6 %) was purchased from Mann Research (New York, NY), δ-T **VI** (91.2%) was purchased from Sigma (St. Louis, MO), and (d)-γ-T **II** (92.6%) was kindly supplied by Tama (Tokyo, Japan). Human hemoglobin from a sample of laked blood was kindly supplied by Dr. C.R. Hille of this institution. Dulbecco's PBS (DPBS), trypan blue, GSH, BSA, insulin, and calf thymus histone Type II-s (NaCl extract precipitated in water followed by acid extraction and precipitation with alcohol) were obtained from Sigma, and FBS from HyClone (Logan, UT).

*Synthesis of tocopheryl quinones and their glutathion-*S*-yl adducts.* α-TQ **III** and γ-TQ **I** were synthesized from parent tocopherols **II** and **IV** (Fig. 1) by oxidation with $FeCl_3$ as previously described (3). δ-TQ **V** was synthesized from its parent tocopherol **VI** by oxidation with $AgNO_3$ in ethanol/water (85:15, vol/vol) to prevent overoxidation, as previously described (4). Quinones were separated as single bands on a silica gel 60 column using petroleum ether/ether (1:1, vol/vol) as the mobile phase and gave single peaks on HPLC, characteristic peaks in NMR and IR spectra, and appropriate M.W. for parent ions in FABMS. Tocopherols and their quinones were stored at –20°C, and working solutions were dissolved in ethanol, stored at 4°C, and monitored routinely by TLC. Ethanolic solutions of δ-TQ polymerized on standing.

The glutathion-*S*-yl adduct **VII** of γ-TQ **I** (Fig. 2) was obtained by adding equimolar amounts of quinone dissolved in 95% ethanol to the monosodium salt of GSH dissolved in 60% ethanol. The precipitate was washed with acetone and purified by preparative HPLC on a Vydac C_4 column (10 mm × 25 cm) using 0.1% TCA in water (A) and 0.1% TCA in acetonitrile (B) in a 10–90% linear gradient (3). The product was identified by FABMS. Monoglutathion-*S*-yl hydroquinone adducts **VIII** of δ-TQ **V** (Fig. 2) were prepared by adding δ-TQ **V** to excess GSH in a 2:3 mol ratio, purified as described, and identified by FABMS (4). The two monoglutathion-*S*-yl positional isomers **VIII** were not separated. The diglutathion-*S*-yl hydroquinone adduct **IX** of δ-TQ **V** (Fig. 2) was prepared by the slow sequential addition of δ-TQ **V** to a mixture containing the monoglutathion-*S*-yl hydroquinone adduct **VIII** and GSH, purified as described, and identified by FABMS (4).

Cell culture. A human acute lymphoblastic leukemia cell line (CEM) was purchased from Centocor (Malvern, PA). Cells were grown in RPMI C1640 supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/mL), and strepto-

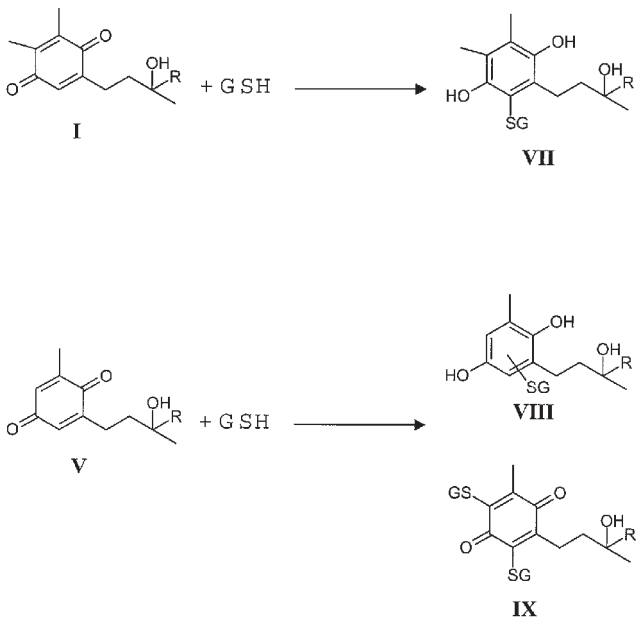

FIG. 2. Structural formulas for Michael adducts **VII**, **VIII**, and **IX** of tocopheryl quinone electrophiles, **I** and **V**, and the thiol nucleophile GSH. Positional isomers of compound **VIII** were not separated and identified.

mycin (100 μg/mL). Cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. Cell viability was determined before each experiment by trypan blue exclusion and was always ≥94%.

Cell treatment. In these experiments, 10 or 20 × 10^6 CEM cells (10^6 cells/mL in C1640 with 10% FBS) were incubated for 4 h at 37°C in 75-cm^2 flasks after treatment with or without γ-TQ **I** as follows: (i) 50 μM γ-TQ **I** added to CEM cells, (ii) 50 μM γ-TQ **I** added to culture medium without cells, and (iii) CEM cells cultured in medium alone (controls). After 4 h, cells were separated from culture medium by centrifugation at 450 × *g* for 7 min. The medium, which no longer contained cells, was transferred to an appropriate tube and kept at 4°C until assayed. The cell pellet was washed once in 10 mL DPBS, centrifuged at 450 × *g*, and the supernatant discarded. The cell pellet was resuspended in 1 mL DPBS, transferred to a microfuge tube, and centrifuged at 13,000 × *g*. The supernatant was discarded and the pellet frozen at –20°C until assayed. Medium incubated with γ-TQ **I** without cells was treated in the same manner.

TMAH thermochemolysis. The reaction was performed as previously described (14,15). The sample, ~50 μg, was added to a 3-mL Pyrex reaction ampoule along with 50 μL of TMAH (25% TMAH in methanol). The mixture was dried under nitrogen, placed on a vacuum line, flame-sealed, and heated in a convection oven at 250°C for 30 min. The ampoule was cooled to room temperature, scored, dipped in liquid nitrogen, cracked open, and extracted with ethyl acetate. Eicosane was added as an internal standard before extraction. The product was analyzed by capillary GC/MS on a Hewlett-Packard 6890 GC using a 30 m × 0.25 mm i.d. fused-silica capillary column with a 5% phenyl methylsilicon-bonded phase (J&W DB-5MS). The oven was programmed to go from 50 to 320°C at 20°C/min (Program A, Figs. 3 and 4) or from 50 to 220°C at 5°C/min and then from 220 to 320°C at 10°C/min (Program B, Figs. 5 and 6). The gas chromatograph was interfaced to a HP 5972A quadrupole scanning mass spectrometer. Data acquisition and analysis were performed using LECO Pegasus II, version 1.33, software.

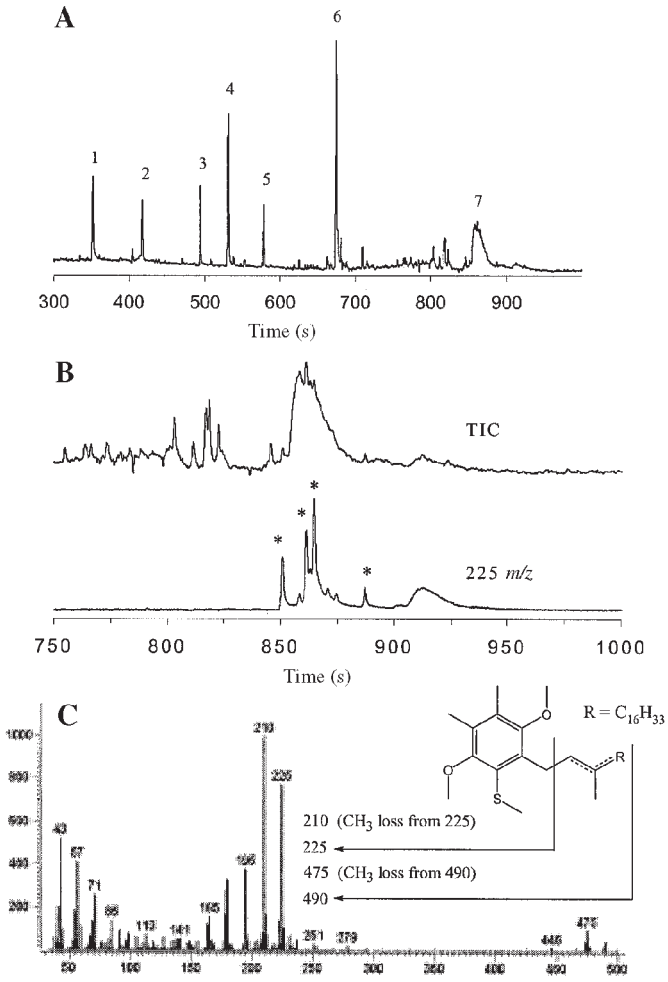

FIG. 3. Chromatograms (temperature Program A) and mass spectrum of adduct **VII**. (A) Full GC/MS chromatogram (total ion current, TIC) after tetramethylammonium hydroxide (TMAH) thermochemolysis, with numbered peaks as listed in Table 1. (B) Selected region of (A) (expanded TIC) and selective ion chromatogram (SIC) *m/z* 225 with characteristic peaks marked by asterisks. (C) MS fragmentation pattern of marked peaks showing the structure of the product with fragmentation pathways noted.

RESULTS

*Thermochemolysis of the quinone adduct of GSH and γ-TQ **I**.* The products obtained from thermochemolysis of the glutathion-*S*-yl adduct **VII** of γ-TQ are shown chromatographically in Figure 3A. Because of the limited amounts of isolated adduct that were used for separate MS confirmation, we prepared a new sample of adduct for TMAH thermochemolysis that constituted the entire mixture plus products. Thus, both adduct and γ-TQ are present in the mixture. Peak 1 (L-proline, 1-methyl-5-oxo, methyl ester) is identified as being from residues of methylated GSH. Other major peaks derive from TMAH thermochemolysis of γ-TQ: Peak 2 is 3-vinyl-5,6-dimethyl-1,4-dimethoxybenzene; peak 3 is prist-1-ene; peak 4 is 6,10,14-trimethyl-2-pentadecanone; peak 5 is eicosane; peak 6 is 4,8,12,16-tetramethylheptadecane-4-olide. Table 1 summarizes the major fragments for peaks 1–6 in Figure 3A and includes the molecular ions (MI), intensities of major fragments as percentages, and the base peak. The region labeled peak 7 (Fig. 3A) is expanded and fragments are identified in Figures 3B and 3C. Peak 7 is due primarily to γ-TQ that has been converted to the hydroquinone and subsequently methylated. Only the aromatic hydroxyls are methylated, and the side-chain hydroxyl remains unmethylated, accounting for the broadness of the peak in the chromatogram. That these peaks all derive from the TMAH thermochemolysis of γ-TQ was determined by analyzing γ-TQ alone. It is clear from the chromatogram that the TMAH reaction mixture contains products that are obtained from the nonadducted γ-TQ.

A cluster of peaks between retention times of 750 and 950 s (Fig. 3B) drew our attention because they appeared only in preparations of adduct **VII** treated with TMAH, and a number of the peaks had similar mass spectra (Fig. 3C). From the

TABLE 1
Mass Spectral Fragmentation and Assignments of GC/MS Peaks from Adduct VII After Tetramethylammonium Hydroxide (TMAH) Thermochemolysis[a,b]

Peak	Assignment	MI	Major MS fragments
1	L-Proline, 1-methyl-5-oxo, methyl ester	157	157(2), **98**(44), 42(14), 41(12)
2	3-Vinyl-5,6-dimethyl-1,4-dimethoxybenzene	192	192(7), **177**(9), 162(4), 49(5), 119(6), 91(9)
3	Prist-1-ene	266	126(2), 111(4), 97(2), 83(4), 69(9), **56**(9), 41(7)
4	6,10,14-Trimethyl-2-pentadecanone	268	124(2), 109(4), 95(4), 85(4), 71(6), 58(10), **43**(12)
5	Eicosane (internal standard)	282	113(2), 99(3), 85(9), 71(13), **57**(18), 43(13)
6	4,8,12,16-Tetramethylheptadecane-4-olide	324	143(3), **99**(13), 69(5), 55(6), 43(9)

[a]Peak numbers correspond to those listed in Figure 3A.
[b]MI is molecular ion (*m/z*); mass spectral fragmentation shown as *m/z* of major fragments with the percentage intensity in parentheses and base peak indicated by boldface type.

mass spectra, we can assign these peaks to the methyl thioether structure shown in Figure 3C, representing a product we can rationalize as being formed by the action of TMAH on the glutathion-*S*-yl adduct of γ-TQ. Analysis of the fragmentation pattern reveals a molecular ion at *m/z* 490, a peak at *m/z* 475 for the molecular ion minus methyl, intense fragmentation ions at *m/z* 225 and 210 (*m/z* 225 − 15), and a homologous series of peaks at low mass (*m/z* 43, 57, 71, 85, 99, 111) representing fragmentation of the phytyl side chain. Most notable is the peak at *m/z* 225, which represents the fragment shown in Figure 3C. Because of its characteristic nature, it can be used for selective ion monitoring. Figure 3B shows the selective ion chromatogram (SIC) trace for *m/z* 225 with four major peaks displayed at 852, 861, 864, and 887 s. These all have nearly identical mass spectra, consistent with the belief that they represent four of the possible major positional isomers of the structure drawn in Figure 3C. A broad peak at 910–925 s shows a molecular ion at 18 mass units higher than *m/z* 490, and we assign this peak as the structure shown in Figure 3C with its hydroxyl group retained on the side chain.

Thermochemolysis of quinone adducts of GSH and δ-TQ V. Products expected from the treatment of the glutathion-*S*-yl adducts of δ-TQ **V**, compounds **VIII** and **IX**, with TMAH are shown in Figure 4. Positional isomers for the GSH adduct **VIII** were not separated and identified. With each compound, the thioether was cleaved and methylated, the aromatic hydroxyl groups were methylated, and the aliphatic hydroxyl group was eliminated with the formation of a double bond. The mass spectra of the compounds expected from TMAH thermochemolysis of the monothioether and dithioether adducts shown in Figure 4B have many of the characteristic features of thioethers of the series. The fragmentation pattern for the TMAH products of **VIII** includes peaks at *m/z* 211 and 196 and a molecular ion at *m/z* 476. The fragmentation pattern expected for the TMAH products of **IX** includes peaks at *m/z* 257, 242, and a molecular ion at *m/z* 522 (Fig. 4B). The SIC plots for *m/z* 211 and 257 are shown in Figure 4A. Complete separation of the individual olefin isomers could not be achieved for either trace. MS fragmentation patterns for **VIII** and **IX** are consistent with patterns for **VII**.

Thermochemolysis of γ-TQ I adducts with specific proteins, hemoglobin, and histone. From 100 to 400 μL of a protein solution (10 to 20 mg protein/mL) was mixed with 100 μL of 11 mM γ-TQ **I** and allowed to stand for 15 h. The mixture was dried and ~50 μg taken for TMAH thermochemolysis. Untreated protein solutions subjected to TMAH thermochemolysis had no peaks in the signature region, 1410 to 1590 s, of the chromatogram (*m/z* 225 SIC, data not shown). When BSA and insulin were treated with γ-TQ **I**, TMAH thermochemolysis also showed no peaks in the signature region of the chromatogram (*m/z* 225 SIC, data not shown). When hemoglobin and histone were treated with γ-TQ **I**, TMAH thermochemolysis showed four peaks characteristic of an γ-TQ **I** adduct in the signature region of the chromatograms for each protein (Fig. 5). The plots shown in this figure are SIC traces for *m/z* 225. Mass spectra of the peaks match well with those shown in Figure 3C for the TMAH thermochemolysis products of the γ-TQ adducts. Note that the signature region representing the *m/z* 225 SIC shows elution peaks at a different retention time window, 1410–1590 s, from what is shown in Figure 3B. In an attempt to achieve better separation of peaks, a different temperature program rate (Program B) from that used previously (Program A) was used in these for plots in Figure 3B.

Thermochemolysis of γ-TQ I adducts with FBS, and with medium and cells separated from CEM tissue culture. Because FBS is a major component of tissue culture medium, 100 μL of FBS was treated with 20 μL of 11 mM γ-TQ **I** and allowed to stand for 15 h. The mixture was subjected to TMAH thermochemolysis, and the chromatogram showed the four characteristic peaks for a γ-TQ **I** adduct in the signature region (Fig. 6A). The SIC at *m/z* 225 confirms that the four peaks, each labeled with an asterisk, are the four isomer products of interest. Untreated FBS had no peaks in the signature region of the chromatogram (data not shown). CEM cells in growth medium were incubated for 4 h with 50 μM γ-TQ **I**, a treatment regimen in which there is an early indication of apoptosis (80%, 4,6-diamidino-2-phenylindole) in cells that are intact (no change in cell number) and in which only 10% of the cells show an indication of membrane damage (ethidium homodimer-1 positive) (5). Medium and intact cells were separated for TMAH thermochemolysis. Both medium (Fig. 6B) and washed cells (Fig. 6C) had the four characteristic peaks of TMAH thermochemolysis for the γ-TQ **I** adduct in the SIC signature region. Medium and cells separated from untreated cultures had no peaks in the signature region of the chromatogram (data not shown).

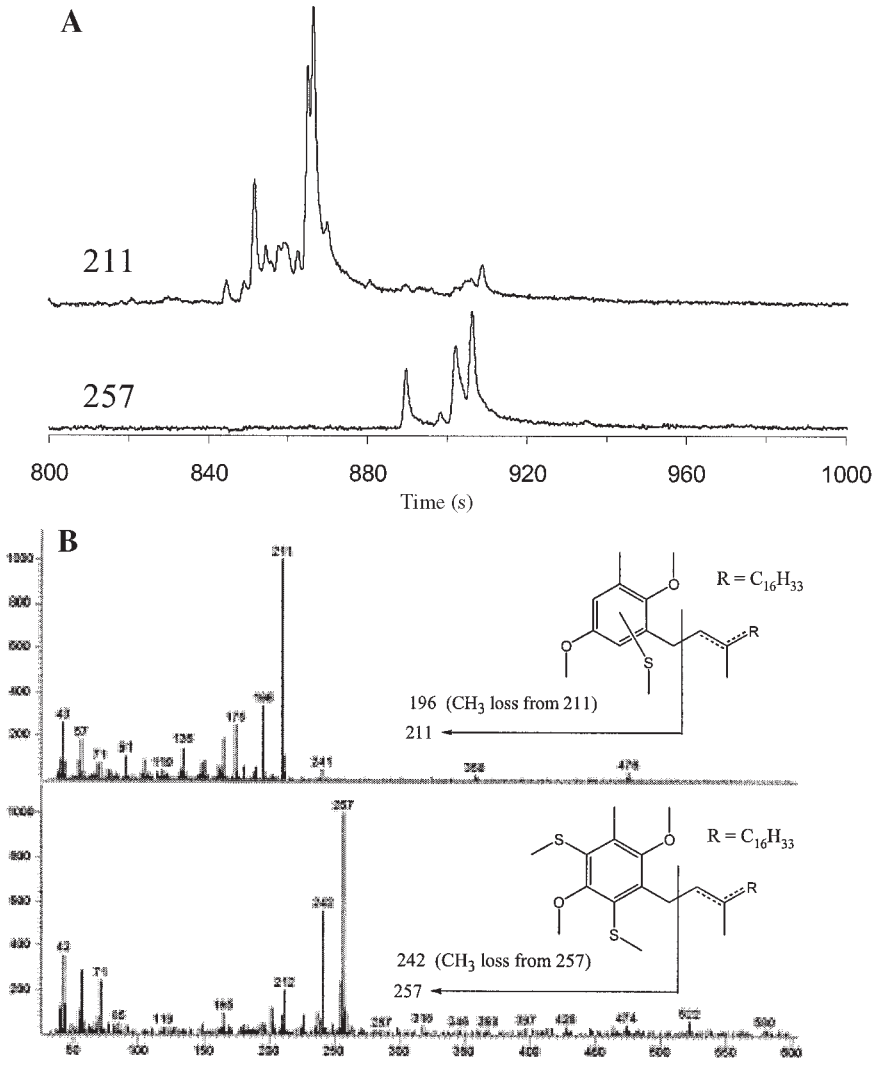

FIG. 4. Chromatograms (temperature Program A) and mass spectra of adducts **VIII** and **IX**. (A) Full GC/MS chromatograms (TIC) after TMAH thermochemolysis of **VIII** (selective ion mode *m/z* 211) and **IX** (selective ion mode m/z 257). (B) MS fragmentation patterns of **VIII** and **IX** showing the structures of the products with fragmentation pathways noted. For abbreviations see Figures 1 and 3.

DISCUSSION

When GSH was used as the nucleophile in model studies with the arylating electrophile γ-TQ **I**, GC/MS after TMAH thermochemolysis showed a characteristic four-peak pattern for the hydroquinone adduct in the signature region of the chromatogram (SIC *m/z* 225). The same four-peak pattern was found when proteins with accessible thiol groups, hemoglobin (16), and histones (17) were treated with γ-TQ **I**, and the same four-peak pattern was found when FBS, complete tissue culture medium that contains FBS, and CEM cells grown in complete tissue culture medium were treated with γ-TQ **I**. No adduct peaks were found anywhere in the SIC when BSA, whose single thiol group is in a restricted environment (18), and insulin (19) were treated with γ-TQ **I**, thus confirming the very low reactivity of the arylating electrophile toward amine group nucleophiles (20) that are present in proteins. Furthermore, untreated hemoglobin and histone, and untreated medium and CEM cells gave no evidence of peaks that give a *m/z* 225 SIC signal. Differences between BSA and donor thiol nucleophiles in FBS should be evaluated in biological studies involving arylating and alkylating electrophiles.

Previous studies from our laboratory are consistent with the hypothesis that cytotoxicity and apoptosis with arylating tocopheryl quinones are the result of Michael additions to nucleophilic thiol groups in cells (3–7). Cytotoxicity and apoptosis are enhanced when intracellular GSH is diminished with BSO and decreased when intracellular thiols are increased with *N*-acetylcysteine. Intracellular GSH levels are diminished when cells are treated with γ-TQ **I**. GSH adducts do not in themselves increase cytotoxicity or apoptosis. We now show that cells treated with γ-TQ **I** generate the signature SIC pattern of thiol Michael adducts after TMAH thermochemolysis. The identification of the signature pattern with charac-

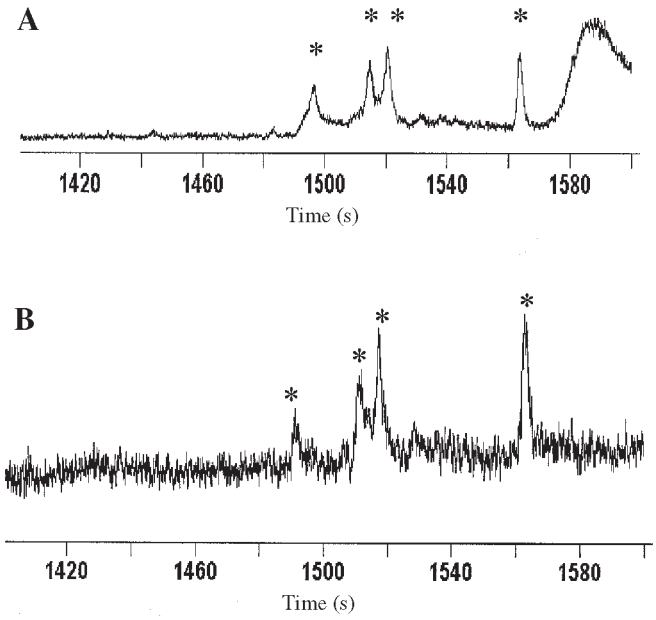

FIG. 5. Chromatograms (temperature Program B) of proteins treated with **I** and then subjected to TMAH thermochemolysis (selective ion mode *m/z* 225). (A) Hemoglobin; (B) histone. For abbreviations see Figures 1 and 3.

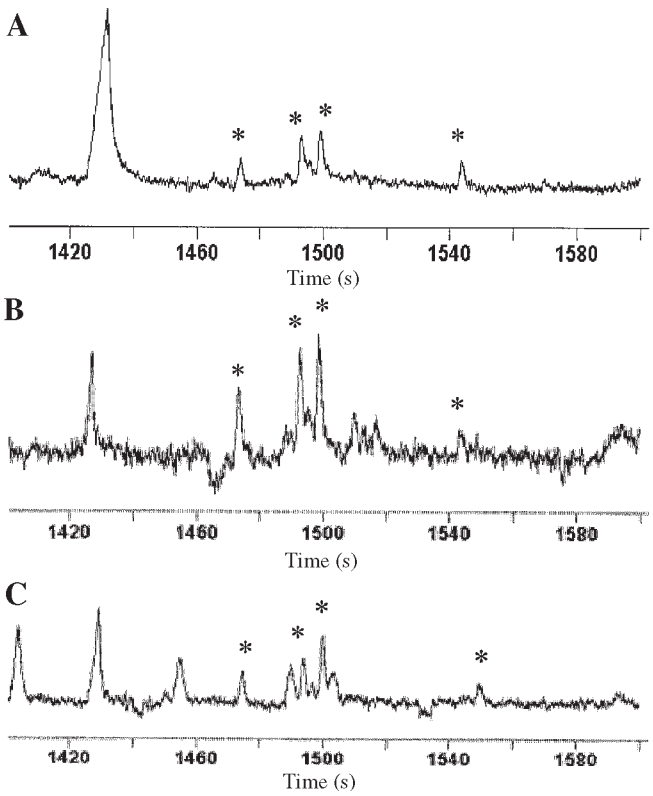

FIG. 6. Chromatograms (temperature Program B) of FBS and drug-sensitive lymphoblastic leukemia cells (CEM) tissue culture treated with **I** and then subjected to thermochemolysis (selective ion mode *m/z* 225). (A) FBS; (B) medium; (C) washed cells. For other abbreviation see Figure 1.

teristic MS fragments (Fig. 3C) in both medium and washed cells demonstrates that it is possible to determine both soluble adducts, which may include glutathion-*S*-yl adducts, and protein adducts, which may include membrane-bound protein adducts, when specific cell fractions are separated.

The intravenous administration of γ-T **II** leads to rapid accumulation in and subsequent depletion from tissues such as liver without a large increase in γ-TQ **I** (1). This distribution pattern may be explained by γ-T **II** depletion through the γ-2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (γ-CEHC) pathway, which occurs after oral administration (21–24), and γ-CEHC should be measured in studies of tocopherol metabolism. The appearance of γ-CEHC does not preclude the formation of γ-TQ **I** Michael adducts, which would not be detected by conventional analyses. γ-CEHC in urine is readily converted to its quinone by oxidation with $FeCl_3$, and yet neither its quinone nor quinone lactone has been found in urine (21) even though the quinone lactone of 2,5,7,8-tetramethyl-2-(β-carboxyethyl)-6-hydroxychroman (α-CEHC) has been found in urine (25). Because the quinone lactone of α-CEHC does not form a Michael adduct, the absence of a γ-CEHC quinone in urine does not preclude the possibility that the quinone is formed *in vivo* and converted to Michael adducts before urinary excretion; this possibility should be explored.

The phenolic antioxidant γ-T **II** is metabolized to γ-TQ **I**, which is both a cytotoxin and a mutagen depending on its concentration and detoxification in the cell (7). The role of γ-T **II** in prostate cancer was discussed in recent reviews (26,27), but a possible role for its quinone metabolite has not been considered. For example, γ-TQ **I** is detoxified by the formation of a glutathion-*S*-yl adduct through a reaction catalyzed by π-class glutathione *S*-transferase, and this enzyme is deficient in prostate cancers (28–30). It will be interesting to examine normal and neoplastic prostate tissue for soluble and protein-bound thiol adducts.

Cellular thiol adducts may have particular importance because γ-TQ **I** is a mutagen in a guanosine phosphoribosyltransferase gene assay but not a genotoxin (7), as would be expected from its low reactivity toward amine group nucleophiles such as those found in DNA. In the present study, we found that γ-TQ **I** forms a Michael adduct with an accessible thiol group in histones, which suggests to us that arylation may be a new mechanism, in addition to methylation, phosphorylation, and acetylation mechanisms, for histone modifications that affect chromatin dynamics (31,32) leading to mutagenesis and carcinogenesis.

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Divergent Pattern of Polyisoprenoid Alcohols in the Tissues of *Coluria geoides*: A New Electrospray Ionization MS Approach

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ABSTRACT: Polyisoprenoid alcohols of the plant *Coluria geoides* were isolated and analyzed by HPLC with UV detection to determine the nature of the polyprenol and dolichol mixture in the organs studied. In roots, a family of dolichols (Dol-15 to Dol-23, with Dol-16 dominating, where Dol-*n* is dolichol composed of *n* isoprene units) was accompanied by traces of polyprenols of similar chain lengths, whereas in hairy roots grown *in vitro*, identical patterns with a slightly broader chain-length range were found. Conversely, in leaves and seeds polyprenols were the dominant form, and their pattern was shifted toward longer chains (maximal content of Pren-19, where Pren-*n* is polyprenol composed of *n* isoprene units). Interestingly, the pattern of dolichols in seeds and leaves (in which Dol-17 dominated) was similar to that found in roots.

Structures of the dolichols and polyprenols isolated were confirmed by the application of a new HPLC/electrospray ionization-MS method, which also offers a much higher sensitivity in detection of these compounds compared to a UV detector. The highest sensitivity was obtained when the $[M + Na]^+$ ions of polyprenols and dolichols were recorded in the selected ion monitoring mode and a small amount of sodium acetate solution was added post-column to enhance the formation of these ions in an electrospray ion source.

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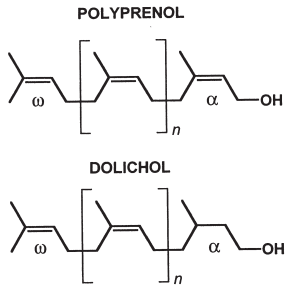
Isoprenoids are among the most numerous and diverse groups of natural products, covering approximately 30,000 compounds identified thus far (1). Among these are linear five-carbon-unit polymers named polyisoprenoid alcohols that occur in almost all living cells. Two main types of hydrocarbon backbones comprise this group: the α-unsaturated polyprenol and the α-saturated dolichol structures (Scheme 1, where *n* indicates the number of internal isoprene units and α and ω stand for terminal isoprene units). Both polyprenols and dolichols are found in cells in the form of free alcohols and esters with carboxylic acids (2). A fraction of polyisoprenoid phosphates also has been detected, and this form is sometimes predominant in dividing cells and *Saccharomyces cerevisiae* (3,4). The biological role of phosphorylated polyisoprenoid alcohols as cofactors in the biosynthesis of glycoproteins and glycosyl PI (GPI) anchor or bacterial peptidoglycans (5,6) is well characterized (dolichols for Eukaryota and polyprenols for Prokaryota). They are also postulated to serve as donors of isoprenoid groups during protein prenylation in rat liver (7,8) and spinach leaves (9). In contrast, the role of free polyisoprenoid alcohols and carboxylic esters is uncertain. On one hand, biophysical studies have shown that isoprenoids increase the permeability and fluidity of model membranes and also enhance membrane fusion (10). On the other hand, there is evidence that these compounds are involved in the transport of endoplasmic reticulum (ER) and vacuolar proteins. The yeast *rer2* mutant [*RER2* encodes *cis*-prenyltransferase, the enzyme responsible for biosynthesis of polyprenyl diphosphate (11)] exhibits disrupted protein transport, resulting in the mislocalization of various ER proteins and abnormalities in the structure of the entire central vacuolar system. The results of recent molecular studies have permitted the identification of the *cis*-prenyltransferase encoding genes in bacteria [*Escherichia coli* (12), *Micrococcus luteus* (13)], the yeast *S. cerevisiae* (11), and the plant *Arabidopsis thaliana* (14). An additional homologous gene, *SRT1,* also has been cloned from *S. cerevisiae,* and the enzymatic activity of SRT1p resulted in the production of dolichols longer than are typical for yeast (15,16). The biochemical and molecular data on *cis*-prenyltransferases have recently been summarized in two comprehensive reviews (17,18).

The end product of *cis*-prenyltransferase, polyprenyl diphosphate, requires saturation of the α-isoprene residue on



SCHEME 1

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Abbreviations: Dol-*n*, dolichol composed of *n* isoprene units; ER, endoplasmic reticulum; HPLC/ESI-MS, high-performance liquid chromatography/electrospray ionization-mass spectrometry; Pren-*n*, polyprenol composed of *n* isoprene units; SIM, selected ion monitoring; TIC, total ion current.

its biosynthetic route toward dolichol. The mechanism of this last chain of events in dolichol biosynthesis has not yet been described in detail. Some experiments performed using rat liver suggest that dephosphorylation of polyprenyl diphosphate is followed by saturation (19), although another study postulated a concomitant synthesis and saturation proceeding *via* condensation with isopentenol (20).

For many years it was generally accepted that α-unsaturated polyprenols are present in bacteria, plant photosynthetic tissues, and wood, whereas dolichols occur in mammalian tissues and yeast cells (2). Only traces of polyprenols accompanying dolichols were detected in pig liver (21). A more detailed examination, together with progress in the development of a new chromatographic 2-D TLC technique (22), showed that this simplified polyprenol vs. dolichol dichotomy is, in fact, far more complicated. Dolichols were found as the predominant form in shoots (23) and seeds (24) of dicotyledonous plants. Small amounts of dolichols were also observed in young roots of *Hevea brasiliensis*. (25). As expected, polyprenols were the dominant form in old leaves, but the ratio of polyprenols to dolichols was species specific (from 2:1 in *H. brasiliensis* to 8000:1 in *Ginkgo biloba*). Detectable amounts of dolichols also occurred in the leaves of angiosperm plants (Ref. 26; Bajda, A., personal communication), and there were equal amounts of polyprenols and dolichols in the case of *Capparis coriaceae*.

Mechanisms of the parallel biosynthesis of polyprenols and dolichols within the same cell have not been studied in detail. The dolichol biosynthesis pathway mentioned above might suggest polyprenols as the precursors of dolichols. However, the existence of two independent *cis*-prenyltransferases responsible for the biosynthesis of polyprenols and dolichols in spinach leaves has been postulated (27).

Both polyprenols and dolichols usually occur as a "family" of prenologs. Dolichol families consist of six to eight members, irrespective of the organism with Dol-16 in yeast cells, Dol-18 in rat, and Dol-19 in humans (where Dol-*n* is dolichol composed of *n* isoprene units) dominating the respective mixtures (28). In some cases, a second family of longer dolichols (with Dol-21 being the most abundant) has been observed in yeast, which could be due to the physiological stimulation of expression of the *SRT1* gene (29). In contrast to dolichols, the diversity of polyprenols is much broader, ranging from Prenol-6 to Prenol-130 (where Prenol-*n* is polyprenol, composed of *n* isoprene units; see the review in Ref. 30 and Chojnacki, T., unpublished report). The polyprenol spectrum is considered to be a species-specific feature. Multifamily mixtures of polyprenols have been isolated from old leaves and needles of several gymnosperm and angiosperm plants (31,32). Differences in the polyprenol pattern of flower buds (in which Pren-11 dominates) and leaves (in which Pren-17 dominates) of Magnoliaceae were also observed (Ranjan, R., and Chojnacki, T., personal communication).

The content of all isoprenoid alcohols increases during the life of the organism, sometimes achieving a 100-fold increase over the neonatal level in the mammalian brain, the highest concentration being found in the endocrine tissues (10). Extensive studies of the polyprenol content in the photosynthetic tissues of plants have shown not only a 10- to 20-fold increase in senescing leaves (33) but also seasonal variations in the case of evergreen plants (34).

Experiments on the occurrence of secondary metabolites in cultured plant tissues confirmed that they could be used as models for biochemical studies. Among others, hairy roots were found to be an abundant source of many classes of secondary metabolites (35).

Coluria geoides (Rosaceae) is a perennial plant native to South Siberia and Mongolia (36). Fresh roots of *C. geoides* are considered a cinnamon substitute; they contain eugenol, which is used in perfumery and as a dental analgesic and which comprises 96% of the oil obtained from this tissue. Thus, an *in vitro* root culture of *C. geoides* has been established (37), and a detailed characterization of the lipid components is required. Tissues of plants belonging to Rosaceae are also known to accumulate high amounts of polyisoprenoid alcohols (30). Indeed, initial experiments performed using cultures of hairy roots from *C. geoides* indicated the presence of polyisoprenoid alcohols in this tissue, although data concerning the structure of these compounds were not presented (38).

The aim of this study was to complete a detailed structural characterization of the isoprenoid alcohols formed in a *C. geoides* root culture. This was achieved using a new HPLC/electrospray ionization (ESI)-MS method.

MATERIALS AND METHODS

Plant material. Seeds, leaves, and roots of *C. geoides* Ledeb. (Rosaceae) were collected from plants grown in the open air in the garden of the Research Institute of Medicinal Plants (Poznan, Poland).

In vitro *cultures*. To obtain hairy roots of *C. geoides,* shoots were inoculated with a fresh culture of *Agrobacterium rhizogenes* strain LBA 9402 (37). Roots were incubated in liquid medium (100 mL of modified B-5, where 0.75% glucose was the sole carbon source) (39) for 21 d in darkness at 22°C on a rotary shaker at 105 rpm.

Isolation of polyisoprenoids. Plant material (seeds, leaves, roots, and hairy roots) of *C. geoides* were dried at room temperature and then extracted with acetone/hexane (1:1, vol/vol) in a Soxhlet apparatus for 48 h. Alkaline hydrolysis of the lipid fraction was performed as described previously (38). Lipids were collected, evaporated, dissolved in hexane, and purified on a silica gel 60 column eluted with hexane containing an increasing concentration of diethyl ether (0–50%). Detectable amounts of polyisoprenoid (according to TLC) were pooled and analyzed by HPLC (40), with modifications.

HPLC-UV analysis of polyisoprenoids. A mixture of purified polyisoprenoids was analyzed on a 4.6 × 60 mm Hypersil ODS (3 μm) reversed-phase column (Knauer, Germany) using a Waters dual-pump apparatus, a Waters gradient programmer, and a UV detector set at 210 nm. For elution, a combination of convex (Waters no. 5, from 0 to 75% B for the initial 20 min; where solvent A was methanol/water, 9:1, vol/vol, and solvent

B was methanol/propan-2-ol/hexane, 2:1:1, by vol) and linear (from 75 to 100% B during the following 10 min) gradients was used; in the last 5 min, re-equilibration back to 0% B was performed. The solvent flow rate was 1.5 mL/min. Pren-16 from *Sorbus suecicca* and Dol-16 from pig liver, together with corresponding natural mixtures of polyprenols and dolichols, were used as qualitative standards. A Dol-23 solution was used as the external standard for quantification of polyisoprenoid alcohols. All standards were from the Collection of Polyprenols (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw).

HPLC-MS analysis of polyisoprenoids. HPLC-MS experiments were carried out using an HP 1100 series HPLC system (Agilent Technologies) coupled to an API 365 triple quadrupole mass spectrometer (Applied Biosystems).

All experiments were performed using a 2 × 200 mm HPLC column filled with Nucleosil 100-C18 (5 μm). Gradient elution was used in the separation experiments. Solvent A was a methanol/propan-2-ol/water mixture (12:8:1, by vol) and solvent B was a hexane/propan-2-ol mixture (7:3, vol/vol). The linear gradient started from 100% of solvent A and changed to 70% of solvent B in 30 or 40 min depending on the experiment. The flow rate was 0.2 mL/min. The UV detector was set at 210 nm.

Isocratic conditions were used to create a calibration curve. The solvent consisted of a mixture of solvent A/solvent B (6:4, vol/vol). Sodium acetate dissolved in solvent B was introduced postcolumn by a syringe pump (flow rate, 5 μL/min) through a T-union into the LC flow before entering the mass spectrometer. The final concentration of sodium acetate in the sample was 10 μg/mL.

Mass spectra were acquired using a standard TurboIon Spray ion source. Nitrogen was used as the nebulizing and curtain gas. ESI was carried out in a positive ion mode. Spray tip voltage was kept at 4500 V. The declustering potential was set to 85 V and the focusing potential to 380 V. These parameters were optimized for the highest intensity of the peak of the $[M + Na]^+$ ion of Dol-16. The data acquired were processed using an Analyst v. 1.1 software package (Applied Biosystems, Foster City, CA). Standard smoothing procedures were applied to the data in all the chromatograms presented.

RESULTS AND DISCUSSION

Chromatographic identification of polyisoprenoid alcohols in C. geoides *tissues*. It was previously shown (38) that hairy roots of *C. geoides* contain a family of polyisoprenoid alcohols. More detailed studies including careful chromatographical separation of these products revealed that the roots of *C. geoides* accumulated a mixture of both saturated and unsaturated alcohols, with dolichols (α-saturated) as the predominant compounds (Scheme 1). The chain lengths of dolichols covered the range from Dol-13 to Dol-29, with Dol-16 being the dominant form. The dolichol family was accompanied by a family of polyprenols with the identical chain-length spectrum (Fig. 1A; Table 1).

The occurrence of dolichols and polyprenols in hairy roots of *C. geoides* cultured *in vitro* raised the question of the content of these compounds in the roots and other organs of plants grown in the soil. As might be expected, the regular roots contained similar mixtures of dolichols and polyprenols, although both families were more narrow (Fig. 1B). Dol-15 to Dol-22 were easily detectable, together with a family of polyprenols with corresponding chain lengths (Table 1).

Examination of the leaves and seeds showed that polyisoprenoid alcohols were also present in these tissues, although notable differences were observed: polyprenols were the predominant alcohols, and the spectrum was shifted toward longer prenologs, i.e., from Pren-16 to Pren-29, with Pren-19 dominating. Traces of dolichols were also observed, yet the pattern was slightly different. Dol-17 was the predominant component of the dolichol family in leaves and seeds (Figs. 1C and 1D; Table 1). This result concerning the proportion of prenol/dolichol in seeds questions the conclusion of Ravi *et al.* (24) that dicotyledonous plants contain exclusively dolichols in the seeds. Total polyisoprenoid content was highest in leaves, reaching approximately 57 μg/g of dry weight (Table 2). The level in other organs was lower, from approximately 4.5 to 16.2 μg/g of dry weight, which is comparable to the values reported previously for plant tissues (41). In roots the total amount of dolichols exceeded that of prenols by 4.5- to 5.5-fold, which is in contrast to seeds and leaves, where the prenol-to-dolichol ratios were 3.4:1 and 3.8:1, respectively (Table 2). One should keep in mind that these numbers were obtained by extrapolation from the HPLC-UV integration data. A more accurate estimation requires a more precise separation of these alcohols than can be achieved with the reversed-phase chromatographical system.

Significance of the different polyisoprenoid patterns in plant organs. The similarity in the dolichol pattern observed in all *C. geoides* tissues could indicate that the biosynthesis of dolichols is more strictly regulated than that of polyprenols in plants. As mentioned above, these data are in agreement with the literature describing the relatively narrow dolichol families in the animal kingdom, irrespective of the organism (28). To discover the full biological implications of our results, further detailed analysis is required. On first inspection, these data could suggest the independent regulation of dolichol and polyprenol biosynthesis in plants. The shift of polyprenol chain length toward longer molecules in leaves and seeds excludes the possibility that they are the direct precursors of dolichols, at least in these tissues. Instead, in leaves and seeds our findings support the work of the Sagami group (27), which demonstrated the existence of two independent *cis*-prenyltransferases responsible for the biosynthesis of polyprenols and dolichols in plants. In this previous study, polyprenols (Pren-11 and Pren-12) were recovered from chloroplasts, whereas dolichols (Dol-14 to Dol-16) were found in microsomes of spinach leaves. Parallel experiments performed *in vitro* confirmed the activity of the appropriate *cis*-prenyltransferases. Whether roots also have two distinct pathways is not yet known. Experiments on the subcellular localization of isoprenoid alcohols in roots are in progress.

Interestingly, in this tissue the pattern of polyprenols and dolichols is very similar, and the possibility cannot be ruled out that in roots accumulated polyprenols are intermediates in the dolichol biosynthetic pathway.

The models just discussed should be also considered in light of the possible involvement of two alternative pathways in the early steps of isoprenoid biosynthesis in higher plant cells. The classical mevalonate pathway operates in the cytoplasm and possibly mitochondria to provide sterols, sesquiterpenes, and ubiquinones, whereas plastidial isoprenoids originate from the mevalonate-independent deoxyxylulose/methyl-erythritol phosphate pathway (42–44). The precise biosynthetic route leading to polyprenols and dolichols awaits elucidation, although extensive labeling from mevalonate suggests that the mevalonate pathway is active in roots (Skorupińska-Tudek, K., Olszowska, O., Chojnacki, T., and Swiezewska, E., unpublished data).

ESI-MS of polyprenols and dolichols. The results described above were based on the chromatographic identification of polyprenols and dolichols and required further confirmation. Several approaches involving MS techniques have been applied successfully, and a comprehensive review summarizing these studies was published recently (see Ref. 41 and references therein). Electron ionization mass spectra of dolichols up to 19 isoprene units published in the 1970s and more recently, after some modification of the method (45), demonstrated molecular peaks and substantial fragmentation, but the sensitivity of this method was not very high. More recently developed soft ionization techniques such as FABMS and field desorption (FD)-MS allowing estimation of polyisoprenoid alcohols require prederivatization to acetates (46) or phosphates (47) to increase the sensitivity. High hydrophobicity and nonvolatility of the polyisoprenoid alcohols caused technical difficulties that led us to test a new MS technique based on the ESI method (48). ESI is generally considered to be unsuitable for nonpolar molecules, but our experience with other classes of compounds indicates that it is possible to use ESI to analyze polyisoprenoid

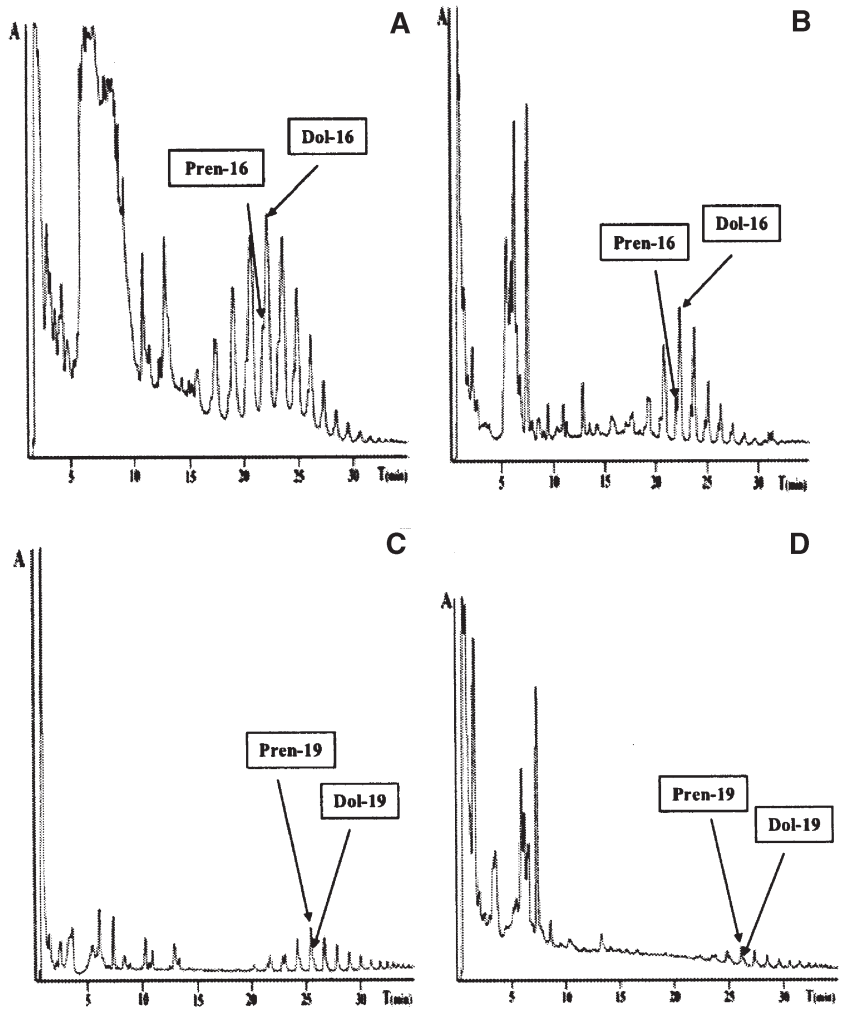

FIG. 1. HPLC-UV spectra of polyisoprenoid alcohols isolated from (A) hairy roots, (B) regular roots, (C) leaves, and (D) seeds of *Coluria geoides*. Lipids were analyzed as described in the Materials and Methods section. Arrows indicate dominating prenols and dolichols. Dol-*n*, dolichol composed of *n* isoprene units; Pren-*n*, polyprenol composed of *n* isoprene units.

TABLE 1
Pattern of Polyisoprenoid Alcohols in Different Organs of *Coluria geoides* Obtained by HPLC-UV analysis[a]

Tissue	Polyprenols (number of isoprene units) Dolichols (number of isoprene units)
Seeds	16 17 18 **19** 20 21 22 23 24 25 26 27 28 29
	16 **17** 18 19 20 21 22
Leaves	15 16 17 18 **19** 20 21 22 23 24 25 26 27 28 29
	15 16 **17** 18
Roots	13 14 15 **16** 17 18 19 20 21 22
	15 **16** 17 18 19 20 21 22 23
Hairy roots	13 14 15 **16** 17 18 19 20 21 22 23 24 25 26 27 28 29
	13 14 15 **16** 17 18 19 20 21 22 23 24 25 26 27 28 29

[a]Numbers in bold indicate the dominating prenologs.

alcohols. To our knowledge, the only similar ESI-MS application described thus far required chemical derivatization of isoprenoid alcohols (45).

Analysis of the spectra of the standards Dol-16 and Pren-16, injected directly as a solution in a methanol/propan-2-ol/water mixture (12:8:1, by vol) with a trace amount of sodium acetate added, showed intense peaks at *m/z* 1132.2 (Fig. 2A) and 1130.3 (Fig. 2B), corresponding to the expected monoisotopic masses of the pseudomolecular ions $[M + Na]^+$ of Dol-16 (1132.0) and Pren-16 (1130.0), respectively. Small signals originating from traces of pseudomolecular ions $[M + K]^+$ (*m/z* 1148.2 and 1146.2, respectively) and $[M + Na + MeOH]^+$ ions (*m/z* 1164.3 and 1162.3, respectively) were also observed (Figs. 2A and 2B). No peaks corresponding to the protonated molecules were observed. By using a series of Dol-16 solutions of known concentration, a calibration curve for this compound was drawn showing a linear detector response ($R^2 = 0.9993$) in the range from 40 pg to 10 ng of the injected standard (data not shown). These promising results encouraged us to consider using the ESI-MS technique for studies of the polyisoprenoid alcohols in biological samples.

Estimation of the structure of putative dolichols and polyprenols following several chromatographic purification steps was possible only after application of the HPLC-MS system, since the signals of interest were not strong enough to be analyzed after direct injection. The first series of experiments was performed using a full-scan technique (mass range 600–2100 Da). The sensitivity of this method was comparable to that obtained by UV detection (Fig. 3), and quite sophisticated processing of the results was necessary. The total ion current (TIC) chromatogram (Fig. 3A) failed to show any peaks in the expected region, but the extracted ion current chromatogram created using masses of the $[M + Na]^+$ ions of polyprenols and dolichols was a considerable improvement (Fig. 3B). The disappointing TIC chromatogram may be explained by a deficiency of sodium ions in the solution entering the ESI ion source. This would also explain the observed shift in peak intensities toward lower chain lengths compared to the UV chromatogram (Fig. 3C). Most probably, compounds entering the ESI ion source first deplete the available sodium ions so that the intensity of the latter peaks is lowered. This effect can be easily overcome by a postcolumn addition of sodium acetate solution (see below). It is also noteworthy that, in contrast to UV detection, the full scan MS method gives unequivocal identification of the polyisoprenoid species corresponding to each peak in the chromatogram and, additionally, makes the identification of other compounds leaving the LC column during the experiment possible. The combined mass spectrum for the time range corresponding to elution of dolichols is presented in Figure 3D.

Much better results were obtained using the selected ion monitoring (SIM) technique with postcolumn addition of a small amount of sodium acetate solution. Two series of $[M + Na]^+$ ions, one corresponding to the dolichol and the second to the polyprenol series (12 to 30 isoprene units), were recorded. The results obtained for all samples analyzed are presented in Figure 4. The plots show overlaid SIM chromatograms obtained for dolichols (normal line) and polyprenols (bold line). Such results can be used to estimate the relative concentrations of the dolichol and polyprenol alcohols in a sample. It should be noted that simple comparison of the peak areas is not appropriate here, because the third isotope ion of a polyprenol has the same mass as the first isotope ion of the related dolichol, so the peak intensities for dolichols are increased. To obtain correct quantitative results, the necessary corrections attributable to the polyprenol isotope ion abundances have to be applied. Generally, the results obtained by using the HPLC-MS technique are in agreement with the HPLC-UV results presented above (Table 2). It should be noted, however, that in the experiments presented in Figure 4, the intensities of the highest peaks are reduced because of the saturation of the MS detector. This was done deliberately to enhance the intensity of the peaks corresponding to

TABLE 2
Content of Polyisoprenoid Alcohols in Different Organs of *C. geoides*[a]

Tissue	Total isoprenoid alcohols (μg/g of dry weight)	Ratio of Pren/Dol
Seeds	16.17	3.4:1
Leaves	56.60	3.8:1
Roots	5.71	0.2:1
Hairy roots	4.43	0.2:1

[a]Lipids were isolated and analyzed as described in the Materials and Methods section. Pren, polyprenol; Dol, dolichol; see Table 1 for other abbreviation.

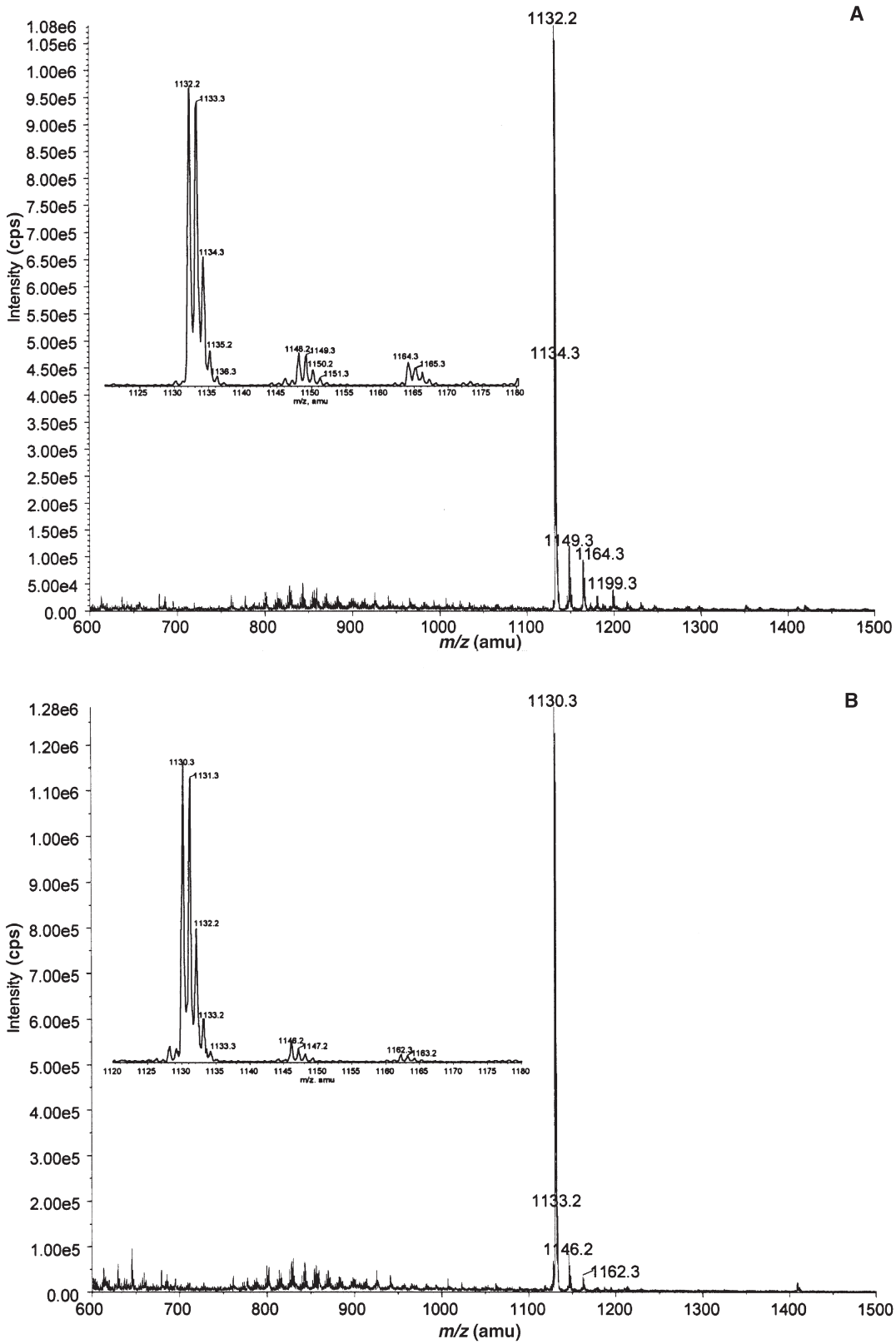

FIG. 2. Electrospray ionization (ESI) mass spectra of (A) Dol-16 and (B) Pren-16 injected as solutions in a methanol/propan-2-ol/water mixture (12:8:1, by vol). In both spectra, $[M + Na]^+$ pseudomolecular ions were observed (*m/z* 1132.2 and 1130.3, respectively), together with small amounts of $[M + K]^+$ ions (*m/z* 1148.2 and 1146.2, respectively) and $[M + Na + MeOH]^+$ ions (*m/z* 1164.3 and 1162.3, respectively). For other abbreviations see Figure 1.

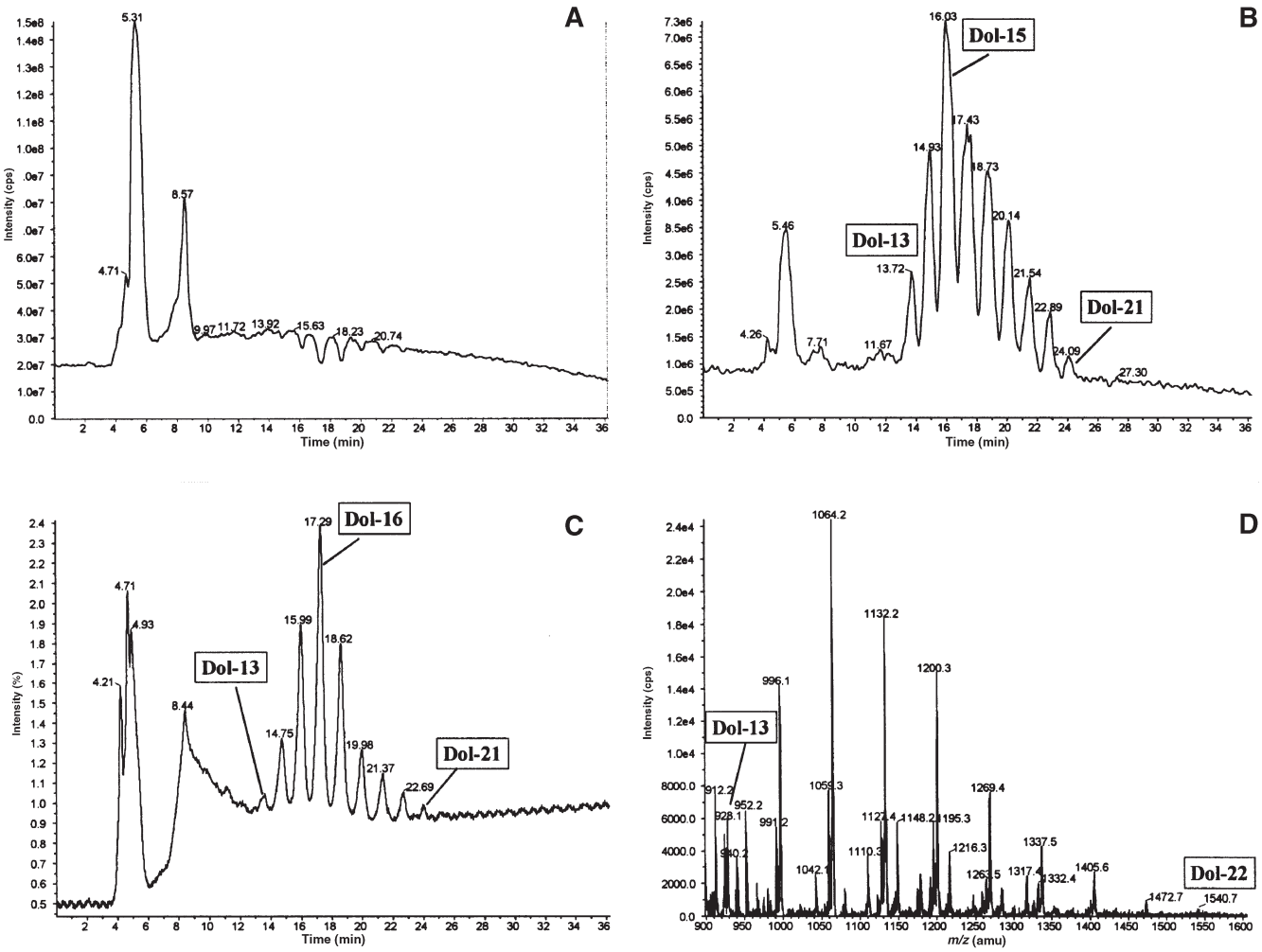

FIG. 3. HPLC-MS of the polyisoprenoid alcohols extracted from hairy roots: (A) total ion current chromatogram; (B) extracted ion current chromatogram; (C) UV (210 nm) chromatogram; (D) combined mass spectrum for the time range from 12 to 28 min. For abbreviations see Figure 1.

polyisoprenoid alcohols present at lower concentrations. After a significant reduction in the amounts of injected samples, the anticipated peak intensities were obtained and were comparable to those measured by UV detection.

As expected, the results obtained by HPLC-MS confirmed the peak assignments, based on co-elution with standards, observed using the HPLC-UV technique. Also, the patterns of polyisoprenoid-derived peaks from the UV and MS detectors were in very good agreement, again confirming the validity of the applied method. However, the HPLC/ESI-MS technique has many advantages over the HPLC-UV method. As mentioned, this method makes possible the unequivocal identification of polyisoprenoid alcohols in a mixture without the necessity of using standards. It also permits the dolichol/polyprenol ratio to be established, even without full chromatographic separation of these species. Additionally, HPLC/ESI-MS using SIM scanning gives significantly enhanced sensitivity and selectivity of detection of polyisoprenoid alcohols. A detailed description of these methods is planned for presentation in a separate publication (Biénkowski, T., Skorupińska-Tudek, K., Hertel, J., Chojnacki, T., Swiezewska, E., and Danikiewicz, W., unpublished data).

The results reported here show that in some organisms the balance between polyprenols and dolichols, compounds of similar structure, varies among tissues. New MS-based methods for their structural analysis provide the opportunity to follow this phenomenon in detail. What remain to be explained are the reasons for the particular saturation state of the α-isoprene unit of isoprenoid alcohols in certain tissues. One hypothesis is based on the greater chemical stability of dolichyl, compared to polyprenyl, derivatives (49) being critical in some physiological conditions. Another possible explanation comes from the postulated differences in the effects of the two groups of isoprenoid alcohols on the fluidity of membranes. The hairy root culture described here might be a useful model in future studies aimed at answering these questions.

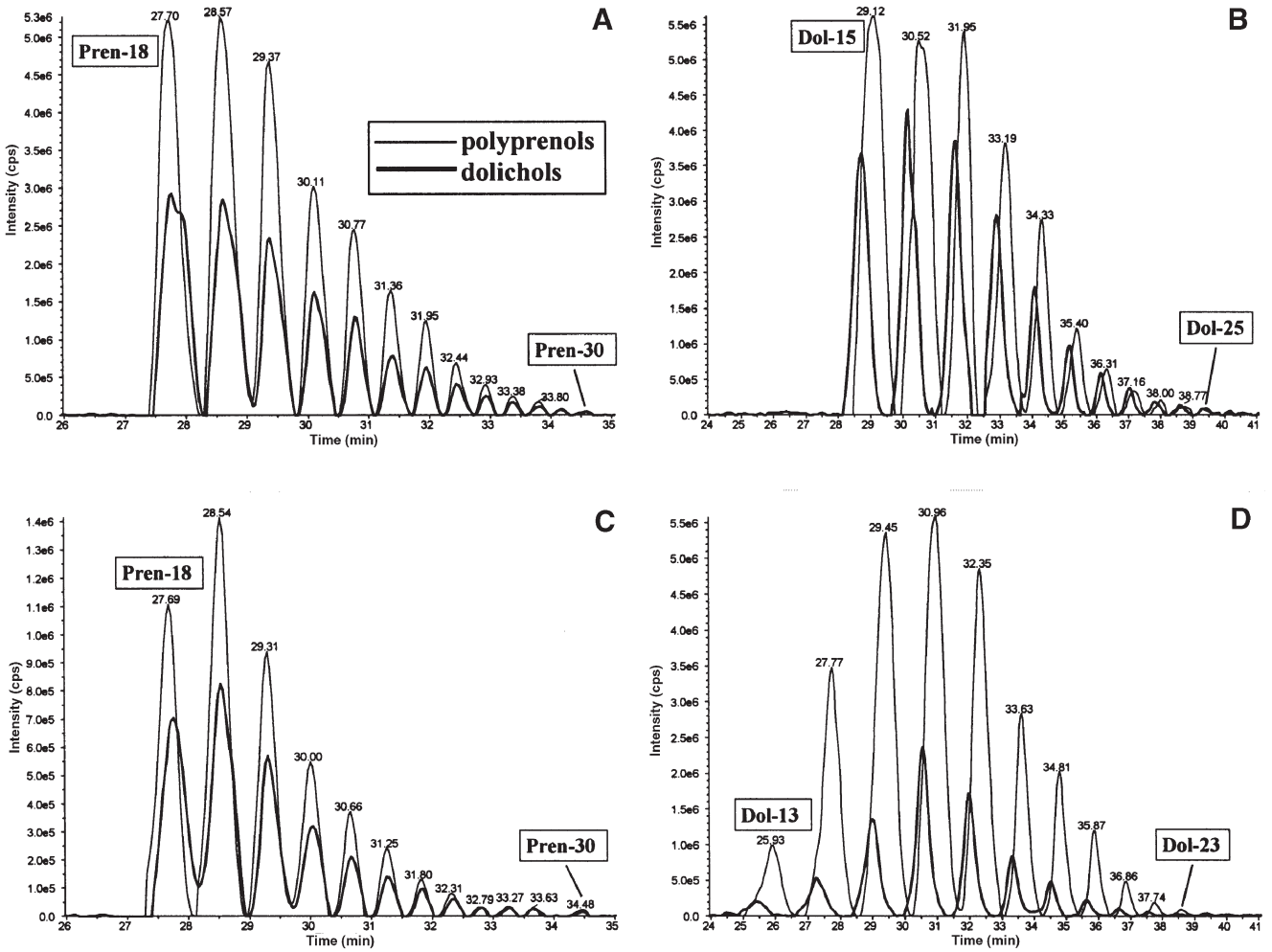

FIG. 4. HPLC-MS chromatograms of the extracts from different organs of *C. geoides*: (A) leaves, (B) roots, (C) seeds, (D) hairy roots. The plots are overlaid mass chromatograms obtained by selected ion monitoring for dolichols (normal line) and polyprenols (bold line). Baseline subtraction was applied. Retention times in chromatograms A and C are shorter compared to chromatograms B and D because of the different gradients used (see the Materials and Methods section). The highest peaks in all chromatograms are truncated because of saturation of the MS detector; see text for full description. For abbreviations see Figure 1.

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Localization of Clavulones, Prostanoids with Antitumor Activity, Within the Okinawan Soft Coral *Clavularia viridis* (Alcyonacea, Clavulariidae): Preparation of a High-Purity *Symbiodinium* Fraction Using a Protease and a Detergent

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ABSTRACT: To investigate the localization of clavulones (CV), prostanoids with antitumor activity, in the Okinawan soft coral *Clavularia viridis,* we developed a method for the isolation of *Symbiodinium* cells from the coral, i.e., treatment of a coral homogenate with a protease, pronase E, and a detergent, Nonidet P-40. The conditions for the treatment were optimized by monitoring the morphology microscopically and the amount of chlorophyll in the *Symbiodinium* fraction (SymF) optically. To evaluate the purity of SymF and a *Symbiodinium*-free coral fraction (CorF), we analyzed them for proteins and lipids using cultivated *Symbiodinium* as a reference. TLC of lipids revealed that SymF contained a greater amount of glycolipids, whereas CorF comprised mostly phospholipids. SDS-PAGE of proteins in SymF and CorF revealed their distinct profiles. Thus, we could obtain each fraction with high purity; we reached the conclusion that CV and arachidonic acid, their possible precursor, are localized exclusively in the insoluble fraction of host coral cells.

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Many marine organisms contain a wide variety of secondary metabolites with potent biotoxic and cytotoxic properties (1–3). Such compounds may act in a variety of ways, probably as a defensive mechanism against predators and pathogens, and as an aid to the organism to compete with neighboring benthic organisms. Marine sessile lower invertebrates, such as corals and sponges, are one of the richest sources of such metabolites (Ref. 4 and reviews therein).

The prostanoids, which are derivatives of prostanoic acid, a C_{20} FA containing a cyclopentane ring, are one of the important groups of bioactive compounds; prostaglandins, mammalian hormones, are also a kind of prostanoid. Various kinds of prostanoids, including prostaglandins (5), clavulones (CV) (6,7), and punaglandins (8), have been detected in some species of soft corals (1). Surprisingly, the accumulation of these prostanoids in corals is comparable with that of mammalian hormones. With regard to why corals include large amounts of compounds that have structures the same as or similar to those of mammalian hormones, allelopathic and ichthyodeterrent roles have been suggested for prostaglandin A_2 of *Plexaura homomalla* (9–11). However, the physiological roles and necessity of prostanoids other than prostaglandin A_2 are unknown. Indeed, neither their distribution in corals nor their seasonal changes are understood.

In *Clavularia viridis*, which inhabits coral reefs around Ishigaki Island, Okinawa, Japan, prostanoids including CV I, II, III, and IV (Scheme 1) (6,7) and halovulones, which contain halogen atoms (12,13) such as chlorovulones (14), have been detected. CV are *C. viridis*-specific prostanoids that have not been detected in other species of *Clavularia*. CV show antitumor and antileukemic activity (15–17), whereas such activity has not been detected for prostanoids from other corals except punaglandins.

As is usual in other corals, dinoflagellates [species of *Symbiodinium* (zooxanthellae)] live symbiotically in *C. viridis*. The symbiotic relationship between host corals and symbiotic algae is an important subject. Fractionation methods of host and symbiont cells have been improved (18) to examine the localization and flux of metabolites within corals. However, separating algal cells unbroken from animal membranes is not easy, since algae are resident within animal host vacuoles that are surrounded by a membrane (19). In this study, we developed a fractionation method for the soft coral involving a protease and a detergent to understand the localization of CV, which are highly accumulated in the soft coral rather than in the symbionts.

EXPERIMENTAL PROCEDURES

Materials. The soft coral *C. viridis* (Alcyonaria, Alcyonacea, Clavulariidae) specimens were collected from a coral reef of Ishigaki Island, Okinawa, Japan (24°19′N, 123°57′E), at a depth of 1–2 m. Some of the samples were frozen on dry ice immediately after collection, transferred to the laboratory, and stored at −30°C until used for experiments. Some of the samples were put into stainless steel containers with seawater immediately after collection, transferred to the laboratory by air, and kept in a tank until experiments.

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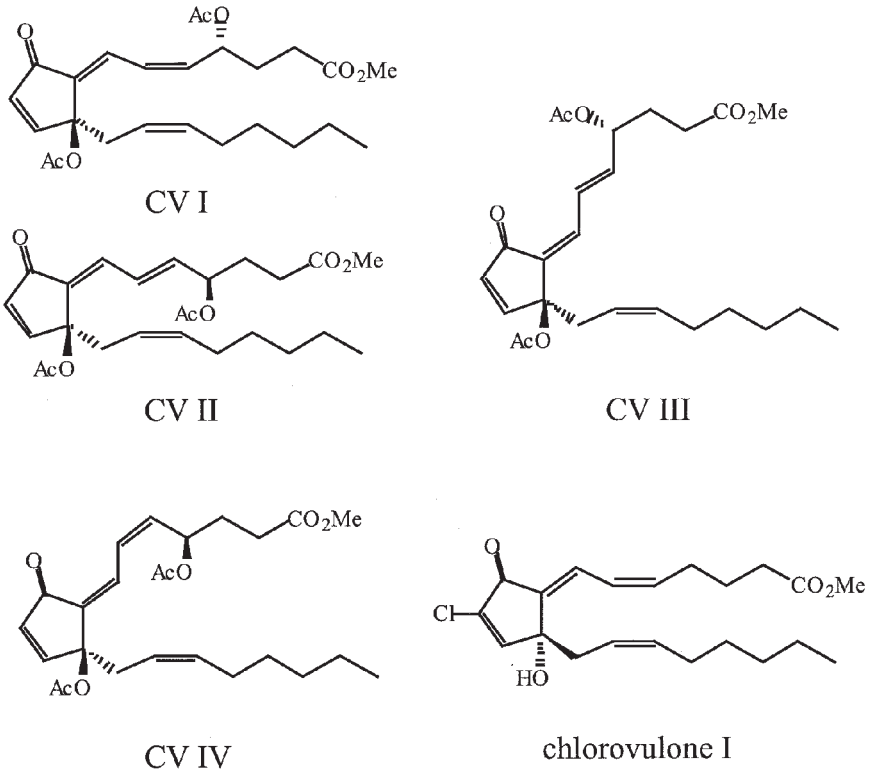
Abbreviations: AA, arachidonic acid; CBP, chlorophyll *a-c*-binding protein; Chl, chlorophyll; CorF, *Symbiodinium*-free coral fraction; CorHo, coral homogenate treated with pronase E; CSym, cultured *Symbiodinium*; CV, clavulone(s); NP-40, Nonidet P-40; PCP, peridinin-Chl-binding protein; ppt, precipitate; sup, supernatant; SymF, *Symbiodinium* fraction.



SCHEME 1

Preparation of soluble and insoluble fractions of coral homogenates. Tentacles (about 1.6 g; frozen corals) were removed from coral polyps using a razor blade and forceps. About 25 vol of homogenization buffer (50 mM Tris-HCl, 10 mM EDTA, 200 mM sorbitol; pH 8.0) was added to the polyps. The sample was homogenized with a glass homogenizer and then sonicated until almost all of the *Symbiodinium* cells had been broken, usually three times for 3 s each at 15 W with a 250D Sonifier (Branson, Danbury, CT). The suspension was ultracentrifuged at 100,000 × *g* for 1 h at 4°C, and then the supernatant (sup; soluble fraction) and precipitate (ppt; insoluble fraction) were analyzed for CV, chlorophyll (Chl) *a*, and protein.

Preparation of a Symbiodinium *fraction (SymF) and a* Symbiodinium*-free coral fraction (CorF).* A homogenate including 1.6 g tentacles (native coral), which was prepared as described above, was incubated in the presence of 2 mg/mL pronase E from *Streptomyces griseus* for 1 h at room temperature. The coral homogenate treated with pronase E (CorHo) was divided into aliquots, and then various concentrations of a detergent, Nonidet P-40 (NP-40), were added to aliquots, followed by incubation for 1 h at room temperature. The samples were then ultracentrifuged at 100,000 × *g* for 1 h at 4°C, and the sup was analyzed for CV and Chl *a*.

For the preparation of CorF, CorHo including about 0.5 g tentacles (native coral) was centrifuged at 160 × *g* for 10 min at 4°C, and the sup was recovered.

For the preparation of SymF, CorHo including about 1 g tentacles (native coral) was treated with 0.4% NP-40 for 1 h at room temperature and then centrifuged at 160 × *g* for 10 min at 4°C. The ppt was washed twice with the homogenization buffer and then resuspended in a small volume of the buffer. The suspension was layered onto a Percoll (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) layer in a centrifuge tube, followed by centrifugation at 20,000 × *g* for 20 min at 4°C. The brown band of *Symbiodinium* cells was collected and resuspended in the buffer. The *Symbiodinium* cells were then precipitated by centrifugation at 160 × *g* for 10 min at 4°C.

Cultures of Symbiodinium. *Symbiodinium* sp. strain CV-I1 isolated from *C. viridis* (20) was cultured at 20°C in IMK medium "DAIGO" (Nihon Pharmacy, Tokyo, Japan) at *ca.* 20 μmol photons m^{-2} s^{-1} provided by cool-white fluorescent tubes with a 14:10 h light/dark regime.

SDS-PAGE (frozen corals). Fractions were extracted with SDS-PAGE sample buffer and then separated by electrophoresis on 12% SDS polyacrylamide gels as previously described (21). The apparent M.W. of polypeptides on the SDS gel were determined using protein markers from Bio-Rad (Precision Protein Standards, Hercules, CA).

Lipid analysis (frozen coral). Total lipids were extracted from the fractions according to the method of Bligh and Dyer (22) and then fractionated by TLC on precoated silica gel plates (5721 Silica gel 60; Merck, Darmstadt, Germany). The solvent system was chloroform/methanol/water (65:25:4, by vol) (23). The spots of phospholipids and glycolipids were visualized by the method of Dittmer and Lester (24) and the anthrone method (25), respectively. FAME were prepared from

the total lipids with 5% anhydrous methanolic HCl for analysis by capillary GLC, as described previously (26).

Determination of the contents of CV, Chl a*, and protein.* CV were extracted from specimens of *C. viridis* and then examined as described previously (27). The amount of Chl *a* was determined by the method of Jeffrey (28). Protein concentrations were determined according to the method of Bradford (29) with BSA as the standard, using the Coomassie Protein Assay Reagent from Pierce Chemicals (Rockford, IL).

RESULTS

Distribution of CV in coral homogenates. First, we investigated the distribution of CV in soluble and insoluble fractions of coral polyps, including ones of *Symbiodinium* (Table 1). The polyps were homogenized by sonication and then centrifuged. Almost all of the CV (98.8%) were detected in the insoluble fraction, where most of the Chl *a* also existed. Proteins were detected in both the soluble (42.2%) and insoluble (57.8%) fractions. Therefore, CV were located in the membranes of either the host or the symbiotic algae.

To clarify in which membranes CV were localized, we tried to solubilize coral membranes with a protease and a detergent without destroying the zooxanthellae cells: The coral membranes were solubilized by treatment of a coral homogenate with 2 mg/mL pronase E, and then with NP-40 at concentrations of up to 2%. Under these conditions, some amount of both CV and Chl *a* was found in the sup after ultracentrifugation at 100,000 × *g* (Fig. 1). On treatment with a low concentration (0.2–0.5%) of NP-40, only a small portion of Chl *a* was solubilized (0–15%), suggesting that hydrophobic substances in the thylakoid membranes of zooxanthellae were extracted to a lesser extent. On treatment with 0.7% NP-40, 80% of CV was solubilized into the sup fraction, whereas about 80% of Chl *a* still remained in the ppt fraction. Microscopic observation of the samples demonstrated that most of the zooxanthellae cells were not broken under these conditions (data not shown). When a higher concentration of NP-40 was applied, up to 2%, the solubility of CV remained constant at 80%, and that of Chl *a* increased linearly with the increase in the concentration of NP-40. Thus, we concluded that most CV were not located in the thylakoid membranes but probably in the coral membranes.

TABLE 1
Contents of Clavulone (CV), Chlorophyll (Chl) *a*, and Protein in the Soluble and Insoluble Fractions[a]

Fraction[b]	CV [mg] (%)	Chl *a* [μg] (%)	Protein [mg] (%)
Homogenate	2.97	108	1.25
40 × *g* sup	1.51	54.7	1.08
100,000 x *g* sup (soluble fraction)	0.01 (1.2)	7.0 (14.2)	0.362 (42.2)
100,000 × *g* ppt (insoluble fraction)	1.17 (98.8)	42.5 (85.8)	0.495 (57.8)
40 × *g* ppt	1.87	72.7	0.465

[a]Variabilities of CV, Chl *a*, and protein were no more than 27, 15, and 13%, respectively.

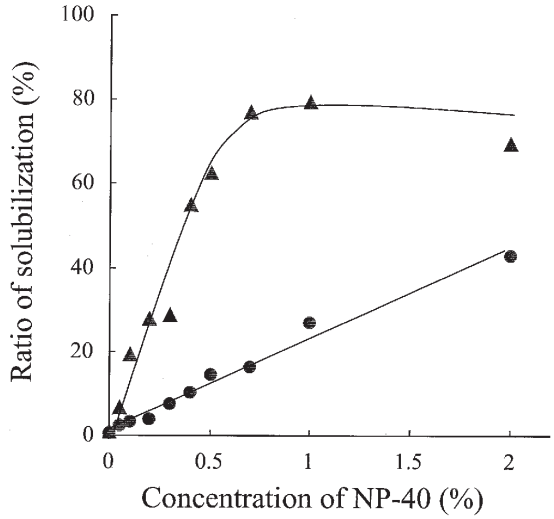


FIG. 1. Solubilization of clavulones (CV) with Nonidet P-40 (NP-40) and pronase E. A coral homogenate was treated with 2 mg/mL pronase E and then with various concentrations of NP-40. The suspension was ultracentrifuged at 100,000 × *g* for 1 h at 4°C, and then CV (▲) and chlorophyll (Chl) *a* (●) in the supernatant were determined as described in the Experimental Procedures section. Chl *a* values are the means of two samples. Each CV result is shown; a separate experiment showed the same pattern as the one here.

Separation of CorF. We then tried to obtain a coral membrane fraction free from *Symbiodinium* to elucidate the localization of CV. The protocol for CorF preparation is shown in Figure 2A. CorHo was centrifuged at 160 × *g*. It was confirmed by microscopy that zooxanthellae cells were not included in the sup (CorF). CV and Chl *a* in the sup (CorF) and ppt after centrifugation were quantified (Table 2). Although only 7.3% of Chl *a* was detected in CorF, 41.8% of CV was found in this fraction. These results suggest that the CV exist in the membranes of the host coral.

Separation of SymF. SymF was also obtained from CorHo, which was treated with 0.4% NP-40 (Fig. 2B). CorHo was centrifuged at 160 × *g*, and then the ppt was washed with the buffer by centrifugation at 40 × *g* three times. The ppt, again suspended in the buffer, was layered over a Percoll solution, and then a density gradient was formed by centrifugation at 20,000 × *g* for 10 min. *Symbiodinium* cells formed a brown

TABLE 2
Contents of CV and Chl *a* in CorHo, CorF, and SymF[a]

Fraction	CV [mg] (%)	Chl *a* [μg] (%)
	Experiment A	
sup (CorF)	2.53 ± 0.14 (41.8)	16 ± 0.4 (7.3)
ppt	3.52 ± 0.06 (58.2)	213 ± 2.2 (92.7)
	Experiment B	
CorHo	8.61 ± 0.16 (100)	238 ± 1.0 (100)
SymF	0.42 ± 0.07 (4.8)	123 ± 0.5 (51.8)

[a]Fractionation was carried out for either CorF (Experiment A) or SymF (Experiment B) to obtain high purity. Data are reported as mean ± SE (n = 3). CorHo, coral homogenate treated with pronase E; CorF, *Symbiodinium*-free coral fraction; SymF, *Symbiodinium* fraction; for other abbreviations see Table 1.

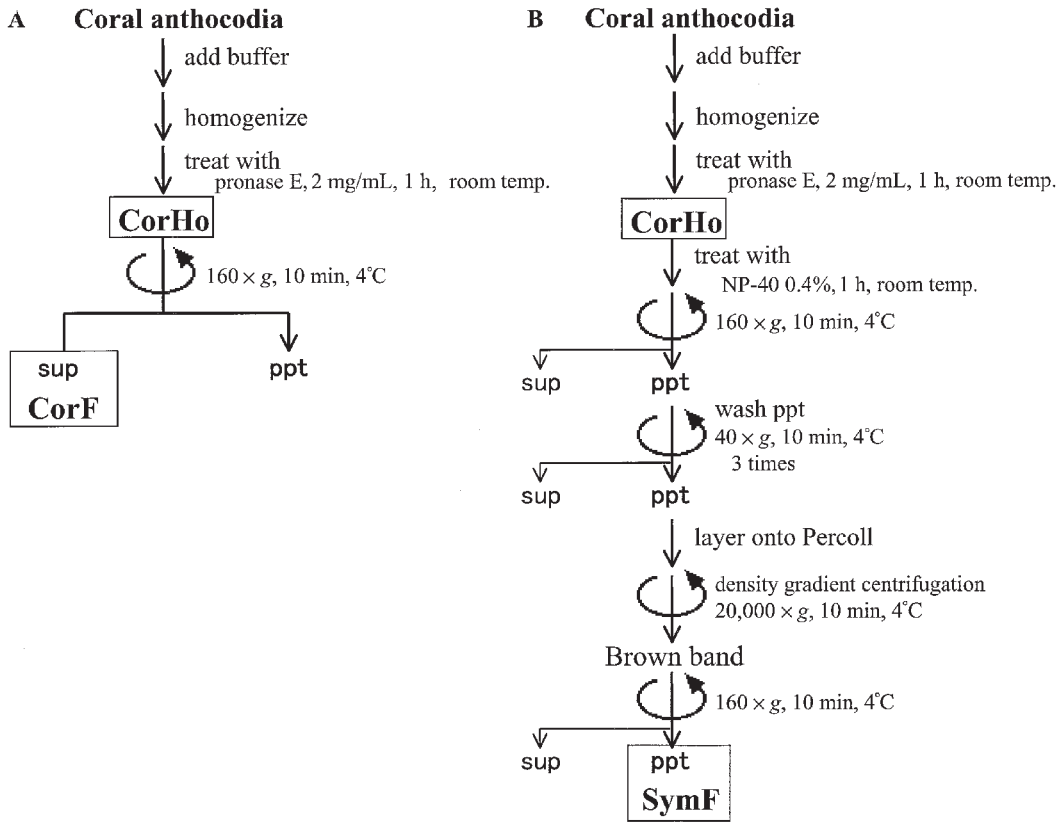


FIG. 2. Protocol for separation of a *Symbiodinium*-free coral fraction (CorF) (A) and a *Symbiodinium* fraction (SymF) (B). CorHo, coral homogenate treated with pronase E; sup, supernatant; ppt, precipitate.

band, whereas coral sclerites were obtained as a white ppt. The brown band (SymF) was recovered, diluted with the buffer, and then collected by centrifugation. Microscopic observation of SymF proved that the *Symbiodinium* cells were not broken and that aggregates of coral membranes were not included (data not shown). Chl *a* and CV in SymF were quantified and compared with those in the CorHo used for the preparation (Table 2). Although the Chl *a* content of SymF was 51.8% that of CorHo, CV were detected in SymF at only a low level (4.8%). This suggests that symbiotic algal cells hardly contain CV or contain much less than the host, if any.

The localization of CV. The contents of CV and Chl *a* in CorHo, CorF, and SymF are summarized in Table 3. Fractionation was performed to obtain high purity. For reference, the contents of *Symbiodinium* sp., which was isolated from *C. viridis* and cultivated as the free-living form, are also shown. Arachidonic acid (AA) is considered to be a precursor of prostanoids such as CV (30,31). The distributions of AA and EPA, whose carbon number per molecule is the same as that of AA, are also shown in the table. The abundance of Chl *a* and EPA was much greater in SymF than in CorF. In contrast, more CV and AA were found in CorF than in SymF, and they were not detected in the cultured *Symbiodinium* (CSym) at all. These results support the conclusion that most CV were localized in the host coral membranes.

Lipid and protein analyses of the fractions. TLC and SDS-PAGE of CorHo, CorF, SymF, and CSym were performed for lipid and protein analyses, respectively (Figs. 3 and 4). Glycolipids were detected in both CorF and SymF, and fraction-specific spots were observed (black and white arrows). For CSym, a glycolipid pattern similar to that for the SymF was found. Blue spots of phospholipids were observed for CorF and CorHo, in which host coral membranes were included, whereas phospholipids were scarcely detected in either CSym or SymF. This indicates that zooxanthellae contain mainly glycolipids and few phospholipids. These results suggest that CorF and SymF were fractionated with high purity.

On SDS-PAGE (Fig. 4), the protease treatment was omitted to prevent proteolysis of the samples. After the samples CSym, CorHo, and SymF were ground with a pestle and mortar, they were ultracentrifuged, and soluble and insoluble fractions were obtained. Major bands around 30 kDa for the soluble fractions and at about 15 kDa for the insoluble fractions were observed

TABLE 3
Contents of CV, Chl *a*, AA and EPA in CorHo, CorF, and SymF[a]

Fraction	CV [mg] (%)	Chl *a* [μg] (%)	AA [mg] (%)	EPA [mg] (%)
CorHo	8.61 (100)	238 (100)	2.38 (100)	0.763 (100)
CorF	2.53 (29.4)	17 (7.0)	0.902 (38.2)	0.079 (10.4)
SymF	0.42 (4.8)	123 (51.7)	0.011 (0.5)	0.329 (43.1)
CSym	0	18	0	0.15

[a]Variabilities of CV, Chl *a*, AA, and EPA were no more than 13, 3, 1, and 12%, respectively. AA, arachidonic acid; CSym, cultured *Symbiodinium*; for other abbreviations see Tables 1 and 2.

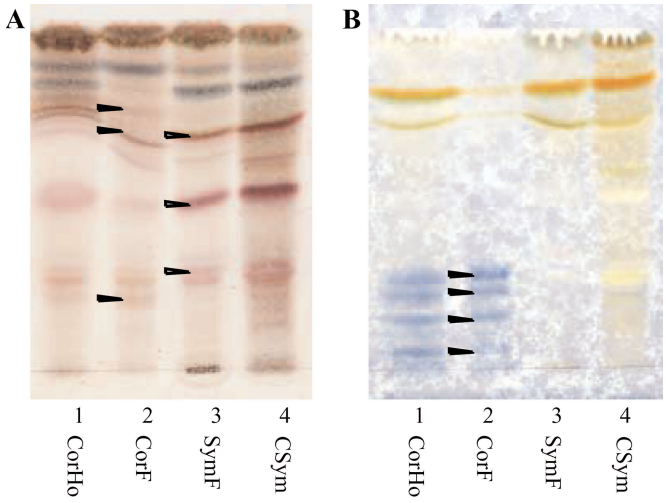


FIG. 3. TLC patterns of total lipids from CorF and SymF. (A) Separation of glycolipids. (B) Separation of phospholipids. The total lipids from CorHo (lane 1), CorF (lane 2), SymF (lane 3), and CSym (lane 4) were separated by TLC as described in the Experimental Procedures section. Black and white arrows indicate host-specific and *Symbiodinium*-specific spots, respectively. For abbreviations see Figure 2.

for *Symbiodinium*-containing samples. The amino acid sequences of the amino-terminals of CSym proteins revealed that the major band for the soluble fraction (indicated by a star) was peridinin-Chl-binding protein (PCP), and that the major insoluble polypeptide (indicated by double stars) is Chl *a*-*c*-binding protein (CBP). For SymF, bands assumed to be PCP and CBP were found as for CSym, although confirmation by means of amino-terminal sequencing was not carried out.

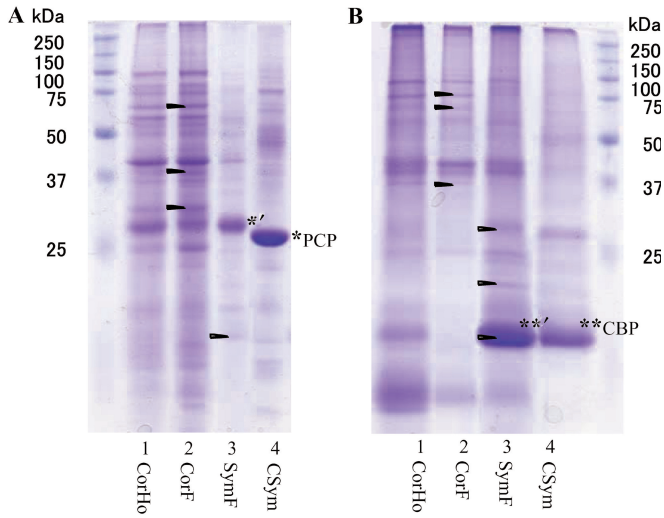


FIG. 4. SDS-PAGE patterns of CorF and SymF. (A) Soluble fraction; (B) insoluble fraction. CorHo (lane 1), CorF (lane 2), SymF (lane 3), and CSym (lane 4) were resolved on a 12% SDS-PAGE gel. The fractionation was performed as described in the Experimental Procedures section. Black and white arrows indicate host-specific and *Symbiodinium*-specific bands, respectively. The amino acid sequences of the amino-terminals of the proteins indicated by * and ** for CSym revealed that the proteins were peridinin-Chl-binding protein (PCP) and chlorophyll *a*-*c*-binding protein (CBP), respectively. Bands in SymF, assumed to be PCP and CBP, are shown by *′ and **′, respectively. For other abbreviations see Figures 1 and 2.

Thus, we concluded that the separation of CorF and SymF was performed successfully, and that almost all CV as well as AA was localized in CorF.

DISCUSSION

The localization and physiological roles of secondary metabolites in marine invertebrates have scarcely been investigated. In this study, we showed that some of the prostanoids, CV, are located in the membrane fraction of the soft coral, *C. viridis*. This is the first report on the localization of prostanoids in a coral.

We also improved the fractionation method to raise the purity of SymF. Only a few reports have appeared on the isolation of symbiotic algae from host corals (18,32,33). Although sucrose density-gradient centrifugation was tried for coral homogenates, the host membranes could not be removed due to aggregation with symbiotic algae and sclerites in the anthocodia. Therefore, either protease or detergent treatment was required for the isolation of SymF and CorF. The lipid and protein profiles of SymF and CorF obtained showed their high purity (Figs. 3 and 4). With the increase in SymF purity from the improvement of the fractionation protocol, the CV abundance in the fraction decreased (data not shown). This also supports the exclusive localization of CV in the host coral cells. As an example of localization of secondary metabolites in host cells but not in zooxanthellae cells, latruculin B has been localized mainly in host sponge cells by Gillor *et al.* (34) using the gold colloid method.

AA is also located in the membrane fraction of host corals. Since AA has been considered as an intermediate of CV synthesis (30,31), CV may be synthesized in coral membranes. Some of the FA synthesized in zooxanthellae through photosynthesis have reportedly been transferred to the host in the form of wax esters and TAG in some of the associations (35,36). In the *C. viridis*/zooxanthellae association, some of the FA in the zooxanthellae might also be transferred to the host to be elongated to AA, which would then be metabolized to CV. Alternatively, the possibility that AA is synthesized in zooxanthellae cells and then immediately transported to the host cells cannot be excluded at present. Strictly speaking, experiments involving isotopes must be performed to determine which photosynthetic products are transferred from zooxanthellae to the coral cells in the process of prostanoid synthesis.

Although the most abundant PUFA in CorF was AA, that in SymF was EPA (data not shown). Pathways of PUFA synthesis have not been elucidated in cnidarian/algal associations. EPA might be synthesized in the zooxanthellae through a pathway of FA elongation, as shown in mammals, or through a polyketide synthase pathway, as found in some marine protists (37).

Lipid analysis by TLC and SDS-PAGE were carried out to confirm the purity of CorF and SymF on fractionation (Figs. 3 and 4). Although lipid and FA analyses of coral and zooxanthellae together have been reported (35,38–43), to our knowledge this is the first investigation of *Symbiodinium*-free

host lipids and fractionated zooxanthellae lipids. We have presented TLC patterns of glycolipids and phospholipids in the host coral and zooxanthellae (Fig. 3). A significant difference was observed between CorF and SymF in both glycolipids and phospholipids. In particular, the levels of phospholipids were very low in SymF.

On protein analyses of the host–zooxanthellae interaction, several reports have been published, e.g., analysis of soluble proteins of zooxanthellae under symbiotic conditions (44), comparison of the protein profile of the host fraction between symbiotic and aposymbiotic conditions (45), and comparison of the symbiont protein expression between symbiotic and free-living conditions (46). However, there have been few reports of the analysis of proteins of fractionated host coral cells and zooxanthellae. In this paper, SDS-PAGE profiles of soluble and insoluble proteins of host cells and zooxanthellae of a soft coral are shown (Fig. 4). For the insoluble fraction, there was little contamination by *Symbiodinium* proteins in CorF, since the band for SymF (indicated by double stars) was not observed for CorF. For the soluble fraction, a band was detected for CorF at the position corresponding to a M.W. equal to that of the band for SymF (indicated by a star). It cannot be concluded at the moment whether this result is due to contamination by the SymF protein or the existence of a protein of the same M.W. in the host soluble fraction.

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METHOD

Light-Scattering Detection of Phospholipids Resolved by HPLC

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ABSTRACT: An improved method for the analysis of phospholipids by normal-phase HPLC is described. Addition of methanol and acetonitrile to a gradient based on 2-propanol/hexane/water promoted a rapid separation of major classes of bovine surfactant phospholipids (PL) by using a conventional silica column. The use of an ELSD permitted an accurate analysis of a mixture of PL. Calibration curves were linear within the range of 5–40 μg with detection limits below 1 μg for PE and PC, and CV ranged from 0.6 to 9.6%. PL present in surfactant homogenates were separated by a solid-phase extraction (SPE) procedure before HPLC analysis. This methodology gave a recovery of 95% and combined SPE–HPLC and quantification of biological PL within a 30-min run. The use of ELSD detection of the eluted compounds was precise, linear, and sensitive.

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For the last decade, HPLC has been the method of choice for the separation and quantification of phospholipid (PL) classes. This methodology has the advantage of avoiding the extensive derivatization needed in GC analysis of PL.

Formerly, detection of peaks in the UV range of 203–210 nm was used. However, this technique could be applied only for qualitative purposes, because it is the amount of double bonds and not the number of molecules that is determined. Therefore, UV detection or derivatization methodology is usually associated with phosphorus quantification (1,2).

To use UV detection, the mobile phase must be UV transparent. Hence, the use of solvent gradients usually causes an undesired baseline drift within the run. An alternative is UV indirect detection. It was successfully applied for the detection of a PI analog, 1-[(1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero)-phospho]-1*D*-3-deoxy-*myo*-inositol. The addition of protriptyline hydrochloride as UV-absorbing reagent in the mobile phase was applied to give rise to detection signals, thus obtaining proper accuracy, precision, and a 5-ng limit of detection (3).

Nowadays, the use of an ELSD eliminates those common problems associated with other HPLC detectors and allows qualitative/quantitative studies of nonvolatile analytes (4). For example, unlike refractive index and low-wavelength UV, ELSD uses multisolvent gradients to improve resolution and perform faster separation of the eluted compounds (5–7). Therefore, an ELSD–HPLC system could analyze underivatized intact lipids, thus saving time and avoiding losses during derivatization and hydrolysis. Evaporative light scattering is based on the detection of nonvolatile molecules carried by a volatile mobile phase. The column effluents are nebulized to an aerosol, followed by volatile compound vaporization and formation of small solute droplets. The laser light scattered by these droplets is detected by a photodiode (8).

The separation of all PL classes from different matrixes by use of a variety of multisolvent gradients had been reported previously (9–12).

Lipids ranging from cholesterol to lysophosphatidylcholine (LPC) can be resolved in a single normal-phase HPLC run using a gradient of increasing polarity. Even nonpolar molecules like hydrophobic pulmonary surfactant proteins can be accurately detected by ELSD (13).

The polarity of different lipid classes constitutes a useful tool allowing PL separation. Solid-phase extraction (SPE) takes advantage of this property and has been used as an alternative to liquid–liquid extraction. A selective elution of the desired compounds can be obtained with SPE by simple fractionation of the analytes through differences in their polarity (14). The aim of this work was to develop an SPE–HPLC combined routine to recover, resolve, and quantify SPE-extracted PL from a complex matrix, such as pulmonary surfactants.

EXPERIMENTAL PROCEDURES

Reagents. n-Hexane, 2-propanol, methanol, and water were HPLC grade from J.T. Baker Inc. (Phillipsburg, NJ). All other chemicals were analytical reagent grade.

L-α-Phosphatidylethanolamine (PE), L-α-phosphatidyl-DL glycerol (PG), L-α-phosphatidyl-L-serine (PS), L-α-phosphatidylcholine (PC), L-α-lysophosphatidylcholine (LPC), and sphingomyelin (SM) with purity of approximately 98% were obtained from Sigma Chemical Co. (St. Louis MO).

To prepare calibration curves, standards were diluted by the appropriate solvent considering its solubility. Duplicate injections of each dilution were used.

Pharmaceutical bovine surfactant was used as a PL source.

SPE procedure. Natural surfactant (1 mL) was extracted with 4 mL of chloroform/methanol (2:1, vol/vol). The organic phase was collected into a conical vial, evaporated to dryness under a nitrogen stream, and made up to 0.5-mL final volume with chloroform. The extract was applied to the top of a normal-phase silica cartridge (LiChrolut Si 60, 500 mg; Merck, Darmstadt, Germany) that had been previously conditioned with 10 mL of chloroform. Sequential elution was performed with 20 mL of chloroform, 5 mL of acetone, and 20 mL of

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Abbreviations: LPC, lysophosphatidylcholine; PG, phosphatidylglycerol; PL, phospholipid; SM, sphingomyelin; SPE, solid-phase extraction.

methanol. To prevent the oxidation of polyunsaturated chains during PL extraction, 0.02% wt/vol BHT was added to the solvents. This procedure separates neutral lipids, glycolipids, and polar lipids (14,15). The methanol extract was dried at 40°C under a nitrogen stream, dissolved in 1 mL of chloroform, and filtered through a 0.2 μm nylon membrane before injection onto the HPLC.

HPLC methodology. The Spectra System HPLC from Thermo Separation Products (TSP) included a P4000 quaternary pump and an autosampler AS3000 with an injection valve of 100-μL sample loop. The separation system consisted of a 3-μm Waters Spherisorb Silica column of 150 × 4.6 mm with a silica guard column. The column oven was set at 30°C. All equipment was connected through an SN4000 interface with the data acquisition system software PC1000 (TSP).

Detector calibration. The evaporative light-scattering system consisted of a ELSD Model 500 detector (Alltech, Deerfield, IL). An interface module converted the ELSD analog signal to digital data that could be processed by the computer. The suitable signal-to-noise ratio was determined by injection and detection of PL standards. A drift tube temperature of 70°C, a gas flow rate of 1.98 standard liters per minute, and a gas pressure of 13.1 psi (0.090 MPa), were the most convenient parameters. High-purity N_2 was used as nebulizer gas. Under these conditions, vaporization of the solute did not occur, and there was also a stable baseline with attenuation factor of 1.

Repeatability and recovery assays. Repeatability was determined for all the analytes, that were present in the surfactant. One batch sample was divided into four aliquots of 1 mL each, which were subjected to the SPE–HPLC procedure. The percent recovery was determined for PC, the component present in the largest amount in the analyzed sample (74–85% of the total PL). Surfactant (1 mL) was enriched with 2 mg of standard and analyzed by using the SPE–HPLC procedure. Five independent samples were prepared.

Calibration curves. A response calibration curve for each class of PL was prepared. Standards were diluted with the appropriate solvent, considering the solubility of the PL. Duplicate 10-μL injections of each dilution were used in the range from 1 to 40 μg for three separate replicates for each analyte. The hypothesis of linearity was tested by using the Linear Regression Model (GLM) procedure, a package program of the Statistical Analysis System (SAS, Cary, NC).

RESULTS AND DISCUSSION

To separate PL of widely differing polarity in a single HPLC-direct phase run, a gradient elution was used, starting with a solvent mixture of low polarity and ending with methanol and acetonitrile.

In a first attempt to separate all PL classes, the classic solvent system of 2-propanol/hexane/water with increased polarity was used. However, the strength of this solvent system was insufficient to elute polar PL even when the water percentage was elevated to 8% (16). PC, SM, and LPC were retained within the column (even after 50 min of elution) under these conditions. Adsorption of PC to the stationary phase of a similar column has been reported (9). A further increase of the water content (above 8%) to solve this problem resulted in a noisy baseline, probably due to partial evaporation of the mobile phase in the nebulizer. This could be resolved by raising the nebulizing temperature. Nevertheless, temperatures over 70°C decreased the detection of eluted peaks. This could be explained by the evaporation of some low-boiling FFA, as described previously (9).

Hence, as shown in Table 1, the first step consisted of a linear gradient from 2-propanol/hexane (40:60, vol/vol) to 2-propanol/*n*-hexane/water (63:35:2, by vol), which separated the PL from traces of neutral lipids. Mobile phases based on hexane/2-propanol/water (5,16,17) and acetonitrile/water (sometimes with addition of methanol) (18) have been used in many laboratories since their introduction and still find almost universal application today (19–22). Gradient polarity was increased by addition of methanol. PG and PE were resolved with 45% methanol; 80% methanol allowed the separation of PS, and separation of PC was achieved with 100% methanol. Further use of acetonitrile in a linear gradient eluted LPC. Before injecting the next sample, it was necessary to re-equilibrate the column by removing the polar solvents with the initial mixture.

Total chromatographic run time was 30 min per sample, consisting of a 25-min run and 5 min additional for equilibration with the initial solvent. Reproducible chromatograms were still obtained after approximately 500 injections onto one silica column.

A typical chromatogram of bovine pulmonary surfactant is presented in Figure 1A. The detection of different peaks was coincident with the retention time of the standards mixture

TABLE 1
HPLC Gradient Scheme

	Time (min)										
	0	5	10	13	13.10	15	17	22	24	25	30
MeOH (%)[a]	0	0	45	45	60	80	100	0	0	0	
ACN (%)	0	0	0	0	0	0	0	0	100	100	0
Isopropanol/hexane (40:60, vol/vol) (%)	100	0	0	0	0	0	0	0	0	0	100
Isopropanol/hexane/H_2O (63:35:2, by vol) (%)	0	100	55	55	40	20	0	0	0	0	0
Flow (mL/min)	1	1	0.8	0.8	1	1	1	1	0.8	0.8	0.8

[a]Percentage of each component of the mobile phase is indicated. ACN, acetonitrile.

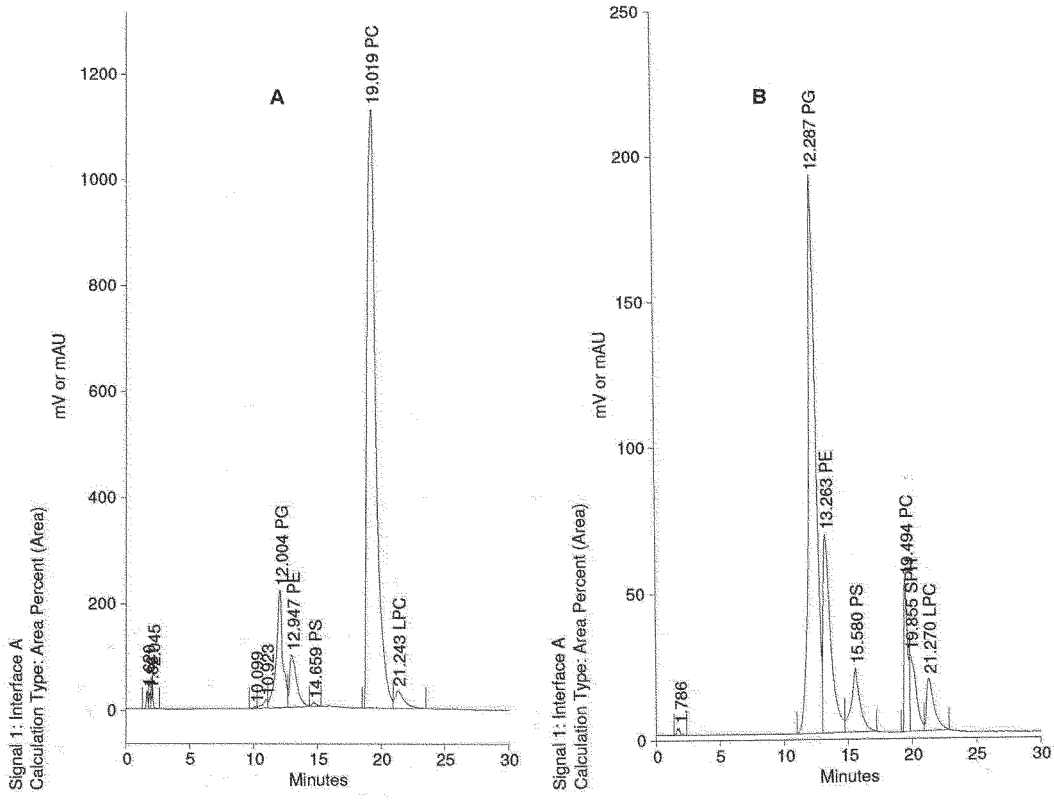

FIG. 1. High-performance liquid chromatogram of a phospholipid (PL) extract (A) and the corresponding standards (B) with ELSD detection. Elution was performed with the gradient indicated in Table 1. PG, phosphatidylglycerol; LPC, lysophosphatidylcholine.

(Fig. 1B). As expected, PC was the major constituent and the peak was significantly higher than the other PL within the sample (Fig. 1A). Thus, a 10-fold dilution of the surfactant was performed in each case for the quantification of PC.

Correspondence of the PL fractions with their respective controls was checked by TLC, confirming the presence of PC, PE, PG, PS, and LPC as the major PL within the surfactant samples.

As peaks were efficiently separated, addition of ions to the mobile phase was not indispensable. However, this practice is required for separation of acidic PL. Interaction of acidic compounds with the stationary phase (which may deteriorate it) could be diminished by addition of organic ions to the mobile phase. Triethylamine and formic acid are commonly used as additives. At the same time, they serve as enhancers of the light-scattering response (23,24). In addition, modifications of the solvent system proposed by Becart *et al.* (25) with ammonium hydroxide in the mobile phase are widely used (26–28).

Calibration curves of the principal PL classes are shown in Figure 2. Standard samples were diluted from 10 mg/mL stock solutions to give concentrations between 1 and 40 µg of PL per 10 µL of injection volume. Each curve was prepared and injected in triplicate. The respective peak areas fitted a linear model within the indicated range. Linearity was observed for the PL from 5 to 40 µg with a correlation coefficient $R^2 > 0.99$ ($P > 0.001$). However, linearity tended to tail off rapidly below 5 µg, as already described by other researchers (5,6,26). The slope was similar for PS, PC, and LPC curves, whereas the slope for PE was approximately three times higher. This is in agreement with other authors who also reported a marked difference between the slope for calibration of PE compared to other PL when using ELSD detection (29,30).

Thus, in accordance with previous investigations, the detector response for natural surfactant samples varied for different PL classes, (31,32). In considering the entire range from 1 to 40 µg, all the curves fitted the power function $y = Cx^{\varepsilon}$, where y corresponded to peak area units, x to PL mass in micrograms, C to a constant, and ε to an exponent. The exponents for the four compounds investigated had a mean value ± SD of 1.51 ± 0.2. Other authors described similar results (26).

The detection limit for the different PL was 1 µg/10 µL of injected volume. Submicrogram quantities were detected for PC and PE (0.3 and 0.5 µg/10 µL of injected volume). Integration of peaks below 1 µg can be achieved by changing the conditions of the integration software. In the case of analyzing quantities below 5 µg, calibration should be done with power functions (26). Indeed, the PE calibration curve in the entire range (0–40 µg) fitted a power function $y = 181{,}100 \cdot x^{1.33}$ with a correlation of 0.9998 (compare with Fig. 2). Unlike HPLC–ELSD detection, LC–MS analysis obtains linear calibration curves for lower concentrations of PC and PE (33).

After optimizing HPLC–ELSD conditions with standard mixtures, the SPE–HPLC procedure was applied for determination

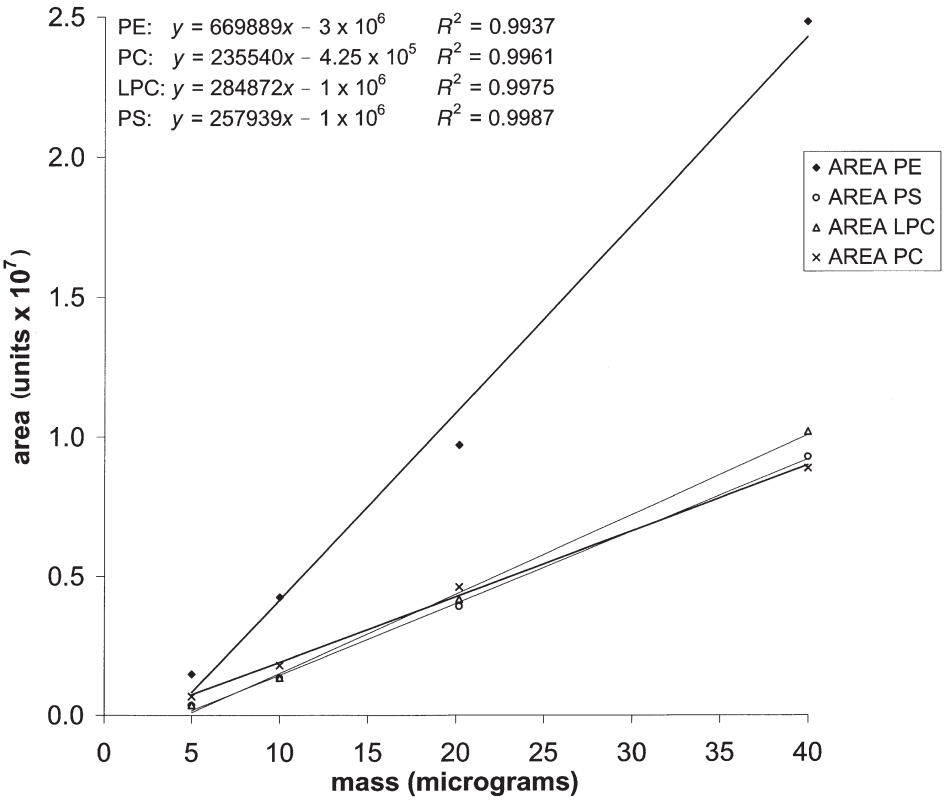

FIG. 2. Calibration curves: mass of PL vs. ELSD response in 10 µL of injection volume. Each point represents the mean value of duplicate injections for three separate replicates. Linearity was obtained within the 5 to 40 µg range ($P < 0.001$) by using the Linear Regression Model (GLM) procedure, a package program of the Statistical Analysis System (SAS, Cary, NC). For abbreviations see Figure 1.

and quantification of PL in pulmonary surfactants. The repeatability of this methodology is shown in Table 2. Results of five independent tests demonstrated a good precision for the analysis of individual PL in surfactant samples. CV ranged from 0.6% for PS to 9.6% for PC. The higher CV values for PC and LPC probably occurred because they were retained more strongly on the column and gave broader peaks than the other analytes. Another possibility for obtaining non-Gaussian peaks is the presence of multiple PL subclasses, which could not be separated by this methodology and which elute together as a single broad peak composed of several molecular species with different FA moieties in their molecules. Molecular species of major PL classes can be separated by reversed-phase HPLC as already described (32,34,35). Recovery was also determined: With added standard, 95% of the PC was recovered in comparison with nonspiked controls. The resultant CV was 4.6% among the replicates.

The analytical procedures detailed in this report allow rapid and accurate analysis of a mixture of PL present in bovine pulmonary surfactants. SPE methodology permitted the isolation of the PL present in this matrix from other lipids, with the advantage of a high recovery. Likewise, it optimized further quantification by HPLC with an ELSD detector. The use of ELSD detection of the eluted compounds was precise, linear, and sensitive, thus avoiding other laborious quantification procedures (12), and consequently allowed simultaneous screening and measurement of a large number of samples by reducing the time required for analysis. This method also can be applied to studies of PL mixtures in different animal tissues, cell cultures, and food products.

TABLE 2
Precision Assay for Samples Analyzed by SPE–HPLC Methodology

	PG	PE	PS	PC	LPC
PL concentration[a] (mg/mL)	0.843 ± 0.0178	0.878 ± 0.0165	0.461 ± 0.0027	21.928 ± 2.1136	0.619 ± 0.0399
CV (% RSD)	2.12	1.88	0.58	9.63	6.44

[a]Results are given as means ± SD of five separate extractions from the same batch of pulmonary surfactant. Experiments were run on different days. SPE, solid-phase extraction; PG, phosphatidylglycerol; LPC, lysophosphatidycholine; RSD, relative SD.

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EFA Supplementation in Children with Inattention, Hyperactivity, and Other Disruptive Behaviors

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ABSTRACT: This pilot study evaluated the effects of supplementation with PUFA on blood FA composition and behavior in children with Attention-Deficit/Hyperactivity Disorder (AD/HD)-like symptoms also reporting thirst and skin problems. Fifty children were randomized to treatment groups receiving either a PUFA supplement providing a daily dose of 480 mg DHA, 80 mg EPA, 40 mg arachidonic acid (AA), 96 mg GLA, and 24 mg α-tocopheryl acetate, or an olive oil placebo for 4 mon of double-blind parallel treatment. Supplementation with the PUFA led to a substantial increase in the proportions of EPA, DHA, and α-tocopherol in the plasma phospholipids and red blood cell (RBC) total lipids, but an increase was noted in the plasma phospholipid proportions of 18:3n-3 with olive oil as well. Significant improvements in multiple outcomes (as rated by parents) were noted in both groups, but a clear benefit from PUFA supplementation for all behaviors characteristic of AD/HD was not observed. For most outcomes, improvement of the PUFA group was consistently nominally better than that of the olive oil group; but the treatment difference was significant, by secondary intent-to-treat analysis, on only 2 out of 16 outcome measures: conduct problems rated by parents (–42.7 vs. –9.9%, $n = 47$, $P = 0.05$), and attention symptoms rated by teachers (–14.8 vs. +3.4%, $n = 47$, $P = 0.03$). PUFA supplementation led to a greater number of participants showing improvement in oppositional defiant behavior from a clinical to a nonclinical range compared with olive oil supplementation (8 out of 12 vs. 3 out of 11, $n = 33$, $P = 0.02$). Also, significant correlations were observed when comparing the magnitude of change between increasing proportions of EPA in the RBC and decreasing disruptive behavior as assessed by the Abbreviated Symptom Questionnaire (ASQ) for parents ($r = –0.38$, $n = 31$, $P < 0.05$), and for EPA and DHA in the RBC and the teachers' Disruptive Behavior Disorders (DBD) Rating Scale for Attention ($r = –0.49$, $n = 24$, $P < 0.05$). Interestingly, significant correlations were observed between the magnitude of increase in α-tocopherol concentrations in the RBC and a decrease in scores for all four subscales of the teachers' DBD (Hyperactivity, $r = –0.45$; Attention, $r = –0.60$; Conduct, $r = –0.41$; Oppositional/Defiant Disorder, $r = –0.54$; $n = 24$, $P < 0.05$) as well as the ASQ for teachers ($r = –0.51$, $n = 24$, $P < 0.05$). Thus, the results of this pilot study suggest the need for further research with both n-3 FA and vitamin E in children with behavioral disorders.

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Attention-Deficit/Hyperactivity Disorder (AD/HD) is the most prevalent psychiatric disorder in children. The behavioral symptoms include inattention and/or impulsivity and hyperactivity (1). AD/HD is thought to arise from multiple causes involving both biological and environmental factors (2–8). In addition to their behavioral abnormalities, children with AD/HD are also reported to exhibit a variety of other associated problems (3), including a higher frequency of health problems than children without AD/HD (9,10). Persons with AD/HD have also been reported to require more medical care than those without AD/HD (11). Among the symptoms that children with behavioral problems or AD/HD reportedly exhibit are several that occur in EFA deficiency (12,13). These symptoms include excessive thirst, frequent urination, dry skin, dry hair, dandruff, brittle nails, and/or hyperfollicular keratoses. Thirst/skin symptoms and blood EFA abnormalities also have been reported in patients suffering from allergy (14–19), rheumatoid arthritis (20,21), end-stage renal disease (22–24), Sjogren's syndrome (25,26), anorexia nervosa (27–29), and learning or psychiatric disorders (30–36). Although abnormal FA metabolism has been reported in all of these disorders, none of the EFA profiles, including anorexia nervosa (37), reveals a primary EFA deficiency, which is characterized by low proportions of n-6 and n-3 PUFA and increased 20:3n-9 in the blood phospholipids. Patients have shown improvements in their thirst/skin symptoms upon supplementation with n-6 and/or n-3 PUFA (14,22–24,38,39).

In previous reports we demonstrated that about 40% of children with AD/HD-type symptoms exhibited thirst/skin symptoms (40,41), and the proportion of arachidonic acid (AA) and DHA in their plasma phospholipids was significantly lower than that observed in other children with and without AD/HD-type symptoms. However, there was no evidence that those children had blood levels of EFA consistent with a primary EFA deficiency or that they consumed less PUFA in their diets than controls (42).

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Abbreviations: AA, arachidonic acid; AD/HD, Attention-Deficit/Hyperactivity Disorder; ALA, α-linolenic acid; ASQ, Conners' Abbreviated Symptom Questionnaire; ASQ-P, Conners' Abbreviated Symptom Questionnaire for Parents; ASQ-T, Conners' Abbreviated Sympton Questionnaire for Teachers; CPT, Conners' Continuous Performance Test; DBD Rating Scale, Disruptive Behavior Disorders Rating Scale; DGLA, dihomo-γ-linolenic acid; HRT, hit reaction time; ITT, intent-to-treat; RBC, red blood cells; WJ-R, Woodcock-Johnson Psycho-Educational Battery-Revised.

Members of both the n-3 and n-6 families of long-chain PUFA were found to be lower than controls in the plasma phospholipids of the children with thirst/skin symptoms and AD/HD-like symptoms (40). The n-3 FA are specifically implicated in maintaining central nervous system function and are known to affect neurotransmitters, peptides, releasing factors, and hormones in the brain (43,44). Animal studies have indicated that long-term n-3 FA deficiency is associated with behavioral abnormalities including decreased exploratory behavior in rats and increased total stereotypy and total locomotion in monkeys (45,46). The n-6 FA series serves as the source for eicosanoid biosynthesis, and deficiency leads to deleterious effects on growth, reproduction, and skin integrity (47).

EFA status and supplementation have been studied in adult psychiatric disorders. Epidemiological studies in the United States and other countries suggest an association between decreased dietary n-3 FA intake and the increase in depression that has been observed in recent years (48). Plasma and red blood cell (RBC) proportions of n-3 FA have been reported to be lower in depressed patients in comparison with healthy adults (49,50). Stoll *et al*. (51) reported that supplementing the diets of patients with bipolar disorder with n-3 FA-enriched PUFA led to a significant improvement in symptoms. Scientists have also studied EFA status and supplementation in patients with schizophrenia. Supplementing the diets of 20 patients hospitalized with chronic schizophrenia with fish oil led to a significant improvement in symptoms (52). However, not all studies using n-3 FA have shown positive effects. For example, supplementation with ethyl EPA did not improve symptoms in a study of patients with schizophrenia (53).

An early FA supplementation study using the n-6 GLA-rich primrose oil with children described as hyperactive reported minimal or no improvement (54). In two recently published double-blind, placebo-controlled intervention trials using n-3 FA-enriched supplements to treat children with AD/HD or AD/HD-like symptoms, contrasting results were reported. In the first, children with specific learning problems and AD/HD-like symptoms were given either a PUFA supplement, containing both n-6 and n-3 FA, or a placebo for 3 mon, and improvements were reported in the supplemented group on 7 of 14 scales for cognitive and behavioral assessments (55). In the second study, children accurately diagnosed with AD/HD consumed DHA-rich supplements for 4 mon with no benefits reported, despite an average 2.6-fold increase in the proportion of DHA in the plasma (56).

To further test whether PUFA supplementation might benefit children with behavioral problems, we report here the results of a pilot intervention trial using a supplement enriched in n-3 but also containing n-6 PUFA. The study sample included children under the care of a clinician for AD/HD and receiving standard therapies. Members of the sample were selected based on their higher frequency of skin/thirst symptoms because we hypothesized that this sample would be more likely to show benefits than a group of children not exhibiting any outward signs or symptoms associated with EFA deficiency.

METHODS

Study participants. Girls and boys ages 6–13 were recruited from the population of central Indiana within a 100-mi radius of West Lafayette. All children were screened by telephone interview with their parents or guardians. Both children and parents gave written permission to participate in the study and to send behavior questionnaires to the children's primary teachers. This study was approved by the Purdue University Committee on the Use of Human Subjects in Research. Children were selected whose parents reported them as diagnosed with AD/HD by their clinical psychologist, psychiatrist, or pediatrician. Parents were asked whether their children had any chronic health problems such as diabetes or kidney disease. Children with chronic health problems were excluded from the study. Parents were also asked to evaluate the following combination of symptoms: excessive thirst, frequent urination, dry hair, dry skin, brittle nails, dandruff, and/or follicular keratoses. This combination of symptoms was quantified using a questionnaire in which each symptom was rated by a parent on a 4-point scale: 0 = not at all, 1 = just a little, 2 = pretty much, and 3 = very much. The total thirst/skin score was calculated as the sum of the scores for each of the seven individual symptoms. Children with AD/HD symptoms were selected for the study only if their total thirst/skin score was 4 or greater. A score of 4 or more was chosen as the cutoff to reflect the presence of one or more severe symptoms or several mild symptoms. Furthermore, in a previous study of plasma phospholipids, AA and DHA concentrations were significantly lower in children with scores of 4 or more than in the controls or in children with AD/HD-like symptoms but few thirst/skin symptoms (40). In that study, 9.3% of controls had scores of 4 or more, whereas 39.6% of children with AD/HD had scores of 4 or more (40). The thirst/skin scores ranged from 0 to 8 in the controls and 0 to 12 in the children with AD/HD (40).

In the study reported here, healthy children without AD/HD-like symptoms and few thirst/skin symptoms were recruited to serve as a reference sample to compare blood FA and tocopherol values. For the intervention, subject recruitment included 193 inquiries regarding the study. Of these, 126 children with AD/HD-like symptoms were screened, and 93 of these children reported frequent thirst/skin symptoms. Participants were excluded from participation in the study for the following reasons: age, distance from the test site, inability to swallow capsules, lack of further interest, or chronic illness (diabetes). Fifty participants were randomized into the two treatment groups. Seventeen participants discontinued participation in the study at different time points after randomization. These included 7 who received the PUFA and 10 who received the placebo. Attrition of participants was due to difficulty contacting working parents, a change in family circumstances (e.g., relocation, divorce), and the refusal or a change in mind about taking supplements or having blood drawn. Analyses of the dropouts compared to the completers for both supplementation groups revealed no statistical differ-

ences for any of the baseline characteristics, thirst/skin symptoms, teacher or parent behavior evaluation scores, or blood FA proportions of AA, EPA, or DHA.

Intervention. The children with AD/HD and thirst/skin symptoms were randomized into two treatment groups, A and B, which were balanced for gender and medication status [none, methylphenidate, methylphenidate plus antidepressant (imipramine, amitriptyline), other medication (pemoline, dextroamphetamine salts, imipramine)]. In a double-blind design, group A (n = 25) received eight capsules of PUFA a day and group B (n = 25) received eight placebo capsules a day for a parallel period of 4 mon. The PUFA supplement (Efalex) was supplied by Efamol Ltd. (Trowbridge, United Kingdom). The FA content of the PUFA supplement (per capsule) was 60 mg DHA, 10 mg EPA, 5 mg AA, 12 mg GLA, and 3 mg vitamin E as a preservative. The placebo contained 0.8 g olive oil in each capsule. Olive oil was chosen as the placebo because it contains very little PUFA and consumption was not expected to affect the blood FA profile. The odor and appearance of the PUFA capsules and the placebo capsules were comparable.

Compliance was monitored by using a separate form completed by the parents that indicated the number of pills consumed each day by their child. Based on the forms returned at the end of the intervention, average compliance was 88%.

Assessment protocol. Fasting blood samples were drawn at baseline and after 2 and 4 mon of treatment. Analysis of the EFA composition of plasma and RBC membranes was conducted as described previously (57–59) with one procedural difference. The methanolysis was carried out using tetramethylguanidine (60). The α-tocopherol concentrations in the plasma and RBC were determined as described previously (61). To assess the effect of supplementation on thirst and skin symptoms, a questionnaire was completed by the parents at baseline and at 0.5, 1, 2, 3, and 4 mon of supplementation.

At baseline and at the end of the intervention period, both parents and teachers completed the Conners' Abbreviated Symptom Questionnaires (ASQ) (62) and the Disruptive Behavior Disorders (DBD) Rating Scale (65), which were the primary outcome measures. Two additional neuropsychological tests were administered: the Conners' Continuous Performance Test (CPT) (66) and eight tests of cognitive ability using the Woodcock-Johnson Psycho-Educational Battery-Revised (WJ-R) (67). The ASQ was chosen as a primary subjective outcome assessment tool because of its widespread clinical use, utility in frequent assessment of progress, and high validity (63,64). The parents' DBD Rating Scale was also chosen as a primary subjective outcome measure because this instrument contains questions that are phrased differently from the ASQ. The DBD Rating Scale assesses four types of behavior: hyperactivity, attention, conduct, and oppositional defiant behavior. If a child was on medication, parents were asked to evaluate their child's behavior off stimulant medication but on any long-acting medications. Teachers also completed the DBD Rating Scale for children in the school environment, and their ratings were for children on medication for those subjects taking any psychostimulant or nonstimulant treatment. For both the ASQ and the DBD Rating Scale, higher scores are indicative of poorer performance.

The CPT and WJ-R Tests of Cognitive Ability were chosen as objective measures of outcome assessment. The CPT is a computer task that requires the subject to hit the space bar every time a letter flashes onto the screen, except for the letter X. It was chosen as an objective measure of inattention and impulsivity (3,66) and also because previous studies have shown that performance on this test is sensitive to interventions that improve attention/behavior (68). The test lasts for over 14 min and children with AD/HD have a difficult time maintaining attention throughout the entire test. The CPT Index reflects overall performance (66); a score of less than 8 is in the normal range, 8 to 11 is borderline, and greater than 11 is considered poor performance. Hit reaction time (HRT) is one of the components of the test that is measured; it represents the elapsed milliseconds between the letter flashing on the screen and the pressing of the space bar. This time is converted into a T-score that represents how each subject performed compared to a large comparative study group. Slow speeds reflect abnormal performance and are indicated by T-scores that are low. Because the test is sensitive to medication status (69,70), we requested that participants refrain from taking stimulant-type medications on the test day. Children on long-term medications did not alter their dosage for the test, and the number of subjects in each group was balanced.

The WJ-R Tests of Cognitive Ability were chosen to assess short-term memory, processing speed, auditory processing, and visual processing (71) because abnormal FA metabolism has been reported to affect perceptual function (72); thus, it was hypothesized that these tests would be sensitive to FA supplementation. Also, a deficit in EFA in primates has been shown to result in impaired visual processing (73), and a previous intervention study with hyperactive children indicated that supplementation with GLA improved the accuracy of short-term memory and response time in a memory distraction task (54). Two different tests for each parameter were conducted, and the results were evaluated in comparison to normalized data for age categories. The results from the two tests were combined to provide an evaluation presented as standard scores, with scores of 100 representing optimal performance for a given age, gender, and educational level.

Parents of the children completed 3-d diet records at baseline and 4 mon after a detailed explanation by investigators using food models to illustrate serving sizes. These records were analyzed for macro and micro components by using the University of Minnesota Nutrition Data System (NDS 95, version 2.8; University of Minnesota, Minneapolis, MN).

Subjects and statistical analysis. Primary analyses were conducted on those subjects who completed the 4-mon intervention and had a minimum compliance of 75%. The total number of subjects at the conclusion of the study was 33—18 in the PUFA group and 15 in the placebo group. These results are reported in the tables. Secondary analyses also were performed using an intent-to-treat (ITT) basis, with the last ob-

servation carried forward for all subjects who were randomized and who had taken at least the first dose of the supplements (74,75). Three of the 50 randomized participants had not taken a single dose; thus, the ITT analysis was based on 47 participants. These ITT results are reported in the tables and discussed, where appropriate, in the text. The baseline characteristics of the two treatment groups were compared by using Student's *t*-test for continuous variables and Fisher's exact test for noncontinuous data. Differences in blood FA composition between the intervention sample and the reference sample were compared using Student's *t*-test. The effect of supplements on FA proportions and α-tocopherol was tested by repeated-measures ANOVA using general linear models followed by *post hoc* testing on differences between means using Tukey's studentized range test, which adjusts $\alpha = 0.05$ for multiple comparisons. The assumptions of normal distribution and homogeneity of variance were met for these analyses. Both the total thirst/skin scores and the behavioral data were converted to the percentage change from baseline scores, [(baseline − 4 mon)/baseline]·100, and the effects of FA supplementation were tested using the Kruskal–Wallis nonparametric test for group differences and the Wilcoxon signed rank test for within-group differences. The associations between the percentage change in blood FA as well as tocopherol and the percentage change in scores on behavioral assessment tests at the conclusion of the study were evaluated using Spearman correlations. These statistical analyses were carried out using SAS (SAS Institute, Cary, NC), with the level of significance at $P < 0.05$.

RESULTS

Sample description. Table 1 summarizes the characteristics at baseline of those participants who were randomized and who had consumed at least one dose of the supplement. No statistically significant differences were found between the two treatment groups with respect to age, height, gender, medication status, frequency of thirst/skin symptoms, or nutrient intake. Mean weight was greater in the placebo group, but the variation was large and no statistical differences were observed. In comparing the two groups for baseline behavioral assessment scores, the ASQ for parents (ASQ-P), the DBD Attention score for parents and the HRT for the CPT were significantly different between the two treatment groups (Table 1). As shown in Table 1, the remaining teacher and parent tests, CPT Index, and WJ-R tests were not different.

Participants with AD/HD-like symptoms and reporting thirst/skin symptoms had significantly lower mean proportions of dihomo-γ-linolenic acid (DGLA, 20:3n-6) ($P < 0.001$), AA (20:4n-6) ($P < 0.006$), α-linolenic acid (ALA, 18:3n-3) ($P < 0.001$), EPA (20:5n-3) ($P < 0.04$), DHA (22:6n-3) ($P < 0.002$), ∑n-3 ($P < 0.02$), and the Δ6-desaturase index ($P < 0.03$) in plasma polar lipids than in the reference sample. Also, the intervention sample differed from the reference sample in the RBC FA composition (Table 2). The mean proportions of linoleic acid ($P < 0.03$) and ALA ($P < 0.02$), the ratio of ∑n-6/∑n-3 ($P < 0.001$), and the Δ6-desaturase index ($P < 0.001$) were significantly lower in the children with AD/HD-like symptoms in comparison with the control group. However, RBC AA ($P < 0.001$), 22:4n-6 ($P < 0.001$), 22:5n-6 ($P < 0.008$), ∑n-6 ($P < 0.001$), EPA ($P < 0.001$), 22:5n-3 ($P < 0.002$), DHA ($P < 0.001$), and ∑n-3 ($P < 0.001$) were significantly higher in the intervention sample in comparison with the reference sample.

Effect of supplementation on blood FA composition. At baseline, no significant differences between those participants receiving the PUFA and those receiving the placebo were observed for plasma and RBC FA (Tables 3 and 4). Several significant changes occurred in plasma and RBC FA composition during the period of supplementation. For both treatment groups, the proportion of plasma AA increased significantly by the fourth month of supplementation with no difference between groups. PUFA supplementation led to a dramatic decrease in both 22:4n-6 and 22:5n-6 by the fourth month. For the n-3 FA series, the proportion of ALA increased almost twofold, and a greater than twofold increase in DHA was observed in the plasma of the children in the PUFA-supplemented group. A great deal of variation was found in the proportion of EPA with supplementation. The ANOVA analysis revealed within-group effects but no between-group effects. The mean proportion of EPA increased in both groups, but no significant difference between the groups was found. In the olive oil placebo group, the content of ALA in the plasma phospholipids increased twofold by the end of the study, but increases in both EPA and DHA were not significant. As a result of supplementation, the ratio of total n-6/total n-3 FA decreased in the PUFA-supplemented group but did not change in the olive oil placebo group.

For saturated FA, both olive oil supplementation and PUFA supplementation led to significant decreases in the proportion of 16:0 but increases in the proportion of 18:0. Correspondingly, the proportion of 18:1n-9 was found to be significantly lower at the end of the supplementation period in the plasma phospholipids of both groups. This occurred in the olive oil placebo group despite the fact that the children in this group were consuming almost five additional grams of oleic acid from the supplement.

For the RBC, similar changes in n-3 FA were observed in the PUFA-supplemented group (Table 4), with twofold increases observed in the proportion of EPA and DHA, and approximately a 50% increase in the proportion of total n-3 FA. Also, the proportion of total n-6 FA and the ratio of total n-6/total n-3 in the RBC decreased dramatically in the PUFA-supplemented group. However, in contrast to the plasma results, the proportion of AA in the RBC decreased in both treatment groups, and the magnitude of the decrease was significantly greater in the PUFA-supplemented group. Unlike the plasma phospholipids, the proportion of 18:0 did not change with supplementation and the proportion of 16:0 increased slightly rather than decreased. Also, no change in the proportion of 18:0 was observed for either group.

Effect of supplementation on blood vitamin E concentration. The PUFA supplement contained 3 mg of added tocoph-

TABLE 1
Characteristics of Two Treatment Groups at Baseline[a]

Characteristic	Olive oil (n = 22)	PUFA (n = 25)	P
Age (yr)[b]	10.1 ± 2.0	9.5 ± 1.7	NS
Height (in.)	59.0 ± 10.2	55.4 ± 5.5	NS
Weight (lb)	91.2 ± 39.4	73.9 ± 23.5	NS
Medication (# of subjects)			
None	5	5	NS
Methylphenidate	12	13	NS
Methylphenidate + other	3	3	NS
Other	2	4	NS
Gender (% female)	13.3	11.1	NS
Total thirst/skin symptoms score[b]	8.8 ± 3.5	8.4 ± 3.2	NS
Nutrient intake[b,c]			
Energy (kcal)	2248 ± 690.5	2054.7 ± 484.2	NS
Total fat (g)	79.7 ± 24.1	77.9 ± 21.7	NS
Total carbohydrate (g)	313.4 ± 125.7	270.6 ± 73.8	NS
Total protein (g)	77.7 ± 26.7	73.8 ± 25.1	NS
Iron (mg)	17.6 ± 7.8	15.3 ± 6.7	NS
Zinc (mg)	11.3 ± 4.7	10.5 ± 4.0	NS
SFA (g)	29.0 ± 9.4	28.9 ± 7.7	NS
MUFA (g)	30.3 ± 9.1	29.4 ± 7.7	NS
PUFA (g)	14.6 ± 5.3	14.1 ± 5.6	NS
Linoleic acid (g)	13.1 ± 4.8	12.8 ± 5.3	NS
Linolenic acid (g)	1.3 ± 0.5	1.1 ± 0.4	NS
Behavioral assessments[b]			
ASQ-Parents	19.9 ± 4.5	16.5 ± 4.9	0.004
ASQ-Teachers	13.1 ± 7.7	10.5 ± 7.4	NS
DBD Questionnaires (Parents)			
Hyperactivity	23.1 ± 5.1	20.6 ± 4.7	NS
Inattention	24.2 ± 2.9	19.5 ± 4.2	
0.0001			
Conduct	8.2 ± 5.3	7.7 ± 5.2	NS
Oppositional/Defiant Disorder	17.3 ± 5.1	14.5 ± 5.4	NS
DBD Questionnaires (Teachers)			
Hyperactivity	10.1 ± 7.7	10.5 ± 8.9	NS
Attention	15.1 ± 9.3	11.7 ± 8.8	NS
Conduct	1.8 ± 2.2	1.9 ± 3.9	NS
Oppositional/Defiant Disorder	6.9 ± 6.0	5.6 ± 5.9	NS
Attention performance: Conners' CPT			
CPT Overall Index	8.9 ± 6.3	8.4 ± 8.2	NS
Hit reaction time (ms)	459.0 ± 74.7	415.8 ± 64.4	0.03
Hit reaction time (T-scores)	35.7 ± 12.0	45.7 ± 8.8	0.002
Cognitive ability: Woodcock-Johnson Tests of Cognitive Ability[d]			
Processing speed	93.2 ± 15.5	99.8 ± 22.6	NS
Short-term memory	95.9 ± 15.9	101.4 ± 17.0	NS
Visual processing	104.8 ± 14.2	107.1 ± 16.1	NS
Auditory processing	96.4 ± 12.4	98.6 ± 12.8	NS

[a]Scores on Conners' Abbreviated Symptom Questionnaire (ASQ)-Parents, Disruptive Behavior Disorders (DBD) Inattention (Parents) and Conners' Continuous Performance Test (CPT) hit reaction time were also significantly different between groups using a completers-only analysis. No other score was significantly different.
[b]Mean ± SD. MUFA, monounsaturated FA; SFA, saturated FA; NS, not significant.
[c]Nutrient analysis of 3-d diet records. Placebo, n = 19; PUFA, n = 23.
[d]Standard scores.

erol acetate in addition to naturally occurring α-tocopherol (0.16 mg) and γ-tocopherol (0.10 mg) in each gel capsule. The olive oil placebo contained no tocopherol acetate but did contain small amounts of α-tocopherol (0.22 mg) and γ-tocopherol (0.009 mg). Supplementation with PUFA led to a greater than 50% increase in plasma and RBC α-tocopherol concentration and a significant decrease in γ-tocopherol concentration in comparison with supplementation with the olive oil placebo (Table 5).

Effect of supplementation on the frequency of thirst/skin symptoms. Parents of children in both the PUFA and olive oil groups reported a decrease in total thirst/skin symptoms but the percentage change from baseline was not significantly different based on either a completers or ITT analysis (Table 6).

TABLE 2
Blood FA Composition and Tocopherol Content (mean ± SD) of Children With AD/HD-like Symptoms and Frequent Thirst/Skin Symptoms vs. Control Children Without AD/HD-like Symptoms and Few Thirst/Skin Symptoms

	Plasma (area%)			RBC (area%)		
	Reference sample 1[a] (*n* = 24)	Intervention sample 2[b] (*n* = 50)	*P*-value	Reference sample 1[a] (*n* = 24)	Intervention sample 2[b] (*n* = 50)	*P*-value
16:0	16.04 ± 5.37	22.04 ± 3.73	0.001	15.82 ± 1.02	12.48 ± 4.29	0.001
18:0	15.70 ± 2.60	14.71 ± 2.44	NS	14.69 ± 0.62	15.01 ± 1.38	NS
18:1	11.70 ± 2.77	13.19 ± 6.27	NS	14.41 ± 0.63	14.13 ± 0.92	NS
18:2n-6	22.91 ± 4.26	21.30 ± 3.12	0.07	14.11 ± 1.00	13.32 ± 1.51	0.02
20:3n-6	3.67 ± 1.41	2.59 ± 0.67	0.001	2.14 ± 0.25	2.15 ± 0.52	NS
20:4n-6	10.52 ± 1.60	8.93 ± 2.52	0.006	18.16 ± 1.31	20.15 ± 2.39	0.001
22:4n-6	0.86 ± 0.46	0.66 ± 0.33	0.04	4.74 ± 0.61	5.66 ± 1.11	0.001
22:5n-6	0.64 ± 0.35	0.53 ± 0.26	NS	1.13 ± 0.14	1.29 ± 0.27	0.008
18:3n-3	0.46 ± 0.23	0.26 ± 0.17	0.001	0.13 ± 0.08	0.09 ± 0.06	0.02
20:4n-3	0.49 ± 0.66	0.46 ± 0.28	NS	0.17 ± 0.23	0.52 ± 0.62	0.009
20:5n-3	0.35 ± 0.36	0.31 ± 0.19	NS	0.31 ± 0.13	0.39 ± 0.07	0.001
22:5n-3	0.95 ± 0.51	0.76 ± 0.28	0.04	2.22 ± 0.28	2.61 ± 0.57	0.002
22:6n-3	2.16 ± 0.64	1.74 ± 0.48	0.002	2.88 ± 0.40	3.52 ± 0.83	0.001
∑n-6	38.58 ± 3.99	34.00 ± 4.64	0.001	40.29 ± 1.32	42.56 ± 3.07	0.001
∑n-3	4.55 ± 1.92	3.76 ± 0.92	0.01	5.72 ± 0.66	7.12 ± 1.55	0.001
Δ6-Desat index[c]	1.54 ± 0.50	2.10 ± 1.19	0.03	0.55 ± 0.06	0.46 ± 0.09	0.001
∑n-6/∑n-3	9.52 ± 2.97	9.46 ± 2.36	NS	7.14 ± 0.92	6.2 ± 1.11	0.001
Tocopherols						
α-Tocopherol (μg/mL)	7.77 ± 2.15	8.33 ± 2.59	NS	5.11 ± 0.97	4.59 ± 1.08	0.05
γ-Tocopherol (μg/mL)	1.59 ± 0.56	1.81 ± 0.49	NS	1.63 ± 0.40	1.45 ± 0.38	NS

[a]Recruited reference sample without Attention-Deficit/Hyperactivity Disorder (AD/HD)-like symptoms and without thirst/skin symptoms. RBC, red blood cells; for other abbreviation see Table 1.
[b]Recruited sample with AD/HD-like symptoms and thirst/skin symptoms for intervention.
[c]Ratio of 18:2n-6/(18:3n-6 + 20:3n-6 + 20:4n-6 + 22:4n-6 + 22:5n6).

However, within the olive oil group the change in scores from baseline to 4 mon decreased 28% ($P < 0.005$), and in the PUFA group the decrease was 44.3% ($P < 0.0002$). Individual thirst/skin symptoms were also analyzed for treatment and within-group changes. At baseline there were no differences in the frequency of any thirst/skin symptoms between groups.

TABLE 3
FA Composition in Plasma at Baseline, 2 mon, and 4 mon (area% of total FA)[a–c]

	Olive oil (*n* = 15)			PUFA (*n* = 18)		
Plasma FA	Baseline	2 mon	4 mon	Baseline	2 mon	4 mon
16:0	20.75 ± 3.67^{x}	18.34 ± 3.67^{x}	11.31 ± 3.38^{y}	22.42 ± 2.74^{x}	20.06 ± 2.01^{x}	10.62 ± 3.41^{y}
18:0	14.93 ± 1.43^{x}	14.10 ± 1.37^{x}	16.80 ± 1.52^{y}	13.61 ± 2.3^{x}	14.64 ± 0.92^{x}	16.58 ± 1.25^{y}
18:1n-9	12.02 ± 3.55	10.65 ± 1.17	9.94 ± 1.36	15.13 ± 9.41^{x}	8.52 ± 1.17^{y}	8.65 ± 1.49^{y}
18:2n-6	20.05 ± 3.77	22.29 ± 1.87	22.12 ± 2.38	21.75 ± 2.61	22.08 ± 2.45	21.37 ± 4.07
20:3n-6	2.80 ± 0.57^{x}	2.83 ± 0.36^{x}	3.80 ± 0.76^{y}	2.31 ± 0.71^{x}	2.49 ± 0.52^{x}	3.60 ± 1.02^{y}
20:4n-6	9.82 ± 2.27^{x}	10.77 ± 1.96^{x,y}	12.53 ± 1.88^{a,y}	8.26 ± 3.07^{x}	9.01 ± 1.28^{x,y}	10.63 ± 1.24^{b,y}
22:4n-6	0.75 ± 0.23	0.69 ± 0.16^{a}	0.66 ± 0.34^{a}	0.61 ± 0.42^{a,x}	0.38 ± 0.18^{b,x,y}	0.15 ± 0.25^{b,y}
22:5n-6	0.59 ± 0.15	0.49 ± 0.17^{a}	0.44 ± 0.27^{a}	0.52 ± 0.39^{x}	0.24 ± 0.17^{b,y}	0.02 ± 0.08^{b,z}
18:3n-3	0.28 ± 0.21^{x}	0.35 ± 0.08^{x}	0.59 ± 0.31^{y}	0.25 ± 0.12^{x}	0.35 ± 0.09^{x,y}	0.47 ± 0.26^{y}
20:5n-3	0.39 ± 0.23	0.34 ± 0.17	0.61 ± 0.49	0.25 ± 0.12^{x}	0.59 ± 0.22^{y}	0.70 ± 0.52^{y}
22:5n-3	0.83 ± 0.29	0.83 ± 0.20	0.81 ± 0.40^{a}	0.70 ± 0.29	0.66 ± 0.11	0.46 ± 0.49^{b}
22:6n-3	1.90 ± 0.62	2.05 ± 0.50^{a}	2.70 ± 0.54^{a}	1.65 ± 0.37^{x}	5.51 ± 1.32^{b,y}	7.65 ± 3.17^{b,z}
∑n-6	34.01 ± 4.50^{x}	37.07 ± 3.01^{x,y}	39.55 ± 2.78^{y}	33.43 ± 5.69	34.21 ± 2.72	35.76 ± 3.67
∑n-3	4.12 ± 0.96	3.66 ± 0.73^{a}	5.17 ± 1.21^{a}	3.62 ± 0.76^{x}	7.16 ± 1.52^{b,y}	9.77 ± 3.05^{b,z}
∑n-6/n-3	8.61 ± 0.04^{x,y}	10.48 ± 2.05^{a,y}	8.19 ± 2.73^{a,x}	9.38 ± 1.42^{x}	5.05 ± 1.45^{b,y}	4.06 ± 1.56^{b,y}

[a]Mean ± SD. Variables were analyzed using repeated-measures ANOVA. Tukey's studentized range test was used to determine statistical differences between means for time and treatment effects. Treatment comparisons are indicated by ^{a,b} and time comparisons by $^{x–z}$ within each group. Means with the same superscripts are not different. $P < 0.05$.
[b]Completers analysis ($n = 33$) GROUP*TIME interactions: 18:0, $P < 0.04$; 18:1, $P < 0.04$; 22:4n-6, $P < 0.06$; 22:5n-6, $P < 0.009$; 22:6n-3, $P < 0.0001$; ∑n-3, $P < 0.0001$; ∑n-6/∑n-3, $P < 0.0001$.
[c]Intent-to-treat analysis ($n = 47$) GROUP*TIME interactions: 18:0, $P < 0.08$; 18:1, $P < 0.07$; 22:4n-6, $P < 0.03$; 22:5n-6, $P < 0.002$; 22:6n-3, $P < 0.0001$; ∑n-3, $P < 0.0001$; ∑n-6/∑n-3, $P < 0.0001$.

TABLE 4
FA Composition of RBC at Baseline, 2 mon, and 4 mon (area% of total FA)[a–c]

	Olive oil (n = 15)			PUFA (n = 18)		
RBC FA	Baseline	2 mon	4 mon	Baseline	2 mon	4 mon
16:0	14.68 ± 3.03	15.39 ± 1.35	16.56 ± 1.09	11.30 ± 4.80^{x}	15.25 ± 0.99^{y}	16.66 ± 0.83^{y}
18:0	14.61 ± 0.76	14.63 ± 1.04	14.47 ± 0.82	14.96 ± 1.78	14.33 ± 0.59	14.32 ± 0.77
18:1n-9	14.19 ± 0.95	14.58 ± 0.63	14.85 ± 1.24	14.22 ± 1.15^{x}	13.58 ± 0.99^{y}	14.36 ± 0.84^{y,z}
18:2n-6	13.22 ± 1.72	13.30 ± 1.26	13.78 ± 1.54	13.04 ± 1.55^{x}	13.51 ± 0.95^{x,y}	13.97 ± 1.05^{y}
20:3n-6	1.91 ± 0.28	1.87 ± 0.17	1.89 ± 0.23	2.10 ± 0.50^{x}	1.73 ± 0.31^{y}	1.77 ± 0.34^{y}
20:4n-6	19.39 ± 1.66	18.58 ± 1.61^{a}	18.17 ± 1.30^{a}	20.89 ± 3.01^{x}	16.71 ± 1.22^{b,y}	15.92 ± 1.21^{b,y}
22:4n-6	5.28 ± 0.77	5.05 ± 0.61^{a}	4.75 ± 0.44^{a}	5.94 ± 1.27^{x}	3.77 ± 0.51^{b,y}	3.16 ± 0.52^{b,y}
22:5n-6	1.20 ± 0.15^{a}	1.10 ± 0.11^{a}	1.03 ± 0.09^{a}	1.36 ± 0.31^{b,x}	0.90 ± 0.11^{b,y}	0.78 ± 0.09^{b,y}
18:3n-3	0.10 ± 0.07^{x}	0.21 ± 0.04^{y}	0.29 ± 0.17^{y}	0.10 ± 0.07^{x}	0.16 ± 0.07^{x,y}	0.17 ± 0.07^{y}
20:5n-3	0.39 ± 0.04	0.38 ± 0.07^{a}	0.40 ± 0.08^{a}	0.41 ± 0.08^{x}	0.69 ± 0.17^{b,y}	0.75 ± 0.19^{b,y}
22:5n-3	2.38 ± 0.32	2.19 ± 0.34	2.17 ± 0.33	2.79 ± 0.72^{x}	2.00 ± 0.23^{y}	1.91 ± 0.20^{y}
22:6n-3	3.39 ± 0.78	3.36 ± 0.54^{a}	3.13 ± 0.54^{a}	3.67 ± 0.83^{x}	7.18 ± 1.04^{b,y}	8.08 ± 1.56^{b,z}
∑n-6	41.00 ± 2.38^{a}	39.91 ± 2.20^{a}	39.62 ± 1.67^{a}	43.32 ± 3.80^{b,x}	36.61 ± 1.99^{b,y}	35.59 ± 1.96^{b,y}
∑n-3	6.72 ± 1.13	6.31 ± 0.81^{a}	6.15 ± 0.74^{a}	7.45 ± 1.62^{x}	10.03 ± 1.17^{b,y}	11.14 ± 1.62^{b,y}
∑n-6/∑n-3	6.25 ± 0.99	6.43 ± 0.89^{a}	6.54 ± 0.93^{a}	6.02 ± 1.07^{x}	3.71 ± 0.55^{b,y}	3.28 ± 0.64^{b,y}

[a]Mean ± SD. Variables were analyzed using repeated-measures ANOVA. Tukey's studentized range test was used to determine statistical differences between means for time and treatment effects. Treatment comparisons are indicated by ^{a,b} and time comparisons by $^{x–z}$ within each group. Means with the same superscripts are not different. $P < 0.05$. For abbreviation see Table 2.
[b]Completers analysis (n = 33) GROUP*TIME interactions: 16:0, $P < 0.027$; 18:1n-9, $P < 0.006$; 20:3n-6, $P < 0.0006$; 20:4n-6, $P < 0.0001$; 22:4n-6, $P < 0.0001$; 22:5n-6, $P < 0.0001$; 20:5n-3, $P < 0.0001$; 22:5n-3, $P < 0.0004$; 22:6n-3, $P < 0.0001$; ∑n-6, $P < 0.0001$; ∑n-3, $P < 0.0001$; ∑n-6/∑n-3, $P < 0.0001$.
[c]Intent-to-treat analysis (n = 47) GROUP*TIME interactions: 16:0, $P < 0.07$; 18:1, $P < 0.01$; 20:3n-6, $P < 0.001$; 20:4n-6, $P < 0.001$; 22:4n-6, $P < 0.0001$; 22:5n-6, $P < 0.0001$; 20:5n-3, $P < 0.0001$; 22:5n-3, $P < 0.0001$; 22:6n-3, $P < 0.0001$; ∑n-6, $P < 0.001$; ∑n-3, $P < 0.0001$; ∑n-6/∑n-3, $P < 0.0001$.

A treatment effect was observed for frequent urination scores that decreased more in the PUFA group (29.7%) ($P < 0.05$), but no other group differences were observed. Within group changes, dry skin scores improved 45.6% in the olive oil group ($P < 0.004$) and 50.1% in the PUFA group ($P < 0.0002$). Dry hair scores improved 36.5% ($P < 0.13$) in the olive oil group and improved 42.6% ($P < 0.004$) in the PUFA group. Although excessive thirst scores improved 33.3% in the PUFA group, this was not significant ($P < 0.06$). There were no within-group differences for dandruff, brittle nails, or follicular hyperkeratosis.

Effects of supplementation on behavior and cognitive functions. The effects of PUFA supplementation on scores for multiple behavioral measures are summarized in Table 6. The primary subjective behavioral outcome measures chosen were the ASQ-P and the DBD Rating Scale for parents. Both treatment effects and within-group changes were evaluated. There was a significant decrease in the ASQ-P score from baseline within both the olive oil group (16.4 %, $P < 0.02$) and the PUFA group (23.2%, $P < 0.006$), but no treatment effect was observed.

A significant improvement in scores at 4 mon compared with baseline was observed within both supplementation groups for the DBD assessment by parents of hyperactivity (37.8–40.2% decrease), attention (29.3–34.7% decrease), and oppositional/defiant behavior (24.3–28.8% decrease), but no treatment effect was observed (Table 6). In contrast, for parents' DBD assessment of conduct, only the PUFA-supplemented group showed significant improvement (42.7%

TABLE 5
Tocopherol Comparisons in Plasma and RBC at Baseline, 2 mon, and 4 mon (μg/mL)[a–c]

	Olive oil (n = 15)			PUFA (n = 18)		
Tocopherols	Baseline	2 mon	4 mon	Baseline	2 mon	4 mon
Plasma α-tocopherol (μg/mL)	8.55 ± 2.12	9.02 ± 2.91^{a}	8.12 ± 2.65^{a}	8.37 ± 1.85^{x}	13.38 ± 3.80^{b,y}	13.05 ± 2.87^{b,y}
Plasma γ-tocopherol (μg/mL)	1.74 ± 0.65^{x}	0.98 ± 0.48^{a,y}	1.01 ± 0.51^{a,y}	2.03 ± 0.45^{x}	0.61 ± 0.39^{b,y}	0.30 ± 0.23^{b,y}
RBC α-tocopherol (μg/mL)	4.65 ± 1.12	6.21 ± 2.46	5.57 ± 1.59^{a}	4.23 ± 0.87^{x}	7.86 ± 2.48^{y}	8.70 ± 1.63^{b,y}
RBC γ-tocopherol (μg/mL)	1.40 ± 0.37	1.54 ± 0.51^{a}	1.81 ± 0.49^{a}	1.44 ± 0.40^{x}	0.83 ± 0.31^{b,y}	0.92 ± 0.30^{b,y}

[a]Mean ± SD. Variables were analyzed using repeated-measures ANOVA. Tukey's studentized range test was used to determine statistical differences between means for time and treatment effects. Treatment comparisons are indicated by ^{a,b} and time comparisons by $^{x–z}$ within each group. Means with the same superscripts are not different. $P < 0.05$. For abbreviation see Table 2.
[b]Completers analysis (n = 33) GROUP*TIME interactions: plasma α-tocopherol, $P < 0.0001$; plasma γ-tocopherol, $P < 0.0001$; RBC α-tocopherol, $P < 0.0001$; RBC γ-tocopherol, $P < 0.0001$.
[c]Intent-to-treat analysis (n = 47) GROUP*TIME interactions: plasma α-tocopherol, $P < 0.001$; plasma γ-tocopherol, $P < 0.0001$; RBC α-tocopherol, $P < 0.0005$; RBC γ-tocopherol, $P < 0.0001$.

TABLE 6
Percentage Change in Scores from Baseline to 4 mon for PUFA and Olive Oil for Completers[a]

	Absolute change (range)	Percentage change[b]	*P* for difference within group	*P* for difference between groups (ITT)[c]
Parent scores				
Thirst/skin symptoms total score				
Olive oil	2.9 (–2 to14)	28.0	0.005	0.25 (0.08)
PUFA	2.9 (–1 to 9)	44.3	0.0002	
ASQ-Parents				
Olive oil	2.9 (–3 to 9)	16.4	0.02	0.29 (0.19)
PUFA	4.3 (–3 to12)	23.2	0.006	
DBD Hyperactivity				
Olive oil	8.5 (1 to 20)	37.8	0.0001	0.68 (0.28)
PUFA	7.9 (3 to 19)	40.2	0.0001	
DBD Attention				
Olive oil	6.6 (0 to 18)	29.3	0.0002	0.46 (0.24)
PUFA	6.8 (0 to 15)	34.7	0.0001	
DBD Conduct				
Olive oil	2.1 (–3 to 9)	9.9	0.09	0.08 (0.05)
PUFA	3.4 (0 to17)	42.7	0.0005	
DBD Oppositional/Defiant Disorder				
Olive oil	4.5 (–7 to14)	24.3	0.02	0.88 (0.55)
PUFA	4.4 (–4 to 20)	28.8	0.002	
Teacher scores (n = 26)[d]				
ASQ-Teachers				
Olive oil	1.9 (–3 to 8)	–2.5	0.71	1.0 (0.94)
PUFA	1.4 (–9 to11)	12.3	0.29	
DBD Hyperactivity				
Olive oil	–0.4 (–14 to 6)	44.3	0.92	0.71 (0.97)
PUFA	2.5 (–9 to18)	44.9	0.32	
DBD Attention				
Olive oil	0.2 (–6 to 9)	–3.4	1.00	0.09 (0.03)
PUFA	4.6 (–2 to17)	14.8	0.009	
DBD Conduct				
Olive oil	0.0 (–1 to 1)	1.9	1.00	0.69 (0.41)
PUFA	0.2 (–5 to 5)	–18.2	0.74	
DBD Oppositional/Defiant Disorder				
Olive oil	0.1 (–4 to 4)	–2.3	0.96	0.53 (0.19)
PUFA	0.8 (–10 to14)	–1.3	0.53	
Conners' CPT (n = 32)				
CPT Index[e]				
Olive oil	–3.9 (-12 to 6)	—	0.006	0.28 (0.13)
PUFA	–0.2 (–15 to 21)	—	0.99	
Hit reaction time (ms)				
Olive oil	–12.3 (–85.5 to 71.8)	–2.4	0.43	0.05 (0.05)
PUFA	25.1 (–63.2 to 105.8)	5.6	0.08	
Hit reaction time (T-score)				
Olive oil	3.1 (–11.3 to 11.2)	12.6	0.12	0.02 (0.12)
PUFA	–3.2 (–19.0 to 13.2)	–9.08	0.16	
Woodcock-Johnson Psycho-Educational Battery-Revised				
Processing speed				
Olive oil	–0.1 (–8 to13)	–1.0	0.69	0.54 (0.66)
PUFA	0.9 (–11 to17)	0.9	0.56	
Short-term memory				
Olive oil	–0.8 (–10 to17)	0.9	0.96	0.55 (0.96)
PUFA	–1.4 (–20 to 26)	–1.5	0.68	
Visual processing				
Olive oil	–4.9 (–23 to10)	–3.7	0.09	0.69 (0.88)
PUFA	–3.7 (–21 to16)	–4.3	0.19	
Auditory processing				
Olive oil	–5.1 (–23 to14)	–5.3	0.09	0.86 (0.74)
PUFA	–5.4 (–21 to 7)	–5.8	0.02	

[a]n = 33 unless otherwise stated. For abbreviations see Table 1.
[b]Percentage change is calculated as (value at baseline – value at 4 mon)/(value at baseline) × 100, using mean values. A positive percentage change indicates improvement, except for CPT (T-scores only) and Woodcock-Johnson tests, which are based on standard scores.
[c]*P*-value completers (*P*-value intent-to-treat).
[d]Teacher evaluations were completed at 2.5–3 mon.
[e]Simple change rather than percentage change due to some zero values at baseline.

decrease, $P < 0.01$); the treatment effect for the completers analysis was not significant ($P < 0.08$), whereas the ITT analysis was significant ($P < 0.05$).

The teachers' assessment of behavior using the ASQ for teachers (ASQ-T) and DBD questionnaires were chosen as secondary outcome measures for several reasons. First, the teachers interacted mostly with the participants on medication; thus, their evaluation of each child's behavior did not agree with that of the parents. Second, the teachers' final assessments during the intervention occurred at the end of the school year, which was a month prior to the end of the intervention period. Finally, data collection at the end of the study was incomplete for the teachers. A treatment effect for the teachers' DBD attention subscale was not significant for the completers but was significant based on the ITT analysis ($P < 0.03$). Within the PUFA group, the attention score improved 14.8% ($P < 0.009$), with no change in the olive oil group. None of the other teacher DBD and ASQ-T outcome measures was affected by supplementation.

For the CPT tests, differences were observed within each group. The average CPT Index score for the children on PUFA supplementation did not change from baseline to 4 mon, whereas that for the placebo group increased ($P < 0.006$). A deterioration in performance on this objective outcome measure was not unexpected, as the CPT often shows a negative practice effect (66). No treatment effect was observed on the CPT Index. Of the components of the test compiled in the index, only HRT showed a treatment effect ($P < 0.05$, completers and ITT). HRT is a highly sensitive index of how quickly targets are processed (66), and a slow HRT is indicative of inattention (76). For the WJ-R tests of cognitive skills, auditory processing improved 5.8% ($P < 0.02$) in the PUFA group. No other group or within-group differences were found.

Effect of supplementation on clinical outcomes. In addition to total scores, the DBD Rating Scale results were also evaluated based on diagnostic criteria, which is typically how these assessment tools are used in clinical practice. The results are summarized in Table 7. At baseline there were no significant differences between the groups in terms of the proportion of participants with scores in the clinical range for each subscale for either the parents or the teachers. With supplementation, treatment effects were observed for the parents' Oppositional/Defiant Disorder subscale. No other clinical outcome was affected by PUFA supplementation.

Association between FA composition and performance on behavioral measures. Because this pilot study was designed, in part, to evaluate the relationship between proportions of PUFA in the blood and the severity of behavioral symptoms, we compared the changes in the proportion of FA and tocopherol in the erythrocytes with the changes in scores on behavioral outcomes. This was done using correlations between the percentage change from baseline, [(baseline − 4 mon)/baseline]·100, for each PUFA and behavioral assessment. Pearson correlations were conducted on normally distributed data and Spearman correlations for the rest not normally distributed, as indicated in Table 8. A negative correlation between a FA or α-tocopherol and a behavioral score indicates that a positive change in the biochemical parameter is associated with a negative change in the behavioral parameter, whereas a positive correlation indicates the reverse. Negative changes signify improvement for all parameters except the Woodcock–Johnson tests. For this outcome measure, increasing values signify better performance. The percentage change in ASQ-P scores was negatively correlated with the percentage change in RBC EPA and positively correlated with RBC AA. Although no correlations were significant for the parents' DBD

TABLE 7
Effects of Supplementation on Clinical Outcomes

	Baseline[a]			4 mon[b]		
	Olive oil (%) $n = 15$	PUFA (%) $n = 18$	P-value[c]	Improved olive oil	Improved PUFA	P-value[c]
Parents DBD[d]						
Hyperactivity	12 (80)	14 (78)	NS	5	9	NS
Attention	13 (86.7)	13 (72)	NS	4	9	0.09
Conduct	4 (26.7)	5 (27.8)	NS	1	5	NS
Oppositional/Defiant Disorder	11 (73.3)	12 (66.7)	NS	3	8	0.02
	$n = 9$	$n = 17$	3 mon[b]			
Teachers DBD[d]						
Hyperactivity	1 (11.1)	5 (29.4)	NS	1	4	NS
Attention	4 (44.4)	6 (35.3)	NS	1	4	NS
Conduct	0 (0)	0 (0)	NS	0	0	NS
Oppositional/Defiant Disorder	2 (22.2)	2 (11.8)	NS	1	1[e]	NS

[a]Participants meeting the criteria for clinical diagnoses based on parent and teacher DBD subscales at baseline.
[b]The number of participants who improved enough that they no longer met the criteria at the end of the study.
[c]Statistical analysis using Fisher's exact test.
[d]Diagnostic criteria were as follows: parents' or teachers' responses of 2 ("pretty much") or 3 ("very much") for at least six questions on the hyperactivity rating scale, at least six questions on the attention rating scale, at least three questions on the conduct rating scale, or at least four questions on the Oppositional/Defiant Disorder rating scale. For abbreviations see Table 1.
[e]Three participants did not meet criteria at baseline but did at 3 mon.

TABLE 8
Correlations[a] Between Percentage Change in EFA and Tocopherols in RBC and Percentage Change in Thirst/Skin Symptoms Score, Behavioral Assessments, and Cognitive Skills at 4 mon (*n* = 31)

	FA				
	AA	EPA	DHA	n-6/n-3	α-Tocopherol
Total thirst/skin symptoms score	–0.05	–0.35[a]	–0.39[a]	0.16	–0.17
ASQ-Parents	0.36[a]	–0.38[a]	–0.27	0.18	–0.23
Parents DBD rating scale					
Hyperactivity	0.16	–0.01	–0.08	–0.10	–0.06
Attention	0.05	–0.15	–0.19	0.08	–0.20
Conduct	0.01	–0.32	–0.20	0.18	–0.07
Oppositional/Defiant Disorder	–0.07	–0.14	–0.13	0.07	–0.21
ASQ-Teachers (*n* = 24)	0.11	0.03	0.08	–0.03	–0.51[a]
Teachers DBD Rating Scale (*n* = 24)					
Hyperactivity	0.12	–0.15	–0.20	0.14	-0.45[a]
Attention	0.17	–0.38	–0.49[a]	0.39	-0.60[b]
Conduct	0.19	–0.15	–0.28	0.13	-0.41[a]
Oppositional/Defiant Disorder	0.34	–0.32	–0.39	0.33	–0.54[b]
CPT (*n* = 30)					
Overall Index	–0.04	–0.02	–0.19	0.08	–0.27
Hit reaction time	0.32	–0.21	–0.31	0.31	–0.46[a]

[a]Spearman correlation coefficients: [a]$P \leq 0.05$, [b]$P \leq 0.01$. Pearson correlations were used when both blood and behavior data were normally distributed. AA, arachidonic acid; for other abbreviations see Table 1.

Rating Scale, all of the subscales of the teachers' DBD Rating Scale correlated negatively with the change in RBC α-tocopherol concentration. This was also true for the ASQ-T. Additionally, the Attention subscale of the teachers' DBD Rating Scale was negatively correlated with the percentage change in the proportion of RBC DHA. RBC α-tocopherol scores were negatively correlated with the HRT of the CPT. Also, a positive correlation was found for the processing speed component of the WJR and the RBC n-6/n-3 ratio.

DISCUSSION

The purpose of this study was to determine whether supplementation with n-3 FA-enriched PUFA might benefit children with AD/HD-like symptoms who reported a high frequency of thirst and skin symptoms. We had previously shown that children with AD/HD-like and thirst/skin symptoms had lower proportions of AA, EPA, and DHA in their plasma phospholipids in comparison with other children with AD/HD not exhibiting these thirst/skin symptoms (42). Data from the literature for the PUFA composition of plasma phospholipids for healthy children vary across publications. For AA, for instance, proportions range from a mean of about 6% for healthy Polish children (77) to a high of about 12.5% for healthy children from the United States (78). For DHA, the proportions in the literature for healthy children range from a low of about 1.9% for American children (78) to a high of about 2.6% as reported from a study of apparently healthy German children (79). For FA proportions in erythrocytes, AA ranges from a low of about 16.4% for healthy German children to a high of 26.3% for normal girls and boys in New Zealand (80). Similarly, proportions of DHA in erythrocytes have been reported to vary from about 2.2% (40) for healthy children in the U.S. Midwest to 6.3% for children from New Zealand (80). In these different publications, the range in the proportions of the two main families of PUFA varies considerably across populations, as much as 100%. Factors that contribute to this variation include differences in methodology as well as genetic background and food intake patterns (81). The variation within a reference sample is much smaller, generally 10–20%; thus, the most appropriate sample for a study is one drawn from the local population having similar characteristics and food intake patterns. For these studies we recruited a reference sample of the same age range and with a similar dietary intake. Proportions of plasma phospholipid FA proportions were statistically lower for the intervention sample in comparison with the reference sample for AA (8.9 vs. 10.5%, Table 2) and DHA (1.7 vs. 2.2%), and this result is similar to our previous report (40). Paradoxically, the proportions of AA and DHA in RBC were greater in the intervention sample compared with the reference sample of healthy children with no behavioral problems (AA: 20.2 vs. 18.2%; DHA: 7.1 vs. 5.7%), an observation that differs from what we previously found. Additionally, a comparison of these values with literature data revealed that the proportions were not outside those reported for some samples of healthy children. Perhaps focusing on the absolute proportions of n-6 and n-3 FA in the plasma and/or the RBC is not as important as focusing on whether a change in the proportions of these FA leads to an improvement in outcome measures.

We chose a supplement that contained the specific preformed PUFA that were low in the plasma phospholipids of the reference sample from a previous study (42). The supplement was enriched in n-3 relative to n-6 FA, but contained both families of EFA. The PUFA supplement contained a greater proportion of DHA than EPA (6:1), and this was desired because we hypothesized that the lower proportion of DHA in the blood was reflecting a decreased proportion of

this important FA in the brain and thus might mediate the abnormal neuronal signaling that results in aberrant behaviors (82). This was based on previous studies demonstrating that changes in the proportion of EFA in plasma and RBC were also reflected in the brain (83). However, although this assumption may be true in children for muscle tissue (84), recent studies suggest it may not be true for brain tissue (85). The relationship between plasma phospholipids, proportions in RBC, and brain chemistry needs to be explored further in these children.

We predicted that the proportions of AA, EPA, and DHA in the blood would increase by supplementing the diet with PUFA. Several studies have shown that proportions of PUFA in the blood can be increased by consumption of preformed PUFA. Consumption of marine oils, rich in n-3 PUFA, has been shown to lead to an increase in the proportions of EPA and DHA in the serum and blood cells of healthy adults (86). Similarly, a study with newborn infants demonstrated that increasing the concentration of DHA in the formula led to an increase in the proportion of this FA in the blood (87). A previous study of school-age children with behavior problems tested the effects of supplementing an oil source rich in the n-6 FA GLA, and found that increasing the proportion of this FA in the diet led to an increase in the amount in the blood (54).

The results from this study show that daily oral supplementation with 480 mg DHA and 80 mg EPA led to at least a doubling in the mean proportion of these two FA in both the plasma phospholipids and the RBC. Supplementation with a combination of the n-6 FA GLA, at 96 mg/d, and AA, at 40 mg/d, along with the n-3 PUFA, led to approximately a 25% increase in the proportion of AA in plasma, but approximately a 25% decrease in the RBC. There was also a decrease in the composition of two other long-chain n-6 FA, 22:4n-6 and 22:5n-6. This decrease in the proportion of n-6 FA in the RBC was probably due to the large increase in the proportion of the n-3 FA in the RBC membranes.

Not only are the proportions of each n-6 and n-3 FA key to influencing membrane function but the ratio between total n-6 and total n-3 is also important (43). The n-6/n-3 ratios in plasma and RBC were significantly influenced by supplementation, which led to significant decreases in the PUFA group (9.35 to 4.06 in plasma, 6.02 to 3.28 in RBC), whereas the ratio in the placebo group remained constant over the 4 mon. This antithetical relationship between n-3 and n-6 PUFA has been noted previously (88,89).

We assessed the frequency of thirst/skin symptoms in selecting a subpopulation of children with behavioral problems on which to test the effects of supplementation. We hypothesized that children exhibiting thirst/skin symptoms would be more likely to show effects of PUFA supplementation than those not reporting these EFA-deficiency-related symptoms. As noted previously, it is unlikely that the thirst/skin symptoms resulted from a primary EFA deficiency in these children. Similar types of thirst/skin symptoms have been reported to occur in patients suffering from a variety of different conditions (14–36). Although improvements have been noted in the frequency of symptoms with PUFA supplementation (14,22–24,38,39), it is unclear whether perturbations in PUFA metabolism are the root cause of thirst/skin symptoms. As reported here, supplementation with either oil led to a decrease in the frequency of these symptoms, but no treatment effect was observed. The magnitude of the decrease in the score, however, did correlate with the magnitude of the increase in the proportions of EPA and DHA in RBC. Since the thirst/skin symptom questionnaire has not been validated, the implications of this result are unclear.

The effect of PUFA supplementation on behavior was evaluated using several types of behavioral and neuropsychological tests. When using a change in score as the criterion, PUFA supplementation led to a significant improvement in behavior over the placebo on only 2 of the 16 outcome measures that were used—DBD-Conduct (Parents) and the DBD-Attention (Teachers)—and by ITT analysis only. One of these tests might be expected to be significant by chance alone (assuming a 5% random occurrence). However, caution should be exercised in interpreting this as a negative outcome. Enough improvements were seen in the children's behavior to warrant further research. This statement is based on the following results. First, fairly substantial improvements in behavior were observed by the parents for both intervention groups, and since the olive oil group also exhibited increases in the proportions of the n-3 FA in the plasma, these behavioral improvements were probably not due to the placebo effect alone. Also, given the changes in key FA in the placebo group, the potential effect size of the active treatment protocol was probably diminished. Second, the two outcome measures, based on scores that improved with the PUFA treatment, are of great importance to both parents (Conduct) and teachers (Attention). Third, when using clinical outcomes as the criteria for evaluating the parents' DBD Rating Scale, more children improved from the clinical range to the nonclinical range with PUFA supplementation. This was significant for the subscale of Oppositional/defiant behavior. Given that clinical outcomes are important to parents and clinicians, this outcome may indicate more in terms of "real-life" potential than an evaluation of the change in scores alone.

A fourth observation was that the results obtained by comparing the magnitude of change in RBC AA, EPA, DHA, and α-tocopherol vs. the magnitude of improvement in outcome measure scores support further research. Significant correlations were observed for proportions of EPA and the ASQ-P, for proportions of DHA and the teachers' DBD Rating Scale for Attention, and for α-tocopherol concentration and all four of the subscales of the teachers' DBD Rating Scale, the ASQ-T, and the CPT HRT. This result suggests that the amount of these FA in cell membranes could influence neurological processes that affect behavior. Previous studies in rats found that altered composition of n-3 FA in neurological membranes due to dietary insufficiency of ALA can influence both serotoninergic and dopaminergic neurotransmission (90,91). n-3 FA insufficiency has been associated with deficits in learning and memory (92), but effects on attention have not

been as clear (93,94). Proportions of n-3 FA in membranes have been reported to influence aggressive behaviors (95–97), as well as to moderate the severity of depressive symptoms (51). Few studies have addressed the relationship between α-tocopherol concentrations in membranes and psychiatric disorders. However, a recent paper (98) reported improvement in schizophrenics using EFA supplements along with vitamins E and C. Thus, further studies are warranted into the mechanistic relationship between the membrane composition of AA, EPA, DHA, and α-tocopherol and the neurological pathways that influence these behaviors.

The preliminary intervention study reported here had several confounding variables or limitations that should be avoided in future studies. First, participants were accepted into the study based on the parents' confirmation of a clinical diagnosis by a qualified doctor, but the diagnosis was not reconfirmed by a collaborating psychiatrist or psychologist. Barkley (3) has reported that symptoms of AD/HD are similar to many other conditions including visual or hearing problems, lead poisoning, anemia, sleep disorders, unrecognized learning disabilities, mild mental retardation, pervasive developmental disorders, depression, and anxiety disorders. In a study of children who had been diagnosed with AD/HD by their own physicians, only 72% qualified as having AD/HD using a structured diagnostic interview (99). Thus, participants can be considered as only "exhibiting AD/HD symptomology." Therefore, future studies should confirm the diagnosis based on DSM-IV criteria (56). Second, the olive oil placebo was "active" in that the supplement did not maintain the baseline plasma phospholipid PUFA composition. To more accurately conduct a treatment effect comparison, the use of a different oil, such as liquid paraffin oil (100), as the placebo in supplementation studies would be more appropriate. Another limitation was the timing of the intervention relative to the school year. Because the recruiting and baseline testing process took 6 mon, the intervention component did not begin until March. This resulted in the final testing outcome assessments being completed at different times for the teachers and parents. Thus, future studies should be designed to start earlier in the school year and be completed prior to the onset of summer vacation. Another factor that may have affected the results is the use of medication. The study protocol did not interfere with each participant's treatment regimen and, as such, some children stopped medication during the summer months, which was the 4-mon testing time point. Statistical testing did not reveal confounding evidence for medication or a change in medication for the behavior outcomes, but this practice may still have have obscured or overwhelmed a treatment effect of the PUFA. Finally, the sample size was small and did not provide adequate power in all cases. Future studies should include a larger sample size and strive for improved retention to provide adequate statistical power for assessing behavioral change.

In summary, children with AD/HD-type symptoms who reported frequent thirst/skin symptoms and under a variety of standard treatments for their behavioral problems participated in a double-blind intervention trial comparing a PUFA supplement enriched in DHA, EPA, and α-tocopheryl acetate vs. olive oil placebo. Supplementation with the PUFA led to up to a twofold increase in the proportions of EPA, DHA, and α-tocopherol in the plasma phospholipids and RBC total lipids, but a small increase was noted in the plasma phospholipid proportions of n-3 FA with olive oil as well. A clear benefit from PUFA supplementation for all behaviors characteristic of AD/HD was not observed; however, treatment effects for conduct (parents) and attention (teachers), together with clinical improvements in Oppositional/defiant behavior, along with significant associations between the magnitude of the change in RBC FA and vitamin E vs. improvements for some behavioral outcome measures support further research into these relationships.

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The Hypotriglyceridemic Effect of Dietary n-3 FA Is Associated with Increased β-Oxidation and Reduced Leptin Expression

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ABSTRACT: To study the mechanisms responsible for the hypotriglyceridemic effect of marine oils, we monitored the effects of high dietary intake of n-3 PUFA on hepatic and muscular β-oxidation, plasma leptin concentration, leptin receptor gene expression, and *in vivo* insulin action. Two groups of male Wistar rats were fed either a high-fat diet [28% (w/w) of saturated fat] or a high-fat diet containing 10% n-3 PUFA and 18% saturated fat for 3 wk. The hypotriglyceridemic effect of n-3 PUFA was accompanied by increased hepatic oxidation of palmitoyl-CoA (125%, $P < 0.005$) and palmitoyl-L-carnitine (480%, $P < 0.005$). These findings were corroborated by raised carnitine palmitoyltransferase-2 activity (154%, $P < 0.001$) and mRNA levels (91%, $P < 0.01$) as well as by simultaneous elevation of hepatic peroxisomal acyl-CoA oxidase activity (144%, $P < 0.01$) and mRNA content (82%, $P < 0.05$). In contrast, hepatic carnitine palmitoyltransferase-1 activity remained unchanged despite a twofold increased mRNA level after n-3 PUFA feeding. Skeletal muscle FA oxidation was less affected by dietary n-3 PUFA, and the stimulatory effect was found only in peroxisomes. Dietary intake of n-3 PUFA was followed by increased acyl-CoA oxidase activity (48%, $P < 0.05$) and mRNA level (83%, $P < 0.05$) in skeletal muscle. The increased FA oxidation after n-3 PUFA supplementation of the high-fat diet was accompanied by lower plasma leptin concentration (–38%, $P < 0.05$) and leptin mRNA expression (–66%, $P < 0.05$) in retroperitoneal adipose tissue, and elevated hepatic mRNA level for the leptin receptor Ob-Ra (140%, $P < 0.05$). Supplementation of the high-fat diet with n-3 PUFA enhanced *in vivo* insulin sensitivity, as shown by normalization of the glucose infusion rate during euglycemic hyperinsulinemic clamp.

Our results indicate that the hypotriglyceridemic effect of dietary n-3 PUFA is associated with stimulation of FA oxidation in the liver and to a smaller extent in skeletal muscle. This may ameliorate dyslipidemia, tissue lipid accumulation, and insulin action, in spite of decreased plasma leptin level and leptin mRNA in adipose tissue.

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Very long chain n-3 PUFA, such as EPA and DHA, potently lower blood triglyceride (TG) concentration (1,2) and ameliorate insulin sensitivity in animals with high-fat (HF) diet induced insulin resistance (3,4). These effects of dietary n-3 PUFA are associated with inhibition of lipogenesis (1,5,6), hepatic TG synthesis and secretion (7–9), and with stimulation of hepatic FA oxidation (10–12), whereas little is known about effects of n-3 PUFA on skeletal muscle fat metabolism. It has been shown that accumulation of TG in skeletal muscle is associated with impaired insulin action (13). There are indications that n-3 PUFA increase acyl-CoA oxidase mRNA, suggesting a direct stimulation of peroxisomal FA oxidation in skeletal muscle (14). Thus, we further evaluated the possible regulatory function of n-3 PUFA on hepatic and muscular FA oxidation, which might be associated with hypotriglyceridemic and insulin-sensitizing effects of dietary fish oil.

Recent evidence suggests that leptin may contribute to the hypotriglyceridemic effect of n-3 PUFA. Leptin may lower the TG content in liver, skeletal muscle, pancreatic β-cells, and adipose tissue *via* decreased TG synthesis and enhanced β-oxidation (15–19). Dietary regulation of plasma leptin levels has been demonstrated. Thus, dietary restriction and refeeding down- and up-regulate plasma leptin levels and Ob gene expression in rodents (20–22). Some authors showed that dietary n-3 PUFA slightly reduced plasma leptin level and leptin mRNA in chow-fed rats (23,24), whereas others showed that n-3 PUFA increased both plasma and adipose tissue leptin levels in insulin-resistant, sucrose-fed rats (25). To further explore this controversial issue, we examined the regulation of plasma leptin and leptin gene expression by dietary n-3 PUFA in insulin-resistant HF diet-fed rats.

The aim of this present study was to investigate the effects of dietary n-3 PUFA on (i) FA oxidation in liver and skeletal muscle, and (ii) circulating leptin levels, leptin gene expression in adipose tissue, and leptin receptor (Ob-Ra) mRNA levels in different tissues of rats with HF diet-induced hypertriglyceridemia and insulin resistance.

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Abbreviations: AOX, acyl-CoA oxidase; CPT-1/-2, carnitine palmitoyltransferase-1 and -2; EHC, euglycemic hyperinsulinemic clamp; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; G3PDH, glyceraldehyde 3 phosphate dehydrogenase; HDT, β-oxidation multienzyme complex composed of 2-enoyl-acyl-CoA **h**ydratase, 3-hydroxyacyl-CoA **d**ehydrogenase, and 3-oxoacyl-CoA **t**hiolase; HF, high-fat; HF/n-3, n-3 PUFA-supplemented HF diet; PUFA, polyunsaturated FA; RT, reverse transcription.

MATERIALS AND METHODS

Chemicals. RNAzol™ B (Tel-Test, Friendswood, TX) was used for total RNA isolations, and a reverse transcription (RT)-PCR kit (Gene Amp; PerkinElmer, Norwalk, CT) and [α-^{32}P]-dCTP (111 TBq/mmol; Amersham, Buckinghamshire, United Kingdom) for measurement of mRNA expression. The labeled substrates [1-^{14}C]palmitoyl-CoA (40–60 mCi/mmol), [1-^{14}C]palmitoyl-L-carnitine chloride (40–55 mCi/mmol), L-[methyl-^{14}C]carnitine hydrochloride (40–60 mCi/mmol) (NEN, Boston, MA), HDT-β-oxidation multienzyme complex composed of 2-enoyl-acyl-CoA **h**ydratase, 3-hydroxyacyl-CoA **d**ehydrogenase, and 3-oxoacyl-CoA **t**hiolase (Asahi, Ohito, Japan), FAD, NAD, CoA, BSA, palmitoyl-CoA, malonyl-CoA, EDTA, and DTT (Sigma-Aldrich Chemie, Deisenhofen, Germany) were all used for enzyme activity measurements. The scintillation liquid ASC2 (Amersham) was used to monitor radioactivity. All other chemicals and solvents were of reagent grade from common commercial sources.

Animals and diets. All experiments were approved by the Institute of Experimental Endocrinology Animal House Ethics Committee. Male Wistar rats (15 wk old, 309 ± 5 g) (AnLab, Prague, Czech Republic) were housed in wire mesh cages in a temperature- (22 ± 2°C) and light-controlled room (12 h light/12 h dark cycle, lights off at 1800). The rats were divided into two groups of 18 animals each. One group was fed the HF diet containing 28% (w/w) saturated fat (26). The other group was fed an n-3 PUFA-supplemented high-fat (HF/n-3) diet, where n-3 PUFA (EPAX 5500 TG; Pronova Biocare, Sandefjord, Norway) represented 10%, and the total fat content remained unchanged (saturated fat 18%). The average intake of EPA and DHA in the HF/n-3 group was 1.85 and 1.12 g/kg/d, respectively. Body weight (weekly) and food consumption (daily) were recorded during the dietary experiment. After 21 d of *ad libitum* feeding, six rats from each dietary group were subjected to surgery for implantation of a cannula (see below). These animals were then housed individually until the euglycemic hyperinsulinemic clamp (EHC) study. Another six animals from each group were sacrificed in the fasted state, and the last six in the fed state. Tissue aliquots from liver, gastrocnemius muscle, and epididymal and retroperitoneal white fat were immediately dissected, frozen in liquid nitrogen, and stored at −70°C until analysis. Aliquots of freshly dissected liver and quadriceps muscle were used immediately for measurement of FA oxidation.

EHC study. EHC were used to measure insulin action *in vivo* (27,28). After 3 wk of consuming different diets, the animals were anesthetized by i.p. injection of xylazine hydrochloride (10 mg/kg) plus ketamine hydrochloride (75 mg/kg) and fitted with chronic artery and jugular cannula (28). After surgery, the feeding continued and assays were performed 72 h after catheter implantation in the unrestrained, sedentary conscious state. Food was removed 16 h before the assay. Continuous infusion of human insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was given for 90 min at a dose of 6.4 mU/kg/min to achieve plasma insulin concentrations of 250 μU/mL. The arterial blood glucose concentration was clamped at the basal fasting level using a variable rate of glucose infusion (27,28).

RNA isolation. Total RNA was isolated from the liver, gastrocnemius muscle, and retroperitoneal white adipose tissue by the guanidinium isothiocyanate method (29), using commercially available RNAzol B (Tel-Test Inc., Friendswood, TX).

Leptin and leptin receptor gene expressions. These were determined by RT-PCR. The RT-PCR reactions were carried out using a Gene Amp RT-PCR kit (PerkinElmer), in a Touchgene thermocycler (Techne, Cambridge, United Kingdom). The reverse transcription reaction was carried out in 20 μL of buffer containing 3 μg DNA, 10 mmol/L Tris HCl (pH 8.3), 50 mmol/L KCl, 5 mmol/L $MgCl_2$, 2.5 μmol/L of oligo $(dT)_{16}$, 1 mmol/L of each dNTP, 20 units of RNAse inhibitor, and 50 units of MuLV-Reverse transcriptase (PerkinElmer) and incubated at 42°C for 15 min. The enzyme was inactivated by heating at 95°C for 5 min. cDNA (10 μL) was amplified in a 50 μL PCR-reaction mixture containing 15 pmol of each primer, 10 mmol/L Tris HCl (pH 8.3), 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.2 mmol/L of each deoxynucleotide 5′ triphosphate, [α-^{32}P]-dCTP (111 MBq/nmol), and 1.75 unit of AmpliTaq® DNA Polymerase (PerkinElmer, Foster City, CA). Leptin cDNA was amplified using primers to murine leptin, product size 250 bp: 5′-AGC-AGT-GCC-TAT-CCA-GAA-AGT-3′, 5′-ATT-CTC-CAG-GTC-ATT-GGC-TAT-3′, Ob-Ra cDNA, product size 236 bp: 5′-ACA-CTG-TTA-ATT-TCA-CAC-CAG-AG-3′, 5′-AGT-CAT-TCA-AAC-CAT-AGT-TTA-GG-3′. To confirm the absence of contaminating genomic DNA, control reactions were performed without reverse transcriptase. As a control for RNA quality and quantity, we used commercially available primers for glyceraldehyde-3-phosphate dehydrogenase (G3PDH), product size 452 bp: 5′-ACC-ACA-GTC-CAT-GCC-ATC-AC-3′, 5′-TCC-ACC-ACC-CTG-TTG-CTG-TA-3′ (Clontech Laboratories, Palo Alto, CA). cDNA were amplified for 33 (leptin and Ob-Ra) and 28 (G3PDH) cycles in all the tissues studied (liver, skeletal muscle, and white adipose tissue) using the following parameters: 94°C for 30 s, 60°C for 30 s, 72°C for 45 s, with a final extension step at 72°C for 7 min. PCR products were analyzed on 2% agarose gel, and cDNA bands corresponding to leptin and/or leptin receptor were excised from the gel and, after 2 h incubation in scintillation liquid, counted in a scintillation analyzer (Beckman LS-6500; Beckman Coulter, Fullerton, CA). Relative abundance of the different mRNA was calculated as the ratio between the PCR products and G3PDH for each sample.

Acyl-CoA oxidase (AOX) gene expression. The relative abundance of AOX mRNA was determined by Northern blot analysis. RNA was size-fractionated by agarose gel electrophoresis and subsequently transferred to supported nitrocellulose membrane (0.45 μm pore size; Hybond C-extra, Amersham). RNA was fixed to the membrane by vacuum baking (2 h, 80°C), and the abundance of each transcript of interest was determined by hybridization with the specific cDNA probe. Probes were radiolabeled using PRIME-IT RmT (Stratagene, La Jolla, CA) and [α-^{32}P]dCTP (Amersham). Prehybridization and hybridization were performed using the Quick-Hyb

hybridization solution (Stratagene). After hybridization, the membrane was exposed to X-ray-sensitive film (Kodak X-OMAT; Sigma, St. Louis, MO), and the resulting autoradiographic signal was quantified by densitometry. The relative abundance of mRNA was calculated as the ratio between AOX mRNA and G3PDH mRNA for each sample.

FA oxidation. FA oxidation was measured as conversion of [1-^{14}C]palmitoyl-CoA and, [1-^{14}C]palmitoyl-L-carnitine (NEN, Boston, MA) into acetyl-CoA in the postnuclear fraction prepared from freshly dissected liver and quadriceps muscle. Activity of the accumulated product was measured as "acid-soluble products" (30). Tissues were homogenized in ice-cold medium [0.25 mmol/L sucrose, 10 mmol/L HEPES (pH 7.4) and 2 mmol/L EDTA] by a Potter–Elvehjem homogenizer. The homogenates were centrifuged for 10 min at 600 × *g*, and the supernatants were assayed for total FA oxidation. The assay medium contained 13.3 mmol/L HEPES (pH 7.3), 83.3 mmol/L KCl, 16.7 mmol/L $MgCl_2$, 13.3 mmol/L DTT, 0.7 mmol/L EDTA, 1.25 mmol/L L-carnitine 0.2 mmol/L NAD, 2.0 mmol/L ATP, 0.4 mmol/L CoA-SH, [1-^{14}C]FA (palmitoyl-CoA or palmitoyl-carnitine, 72 kBq/nmol), and 1 mg of tissue protein from postnuclear fractions.

Carnitine palmitoyltransferase-1 (CPT-1) and CPT-2 activities. CPT-1 activity was measured (31) by using radioactive [1-^{14}C]L-carnitine (NEN) as a substrate, and production of labeled acyl-carnitine was measured after extraction to butanol. Mitochondrial fractions were prepared by homogenization of the frozen powdered tissues with the Ultra-Turrax homogenizer (T8; IKA, Staufen, Germany) in ice-cold medium containing 62.5 mmol/L sucrose, 2.5 mmol/L HEPES (pH 7.4), and 0.25 mmol/L EDTA, followed by centrifugation for 10 min at 600 × *g*. Protein concentration in the resulting supernatant was measured using a Bio-Rad protein kit (Bio-Rad, Richmond, CA). The assay for CPT-1 contained 20 mmol/L HEPES (pH 7.4), 50 mmol/L KCl, 5 mmol/L KCN, 100 μmol/L palmitoyl-CoA, 10 mg/mL BSA, and 0.6 mg/mL tissue proteins. The reaction was started by [methyl-^{14}C]L-carnitine, 72 kBq/nmol, and stopped after incubation for 6 min at 30°C by adding HCl. To test the sensitivity of CPT-1 to malonyl-CoA, malonyl-CoA in a final concentration of 20 μmol/L was added prior to the start of the reaction. Assay conditions for CPT-2 were identical to the description above, except that BSA was omitted and Triton X-100 was included during incubation.

AOX activity. The enzymatic activity of peroxisomal AOX was measured in peroxisomal fractions using an assay coupled with the β-oxidation multienzyme complex HDT (Asahi Chemical Industry) (32). [1-^{14}C]Palmitoyl-CoA was used as a substrate, and the amount of the acyl-CoA resulting from peroxisomal oxidation was measured as acid-soluble product. Frozen powdered tissues were homogenized by Ultra-Turrax in an ice-cold homogenization buffer containing 100 mmol/L potassium phosphate buffer (pH 7.5) and 2.5 mmol/L FAD and centrifuged (10 min, 12,000 × g, 4°C). Protein concentration in the resulting supernatant was measured using the Bio-Rad protein kit (Bio-Rad). The assay for AOX contained 100 mmol/L potassium phosphate buffer (pH 7.5), 2.5 μmol/L FAD, 200 μmol/L NAD, 50 μmol/L CoA, 200 μg/mL BSA, 3 U/mL HDT β-oxidation multienzyme complex, and 120 kBq/nmol [1-^{14}C]palmitoyl-CoA.

Routine analytical methods. In serum the levels of glucose (Beckman glucose analyzer 2; Beckman Instruments, Fullerton, CA), TG (Triglyceridy DST-P; DOT-diagnostics, Prague, Czech Republic), nonesterified FA (RANDOX, Ardmore, United Kingdom), and insulin (rat insulin RIA kit; Linco Research Inc., St. Charles, MO) were measured. Plasma leptin levels were determined by the rat leptin RIA kit (Linco Research Inc.).

Statistical analyses. The results were expressed as means ± SEM (n = 5 or 6). Differences between groups were analyzed using a paired t-test. Threshold for significance was set to $P < 0.05$.

RESULTS

Replacement of one-third (10%) of the saturated fat in the HF diet by n-3 PUFA reduced body weight gain by 28% ($P < 0.05$) and tended to decrease the post-dissection weights of epididymal and retroperitoneal fat (Table 1). Animals fed the n-3 PUFA-enriched HF diet consumed 15% less than their HF-fed counterparts. In spite of reduced consumption, the food efficiency (the ratio between body weight gain and cumulative food intake) tended to be lower in the n-3 PUFA-supplemented group (Table 1). Dietary intake of n-3 PUFA reduced plasma (by 71%, $P < 0.05$) and hepatic concentrations of TG (by 64%, $P < 0.05$); reduced circulating nonesterified FA (by 42%, $P < 0.05$), which were elevated in HF-fed animals; and normalized fasting insulinemia in these insulin-resistant rats (Table 1). However, there was no change of fasting plasma glucose level after 3 wk of n-3 PUFA feeding (Table 1). Measurement of *in vivo* insulin action by EHC showed improved insulin sensitivity in animals fed the n-3 PUFA–supplemented HF diet as indicated by increased glucose infusion rate (see Table 1).

TABLE 1
Effect of Dietary n-3 PUFA on Body Weight, Food Intake, Plasma/Tissue Lipids, Insulin, Glucose, and *in vivo* Insulin Action in Rats[a]

	HF	HF/n-3
Body weight gain (g)	89.2 ± 8.7[a]	63.7 ± 7.6[b]
WAT epi weight (g)	5.3 ± 0.4	4.6 ± 0.5
WAT rtp weight (g)	5.5 ± 0.4	4.9 ± 0.5
Food consumption (g/kg/d)	59 ± 1[a]	49 ± 2[b]
Food efficiency[b]	0.21 ± 0.02	0.18 ± 0.02
Plasma TG (mmol/L)[c]	4.2 ± 0.1[a]	1.2 ± 0.3[b]
Liver TG (μmol/g)	39.7 ± 6.6[a]	14.4 ± 2.4[b]
Serum NEFA (mmol/L)	1.2 ± 0.1[a]	0.7 ± 0.2[b]
Plasma insulin (μU/mL))	23.4 ± 5.0[a]	11.2 ± 2.2[b]
Plasma glucose (mmol/L)	6.4 ± 0.2	6.0 ± 0.2
GIR (mg/kg/min)	15.7 ± 0.4[a]	23.2 ± 0.9[b]

[a]Data represent means ± SEM from 6–8 rats. Values with different superscript letters differ significantly by paired t-test ($P < 0.05$). WAT, white adipose tissue; epi, epididymal; rtp, retroperitoneal; NEFA, nonesterified FA; GIR, glucose infusion rate (index of *in vivo* insulin sensitivity); HF diet, high-fat diet; HF/n-3 diet, n-3 PUFA-supplemented high-fat diet.
[b](Body weight gain)/(total food consumed).
[c]Plasma taken in the fed state.

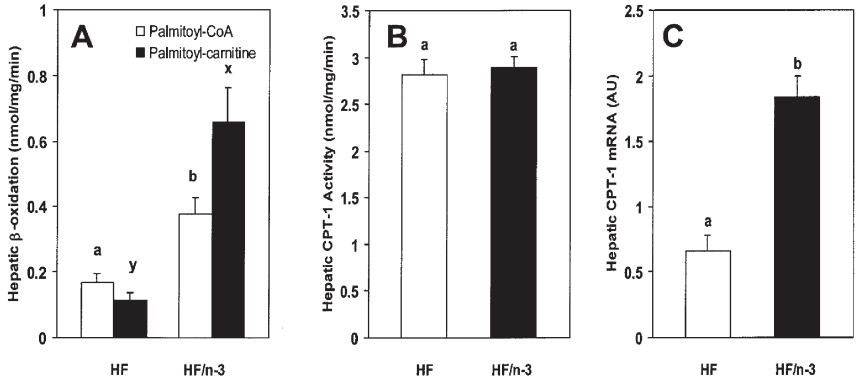

FIG. 1. Effect of dietary n-3 PUFA on hepatic palmitoyl-CoA and palmitoyl-L-carnitine oxidation (A), carnitine palmitoyltransferase-1 (CPT-1) enzymatic activity (B), and CPT-1 mRNA expression (C). Data represent means ± SEM from 5 or 6 rats. Values without a common superscript are significantly different ($P < 0.05$). HF diet, high-fat diet; HF/n-3 diet, n-3 PUFA-supplemented HF diet.

Intake of n-3 PUFA elevated hepatic FA utilization as shown by increased oxidation of palmitoyl-CoA and palmitoyl-L-carnitine (Fig. 1A). Activities of the enzymes involved in transport of FA into the mitochondria, CPT-1 and CPT-2, were regulated in a specific manner. Whereas the CPT-2 enzyme activity ($P < 0.05$) (Fig. 2A) and mRNA (Fig. 2B) levels were increased approximately twofold with n-3 PUFA, CPT-1 activity (Fig. 1B) remained unchanged in spite of high gene expression (Fig. 1C). In parallel with increased CPT-2-dependent mitochondrial FA oxidation, intake of n-3 PUFA increased activity (Fig. 2C) and gene expression (Fig. 2D) of peroxisomal AOX, the key enzyme of the peroxisomal FA oxidation pathway. These data suggest that n-3 PUFA supplementation elevates both mitochondrial and peroxisomal FA oxidation in liver, whereas CPT-1 activity is unchanged.

Dietary n-3 PUFA did not change mitochondrial FA oxidation in skeletal muscle, as suggested by the results of palmitoyl-CoA and palmitoyl-L-carnitine oxidation, and as further supported by CPT-1 and CPT-2 enzyme activity measurements (data not shown). Dietary n-3 PUFA, however, increased skeletal muscle peroxisomal oxidation, as indicated by elevated enzyme activity (Fig. 3A) and gene expression (Fig. 3B) of acyl-CoA oxidase.

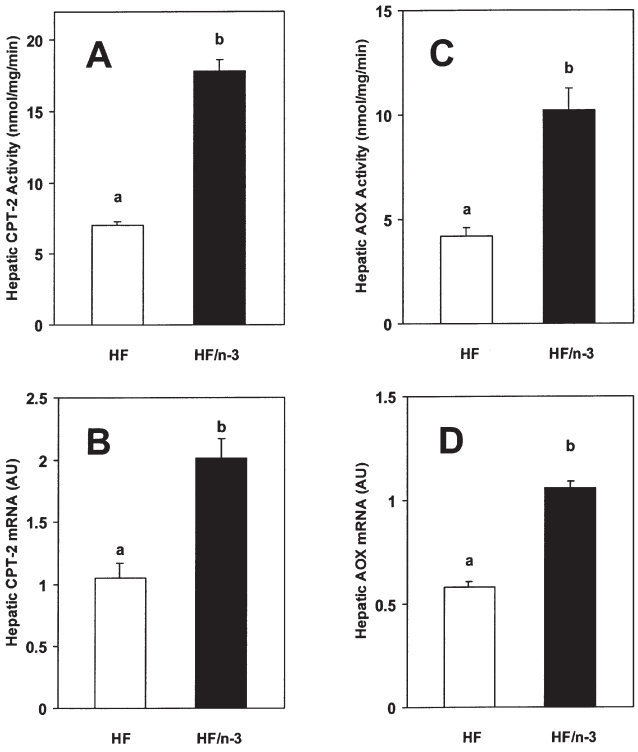

FIG. 2. Effect of dietary n-3 PUFA on hepatic CPT-2 and acyl-CoA oxidase (AOX) enzymatic activities (A, C) and mRNA levels (B, D). Data represent means ± SEM from 5 or 6 rats. Values without a common superscript are significantly different ($P < 0.05$). For other abbreviations see Figure 1.

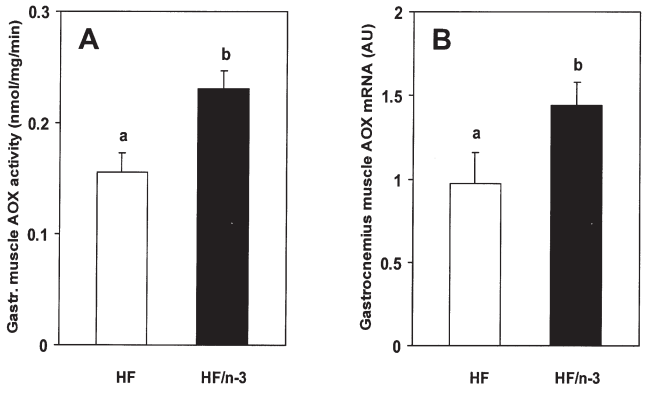

FIG. 3. Effect of dietary n-3 PUFA on the gastrocnemius muscle AOX enzymatic activity (A) and gene expression (B). Data represent means ± SEM from 5 or 6 rats. Values without a common superscript are significantly different ($P < 0.05$). See Figures 1 and 2 for abbreviations.

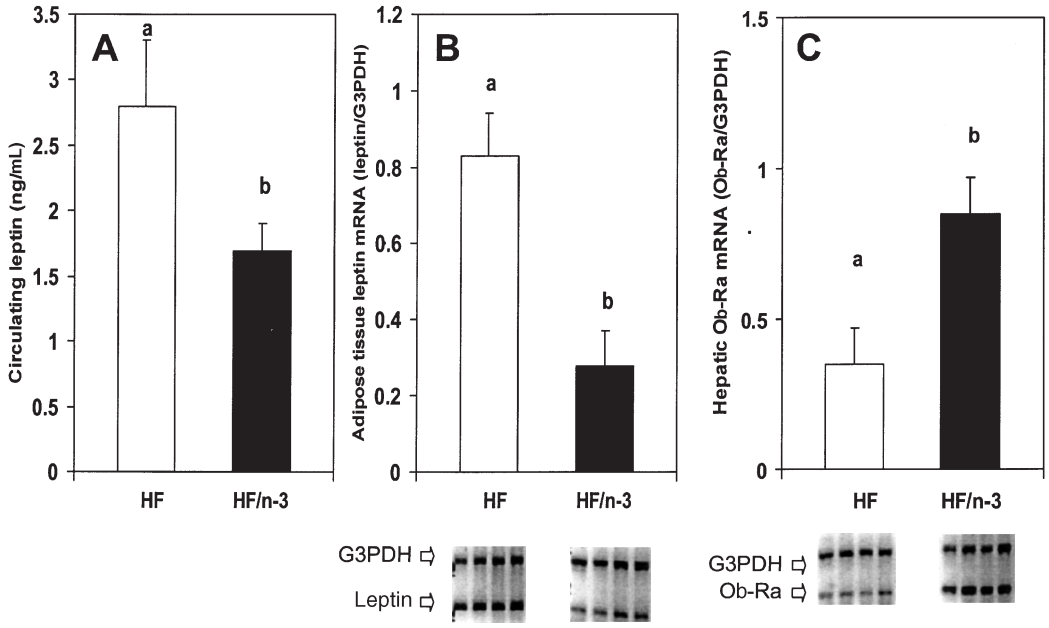

FIG. 4. Effect of dietary n-3 PUFA on circulating leptin (A), retroperitoneal white adipose tissue leptin mRNA (B), and hepatic leptin receptor Ob-Ra mRNA (C) expressions. Panels below the figures represent the reverse transcription-PCR products resolved on 1% agarose gel with 0.001% ethidium bromide; amplification of the transcript for leptin (B) or leptin receptor (C) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were carried out in the same tube. Gene expression was determined as incorporation of [α-^{32}P]dCTP into the amplified DNA (see Materials and Methods section for detailed description). Data represent means ± SEM from 5 or 6 rats. Values without a common superscript are significantly different ($P < 0.05$). Ob-Ra, short form of leptin receptor; for other abbreviations see Figure 1.

n-3 PUFA lowered both plasma leptin concentration (Fig. 4A) and leptin mRNA (Fig. 4B) in retroperitoneal adipose tissue. Dietary supplementation of n-3 PUFA increased hepatic Ob-Ra gene expression (Fig. 4C), whereas the Ob-Ra mRNA level was unchanged in gastrocnemius muscle and white adipose tissue (data not shown).

DISCUSSION

Our data suggest that the hypotriglyceridemic effect of dietary n-3 PUFA is related to increased hepatic FA oxidation as shown by stimulation of palmitoyl-CoA oxidation. Mitochondrial oxidation of palmitoyl-CoA is regulated by CPT-1-mediated transport into the mitochondrial matrix (33). However, the enzyme activity of CPT-1 was not changed by n-3 PUFA supplementation. Elevated palmitoyl-CoA oxidation may therefore be due to increased peroxisomal FA oxidation. This is also in line with our observation that dietary n-3 PUFA increased the activity and mRNA level of AOX, the key enzyme of peroxisomal FA oxidation. In addition to AOX, n-3 FA elevated hepatic CPT-2 activity, suggesting an intriguing role for peroxisomes in mediating CPT-1-independent mitochondrial FA oxidation. Long-chain acyl carnitines from peroxisomes may enter mitochondria directly and could be activated (formation of acyl-CoA) by CPT-2 for further oxidation. Another experimental support for this hypothesis comes from comparison of palmitoyl-L-carnitine and palmitoyl-CoA oxidation rates, where palmitoyl-L-carnitine seems to be the preferred oxidative substrate in animals fed n-3 PUFA. Results of a study by Ide *et al.* (11) are in agreement with our observations, with one exception. In a very similar experiment they found a 50% elevated CPT-1 activity after feeding n-3 PUFA. However, CPT-2 and AOX responded to dietary n-3 PUFA by more than a fourfold increase in enzyme activity, suggesting only a minor role for CPT-1 in PUFA-stimulated hepatic FA oxidation (11). Thus, the mitochondrial oxidation of long-chain acyl-carnitines released from peroxisomes, which is independent of CPT-1 (12,34), might represent an effective n-3 PUFA-activated mechanism for increasing mitochondrial long-chain FA oxidation. Several recent reports indicate that CPT-1 may not always play a key role in regulation of mitochondrial FA oxidation (35–38). In agreement with some earlier studies (10,12,35,39), our data suggest that increased hepatic FA oxidation participates in the hypotriglyceridemic action of n-3 PUFA.

Accumulation of TG in skeletal muscle is associated with impaired insulin action (13). As shown in our study and many others, dietary n-3 PUFA improve *in vivo* insulin sensitivity (3,4). To evaluate the mechanisms by which marine n-3 PUFA may protect against fat-induced insulin resistance, we measured mitochondrial and peroxisomal FA oxidation in gastrocnemius muscle of HF diet-fed rats. We did not observe an effect of dietary n-3 PUFA on mitochondrial oxidation of palmitoyl-CoA or palmitoyl-L-carnitine. In addition, neither CPT-1 nor CPT-2 enzyme activities were changed in gastrocnemius muscle. On the other hand, evidence exists that n-3 PUFA may elevate the CPT-1 enzyme activity and lower the sensitivity of CPT-1 to

malonyl-CoA inhibition in the soleus muscle (40). In our study we observed that dietary n-3 PUFA increased peroxisomal FA oxidation in gastrocnemius muscle in spite of the unchanged gating system for long-chain FA in the mitochondrial inner membrane. This is indicated by a 30% increase of the enzyme activity and mRNA level of acyl-CoA oxidase, and it is in agreement with previous measurements of AOX gene expression (14).

It has recently been shown that the adipose tissue-derived hormone leptin may directly influence FA oxidation (15,16,41). Leptin may protect nonadipose tissues such as skeletal muscle, liver, and pancreas against products of the nonoxidative metabolism of FA by stimulating breakdown and inhibiting synthesis (15,16). It has been shown that HF diets increased plasma leptin levels in rats (22, 42), whereas we demonstrate that n-3 PUFA suppressed plasma leptin concentration and adipose tissue leptin gene expression in insulin-resistant HF-fed rats, which is in line with previous observations (23,24). A recent report by Peyron Caso *et al.* (25) shows dissociation of plasma leptin concentration and adipose tissue mass, because a 10% reduction in total fat mass due to PUFA treatment was associated with enhancement rather than reduction of plasma leptin levels. The issues of mutual metabolic regulations of leptin and insulin and their association with obesity and diabetes are extensively studied and presently not fully understood. Insulin may influence leptin gene expression (21,42–44). Results of this study showed that dietary n-3 PUFA caused a 40–50% reduction in plasma levels of leptin and insulin in animals with hyperleptinemia and hyperinsulinemia induced by HF diet. The reduction in plasma leptin levels might be due to a direct effect of n-3 PUFA on leptin gene expression (24) or an indirect effect due to reduced insulin levels or body weight. In addition, we found that dietary intake of n-3 PUFA increased hepatic Ob-Ra receptor gene expression. Up-regulation of hepatic Ob-Ra receptor, which binds leptin in plasma as well as on the plasma membrane, might be associated with enhanced clearance of circulating leptin after dietary n-3 PUFA supplementation.

In summary, we demonstrated that dietary n-3 PUFA stimulate CPT-1-independent (mostly peroxisomal) FA oxidation in both liver and skeletal muscle, probably playing an important role in lowering plasma lipids concentrations. The increased FA oxidation after n-3 PUFA supplementation of the HF diet was accompanied by lower plasma leptin concentration, decreased leptin mRNA expression, increased hepatic leptin receptor mRNA, and enhanced *in vivo* insulin sensitivity.

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Interactive Effects of Dietary Palm Oil Concentration and Water Temperature on Lipid Digestibility in Rainbow Trout, *Oncorhynchus mykiss*

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ABSTRACT: An experiment was conducted to evaluate the interactive effects of dietary crude palm oil (CPO) concentration and water temperature on lipid and FA digestibility in rainbow trout. Four isolipidic diets with 0, 5, 10, or 20% (w/w) CPO, at the expense of fish oil, were formulated and fed to groups of trout maintained at water temperatures of 7, 10, or 15°C. The apparent digestibility (AD) of the FA, measured using yttrium oxide as an inert marker, decreased with increasing chain length and increased with increasing unsaturation within each temperature regimen irrespective of CPO level fed to the fish. PUFA of the n-3 series were preferentially absorbed compared to n-6 PUFA in all diet and temperature treatments. Except for a few minor FA, a significant ($P < 0.05$) interaction between diet and temperature effects on FA digestibility was found. Increasing dietary levels of CPO lead to significant reductions in the AD of saturates and, to a lesser extent, also of the other FA. Lowering water temperature reduced total saturated FA digestibility in trout regardless of CPO level. Based on the lipid class composition of trout feces, this reduction in AD of saturates was due in part to the increasing resistance of dietary TAG to digestion. Increasing CPO level and decreasing water temperature significantly increased TAG content in trout fecal lipids, with saturates constituting more than 60% of the FA composition. Total monoene and PUFA digestibilities were not significantly affected by water temperature in fish fed up to 10% CPO in their diet. The potential impact of reduced lipid and FA digestibility in cold-water fish fed diets supplemented with high levels of CPO on fish growth performance requires further research.

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Fish oil is an important ingredient in the formulation of commercial aquaculture feeds, supplying dietary energy and EFA to the farmed aquatic animal. The use of fish oils in aquafeeds has grown in recent years because of the rapid development of the global aquaculture industry and the technological advances in fish feed manufacturing, which allows the production of high-lipid diets. Aquafeeds currently use about 70% of the global supply of fish oil, and by the year 2010, fish oil used in aquaculture is estimated to reach about 97% of the world supply (1). Of the cultured aquatic animals, salmonid fishes such as the Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) currently account for more than 55% of the total fish oil used in aquaculture (1). Fish oil is produced from small marine pelagic fish and represents a finite fishery resource. Over the past decade, global fish oil production has reached a plateau and is not expected to increase beyond current levels. The rapidly growing aquaculture industry cannot continue to rely on finite stocks of marine pelagic fish for fish oil supply.

Much research is currently taking place in evaluating alternatives to fish oil, especially in the diets of salmonid fishes (2–7). One potential substitute for fish oil in commercial salmonid feeds is palm oil, which is currently the second-largest volume of vegetable oil produced in the world. The potential use of palm oil in the diets of various fish species and the advantages palm oil has over other vegetable oils in aquafeeds have been reviewed by Ng (8,9). Research into the use of crude palm oil (CPO) in the diets of Atlantic salmon (3–5) and rainbow trout (6) had reported growth and feed utilization efficiency comparable to that in fish fed equivalent levels of dietary marine fish oils. Despite the encouraging growth data, some salmon feed companies have expressed concerns over the use of CPO because it contains about 50% saturated FA and 40% monoenes (10), which may reduce FA digestibility and subsequent energy availability, especially when such CPO-based diets are fed to salmon during the winter/cold season. The digestibilities of saturated and monounsaturated FA in salmonids have been reported to be lower compared to PUFA (11–13). The apparent digestibility (AD) of FA also is reportedly influenced by dietary lipid source and the level of dietary saturates (6). The effects of increasing dietary levels of CPO on FA digestibility in fish have not been reported.

Evidence for the influence of water temperature on lipid and FA digestibility in salmonids is not conclusive. Austreng *et al.* (14) reported no significant differences in lipid and FA digestibility in rainbow trout reared at 3 or 11°C. FA digestibility varied by less than 5% in Atlantic salmon fed increasing dietary levels of soybean oil and maintained at water temperatures of 5 or 12°C (15). In contrast, Olsen and Ringo (16) reported that the digestibility of saturated FA was significantly reduced in Arctic charr, *Salvelinus alpinus*, maintained at 0.6°C compared to fish maintained at 10°C, but the digestibilities of monoenes and PUFA were hardly affected by the change in

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Abbreviations: AD, apparent digestibility; CPO, crude palm oil; PL, phospholipid; SROL, sterol; SE, sterol ester.

water temperature. Windell *et al*. (17) reported a significant decrease in lipid digestibility in small (about 19 g) rainbow trout reared in water temperatures of 7°C compared to fish in water temperatures of 11 or 15°C, but this reduction in lipid digestibility was not observed in medium (about 207 g)- or large (about 586 g)-sized fish at the same temperature.

The present study was designed to evaluate the interactive effects, if any, of various dietary CPO levels and water temperature on lipid and FA digestibility in rainbow trout. An accurate knowledge of the apparent FA digestibility at all the relevant water temperatures is required to optimize the inclusion of CPO in salmonid diets for use throughout the year.

MATERIALS AND METHODS

Experimental diets. Four practical extruded diets (3 mm diameter) were formulated (BioMar Ltd., Grangemouth, United Kingdom) by using the same basal ingredients and varying only their CPO content, added at 0, 5, 10, or 20% (w/w) of the diet, at the expense of marine fish oil (Table 1). Yttrium oxide was added to the experimental diets (0.2 g/kg) as an inert marker for calculations of AD of FA (18). The diets were designed to meet all the known nutritional requirements of salmonid fish including n-3 EFA (19). CPO was properly melted and thoroughly mixed with the fish oil before the oil mixture was used to coat the extruded pellets. The ingredient formulation and proximate composition of the experimental diets are shown in Table 1. The FA composition of the experimental diets is shown in Table 2, and the lipid class composition is in Table 3.

Experimental procedure. Rainbow trout, with a mean initial body weight of about 45 g, were obtained from a local trout hatchery and stocked into three indoor 1000-L tanks on arrival at the Aquaculture Research Unit (University of Stirling, Scotland). Over a 2-wk period, the water temperature of two of the tanks was gradually increased to 10 ± 1 and 15 ± 1°C, respectively, while the third tank was maintained at the ambient water temperature of 7 ± 1°C. The indoor tanks were subjected to a 12-h light/12-h dark photoperiod regime. All fish were fed a commercial trout pellet (BioMar Ltd., Grangemouth, United Kingdom) during this acclimation period. After 2 wk, each group of temperature-acclimatized fish was randomly distributed into four circular tanks of 100-L capacity (at 40 fish/tank) supplied with flow-through water, of the same temperature, at 1 L/min. Fish maintained at each temperature grouping were then fed one of the four experimental diets for another 2 wk before the commencement of feces collection. Fish were fed three times daily (last feeding at 1800 h) to satiation.

After 2 wk, samples of feces were collected from trout maintained at the three different water temperatures and fed their respective dietary treatment of increasing CPO levels at

TABLE 1
Ingredient[a] and Proximate Composition of the Rainbow Trout Experimental Diets

	Dietary crude palm oil[b] (%)			
Nutrient	0	5	10	20
	(g/100 g diet)			
Moisture	6.5	8.2	7.2	6.9
Crude protein	47.9	46.5	47.8	46.7
Crude lipid	21.2	20.1	20.9	21.2
Ash	7.1	7.1	7.0	7.0
Gross energy (kJ/g)	22.8	23.6	23.8	23.6

[a]Main ingredients of all four diets were fish meal (34.3%), Hi Pro soybean meal (12.7%), corn gluten (10%), wheat gluten (10%), wheat flour (10%), and 3.4% of amino acid, vitamin, and mineral premixes of which the exact formulation is proprietary to BioMar Ltd. (Grangemouth, United Kingdom).
[b]Crude palm oil (United Plantations, Teluk Intan, Malaysia) was added at the expense of marine fish oil (Norsemeal Ltd., London, United Kingdom).

TABLE 2
FA Composition of the Rainbow Trout Experimental Diets

	Dietary crude palm oil (%)			
FA[a]	0	5	10	20
	(% of total FA)			
14:0	6.4	4.6	3.7	1.6
15:0	0.6	0.4	0.3	0.2
16:0	18.6	26.7	30.6	37.9
16:1n-7	6.7	4.5	3.5	1.0
18:0	3.4	3.8	4.2	4.2
18:1n-9	11.1	20.2	24.2	35.9
18:1n-7	2.5	1.9	1.8	0.6
18:2n-6	5.9	8.1	9.1	11.8
18:3n-3	1.3	1.0	0.9	0.6
18:4n-3	2.9	1.9	1.4	0.2
20:1n-9	5.4	3.5	2.8	0.4
20:4n-6	0.6	0.4	0.3	0.2
20:4n-3	0.6	0.4	0.3	0.1
20:5n-3	9.2	6.0	4.7	1.2
22:1n-11	7.2	4.5	2.5	0.4
22:5n-3	1.2	0.8	0.7	0.3
22:6n-3	11.3	7.9	6.4	2.7
24:1	0.6	0.4	0.3	0.1
Total saturates	29.3	35.8	39.1	44.1
Total monoenes	34.7	35.6	35.5	38.5
Total PUFA	36.0	28.5	25.4	17.4
Total n-3	26.9	18.4	14.6	5.1
Total n-6	7.2	9.0	9.9	12.1
n-3/n-6	3.7	2.0	1.5	0.4

[a]Some minor FA are not shown, including 16:1n-9, 16:2, 16:3, 16:4, 18:2n-3, 18:3n-6, 20:0, 20:1n-7, 20:2n-6, 20:3n-3, 22:1n-9, and 22:5n-6.

TABLE 3
Lipid Class Composition of the Rainbow Trout Experimental Diets

	Dietary crude palm oil (%)			
Lipid fraction[a]	0	5	10	20
	(% of lipid)			
PC	3.2	3.4	3.6	4.3
PE	1.4	1.7	1.9	2.2
SROL	11.6	13.3	13.5	16.2
TAG	62.2	60.8	60.6	57.8
FFA	12.9	12.7	12.6	12.9
SE	8.7	8.1	7.8	6.6
Total PL	4.6	5.1	5.5	6.5
Total NL	95.4	94.9	94.5	93.5

[a]Lipid fractions are PC, PE, sterol (SROL), TAG, FFA, and sterol ester (SE). PL denotes phospholipids and NL denotes neutral lipids. PS, PI, phosphoglycerol and cardiolipin (PG + CL) were not detected. Trace amounts of DAG and MAG were detected.

each temperature regime. In the morning after the last feeding of the previous day (about 12–15 h elapsed time), all fish from each tank were lightly anesthetized with tricaine methanesulfonate (MS-222; Argent, Redmond, WA), and fecal samples were collected from the hindgut region by gently squeezing the ventral abdominal area (20). Samples from 40 fish were pooled by tank and stored at −20°C prior to analysis of lipid content, lipid class, FA composition, and yttrium oxide. After fecal stripping, all fish were returned to their respective tanks and allowed to feed on the experimental diet for three more days. The whole process was then repeated to collect a duplicate sample of feces. All fish-handling procedures were conducted in accordance with the British Home Office guidelines regarding research on experimental animals.

Chemical, FA, and lipid class analysis. The nutrient composition (moisture, protein, lipid, and ash) of the four experimental diets was determined by proximate analysis as previously described by Bell *et al.* (5). Gross energy was determined using an adiabatic bomb calorimeter (Gallenkamp autobomb). Yttrium oxide (Y_2O_3) was analyzed by an inductively coupled plasma emission spectrophotometer at AKVAFORSK (Institute of Aquaculture Research, A/S, Ås, Norway). Dried diet and feces were combusted at 550°C overnight in glass scintillation vials and boiled in HCl/HNO_3 (2:1, vol/vol) until colorless before Y_2O_3 analysis.

Total lipid was extracted from pooled fish feces with chloroform/methanol (2:1, vol/vol) in an Ultra-Turrax tissue homogenizer (Fisher Scientific, Loughborough, United Kingdom) and nonlipid impurities were removed by washing with 88 g/L KCl (21). The total lipid content of fecal samples was determined gravimetrically after evaporation of solvent under a stream of oxygen-free nitrogen and overnight desiccation *in vacuo*. FAME were prepared by acid-catalyzed transesterification of total lipid according to the method of Christie (22) with 17:0 being added at 10% of the total lipid weight as an internal standard for quantitative measurement of individual FA present in the diets and feces. Extraction and purification of FAME were performed as described by Ghioni *et al*. (23). FAME were resolved and analyzed by GLC (Fisons GC8000 Series; ThermoFinnigan, Hemel Hempstead, Herts, United Kingdom) using a 30 m × 0.32 mm i.d. fused-silica capillary column (CPWax 52CB; Chrompack, Middelburg, The Netherlands). Hydrogen was used as a carrier gas, and temperature programming was from 50 to 150°C at 40°C/min and then to 230°C at 2.0°C/min. FAME were identified by comparing retention times with those of known standards and by reference to published data (10,24).

Lipid class composition of the experimental diets and fish feces was determined according to the procedures described by Olsen and Henderson (25). Lipid classes were separated on high-performance TLC plates (HPTLC silica gel 60; Merck, Poole, Dorset, United Kingdom) and detected by spraying the plate with 3% cupric acetate in 8% phosphoric acid followed by charring at 160°C for 20 min. Quantification was performed using scanning densitometry on a CAMAG TLC Scanner 3 (Muttenz, Switzerland).

Digestibility calculations and statistical analysis. The apparent digestibility (% AD) of dietary FA (dry weight basis) was calculated as: $100 - [100 \times (Y_2O_3$ conc. in feed/Y_2O_3 conc. in feces) × (FA conc. in feces/FA conc. in feed)]. The concentrations of individual FA in diets and feces were calculated based on the relative proportion of each FA compared to a known amount of the internal standard (17:0) added and the total lipid determined in the samples. ANOVA using the full factorial model (effects of diet, temperature, and interaction) was conducted using the General Linear Models (GLM) procedure of the SAS computer software (SAS Institute, Cary, NC). Differences between means were assessed by Duncan's multiple range test (26) and effects with a probability of $P < 0.05$ were considered significant.

RESULTS

FA and lipid class composition of diets. The four experimental diets were essentially isonitrogenous, isolipidic, and isoenergetic (Table 1). However, the FA composition of the diets was distinctly different and consistent with the increasing levels of supplemented CPO (Table 2). The diet with fish oil as the sole lipid source contained the highest and the lowest concentrations of n-3 and n-6 PUFA, respectively, compared to other diets. The n-3/n-6 ratios were 3.7, 2.0, 1.5, and 0.4 for diets with 0, 5, 10, and 20% CPO, respectively. With increasing dietary CPO, total saturated (in particular, 16:0) and monounsaturated (in particular, 18:1n-9) FA concentrations were increased in the diets. The increase in total dietary saturates was more pronounced compared to monoenes since fish oil also contains significant amounts of monoenes such as 16:1n-7, 20:1n-9, and 22:1n-11.

There was a slight decrease in percent TAG and sterol ester (SE) and a corresponding increase in percent sterol (SROL) with increasing CPO in the trout diets (Table 3). The concentrations of FFA remained the same. Total phospholipids (PL) were about 2% higher in diets with 20% CPO compared to the control diet with 20% fish oil (0% CPO).

FA and lipid class composition of feces. Total lipid in feces was not significantly affected by decreasing water temperatures (15 to 7°C) for rainbow trout fed 0 or 5% dietary CPO (Table 4). However, in fish fed 10 or 20% CPO diets, fecal lipid content was significantly higher in fish maintained at water temperatures of 7°C compared to fish maintained at 15°C. For example, feces from trout fed a 20% CPO diet in water temperatures of 7°C contained 37.2% lipid (dry weight basis) compared to 22.5% lipid from fish fed the same diet but maintained at 15°C. A significant interaction effect between diet and temperature was evident for the total lipid content of feces.

Lipid class composition of the excreted fecal lipid was markedly different among the various dietary and temperature treatments (Table 4). FFA was the major lipid class found in all feces. With increasing dietary CPO, the percentage of FFA in fecal lipids decreased, and the percentage of TAG increased. At each dietary CPO level, there was a trend of decreasing percentage FFA and increasing percentage TAG in the fecal lipid associated with decreasing water temperatures. In fish maintained at a water temperature of 7°C and fed the 20% CPO diet, almost equal proportions of TAG and FFA were observed. Diet

TABLE 4
Total Lipid and Lipid Class Composition of Feces Collected from Trout Fed Diets Containing Increasing Levels of Crude Palm Oil (CPO) and Maintained at Three Different Water Temperatures[a]

CPO (%)	Temp. (°C)	Lipid class (%)[b] SROL	TAG	FFA	SE	Total lipid (%)
0	15	25.3 ± 5.9	7.2 ± 1.0^{h}	62.3 ± 2.3^{a}	5.2 ± 3.0	21.6 ± 0.4^{c,d}
0	10	23.1 ± 2.8	12.0 ± 2.2^{f,g}	60.3 ± 2.7^{a}	4.7 ± 2.1	18.8 ± 0.3^{d}
0	7	26.2 ± 4.2	18.0 ± 1.0^{d,e}	49.1 ± 7.0^{a,b}	6.8 ± 1.9	17.8 ± 2.8^{d}
5	15	26.9 ± 3.5	9.7 ± 1.5^{g,h}	54.4 ± 7.2^{a,b}	9.0 ± 2.2	20.1 ± 2.7^{d}
5	10	20.3 ± 0.1	14.7 ± 0.5^{e,f}	59.4 ± 3.5^{a}	5.7 ± 3.1	20.0 ± 1.0^{d}
5	7	22.8 ± 3.8	21.0 ± 1.6^{c,d}	49.5 ± 5.4^{a,b}	6.6 ± 3.2	20.1 ± 1.6^{d}
10	15	27.4 ± 4.2	10.2 ± 0.9^{g,h}	55.0 ± 3.2^{a,b}	7.5 ± 0.2	20.0 ± 1.0^{d}
10	10	19.6 ± 1.8	21.6 ± 0.1^{c,d}	52.2 ± 3.7^{a,b}	6.5 ± 2.1	22.6 ± 1.0^{c,d}
10	7	22.7 ± 4.2	24.1 ± 2.2^{b,c}	47.0 ± 3.7^{a,b}	6.2 ± 1.7	25.8 ± 1.9^{b,c}
20	15	24.6 ± 2.3	20.1 ± 1.3^{c,d}	48.9 ± 1.4^{a,b}	6.4 ± 2.2	22.5 ± 2.5^{c,d}
20	10	17.7 ± 0.6	27.6 ± 0.2^{b}	48.2 ± 1.3^{a,b}	6.5 ± 1.0	28.3 ± 1.6^{b}
20	7	13.4 ± 1.6	33.8 ± 1.6^{a}	38.2 ± 0.1^{b}	14.6 ± 3.4	37.2 ± 1.2^{a}
Two-way ANOVA, P^{c}						
Diet		0.1535	0.0001	0.0447	0.3388	0.0001
Temperature		0.0671	0.0001	0.0269	0.2948	0.0114
Diet × temp. interaction		0.6996	0.2443	0.9554	0.3931	0.0043

[a]Values (mean ± SE of duplicate pooled fecal samples from 40 fish) in the same column with different roman letter superscripts are significantly different ($P < 0.05$).
[b]See footnote of Table 3. No phospholipids, DAG, or MAG were detected in fecal samples. For abbreviations see Table 3.
[c]Significance probability associated with the *F*-statistic.

TABLE 5
FA Composition of Feces Collected from Trout Fed Increasing Dietary Levels of Crude Palm Oil (CPO) and Maintained at Three Different Water Temperatures[a]

	0% CPO			5% CPO			10% CPO			20% CPO			Pooled
FA[b]	15°C	10°C	7°C	15°C	10°C	7°C	15°C	10°C	7°C	15°C	10°C	7°C	SEM
							(% of total FA)						
14:0	7.8^{b}	9.0^{a}	9.4^{a}	4.1^{c,d}	4.6^{c}	4.6^{c}	3.4^{e}	3.6^{d,e}	3.5^{e}	1.3^{f}	1.4^{f}	1.4^{f}	0.8
15:0	1.0^{b}	1.0^{a,b}	1.1^{a}	0.6^{c}	0.5^{c,d}	0.6^{c}	0.4^{c,d}	0.4^{d}	0.4^{d}	0.2^{e}	0.1^{e}	0.1^{e}	0.1
16:0	40.9^{f}	41.8^{f}	47.4^{e}	53.6^{c,d}	51.9^{d}	57.4^{a,b,c}	55.8^{b,c,d}	57.0^{a,b,c}	60.4^{a,b}	60.8^{a}	57.9^{a,b,c}	57.1^{a,b,c}	2.0
16:1n-7	3.1^{a}	2.7^{a}	2.0^{b}	1.6^{b,c,d}	1.9^{b,c}	1.4^{c,d}	1.4^{c,d}	1.4^{c,d}	1.2^{d}	0.4^{e}	0.4^{e}	0.5^{e}	0.2
18:0	9.8^{b}	9.6^{b,c}	11.2^{a}	9.4^{b,c,d}	7.9^{e,f}	8.6^{c,d,e}	9.0^{b,c,d,e}	8.2^{e,f}	8.2^{d,e,f}	7.4^{f,g}	6.4^{g,h}	6.1^{h}	0.4
18:1n-9	7.4^{d}	6.9^{d}	5.5^{d}	12.1^{c}	14.2^{c}	12.4^{c}	14.2^{c}	14.3^{c}	14.2^{c}	23.4^{b}	26.9^{a}	27.4^{a}	2.1
18:1n-7	1.9^{a}	1.8^{a}	1.4^{b}	1.4^{b}	1.4^{b,c}	1.2^{c,d}	1.1^{c,d}	1.1^{c,d}	1.1^{d}	ND	ND	ND	0.2
18:2n-6	2.8^{e}	3.2^{d,e}	2.9^{e}	3.9^{b,c,d}	4.3^{a,b,c}	3.6^{c,d}	3.6^{c,d}	3.7^{b,c,d}	3.2^{d,e}	3.7^{b,c,d}	4.4^{a,b}	5.0^{a}	0.2
18:3n-3	0.5^{a}	0.5^{a}	0.4^{a,b}	0.4^{a,b}	0.4^{a,b}	0.3^{b,c,d}	0.4^{b,c}	0.3^{b,c,d}	0.2^{b,c,d}	0.2^{d}	0.2^{d}	0.2^{d}	0.03
18:4n-3	0.3	0.2	0.1	0.1	0.2	0.1	0.1	0.1	Tr	ND	ND	ND	0.03
20:0	0.8^{b}	0.8^{b}	1.0^{a}	0.8^{b,c}	0.6^{c,d}	0.6^{c,d}	0.7^{b,c}	0.6^{c,d}	0.6^{c,d}	0.6^{c,d}	0.5^{d}	0.4^{d}	0.04
20:1n-9	5.7^{a}	5.2^{a}	4.1^{b}	2.8^{c,d}	2.8^{c,d}	2.1^{d,e}	2.2^{c,d}	2.1^{d,e}	1.4^{e}	0.4^{f}	0.3^{f}	0.3^{f}	0.5
20:4n-6	0.1	0.1	0.1	0.1	0.1	Tr	0.1	0.1	Tr	ND	ND	ND	0.01
20:4n-3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	ND	ND	ND	ND	0.02
20:5n-3	1.0^{a}	0.9^{a,b}	0.4^{b,c,d}	0.4^{c,d}	0.7^{a,b,c}	0.4^{c,d}	0.4^{c,d}	0.5^{a,b,c,d}	0.4^{b,c,d}	0.1^{d}	0.1^{d}	0.2^{d}	0.08
22:1n-11	8.5^{a}	8.3^{a}	6.4^{b}	4.1^{c}	3.8^{c}	2.9^{d}	3.1^{d}	2.7^{d}	2.0^{e}	0.3^{f}	0.2^{f}	0.2^{f}	0.8
22:5n-3	0.3^{a}	0.2^{a,b}	0.1^{b}	0.2^{a,b}	0.2^{a,b}	0.1^{b}	0.2^{a,b}	0.2^{a,b}	Tr	Tr	Tr	Tr	0.02
22:6n-3	3.0^{a,b}	3.1^{a}	2.0^{c}	1.9^{c}	2.2^{b,c}	1.5^{c,d}	1.6^{c,d}	1.6^{c,d}	1.6^{c,d}	0.8^{d}	0.7^{d}	0.8^{d}	0.2
24:1	2.1^{a}	1.8^{b}	1.9^{a,b}	1.1^{c}	0.9^{d}	0.8^{d}	0.9^{d}	0.7^{d,e}	0.6^{e}	0.2^{f}	0.1^{f}	0.1^{f}	0.2
Total saturates	60.3^{e}	62.2^{d,e}	70.2^{a,b,c}	68.3^{a,b,c}	65.6^{c,d,e}	71.9^{a,b}	69.4^{a,b,c}	69.9^{a,b,c}	73.2^{a}	70.2^{a,b,c}	66.2^{b,c,d}	65.2^{c,d,e}	1.1
Total monoenes	30.5^{a}	28.2^{a,b}	22.8^{c,d}	24.0^{c,d}	25.6^{b,c}	21.4^{d}	23.6^{c,d}	22.9^{c,d}	21.0^{d}	24.8^{b,c,d}	28.2^{a,b}	28.6^{a,b}	0.9
Total PUFA	9.2^{a,b}	9.6^{a}	7.1^{a,b,c,d,e}	7.6^{a,b,c,d}	8.8^{a,b,c}	6.7^{b,c,d,e}	6.9^{b,c,d,e}	7.2^{a,b,c,d,e}	5.8^{d,e}	5.0^{e}	5.6^{d,e}	6.2^{c,d,e}	0.4
Total n-3	5.4^{a}	5.4^{a}	3.5^{b}	3.2^{b}	3.9^{a,b}	2.6^{b,c}	2.8^{b,c}	3.0^{b,c}	2.4^{b,c}	1.2^{c}	1.1^{c}	1.2^{c}	0.4
Total n-6	3.5^{d}	3.8^{b,c,d}	3.4^{d}	4.2^{a,b,c,d}	4.7^{a,b}	3.9^{b,c,d}	3.9^{b,c,d}	4.0^{b,c,d}	3.4^{d}	3.8^{c,d}	4.4^{a,b,c}	5.0^{a}	0.2
n-3/n-6	1.5^{a}	1.4^{a}	1.0^{b}	0.8^{b}	0.8^{b}	0.6^{b,c}	0.7^{b,c}	0.7^{b,c}	0.7^{b,c}	0.3^{c,d}	0.2^{d}	0.2^{d}	0.1

[a]Values are the mean of duplicate pooled fecal samples from 40 fish. Mean values in the same row with different superscripts are significantly different ($P < 0.05$). Tr = trace (<0.1 g/100 g FA); ND = nondetectable (<0.01 g/100 g FA).
[b]Some minor FA are not shown (16:2, 16:3, 16:4, 18:2n-3, 18:3n-6, 20:1n-7, 20:2n-6, 22:1n-9, and 22:5n-6).

TABLE 6
Apparent Digestibility (%) of Total Lipid and FA in Rainbow Trout Maintained at Three Different Water Temperatures and Fed Diets Supplemented with Increasing Levels of Crude Palm Oil (CPO)[a]

	0% CPO			5% CPO			10% CPO			20% CPO			Pooled
FA	15°C	10°C	7°C	15°C	10°C	7°C	15°C	10°C	7°C	15°C	10°C	7°C	SEM
14:0	85.5^{a}	74.7^{b,c,d}	76.9^{b,c}	83.6^{a}	75.2^{b,c,d}	74.7^{b,c,d}	80.7^{a,b}	73.0^{c,d}	68.9^{d}	76.6^{b,c}	60.5^{e}	41.9^{f}	3.4
15:0	79.9^{a}	67.2^{b,c}	68.6^{b,c}	75.4^{a,b}	68.8^{b,c}	66.6^{b,c}	73.1^{a,b}	64.7^{b,c}	58.6^{c}	73.1^{a,b}	60.5^{c}	38.3^{d}	3.1
16:0	73.8^{a}	59.1^{b,c}	59.3^{b,c}	61.7^{b,c}	51.5^{c,d}	45.5^{d}	61.9^{b}	49.0^{d}	34.3^{e}	54.8^{b,c,d}	30.8^{e}	0.0^{f}	5.6
16:1n-7	94.4^{a}	92.7^{a}	95.3^{a}	93.0^{a}	89.4^{a}	92.2^{a}	91.8^{a}	89.0^{a}	88.3^{a}	88.3^{a}	79.6^{b}	67.5^{c}	2.2
18:0	65.7^{a}	48.9^{b,c}	47.5^{b,c}	52.2^{b,c}	47.7^{b,c}	42.4^{c,d}	55.4^{a,b}	47.1^{b,c}	34.8^{d}	50.5^{b,c}	31.1^{d}	2.0^{e}	4.6
18:1n-9	91.9^{a}	88.9^{a,b}	92.2^{a}	88.6^{a,b}	82.5^{c,d}	84.4^{b,c,d}	87.8^{a,b,c}	83.8^{b,c,d}	80.5^{d}	81.3^{d}	65.7^{e}	48.8^{f}	3.6
18:1n-7	90.9^{b}	86.9^{b,c}	91.0^{b}	85.5^{b,c,d}	82.3^{c,d}	84.8^{b,c,d}	86.5^{b,c}	82.8^{c,d}	79.4^{d}	100.0^{a}	100.0^{a}	100.0^{a}	2.1
18:2n-6	94.1^{a}	90.1^{b,c,d}	92.3^{a,b}	90.9^{b,c,d}	86.6^{e}	88.6^{c,d,e}	91.8^{a,b,c}	88.8^{c,d,e}	88.2^{d,e}	90.9^{b,c,d}	82.7^{f}	71.5^{g}	1.7
18:3n-3	95.6^{a}	93.2^{a,b}	95.6^{a}	93.0^{a,b}	90.2^{b}	92.9^{a,b}	92.2^{b}	90.1^{b}	90.2^{b}	90.8^{b}	84.9^{c}	76.7^{d}	1.5
18:4n-3	98.6	98.4	99.2	99.0	97.5	98.8	98.5	97.2	99.2	100.0	100.0	95.8	0.4
20:0	63.3^{a}	46.4^{b,c,d}	41.5^{b,c,d}	48.3^{b,c}	43.9^{b,c,d}	38.1^{c,d}	53.1^{a,b}	45.3^{b,c,d}	35.9^{c,d}	33.8^{d}	9.7^{e}	0.0^{e}	6.8
20:1n-9	87.5^{a,b}	82.9^{a,b,c}	88.2^{a}	85.0^{a,b}	80.5^{a,b,c}	85.3^{a,b}	83.7^{a,b,c}	79.0^{b,c}	82.2^{a,b,c}	75.6^{c}	64.0^{d}	51.4^{e}	3.1
20:4n-6	97.3^{a,b,c,d}	96.3^{a,b,c}	97.9^{a,b,c}	96.6^{b,c,d}	94.9^{c,d}	98.0^{a,b,c}	95.5^{b,c,d}	94.4^{d}	98.1^{a,b}	100.0^{a}	100.0^{a}	100.0^{a}	0.6
20:4n-3	96.2^{b,c,d}	96.3^{b,c,d}	98.0^{a,b}	96.8^{a,b,c}	94.3^{c,d}	92.8^{d}	96.1^{b,c,d}	93.2^{d}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	0.8
20:5n-3	98.7^{a,b}	98.1^{a,b}	99.2^{a}	98.6^{a,b}	97.1^{b}	98.5^{a,b}	98.2^{a,b}	97.0^{b}	96.9^{b}	97.2^{a,b}	94.9^{c}	90.9^{d}	0.7
22:1n-11	86.2^{a}	79.5^{a,b,c}	86.0^{a}	83.0^{a,b}	79.4^{a,b,c}	83.7^{a}	74.0^{b,c,d}	70.2^{d}	73.1^{c,d}	79.2^{a,b,c}	69.1^{d}	60.3^{e}	2.3
22:5n-3	97.1^{a,b}	96.3^{a,b}	98.2^{a}	96.7^{a,b}	94.6^{b,c}	96.7^{a,b}	95.7^{a,b,c}	94.1^{b,c}	98.1^{a}	95.0^{a,b,c}	92.7^{c}	87.7^{d}	0.8
22:6n-3	96.8^{a}	95.0^{a,b,c}	97.2^{a}	95.6^{a,b}	93.2^{b,c,d}	95.2^{a,b}	94.9^{a,b,c,d}	92.9^{b,c,d}	91.8^{c,d}	91.8^{c,d}	88.0^{e}	80.7^{f}	1.3
24:1	63.0^{a}	49.0^{a,b,c}	53.7^{a,b}	46.3^{a,b,c}	46.2^{a,b,c}	47.1^{a,b,c}	42.0^{b,c}	39.6^{b,c}	34.8^{c,d}	54.5^{a,b}	39.8^{b,c}	21.2^{d}	3.1
Total saturates	75.4^{a}	61.4^{b}	61.8^{b}	63.6^{b}	54.3^{b,c}	49.2^{c}	63.0^{b}	51.2^{c}	37.8^{d}	55.1^{b,c}	31.9^{d}	1.2^{e}	5.6
Total monoenes	89.5^{a}	85.2^{a,b,c}	89.6^{a}	87.1^{a,b}	82.1^{b,c}	84.8^{a,b,c}	86.2^{a,b,c}	82.3^{b,c}	80.3^{c}	81.5^{b,c}	66.5^{d}	50.1^{e}	3.3
Total PUFA	96.9^{a}	95.1^{a,b}	96.9^{a}	94.9^{a,b,c}	92.3^{c,d}	94.1^{b,c,d}	94.4^{a,b,c,d}	92.2^{c,d}	92.4^{c,d}	91.7^{d}	85.3^{e}	75.7^{f}	1.7
Total n-3	97.5^{a}	96.3^{a,b,c}	98.0^{a}	96.7^{a,b}	94.7^{b,c,d}	96.4^{a,b,c}	96.1^{a,b,c}	94.3^{c,d}	94.6^{b,c,d}	93.5^{d}	90.2^{e}	84.3^{f}	1.1
Total n-6	94.1^{a}	90.3^{b,c}	92.5^{a,b}	91.1^{a,b,c}	86.9^{d}	89.0^{c,d}	91.8^{a,b,c}	88.8^{c,d}	88.7^{c,d}	91.0^{a,b,c}	82.9^{e}	71.9^{f}	1.7
Total FA	88.0^{a}	81.8^{b}	84.1^{a,b}	80.9^{b}	75.1^{c,d}	74.7^{c,d}	79.2^{b,c}	72.6^{d}	66.8^{e}	71.6^{d,e}	54.4^{f}	32.9^{g}	4.4
Total lipid	81.9^{a,b}	82.0^{a,b}	82.5^{a}	81.5^{a,b}	80.3^{a,b}	79.3^{a,b}	82.1^{a,b}	78.5^{a,b}	75.1^{b}	76.8^{a,b}	65.4^{c}	51.5^{d}	2.7

[a]Values are the mean of duplicate pooled fecal samples from 40 fish. Mean values in the same row with different superscripts are significantly different ($P < 0.05$).

and temperature did not significantly affect SROL and SE composition. No interaction between diet and temperature was found for all lipid classes. No PL, DAG, or MAG were detected in any fecal samples.

Compared to the diets, total saturated FA concentrations were relatively higher in the feces, whereas the PUFA were present in much lower amounts (Table 5). Among the PUFA, n-3 FA were relatively more reduced in the feces compared to n-6 FA. The proportion of total monoenes was slightly lower in the feces compared to that found in the diets. The FA composition of the feces was very different from that of the diet, and the differences between the diets were not always maintained in the corresponding fecal samples. For example, the concentration of 16:0 in the 20% CPO diet is more than double the amount found in the 0% CPO diet (Table 2), but this difference was much smaller in the corresponding fecal samples (Table 5).

AD of FA. The AD of individual FA within each temperature regimen generally decreased with increasing FA chain length and increased with increasing unsaturation of the FA irrespective of diet (Table 6). For example, the AD of saturates in fish fed the 0% CPO diet at a water temperature of 15°C decreased from 85.5% for 14:0 to 63.3% for 20:0 (increasing chain length), and for the 18-carbon FA increased from 65.7% for 18:0 to 98.6% for 18:4n-3 (increasing unsaturation). The AD of PUFA was the highest, followed by mononenes and then saturated FA. AD of total n-3 PUFA was consistently higher compared to total n-6 PUFA at each corresponding diet and temperature treatment.

Diet significantly affected the AD of all FA with the exception of 18:4n-3 (Table 7). Water temperature significantly affected the AD of all FA except 18:1n-7, 18:4n-3, 20:4n-3, and 22:5n-3. An interaction between diet and temperature effects was found for all the FA except 18:1n-7, 18:4n-3, 20:0, 20:4n-6, and 24:1. Digestibility of saturates was negatively correlated with the dietary CPO level (Table 6). For example, in fish maintained at 15°C, the AD of 16:0 decreased from 73.8% (fed 0% CPO) to 54.8% (fed 20% CPO). For fish maintained at 7°C, this reduction in AD was more drastic (59.3 to 0.0%). The AD of monoenes and PUFA also decreased with increasing dietary CPO at each water temperature regimen but to a much lesser extent than the saturates. Total lipid digestibility was not significantly reduced in rainbow trout raised at 15°C and fed increasing dietary CPO.

Lowering the water temperature significantly reduced total saturated FA digestibility in trout regardless of dietary treatment. However, total monoene and PUFA digestibilities were

TABLE 7
Summary of Two-Way ANOVA of the Effect of Diet, Temperature, and Their Interaction on the Apparent Digestibility of FA in Rainbow Trout

FA	Source of variation (*F* value, *P*)[a]		
	Diet	Temperature	Diet × temp. interaction
14:0	64.61, 0.0001	71.71, 0.0001	11.70, 0.0002
15:0	11.94, 0.0006	28.27, 0.0001	4.01, 0.0195
16:0	69.98, 0.0001	85.57, 0.0001	9.64, 0.0005
16:1n-7	26.30, 0.0001	7.14, 0.0091	4.89, 0.0095
18:0	27.61, 0.0001	43.29, 0.0001	5.74, 0.0057
18:1n-9	136.25, 0.0001	45.18, 0.0001	20.38, 0.0001
18:1n-7	44.73, 0.0001	1.94, 0.1868	1.08, 0.4273
18:2n-6	65.56, 0.0001	53.49, 0.0001	21.27, 0.0001
18:3n-3	76.61, 0.0001	23.35, 0.0001	15.42, 0.0001
18:4n-3	0.07, 0.9773	0.45, 0.6464	1.37, 0.3019
20:0	56.01, 0.0001	31.67, 0.0001	2.17, 0.1193
20:1n-9	51.61, 0.0001	8.48, 0.0051	6.35, 0.0033
20:4n-6	11.97, 0.0006	5.42, 0.0210	0.93, 0.5065
20:4n-3	14.38, 0.0003	3.24, 0.0749	4.32, 0.0149
20:5n-3	31.64, 0.0001	10.26, 0.0025	7.80, 0.0014
22:1n-11	21.15, 0.0001	5.78, 0.0174	3.44, 0.0325
22:5n-3	18.75, 0.0001	3.19, 0.0777	6.42, 0.0032
22:6n-3	58.64, 0.0001	15.16, 0.0005	9.18, 0.0007
24:1	7.84, 0.0037	6.42, 0.0127	2.61, 0.0741
Total saturates	83.83, 0.0001	83.88, 0.0001	10.92, 0.0003
Total monoenes	89.69, 0.0001	31.89, 0.0001	16.98, 0.0001
Total PUFA	129.44, 0.0001	36.50, 0.0001	24.03, 0.0001
Total n-3	78.17, 0.0001	16.46, 0.0004	12.05, 0.0002
Total n-6	63.93, 0.0001	48.13, 0.0001	20.86, 0.0001
Total FA	168.12, 0.0001	73.27, 0.0001	20.46, 0.0001
Total lipid	42.86, 0.0001	15.90, 0.0004	7.57, 0.0016

[a]Significance probabililty (*P*) associated with the *F*-statistic. Degrees of freedom (DF): diet, DF = 3; temperature, DF = 2; diet × temperature, DF = 6.

not significantly affected by lowering water temperature in fish fed up to 10% CPO, and a significant decrease in AD was apparent only in fish fed the 20% CPO diet. The decrease in total lipid digestibility was less than 7% when water temperature dropped from 15 to 7°C in fish fed up to 10% CPO, but a 25% decrease was observed in fish fed the 20% CPO diet.

DISCUSSION

The observed lipid and FA digestibility values in rainbow trout fed the 0% CPO diet is comparable to that reported in other studies with salmonids fed similar fish oil-based diets (11,14,17). Consistent with other studies (11,13,14), FA digestibility in rainbow trout in the present study decreases with increasing chain length but increases with increasing degree of unsaturation. The preferential absorption by rainbow trout of PUFA, followed by monoenes and then by the saturated FA has been reported in other studies (6,11–13). It was interesting to note that the absorption of total n-3 PUFA was consistently higher than that of total n-6 PUFA irrespective of dietary FA composition, probably indicating the higher importance of preformed n-3 HUFA (highly unsaturated FA) to the nutritional requirements of rainbow trout (18). Despite the lack of extensive data, variations in the digestibility of individual FA in fish have been attributed to the m.p. of the FA [with digestibility decreasing with increasing m.p. (11,14)], lipolytic enzyme specificity for PUFA (27), and the tendency for long-chain saturated and monounsaturated FA to form insoluble soaps with divalent cations in the gut (28).

In general, increasing dietary CPO levels markedly reduced the AD of saturated FA in rainbow trout in the present study. The overall AD of monoenes and PUFA was also affected by increasing CPO levels, especially in trout fed the 20% CPO diet. Using various combinations of fish oils, plant oils, and lard in rainbow trout diets, Caballero *et al*. (6) similarly concluded that an increase in the dietary level of saturated FA would lead to a reduction in the AD of the saturates and, to a lesser extent, also of the other FA. Dietary oils, such as soybean oil, with low levels of saturated FA have very little effect on FA digestibility even when incorporated at high levels (30%) in salmonid diets (15).

When water temperatures were lowered, total monoene and PUFA digestibility were not significantly reduced in trout fed up to 10% CPO in their diets, and a significant drop in digestibility was observed only in fish fed 20% dietary CPO. Olsen and Ringo (16) also reported the lack of temperature influence on monoene and PUFA digestibility in Arctic charr. In the present study, despite significant reductions in the AD of saturated FA, total lipid and FA digestibilities were reduced by less than 7 and 13%, respectively, when water temperature was lowered from 15 to 7°C in fish fed diets with up to 10% CPO. It would seem that the increased gastrointestinal holding time (thereby increasing the time of contact with digestive enzymes

and for absorption of nutrients) in rainbow trout following low-temperature adaptation (29) might be able to offset some of the reduction in lipolysis and absorption rates. However, when the levels of dietary saturates are very high, such as that found in the 20% CPO diet, such compensatory effects are no longer sufficient to minimize the impact on nutrient digestibility of lowered water temperatures.

Luminal micellar solubilization of FA is essential for uptake by enterocytes. Although PUFA are well solubilized over a wide temperature range, the solubilization of saturates decreases with increasing chain length and decreasing temperature (30). In the feces collected in the present study (Table 5), more than 60% of the nonabsorbed fecal lipids were saturated FA but less than 10% were PUFA. The increasing concentrations of undigested TAG in the feces of rainbow trout (Table 4), together with the observation of floating orange-colored oil-like substances on the water surface of the tank maintained at 7°C with fish fed the 20% CPO diet, seem to indicate the increasing solidification of dietary oils in the digestive tract of trout when fed high levels of dietary CPO in decreasing water temperatures. In fish, under normal circumstances, dietary TAG are largely hydrolyzed into FFA and glycerol owing to the presence of two lipases, one with 1,3-specificity and the other a nonspecific lipase (31). In the present study, the reduction in AD of FA, especially the saturates, was due in part to the increasing resistance of dietary TAG to digestion with increasing dietary CPO content and decreasing water temperature. The lack of temperature effects on lipid and FA digestibility reported in some studies (14,15,17) might therefore be due to the fact that the dietary lipid used did not contain high levels of dietary saturates.

The present study showed that total lipid and FA digestibilities were not drastically reduced in rainbow trout fed diets with up to 10% CPO even when water temperatures were lowered to about 7°C. However, at 20% dietary CPO levels, lipid and FA digestibilities were significantly reduced, thereby possibly affecting energy availability to fish fed such diets. Nevertheless, good growth and feed utilization efficiency were still observed in salmonid fish in all the feeding trials conducted to date, despite using diets supplemented with more than 10% dietary CPO and raising them in a wide range of water temperatures (3–6). Caballero *et al*. (6) reported no negative effects on the growth performance of rainbow trout fed diets supplemented with 10% CPO at water temperatures of 12°C. Atlantic salmon fed diets with about 15% CPO showed growth, survival, body traits, and fillet quality similar to fish fed diets containing only added fish oil during the grow-out phase in net pens (temperature 5–15°C) (4). No negative growth effects were observed in Atlantic salmon fed diets with CPO added at up to 24% of the diet over a period of 30 wk with water temperatures ranging from 5.9 to 14.7°C (5). Torstensen *et al.* (3) reported comparable growth performance of Atlantic salmon raised at water temperatures of 8.0 ± 0.4°C and fed high-lipid diets (30% lipid) containing either CPO or capelin oil as the sole lipid source.

It should be noted that the size of rainbow trout used in the present study was much lower than that of the fish used in the growth trials (3–6) listed above. Windell *et al*. (17) reported that only small rainbow trout (about 19 g) showed a significant reduction in nutrient digestibility when water temperatures were lowered from 15 to 7°C. No significant temperature effects on nutrient digestibility were observed in fish of 207 or 586 g. Similarly, Lee (32) reported that lipid and energy digestibilities were reduced by lowering water temperatures only in juvenile Korean rockfish, *Sebastes schlegeli*, of 30 g, and not in adult fish of 300 g. However, a significant effect of water temperature on FA digestibility in adult Korean rockfish was observed when highly saturated beef tallow was added as a lipid source. Data on the variation in FA digestibility over the wide range of water temperatures encountered in the grow-out farms of salmonid fishes of various sizes is currently lacking.

Torstensen *et al*. (3) reported that the AD of FA in Atlantic salmon fed palm oil-supplemented diets was significantly lower than in fish fed fish oil- or sunflower oil-supplemented diets. To explain why this reduced digestibility did not affect growth of the fish, attempts were made to measure total mitochondrial β-oxidation in various fish tissues since FA such as 16:0 and 18:1n-9 (which is abundant in CPO) are reportedly preferred substrates for β-oxidation (33). Unfortunately, as pointed out by the authors themselves, some technical difficulties inherent in the experimental design, such as the use of palmitoyl-CoA instead of palmitoyl-carnitine and the high dietary lipid levels used, may have masked the results, leading the authors to conclude that the different dietary FA compositions did not significantly affect β-oxidation capacity. The preferential use of saturated and monounsaturated FA for the production of energy in the mitochondrial system of fish deserves further investigation. Caballero *et al.* (6) reported that, despite a better AD of 16:0 in rainbow trout fed a CPO-supplemented diet compared to a diet with added lard, the low content of 16:0 found in the liver tissue of fish fed the CPO-supplemented diet suggested that this FA may have been preferentially oxidized to obtain energy, which explains the good growth in fish fed the CPO diet.

Besides fish size and preferential catabolism of 16:0 and 18:1 for energy, other factors, such as the high-lipid feeds used in commercial salmon farming, might also be a factor in ensuring good growth performance despite lowered lipid and FA digestibility when high dietary CPO levels are used. Nevertheless, the results of the present study show that the inclusion of CPO, as a replacement for fish oil, up to 10% in salmonid feeds results in negligible differences in FA digestibility and should not reduce growth performance of fish at the range of water temperatures generally encountered in the grow-out phase. However, inclusion of higher levels of CPO in the diets of cold-water species may result in some problems with FA digestibility. The potential for CPO use in warm-water fishes would be significantly greater (8,10).

In conclusion, CPO remains a potential partial alternative to marine fish oils in salmonid diets, considering the lower cost and long-term production sustainability of this oil source. Apart from its academic value, the lipid and FA digestibility data presented in this study will enable feed formulators to better calculate for dietary energy availability and to optimize the use of CPO in salmonid diets according to environmental temperatures.

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Effect of Orlistat on Fat Absorption in Rats: A Comparison of Normal Rats and Rats with Diverted Bile and Pancreatic Juice

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ABSTRACT: Orlistat is a specific inhibitor of pancreatic and gastric lipases leading to decreased absorption of fat. In the present study, we measured the effect of orlistat on lymphatic fat transport in rats following intake of oils very different in FA composition and TAG structure, and compared this with the transport in normal rats and rats with fat malabsorption. Rats were subjected to cannulation of the main mesenteric lymph duct, and a feeding catheter was inserted into the stomach. In addition, malabsorbing rats were cannulated in the common bile and pancreatic duct. Emulsified safflower, fish, and randomized oils were administered, and lymph was collected for 24 h and analyzed for FA composition. Administration of 25 mg orlistat together with the dietary oils resulted in very small changes from baseline lymphatic transport, indicating that inhibition of the fat absorption was almost complete and furthermore that the source of fat had no influence on the inhibitory effect of orlistat. Orlistat did not interfere with the absorption of the hydrolysis products, since high absorption of *sn*-2 MAG and FFA (oleic acid) mixed with orlistat was observed. The baseline lymphatic transport in the orlistat group was higher than in the malabsorbing group, but this was the result of generally lower transport of endogenous FA in the malabsorbing group, presumably caused by the absence of bile FA. The transport of FA in normal rats was severalfold higher than the transport after orlistat addition and in malabsorbing rats. Thus, this study showed that orlistat inhibited fat hydrolysis, and thereby lymphatic absorption, almost completely independently of the fat administered.

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Pancreatic lipase catalyzes the intestinal hydrolysis of ingested TAG with preference for the *sn*-1,3 positions, resulting in *sn*-2 MAG and FFA (1,2). The major route of absorption for the long-chain FA and *sn*-2 MAG is through the enterocytes, with high conservation of the FA located in the *sn*-2 position of the dietary fat (3), whereas short- and medium-chain FA are absorbed primarily *via* the portal vein (4). In the enterocytes, the *sn*-2 MAG are reesterified with FA of exogenous and endogenous origin to form a new population of TAG, and these are packed into chylomicrons and secreted to the lymph.

Orlistat is a chemically synthesized, hydrogenated derivative of lipstatin, a naturally occurring lipase inhibitor produced by *Streptomyces* strains (5). It is a very lipophilic molecule that inhibitis gastric and pancreatic lipases by forming a covalent bond to the active-site serine of the lipase (6–8), thereby leading to decreased fat absorption. The effect of orlistat on fat absorption in obese subjects was investigated in a range of clinical trials (9–13). In comparison with a placebo, orlistat induced larger weight losses in combination with hypocaloric diets and smaller weight regains in combination with weight-maintenance diets. Furthermore, orlistat treatment improved obesity-related risk factors to a greater extent than the placebo. A retrospective analysis of the dose–response relationship of orlistat on fecal fat excretion in humans revealed that 32% was the maximum obtainable fat excretion. At doses higher than 400 mg/d, fat excretion showed a tendency to plateau (14). In animal studies a higher inhibition of fat absorption was observed (15,16).

The aim of the present study was to measure the effect of orlistat on lymphatic fat transport after administration of three very different oils: a safflower oil, a fish oil, and an oil made by random interesterification of fish oil and 10:0. The oils thus differed in FA chain lengths, FA composition, and TAG structure. This absorption was compared with the absorption in normal rats and in rats with fat malabsorption. The lymphatic system is the major route for fat absorption, and to our knowledge, lymphatic absorption together with orlistat administration was investigated in only one other study in which only the absorption of triolein was measured (16). Studies by Isler *et al.* (17) showed that administration of 10 µmol/kg (~5 mg/kg) orlistat to mice resulted in elimination of 20% of the administered fat in feces, 100 µmol/kg (~49.6 mg/kg) orlistat resulted in elimination of 70% of the administered fat, and 1000 µmol/kg (~496 mg/kg) resulted in elimination of 80% of the administered fat. In the present study each rat was given 25 mg orlistat (~90 mg/kg) together with the oil, and on the basis of the above-mentioned study, we expected the inhibition to be close to 70%. A minor experiment was conducted in which 1 mg (~3–4 mg/kg) of orlistat mixed with the randomized oil was administered to rats to investigate the effect of a supposed relatively low inhibition of the pancreatic lipase.

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Abbreviations: FFA, oleic acid; 2 MG, 2-monopalmitoylglycerol.

MATERIALS AND METHODS

Oils. The following oils were used for the experiment: safflower oil (Róco, Copenhagen, Denmark), fish oil (Aarhus Olie, Aarhus, Denmark), and a randomized oil produced by chemical interesterification of fish oil and tridecanoin (Grünau GmbH, Illertissen, Germany) by using sodium methoxide as catalyst, resulting in random distribution of FA in the TAG.

Animal experiment. Male Wistar rats (Møllegård Breeding Centre, Ll. Skensved, Denmark) weighing 260–300 g were used for the experiment. They were fed a standard rat chow diet (Altromin No. 1324; Chr. Petersen A/S, Ringsted, Denmark) in the animal department for at least 10 d before surgery. The experiment was approved by the Danish Committee for Animal Experiments.

In normal rats, a catheter was inserted in the main mesenteric lymph duct (18), whereas malabsorbing rats were cannulated in the common bile and pancreatic ducts as well (19). This was performed to divert both bile and pancreatic juice, thereby making the rats malabsorbing. Details about lymph collection have been reported previously (18,19). Briefly, on the postoperative day the rats received an emulsion composed of oil and taurocholate/choline, and lymph was collected for the next 24 h. Each of the three oils was administered to three groups of rats (n = 5–7 in each group): normal, malabsorbing, and normal rats to which 25 mg (~90 mg/kg) of orlistat (F. Hoffmann-La Roche Ltd., Basel, Switzerland) was added to the oil before emulsification. One group of rats (n = 3) received 1 mg (~3–4 mg/kg) of orlistat mixed with the randomized oil. Normal rats were administered with 270 mg oil, and malabsorbing rats and normal rats that were administered with orlistat also received either 450 mg oil added in the fish and randomized oil groups or 305 mg in the safflower oil groups. Malabsorbing rats and rats receiving orlistat were administered more oil to ensure that the lymph contained measurable amounts of fat.

A group of rats (n = 4) received a bolus of 115 mg 2-monopalmitoylglycerol (2-MG; Larodan Fine Chemicals AB, Malmö, Sweden), 195 mg oleic acid (FFA; Sigma-Aldrich Corp., St. Louis, MO), and 25 mg orlistat to investigate whether orlistat had any influence on the lymph absorption model as such. The amounts of fat administered corresponded to the amounts administered to rats receiving safflower oil and orlistat.

FA composition of oils and lymph samples. After addition of an internal standard (15:0 or 17:0 as methyl esters), total lipids were extracted from the lymph with chloroform and methanol (20). The FA composition was determined after transesterification catalyzed by KOH in methanol (21), and the FAME were analyzed by GLC as described previously (18). The TAG structure of oils was determined by Grignard degradation (22). The FA composition in TAG and in *sn*-2 MAG is shown in Table 1.

Calculations and statistical analyses. Results were expressed as means ± SEM. Recoveries of FA were calculated as the ratio between the amount of a FA that accumulated in lymph at 24 h and the amount of that FA administered. Differences between groups were tested using one-way ANOVA, and the Newman–Keuls method was used to determine the exact nature of the differences (Prism, version 3.02; GraphPad Software Inc., San Diego, CA).

TABLE 1
FA Composition in TAG and *sn*-2 MAG[a]

	mol%					
	Randomized oil		Fish oil		Safflower oil	
FA	TAG	*sn*-2	TAG	*sn*-2	TAG	*sn*-2
10:0	47.0	48.9	—	—	—	—
14:0	4.4	4.8	9.0	12.6	0.1	—
16:0	9.6	10.3	19.3	25.7	7.4	0.3
16:1	3.6	3.6	7.4	7.8	0.1	0.1
18:0	1.4	1.4	2.5	0.6	2.4	0.1
18:1n-9	5.4	5.6	10.3	4.7	13.0	13.6
18:1n-7	1.5	1.4	2.2	0.9	0.4	—
18:2n-6	1.0	1.1	1.9	1.6	74.2	83.7
18:3n-3	0.8	1.0	1.5	1.7	0.6	0.5
18:4n-3	2.5	2.3	4.8	4.7	—	—
20:1n-9	3.6	3.7	6.8	1.7	0.2	—
20:5n-3	6.0	5.3	10.6	12.9	—	—
22:1n-11	5.7	5.5	10.4	2.1	—	—
22:6n-3	7.6	5.1	12.4	21.0	—	—
Others	—	—	0.9	2.0	1.6	1.7

[a]Values are the mean of two determinations for FA composition and three determinations for *sn*-2 MAG. A dash (—) means not detected.

RESULTS

Absorption of total FA. Figures 1–3 show the lymphatic transport of total FA in rats receiving randomized, fish, and safflower oils, respectively. A similar absorption pattern was observed after administration of all three oils to the different groups of rats. The FA transport in normal rats increased during absorption, reaching a maximum 2–5 h after oil administration. This transport was severalfold higher than the transport in the other groups. Lymphatic transport in the groups with orlistat added and in the malabsorption group did not change from baseline after oil administration. Visual inspec-

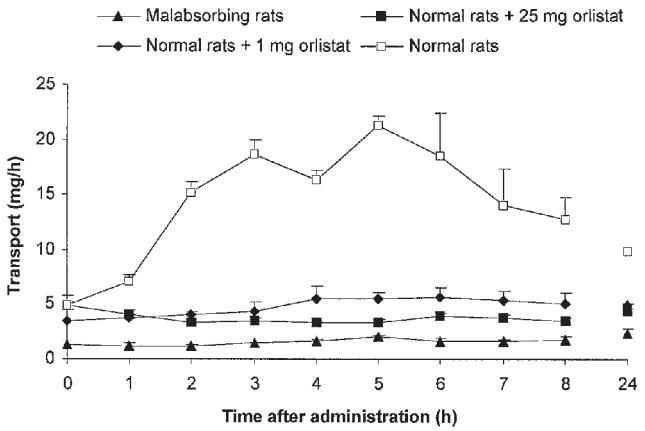

FIG. 1. Lymphatic transport of total FA (mg/h) after administration of the randomized oil to normal, malabsorbing, and normal rats administered with either 1 or 25 mg orlistat. Values are means ± SEM (n = 3–7).

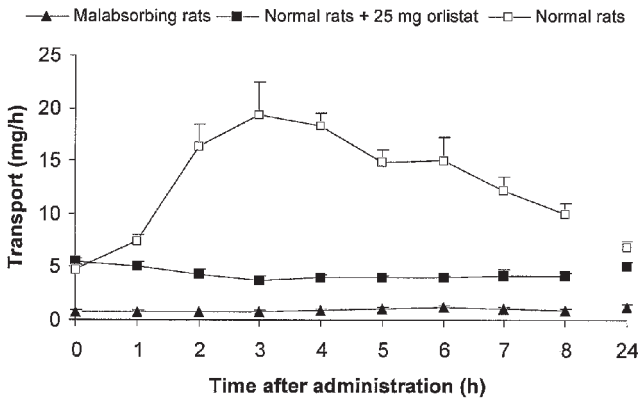

FIG. 2. Lymphatic transport of total FA (mg/h) after administration of the fish oil to normal, malabsorbing, and normal rats administered with 25 mg orlistat. Values are means ± SEM (n = 5–7).

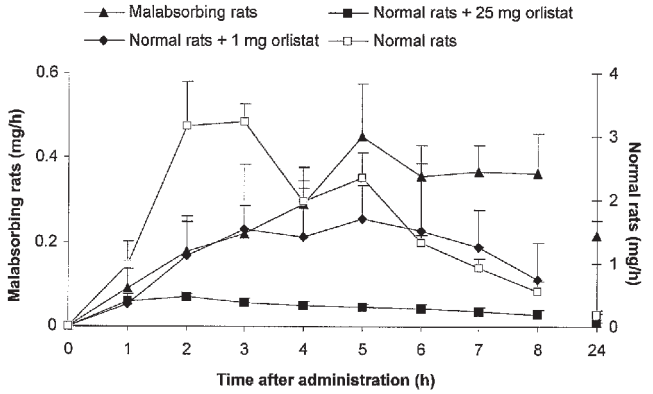

FIG. 4. Lymphatic transport of 10:0 (mg/h) after administration of the randomized oil to normal, malabsorbing, and normal rats administered with either 1 or 25 mg orlistat. Values are means ± SEM (n = 3–7). Notice the different scale for the normal rats.

tion during absorption revealed that the color of the lymph in the group with normal absorption changed from clear to white, whereas this was not observed in the other groups, indicating that only limited fat absorption took place in these groups.

Absorption of lipid hydrolysis products. To investigate whether orlistat interfered with the fat absorption model as such, a group of rats was administered 2-MG, FFA, and orlistat. The total FA transport increased from 8.6 mg/h at baseline to 35.5 mg/h at maximum 2 h after administration, showing high absorption of lipid hydrolysis products.

Absorption of individual FA. For the absorption of individual FA, almost no changes in FA transport were observed in the 25-mg orlistat group. This is illustrated by the transport of the purely exogenous FA 10:0 after administration of the randomized oil (Fig. 4). The transport of 10:0 in the 25-mg group, with a maximum at 0.07 mg/h 2 h after administration, was significantly lower than the transport in the other groups ($P < 0.05$). This indicated almost complete inhibition of the pancreatic lipase, leading to very low hydrolysis of the administered TAG. The transport of 10:0 in the 1-mg orlistat group reached a maximum at 0.26 mg/h 5 h after administration, indicating that at this orlistat concentration the lipase was not completely inhibited. When orlistat was administered at the low concentration, large variations in absorption profiles occurred between individual rats. The maximum transport of 10:0 in the malabsorption group was observed at 5 h (0.45 mg/h); this maximum was late and had a low value compared with that of normal rats, who reached the maximum between 2–3 h (3.2 mg/h). Similar absorption profiles were observed for other FA in rats administered the three different oils (data not shown). The FA composition of total lipids in the lymph samples after administration of the three different oils in combination with orlistat was almost identical (data not shown).

Recovery of FA. As shown in Table 2, recovery of exogenous 10:0 after administration of the randomized oil was 0 ± 0% in the 25-mg orlistat group. This amount was significantly lower ($P < 0.05$) compared with the normal and malabsorbing groups, emphasizing the almost total inhibition of absorption. Recovery of the almost purely exogenous FA 20:5n-3 was significantly lower for both the 25-mg orlistat group and the malabsorbing group compared with normal rats ($P < 0.05$). After administration of the three different oils, recovery of total FA was highest in normal rats, followed by those with orlistat added and the malabsorbing rats. That recovery was higher in those with orlistat added compared with the malabsorbing rats was the result of the generally higher lymphatic transport in the orlistat group, as observed in Figures 1–3. Since almost no exogenous FA were absorbed in the orlistat group, the transported FA must have been of endogenous origin, such as bile FA. This can also be observed from the recovery calculations of 18:2n-6 after safflower oil administration. The recovery of this FA in the group with orlistat added was higher compared with the recovery of 10:0 and 20:5n-3 in the other oil groups, showing the generally higher endogenous contribution of 18:2n-6 to lymph. The baseline transport in the malabsorption group was significantly lower than in the other groups (1–2 mg/h compared with 4–6 mg/h). This could be explained partly by the diversion of bile, since bile FA contribute 1.5–3 mg/h to the total FA transported in lymph (Fruekilde, M.-B., personal communication).

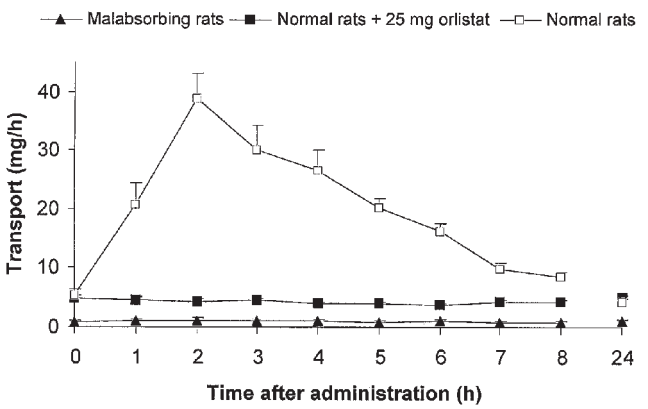

FIG. 3. Lymphatic transport of total FA (mg/h) after administration of the safflower oil to normal, malabsorbing, and normal rats administered with 25 mg orlistat. Values are means ± SEM (n = 6–7).

TABLE 2
Recovery of Individual and Total FA 24 h After Oil Administration[a]

FA	Recovery (%)		
	Normal rats	Malabsorbing rats	Normal rats + 25 mg orlistat
Randomized oil			
10:0	18 ± 2[a]	3 ± 1[b]	0 ± 0[c]
20:5n-3	55 ± 4[a]	5 ± 0[b]	3 ± 0[b]
Total FA	105 ± 4[a]	11 ± 1[b]	22 ± 1[c]
Fish oil			
20:5n-3	31 ± 3[a]	3 ± 1[b]	2 ± 0[b]
Total FA	83 ± 5[a]	6 ± 1[b]	26 ± 2[c]
Safflower oil			
18:2n-6	63 ± 4[a]	4 ± 1[b]	14 ± 1[c]
Total FA	89 ± 6[a]	7 ± 2[b]	37 ± 2[c]

[a]Data are expressed as mean ± SEM of 5–7 rats. Values in rows not sharing a superscript letter are significantly different ($P < 0.05$).

DISCUSSION

Overall, the results showed that orlistat in this fat absorption model inhibited the absorption almost completely. In addition, the results showed that the source of fat had no influence on the ability of orlistat to inhibit fat absorption since the absorption of oils differing in FA composition and TAG structure—such as the randomized, fish, and safflower oils—was inhibited to a similar degree. Normally, the FA composition of lymph lipids changes during absorption according to the FA composition of the oil administered (23), but this was not observed in the orlistat groups in the present study, indicating the endogenous nature of the FA transported in lymph in these groups. Furthermore, the low fat transport in the orlistat groups was the result of an almost complete inhibition of pancreatic lipase, resulting in low fat hydrolysis rather than orlistat interfering with the absorption hydrolysis products, since administration of orlistat together with TAG hydrolysis products (2-MG and FFA) resulted in high fat absorption. In the group of rats made malabsorbing by surgery, the low transport of FA was the result of their shortage of bile and pancreatic juice.

The inhibition seemed to be dose-dependent, with higher inhibition in the 25-mg group (~90 mg/kg) compared with the 1-mg group (~3–4 mg/kg), although the absorption was still marginal compared with rats with normal absorption. Dose-dependent inhibition of triolein absorption in mice was demonstrated by Weibel *et al*. (5) by using lipstatin purified from *S. toxytricini*. At the maximum dose of 60 mg/kg, the lipid absorption measured as feces excretion of labeled fat was reduced to 21%.

In the present experiment, the absorption of a purely exogenous FA (10:0) was inhibited 100% when orlistat was added compared with the absorption in normal rats. The recovery of 10:0 in lymph from normal rats was far from 100% since short- and medium-chain FA were partly absorbed *via* the portal vein, depending on the chain length of the FA (4,18). In previously performed orlistat experiments, the thoracic duct absorption of triolein in rats in the presence of orlistat (10^{-4} M) decreased to 30% of the absorption in control rats (16), whereas the amount absorbed by mice after addition of 49.6 mg/kg orlistat decreased to 20% when the amounts of fat excreted in feces were measured (15). The discrepancy between the lower inhibition in these experiments and the almost complete inhibition of fat absorption in the present experiment could be caused partly by differences in the mode of orlistat addition. In our experiment, orlistat was mixed with the oil and emulsified before administration of a single bolus directly into the stomach, whereas in the feces excretion experiment, orlistat was given immediately after a test meal (5,15). This could result in differences in the effectiveness of the inhibitor. This was further supported by Isler *et al.* (17), who showed that orlistat was more effective at inhibiting TAG absorption when it was administered in meals rather than after meals, indicating that the equilibration between lipid phases in the stomach may not always be complete.

The results obtained in the present study—in which fat absorbed in the small intestine was drained and the inhibition of fat absorption was almost complete—could suggest that the large intestine contributes to the absorption of fat to a higher degree than normal when pancreatic lipase is inhibited. Although the transport of total FA in the malabsorption group was lower than that in the other groups, part of the oil administered was absorbed, as exemplified by the transport of 10:0 in this group compared with rats receiving 25 mg orlistat. This means that in malabsorption situations, a larger part of the intestine could be involved in fat absorption, eventually following degradation to shorter-chain FA by the gut bacteria and thereby partly counterbalancing the lower activity of the pancreatic lipase.

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Dietary α-Linolenic Acid Increases the n-3 PUFA Content of Sow's Milk and the Tissues of the Suckling Piglet

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ABSTRACT: α-Linolenic acid (18:3n-3) is a precursor to DHA (22:6n-3), which is essential for normal growth and development in the infant. This study was undertaken to assess how a raised 18:3n-3 intake in sows affects the n-3 PUFA content of the suckling piglet. Sows consumed a high-18:3n-3 or control diet (n-3 PUFA/n-6 PUFA, 0.5 vs. 0.05, respectively) for 10 d prior to parturition and for 14 d postpartum. Piglets suckled from their mothers until 14 d of age, when they were sacrificed. Sows consuming the high-18:3n-3 diet had 141% more 18:3n-3 and 86% more 22:6n-3 in their milk compared to control sows. There was no difference in the proximate composition of the piglets. The n-3/n-6 PUFA ratio was 82% higher in the milk of sows consuming the high-18:3n-3 diet compared to controls. Piglets suckling from sows consuming the high-18:3n-3 diet had 423% more 18:3n-3 in the carcass as well as a 460% higher n-3/n-6 PUFA ratio than controls. The piglets suckling from sows consuming the high-18:3n-3 diet had 333% more 18:3n-3 and 54% more 22:6n-3 in the liver, as well as a 114% higher n-3/n-6 ratio than control piglets. Piglets suckling from sows consuming a high-18:3n-3 diet also had 24% more 22:6n-3 and a 33% higher n-3/n-6 ratio in the brain compared to control piglets. A high 18:3n-3 intake in the sow increases not only the 18:3n-3 but also the 22:6n-3 content of sow's milk and the tissues of the suckling piglet.

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n-3 PUFA are essential for normal growth and development. DHA (22:6n-3) is found in high concentrations in the retina and neuronal membranes (1–3). Deficiency of n-3 PUFA during early development results in visual and neurological dysfunction and may have adverse long-term sequelae (4,5). Infants not consuming breast milk or formula containing 22:6n-3 currently rely on their stores of 22:6n-3 or their ability to elongate and desaturate α-linolenic acid (18:3n-3) from infant formula to form 22:6n-3 . Sevenfold more 22:6n-3 enters the neonatal baboon brain when provided directly as 22:6n-3 compared to the amount made endogenously (9). Stable isotope studies in humans suggest that the mother's dietary intake of linoleic acid (18:2n-6) contributes only low amounts of arachidonic acid (20:4n-6) to milk 20:4n-6 content (2.2–3.2%) (10). Whether the same is true of 18:3n-3 contributing to milk 22:6n-3 is not known (11,12). However, a recent 18:3n-3 feeding trial in lactating women suggests feeding 18:3n-3 to lactating women does not increase 22:6n-3 in milk (13). Several factors may limit the ability of 18:3n-3 to form 22:6n-3, including the high partitioning of 18:3n-3 toward oxidation relative to its elongation/desaturation to 22:6n-3 (14).

A recent workshop recommended an increased intake of 22:6n-3 during pregnancy and lactation but no increases for 18:3n-3 (15). Piglets have been used extensively as a model to study the role of PUFA in early development. Piglets from sows fed diets high in fish oil have a higher n-3 PUFA content in their tissues (16). Piglets consuming artificial formula high in EPA (20:5n-3) from birth also have increased tissue n-3 PUFA content (17). Feeding 22:6n-3 to the sow increases the 22:6n-3 content of the milk and the tissues of the suckling piglet (18). Several studies have reported that feeding artificial formula rich in 18:3n-3 to piglets increases the n-3 PUFA composition of the piglet tissues (19–21). In addition, several studies have reported that pigs born to sows consuming fish oil during pregnancy have an increased n-3 PUFA content (16,22–24). However, it is not known whether a raised 18:3n-3 intake in the sow affects n-3 PUFA accumulation in the tissues of the suckling piglet.

Higher 18:3n-3 intake in lactating women does not necessarily raise 22:6n-3 in milk, suggesting it is important to model whether 18:3n-3 supplementation during pregnancy and lactation is beneficial to the developing neonate (13). Few data are available on this subject. Our objective was therefore to investigate whether feeding sows a high-18:3n-3 diet just prior to parturition and during lactation would increase the n-3 PUFA composition of the suckling piglet. The study was approved by the Shur-Gain Agresearch Animal Care Committee.

METHODS

Animals and diets. Sixteen pregnant Yorkshire-Landrace sows consumed either a control diet or a diet high in 18:3n-3, starting approximately 8–10 d prior to parturition and continuing until day 14 of lactation (Table 1). The diets consisted of base commercial pig feed (Shur-Gain Feed #694; Guelph, Ontario, Canada) containing 469 g/kg of corn, 117.3 g/kg of soybean meal, 100 g/kg of wheat middlings, 100 g/kg of bakery meal, 176.6 g/kg of barley, 50 g/kg of meat meal, 30 g/kg of bran, 7.5 g/kg of limestone, 15 g/kg of dicalcium phosphate, 3.3 g/kg of salt, and 8.9 g/kg of a vitamin/mineral/trace

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Abbreviations: arachidonic acid, 20:4n-6; DHA, 22:6n-3; docosapentaenoic acid, 22:5n-3; EPA, 20:5n-3; linoleic acid, 18:2n-6; α-linolenic acid, 18:3n-3.

TABLE 1
Total FA Composition of Control and High-18:3n-3 Sow Diets

FA	Control	High-18:3n-3
16:0	14.01[a]	13.3
18:0	4.6	5.6
Sum SFA[b]	19.0	19.4
18:1n-9	28.0	26.8
Sum MUFA[b]	29.7	28.9
18:3n-3	2.3	17.9
Sum n-3 PUFA[b]	2.3	17.9
18:2n-6	48.6	33.5
20:4n-6	0.1	0.1
Sum n-6 PUFA[b]	49.1	33.9
Total FA (mg/g)	58.4	54.6

[a]Percentage of total FA >12C.
[b]Sum includes more FA than those shown. SFA, saturated FA; MUFA, monounsaturated FA.

amino acid supplement. The control diet consisted of the base pig feed supplemented with 22.5 g/kg corn oil, whereas the high-18:3n-3 diet consisted of the same base pig feed supplemented with 2.5 g/kg tallow and 20 g/kg flaxseed oil. The diets varied only in oil source and hence only in FA composition. Piglets suckled from their mothers until day 14. The median-weight piglet from each sow was anesthetized and sacrificed (nonfasted) at 14 d of age. Blood, viscera, liver, and brain were harvested to evaluate organ FA profiles. The carcass is defined as all tissues minus the brain, liver, and viscera. The carcass and liver were homogenized separately using a Hobart grinder with a 2.54-cm die. A minimum of 80 g of carcass was freeze-dried for proximate analysis, whereas 5 g was frozen at –20°C for FA analysis. After injection with oxytocin, milk samples were collected by hand from one teat at 7 d postpartum from the sows and frozen at –20°C for analysis.

Tissue composition. Carcass dry weight was determined after 2 h at 100°C in a drying oven. Dried samples were then analyzed for fat, protein, and ash according to AOCS method Ba 3-38 (25) and AOAC methods 990.03 and 925.23 (26), respectively.

Lipid extraction and FA analysis. Milk, plasma, and tissue total lipids were extracted using chloroform/methanol (2:1, vol/vol). Heptadecanoic acid (Sigma, St. Louis, MO) was added as an internal standard to an aliquot of the plasma and tissue total homogenate to quantify total lipids. Total lipids were then extracted and the FA were converted to FAME using 14% boron trifluoride-methanol (Sigma) under nitrogen (27). FAME were analyzed by GLC using a capillary column (DB-23, 30 m × 0.25 mm i.d.; J&W Scientific, Folsom, CA), in a Hewlett-Packard 5890A gas–liquid chromatograph (Palo Alto, CA) with automated sample delivery and injection (Hewlett-Packard 7671A) and peak integration (Hewlett-Packard 3393 integrator) using a three-stage temperature program from 2 min at 50°C, to 180°C at 10°C/min, to 220°C at 5°C/min for 5 min (27). FA were identified by comparing the retention times of samples to authentic standards (Nu-Chek-Prep, Elysian, MN; Matreya, Pleasant Gap, PA) and were quantified on the basis of the proportion of each FA on the chromatogram relative to the internal standard added to each sample.

TABLE 2
Sow Milk Total FA Composition of Sows Consuming the Control and High-18:3n-3 Diet

FA	Control	High-18:3n-3
16:0	23.9 ± 2.7	25.2 ± 4.9
18:0	4.3 ± 0.7	5.1 ± 0.6*
Sum SFA[b]	31.9 ± 3.3	34.0 ± 5.4
18:1n-9	33.2 ± 3.6	32.3 ± 5.8
Sum MUFA[b]	44.8 ± 3.8	43.6 ± 3.8
18:3n-3	1.7 ± 1.9	4.1 ± 1.7*
20:5n-3	0.1 ± 0.1	0.1 ± 0.1
22:6n-3	0.07 ± 0.02	0.13 ± 0.03*
Sum n-3 PUFA[b]	2.8 ± 2.7	4.3 ± 2.6*
18:2n-6	19.4 ± 2.8	16.1 ± 2.8*
20:4n-6	0.9 ± 0.2	0.9 ± 0.4
Sum n-6 PUFA[b]	21.1 ± 3.2	17.8 ± 3.5*

[a]Percentage of total FA >12C; $n = 8$ sample per group; mean ± SD. Sows began consumption of the appropriate diet 8–10 d prior to parturition and milk was collected 7 d postpartum.
[b]Sum includes more FA than those shown. *$P < 0.05$ vs. control. For abbreviations see Table 1.

Data analysis. All data are expressed as means ± SD. Statistical comparisons between dietary treatments were made using Student's *t*-test. All statistics were performed using the Statistical Analysis System, version 8.01 (SAS Institute, Cary, NC).

RESULTS

Sows consuming a high-18:3n-3 diet produced milk with more 18:0, 18:3n-3, and 22:6n-3 (19, 141, and 86%, respectively) and with 17% less 18:2n-6 (Table 2) vs. sows consuming the control diet. Suckling from sows consuming a high-18:3n-3 diet did not significantly affect the body weight (4.6 ± 0.7 vs. 4.5 ± 0.7 kg for the control and high-18:3n-3 diets, respectively) or the proximate composition (moisture, protein, fat, ash, calcium, phosphorus, and potassium) of the piglet carcass (Table 3). Piglets suckled from sows consuming a high-18:3n-3 diet had higher levels of plasma and liver total lipids of 18:3n-3, 20:5n-3, and 22:6n-3 and lower levels of 20:4n-6 (Table 4). There was no change in the total FA content of the liver. Piglets that suckled from sows consuming a high-18:3n-3 diet had the increased brain total lipids docosapentaenoic acid (22:5n-3) and 22:6n-3 (78 and 24%, respectively) with 12% less 22:4n-6 and 22% less 22:5n-6 (Table 5). Piglets that suckled from sows

TABLE 3
Carcass Proximate Composition of 14-d-old Piglets That Suckled from Sows Consuming a Control or High-18:3n-3 Diet

Component	Control	High-18:3n-3
Moisture	65.9 ± 2.1[a]	66.4 ± 2.9
Protein	16.7 ± 1.1	16.0 ± 0.9
Fat	13.2 ± 2.9	14.1 ± 3.5
Ash	3.4 ± 0.5	2.9 ± 0.6
Calcium	0.97 ± 0.19	0.81 ± 0.2
Phosphorus	0.64 ± 0.09	0.54 ± .013
Potassium	0.23 ± 0.01	0.21 ± 0.03

[a]Percentage of carcass wet weight; $n = 8$ per group; mean ± SD; no differences detected, $P > 0.05$.

TABLE 4
Plasma and Liver Total FA Composition of Piglets Suckling from Sows Consuming a Control or High-18:3n-3 Diet

	Plasma		Liver	
FA	Control	High-18:3n-3	Control	High-18:3n-3
Sum SFA[a]	35.7 ± 2.4[b]	37.1 ± 10.1	36.5 ± 3.3	36.4 ± 3.0
Sum MUFA[a]	23.1 ± 2.2	23.4 ± 4.3	16.4 ± 1.8	16.4 ± 2.9
18:3n-3	0.7 ± 0.6	2.7 ± 1.1*	0.3 ± 0.1	1.3 ± 0.5*
20:5n-3	0.2 ± 0.1	0.6 ± 0.3*	0.1 ± 0.1	0.9 ± 0.4*
22:6n-3	1.1 ± 0.4	1.4 ± 0.3*	3.7 ± 0.5	5.7 ± 0.8*
Sum n-3 PUFA[a]	2.6 ± 1.2	5.7 ± 1.8*	5.5 ± 0.7	10.4 ± 1.5*
18:2n-6	27.8 ± 2.2	25.7 ± 5.2	17.9 ± 1.7	17.5 ± 1.4
20:4n-6	8.7 ± 0.6	6.4 ± 1.3*	20.0 ± 1.5	16.7 ± 1.5*
Sum n-6 PUFA[a]	38.5 ± 2.9	33.8 ± 6.2*	41.5 ± 2.1	36.7 ± 1.6*
Total FA[c]	1.9 ± 0.7	2.5 ± 0.8	25.4 ± 3.3	25.0 ± 3.2

[a]Sum includes more FA than those shown. *$P < 0.05$ vs. control. For abbreviations see Table 1.
[b]Percentage of total FA >12C; $n = 8$ per group; mean ± SD.
[c]Plasma (mg/mL) and liver (mg/g), respectively.

consuming the high-18:3n-3 diet also had a 13% higher brain total lipid content. Piglets suckling from sows consuming a high-18:3n-3 diet had 423% more 18:3n-3, 1000% more 20:5n-3, and 19% less 18:2n-6 in their carcass total lipids (Table 6). There was no difference in the total lipid contents of the piglet carcass. Piglets suckling from sows consuming a high-18:3n-3 diet had a higer n-3/n-6 PUFA ratio in plasma, liver, brain, and carcass (150, 114, 33, and 460%, respectively), and sows had a higher n-3/n-6 PUFA ratio in milk (82%) (Fig. 1).

DISCUSSION

In this study, feeding sows a high-18:3n-3 diet increased the 18:3n-3 and 22:6n-3 content and decreased the 18:2n-6 content, but did not affect the 20:4n-6 content of the milk. An increase in the 22:6n-3 content of the milk with no decrease in the milk 20:4n-6 content has been reported after feeding sows diets supplemented with fish oil, but this increase has not been reported previously with 18:3n-3 supplementation (18). Women supplemented with 18:3n-3 had increased 18:3n-3, 20:5n-3, and 22:5n-3 but no changes in 22:6n-3 or 20:4n-6 in their milk (13).

Piglets suckling from sows consuming the high-18:3n-3 diet had higher total lipid levels of 18:3n-3 and 22:6n-3 in the plasma, liver, and brain, whereas 20:4n-6 was decreased in all of these tissues except the brain. This is consistent with previous findings of piglets suckling from sows consuming fish oils and receiving 18:3n-3 from artificial formula, but has not been reported previously in pigs suckling from sows receiving 18:3n-3 supplementation (16–24). Rat pups suckling from dams consuming a high-18:3n-3 diet have lower brain total lipid 20:4n-6 with no changes in brain or whole body 22:6n-3 (28). Human infant brain 20:4n-6 appears to be less influenced than 22:6n-3 by 18:3n-3 intake than the previously mentioned studies (29). In our study, piglets suckling from sows consuming the high-18:3n-3 diet had increased carcass 18:3n-3 but not carcass 22:6n-3. A similar result also was reported in guinea pigs (30). As discussed by Fu and Sinclair (30), the lack of increase in carcass 22:6n-3 may be due to the high β-oxidation of 18:3n-3 relative to its conversion to 22:6n-3 (14,31–33).

TABLE 5
Brain Total FA Composition of Piglets Suckling from Sows Consuming a Control or High-18:3n-3 Diet

FA	Control	High-18:3n-3
Sum SFA[a]	42.5 ± 2.8[b]	42.3 ± 4.3
Sum MUFA[a]	29.3 ± 4.3	30.0 ± 4.8
18:3n-3	ND	ND
20:5n-3	ND	ND
22:5n-3	0.32 ± 0.06	0.57 ± 0.14*
22:6n-3	7.2 ± 1.2	8.9 ± 1.0*
Sum n-3 PUFA[a]	17.5 ± 1.2	8.3 ± 1.0*
18:2n-6	2.1 ± 0.4	1.8 ± 0.1
20:4n-6	11.8 ± 1.5	11.1 ± 1.3
22:4n-6	5.0 ± 0.7	4.4 ± 0.4*
22:5n-6	2.7 ± 0.6	2.1 ± 0.7*
Sum n-6 PUFA[a]	23.0 ± 3.0	19.1 ± 2.1*
Total FA[c]	2.66 ± 0.35	3.00 ± 0.31*

[a]Sum includes more FA than those shown. *$P < 0.05$ vs. control. ND, not detected; for other abbreviations see Table 1.
[b]Percentage of total FA >12C; $n = 8$ per group; mean ± SD.
[c]Brain (mg/g).

TABLE 6
Carcass Total FA Composition of Piglets Suckling from Sows Consuming a Control or High-18:3n-3 Diet

FA	Control	High-18:3n-3
Sum SFA[a]	31.5 ± 4.0[b]	33.7 ± 5.2
Sum MUFA[a]	45.7 ± 2.6	44.0 ± 3.8
18:3n-3	0.83 ± 0.17	4.34 ± 1.21*
20:5n-3	0.01 ± 0.03	0.11 ± 0.08*
22:6n-3	0.10 ± 0.02	0.15 ± 0.09
Sum n-3 PUFA[a]	1.1 ± 0.2	4.9 ± 1.4*
18:2n-6	19.4 ± 2.0	15.8 ± 1.8*
20:4n-6	1.1 ± 0.3	0.9 ± 0.5
Sum n-6 PUFA[a]	21.7 ± 2.4	17.4 ± 2.2
Total FA[c]	138.8 ± 33.3	144.5 ± 26.6

[a]Sum includes more FA than those shown. *$P < 0.05$ vs. control. For other abbreviations see Tables 1 and 5.
[b]Percentage of total FA >12C; $n = 8$ per group; mean ± SD.
[c]Carcass (mg/g).

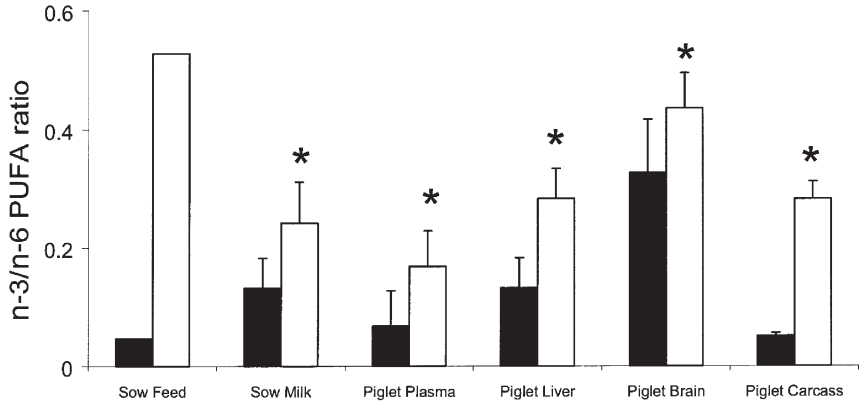

FIG. 1. n-3/n-6 PUFA ratio in sow feeds, milk, and the tissues of the suckling piglet. $^*P < 0.05$ vs. control diet; $n = 8$ per group, mean ± SD. (■) Control diet; (□) high-18:3n-3 diet.

All piglet tissues (especially the carcass) had a 33–460% increase in the n-3/n-6 PUFA ratio in response to suckling from sows consuming a high-18:3n-3 diet (Fig. 1). Interestingly, the n-3/n-6 PUFA ratio of the brain was increased in response to suckling from sows consuming a high-18:3n-3 diet. This was largely due to an increase in brain 22:6n-3 content, which has been observed in piglets suckling from sows consuming fish oil (18). It has been shown that piglet brain 22:6n-3 is influenced by the n-3 PUFA composition of the formula (17,19,20). Our data, along with that from piglets suckling from sows consuming fish oil, show that piglet brain 22:6n-3 is also influenced by the n-3 PUFA composition of the milk (18).

The results of this study suggest that a high 18:3n-3 intake during lactation can increase the 22:6n-3 content of the milk and some of the tissues of the suckling piglets. This study did not attempt to measure whether the 22:6n-3 accumulation in the piglet was due to the increased 18:3n-3 or 22:6n-3 content of the milk. Also, because sows began consumption of a high-18:3n-3 diet 8–10 d prior to parturition, it is not known how much 22:6n-3 PUFA the piglet received from the mother and how much it synthesized itself. However, owing to the rapid weight gain and fat accumulation from birth to 14 d of age, most of the n-3 PUFA accumulation would be expected to occur during lactation. If our data were applicable to humans, it would suggest that since 18:3n-3 is abundant in flaxseed and soybean oils, pregnant vegetarians and others not consuming fish also may be able to raise tissue and milk contents of n-3 PUFA by incorporating more 18:3n-3 into their diets (34).

In conclusion, dietary 18:3n-3 can increase the n-3 PUFA content of the sow's milk, which in turn increases the n-3 PUFA content of the suckling piglet tissues, including the brain. In contrast to what has been reported in rats (28), but similar to what has been observed with sows consuming fish oil and piglets consuming 18:3n-3 (16,18,20,22), this study suggests that during pregnancy and lactation, 18:3n-3 can increase both the 18:3n-3 and 22:6n-3 content of the piglet tissues.

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Stearic Acid Stimulates FA Ethyl Ester Synthesis in HepG2 Cells Exposed to Ethanol

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ABSTRACT: FA ethyl esters (FAEE) are nonoxidative metabolites of ethanol produced by the esterification of FA and ethanol. FAEE have been implicated as mediators of ethanol-induced organ damage *in vivo* and *in vitro,* and are markers of ethanol intake. Upon ethanol intake, FAEE are synthesized in the liver and pancreas in significant quantities. There is limited information on the stimulation of FAEE synthesis upon addition of exogenous FA *in vitro*. HepG2 cells were incubated with ethanol alone, ethanol with 25 μM linoleate, and ethanol with 25 μM stearate. The amount of FAEE in human hepatoblastoma (HepG2) cells was determined 1–3 h after ethanol and FA addition. Stearate increased the FAEE concentration in HepG2 cells when incubated with the cells for 1 h, whereas linoleate did not increase the cellular FAEE concentration at any time. Ethyl palmitate, ethyl stearate, and ethyl oleate were the predominant FAEE species identified in all cases, independent of the specific supplemental FA added to the medium.

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Alcoholism is a major health problem in many countries, and millions of individuals worldwide are adversely affected by alcohol abuse (1). The mechanism by which alcohol induces organ damage is not fully understood. Ethanol can be metabolized by oxidative and nonoxidative pathways. In the oxidative pathway, ethanol is converted in the liver to acetaldehyde by the action of alcohol dehydrogenase, P450 2E1, or catalase (2). Acetaldehyde is then oxidized to acetate by the action of aldehyde dehydrogenase. Alternatively, ethanol can be metabolized nonoxidatively to fatty acid ethyl ester (FAEE) by the action of acyl-CoA:ethanol acyltransferase, FAEE synthase, or other enzymes (3–7). Ethanol can also be metabolized nonoxidatively to phosphatidylethanol (8).

FAEE are present in the blood after ethanol ingestion. They have been implicated as mediators of ethanol-induced organ damage because of their presence in the organs commonly damaged by ethanol abuse—notably, the liver, the pancreas, the heart, and the brain (9–11). In a previous study of individuals consuming ethanol to intoxication at a controlled rate, the peak plasma or serum FAEE concentration of the population of seven individuals (taken as a group) was 1800 nmol/L (12). FAEE have also been found in tissues. Refaai *et al.* (13) recently showed that FAEE are present in the livers of autopsied individuals, who were intoxicated at the time of their death, in a range of FAEE concentrations from 1235 to 568,723 pmol/g tissue.

FAEE-mediated cytotoxicity has been demonstrated in several studies *in vivo* and *in vitro*. FAEE (300–600 μmol/L ethyl oleate and 400–800 μmol/L ethyl arachidonate) decreased protein synthesis and cell proliferation in intact human hepatoblastoma (HepG2) cells (14).

FAEE (11 μM) were shown to cause pancreatic injury in the rat *in vivo* (15). FAEE in emulsions (62.5–750 μM) increased the fragility of isolated lysosomes (16). FAEE (25–200 μM) in emulsions also disrupted oxidative phosphorylation in isolated mitochondria (17). Kabakibi *et al.* (18) found a linear association for HepG2 cells between concentrations of ethanol (0–300 mM) and FAEE concentration. In addition, the FAEE concentration increased linearly over 9 h in the presence of 170 mM ethanol.

In studies involving HepG2 cells incubated for 16 h with FA and subsequently for 7.5 h with both FA and 250 mM ethanol (a supraphysiologic ethanol level to maximize FAEE production), we demonstrated that the synthesis of ethyl oleate, ethyl linoleate, and ethyl arachidonate in HepG2 cells can be influenced by the extracellular concentrations of their corresponding FA. For example, the addition of oleate to the medium of cultured HepG2 cells results in an increase of ethyl oleate. Unlike oleate, linoleate, and arachidonate, the addition of palmitate to the culture medium in increasing concentrations (25–100 μM) had no effect on ethyl palmitate synthesis, and the basis for this finding remains unexplained (19).

In the present study, we set out to determine whether brief exposure (1–3 h) to FA and a physiologic concentration of ethanol (100 mM) affected FAEE synthesis by HepG2 cells. In the studies mentioned above (18,19) we exposed HepG2 cells to a higher concentration of ethanol (≥100 mM) or longer incubation times (≥8 h) as compared to the present study. In this work, we investigated the effect of short-term ethanol exposure to learn about early changes in FAEE production that we had not previously investigated. The results show a selective increase in total cellular FAEE induced by 25 μM stearate.

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Abbreviations: E16:0, ethyl palmitate; E18:0, ethyl stearate; E18:1, ethyl oleate; FAEE, fatty acid ethyl esters; HepG2, human hepatoblastoma (cell line); SFM, serum-free medium; SPE, solid phase extraction.

MATERIALS AND METHODS

Materials. All FA were purchased from Nu-Chek-Prep (Elysian, MN). Essentially FA-free BSA was purchased from Sigma Chemical Co. (St. Louis, MO), 99% FA-free albumin. All tissue culture reagents were obtained from Gibco Life Technologies (Grand Island, NY).

Cell culture. HepG2 cell cultures were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in Dulbecco's minimal essential medium containing 10% FCS, 200 U/mL penicillin, 200 μg/mL streptomycin, and 0.29 mg/mL L-glutamine (complete medium) at 37°C and 5% CO_2.

Methods. To determine the effect of stearate and linoleate on total FAEE synthesis, HepG2 cells were treated with 100 mM ethanol, 100 mM ethanol with 25 μM stearate, or 100 mM ethanol with 25 μM linoleate, and then incubated for 1, 2, or 3 h. We showed in a previous study that free stearate and linoleate concentrations in plasma of nonfasting and of fasting healthy individuals were 16.0 ± 7.8 and 24.0 ± 23.5 μmol (mean ± SEM) and 30.5 ± 7.7 and 54.0 ± 28.8 μmol/L, respectively (20).

In this study, 25 μM stearate (18:0) or 25 μM linoleate (18:2) (17.8 μL of a 10 μg/μL stock solution and 14.0 μL of a 10.5 μg/μL stock solution, respectively) were dried onto the wall of a conical tube under a stream of nitrogen. The FA were then resuspended in 25 mL (for stearate), and 21 mL (for linoleate) of serum-free medium (SFM) containing 25 μM albumin to produce a 1:1 molar ratio of FA (25 μM)/albumin (25 μM), and 100 mM ethanol. The controls contained no supplemental FA in SFM. The mass of stearate or linoleate in the medium of the culture dish was 50 nmol (2-mL vol of 25 μM stearate or linoleate). The total mass of cellular stearate in the dish was approximately 150 nmol, and the total mass of cellular linoleate was approximately 20 nmol. The medium was incubated with HepG2 cell monolayers (approximately 90% confluent) for 1, 2, or 3 h at 37°C. At the end of the incubation period, the medium was aspirated, the cells were rinsed gently twice with PBS, and the cells were harvested by scraping into 1 mL ice-cold PBS with a rubber policeman. A 0.5-mL portion of this cell suspension was used for FAEE quantification, and a 0.25-mL aliquot was used for cellular FA composition studies as described below.

FAEE extraction from HepG2 cells and GC–MS identification and quantification of FAEE. From the harvested HepG2 cells, protein was precipitated with 2 mL of acetone after the addition of 50 μL (1 nmol) of ethyl heptadecanoate (17:0) as an internal standard. FAEE were then extracted from the cells with 8 mL of hexane and subsequently isolated by solid-phase extraction (SPE). The SPE procedure for FAEE purification was a method modified from that described by Kaluzny *et al.* (21). The aminopropyl-silica columns (Bond-Elut LCR; Varian Inc., Harbor City, CA) were placed on a Vac-Elut vacuum apparatus set at 10 kPa (22). The Bond-Elut columns were first conditioned with 4 mL of dichloromethane, followed by 4 mL of hexane. Immediately after the solvent reservoir became empty, 200 μL of sample suspended in hexane was applied to the column, followed by 4 mL of hexane and an additional 4 mL of dichloromethane. The hexane and dichloromethane fractions were then combined into one tube, evaporated under nitrogen, and resuspended in a small amount of hexane for GC–MS analysis, which was performed on a Hewlett-Packard 5890 gas chromatograph coupled to a 5971 mass spectrometer (Hewlett-Packard, Wilmington, DE) equipped with a SUPELCOWAX 10 capillary column as previously described (23–25).

FA composition of HepG2 cell lipids. FA from HepG2 cells were methylated according to Moser and Moser (26). Briefly, 250 μL of a cell suspension was mixed with a 1-mL methanol/dichloromethane (3:1, vol/vol) solution. After addition of an internal standard (7.5 nmol of heptadecanoic acid), the mixture was treated with 200 μL acetyl chloride and incubated at 75°C for 1 h. After cooling, the solution was neutralized with 4 mL of 7% K_2CO_3, and the lipids were extracted into hexane. The hexane fraction was washed with acetonitrile and concentrated under nitrogen. The FAME mixture was then resuspended in hexane and analyzed by GC–MS. The injector and detector were maintained at 260 and 280°C, respectively. The oven temperature was maintained at 150°C for 2 min, ramped at 10°C/min to 200°C and held for 4 min, ramped again at 5°C/min to 240°C and held for 3 min, and then finally ramped to 270°C at 10°C/min and maintained for 5 min. The carrier gas flow rate was maintained at a constant 0.75 mL/min throughout isolation. Peak identification was based upon the normal retention times and on spectral comparison with known standards entered in our database library.

Statistical analysis. Regression analysis was used to assess potential correlations. The unpaired Student's *t*-test was used to evaluate differences between two groups.

RESULTS

Figure 1 shows that HepG2 cells incubated for 1 h with 25 μM stearate (18:0) and 100 mM ethanol produce significantly higher cellular FAEE concentrations, when compared to cells incubated with 25 μM linoleate with 100 mM ethanol or with 100 mM ethanol alone. Although no significant difference was observed at 2 and 3 h of incubation, the trend suggests persistence of increased cellular FAEE amounts in the stearate-treated cells at 2 and 3 h of exposure. Figure 1 also shows that incubating HepG2 cells with 25 μM linoleate and ethanol did not elevate cellular FAEE concentrations over baseline. HepG2 cells not exposed to ethanol do not synthesize detectable amounts of FAEE (18).

Table 1 shows FA profiles of HepG2 cells at baseline (0 h) and after 1, 2, and 3 h of incubation with ethanol alone. At no time did ethanol induce a change in the FA composition of the cells.

Figure 2A shows the mass of FAEE species produced after 1 h of incubation with ethanol, ethanol with 25 μM stearate, and ethanol with 25 μM linoleate. Regardless of the FA supplemented, the FAEE species were ethyl palmitate (E16:0), ethyl stearate (E18:0), and ethyl oleate (E18:1). The mass of

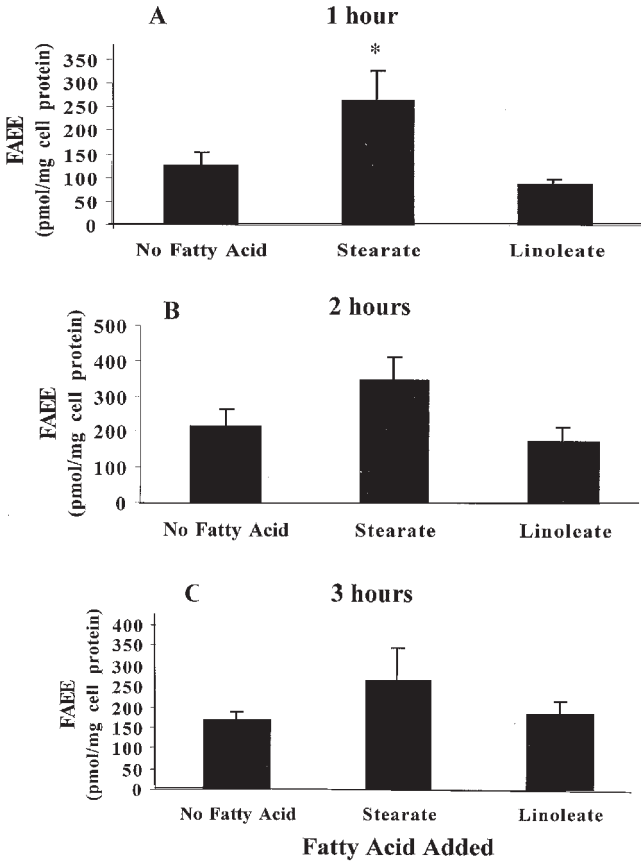

FIG. 1. Stimulation of FA ethyl ester (FAEE) synthesis by HepG2 cells after ethanol administration with stearate. HepG2 cells were treated with serum-free medium containing 100 mM ethanol, 100 mM ethanol with 25 μM linoleate, or 100 mM ethanol with 25 μM stearate in 35-mm Petri dishes and incubated for (A) 1, (B) 2, and (C) 3 h. The cellular lipids were extracted by acetone/hexane. FAEE were isolated by solid phase extraction and quantified by GC–MS as described in the Materials and Methods section. FAEE were expressed as total amount of FAEE in pmol/mg protein. The FAEE species detected were ethyl palmitate, ethyl stearate, ethyl oleate, and, in two samples, trace amounts of ethyl linoleate. The data are combined from four identical experiments and represent the mean ± SEM, $n = 9–12$ per bar. $^*P < 0.05$ for comparisons between FAEE production for cells treated with ethanol and no FA added, cells treated with ethanol and stearate added, and cells treated with ethanol and linoleate added.

each of the three FAEE species was higher with stearate when compared to linoleate or no exogenous FA. Figure 2B shows the percentage of total FAEE for the three FAEE species detected. Despite the higher mass of the three FAEE species in stearate-stimulated cells, there was no difference in the percentage distribution of FAEE species whether the cells were treated with stearate, linoleate, or no FA. In all three conditions, the percentage (relative to total FAEE) of E16:0 was the highest, followed by E18:1, and then E18:0 after 1 h incubation.

DISCUSSION

The major finding from this study is that the addition of 25 μM stearate, a highly physiologic concentration of a common

TABLE 1
FA Profiles[a] of HepG2 Cells After 0–3 h of Incubation with 100 mM Ethanol

FA	Incubation time with ethanol			
	0 h	1 h	2 h	3 h
	(nmol FA/mg protein HepG2 cells)			
14:0	30 ± 5	40 ± 1.7	41 ± 3.1	39 ± 0.9
16:0	302 ± 33	327 ± 19	325 ± 19	333 ± 6.3
16:1n-7	84 ± 9	94 ± 3.5	102 ± 4.9	109 ± 2.7
18:0	101 ± 9	119 ± 5.7	111 ± 6.4	113 ± 3.6
18:1n-9	263 ± 27	285 ± 8.7	295 ± 15	291 ± 10
18:1n-7	118 ± 13	139 ± 5.7	142 ± 5.7	135 ± 7.4
18:2	13 ±1.7	15 ± 0.6	14.3 ± 0.5	13.5 ± 0.6
20:0	4.0 ± 0.6	5.4 ± 0.2	4.0 ± 0.4	5.3 ± 0.3
20:1n-9	6.8 ± 1.3	9.3 ± 0.6	8.3 ± 0.5	9.0 ± 0.7
20:1n-7	3.5 ± 0.6	4.8 ± 0.3	5.0 ± 0.4	4.8 ± 0.5
20:3n-9	6.0 ± 0.7	6.5 ± 0.3	5.3 ± 0.5	6.8 ± 0.5
20:3n-6	4.0 ± 0.6	4.3 ± 0.5	3.8 ± 0.5	5.3 ± 0.5
20:4n-6	22 ± 1.8	22 ± 0.7	21 ± 0.6	21.8 ± 0.9
22:0	3.3 ± 0.3	3.8 ± 0.3	3.8 ± 0.5	2.8 ± 0.3
Total FA	960 ± 93	1073 ± 21	1080 ± 46	1089 ± 27

[a]Data are representative of two identical experiments ($n = 2$ for each) and represent the mean ± SEM of four samples.

dietary FA, to the culture medium of HepG2 cells stimulated an increase of the total cellular FAEE after only 1 h, in the presence of 100 mM ethanol. This ethanol concentration is physiologically attainable by ethanol abusers. This is a potentially important observation because food rich with stearate ingested with alcohol may result in greater production of FAEE, which have been shown to be toxic ethanol metabolites (14–17). The addition of stearate induced the synthesis of the same FAEE species produced in lower amounts by cells incubated with ethanol alone. As shown in Table 1, palmitate and oleate were the most abundant FA in the HepG2 cells in these studies. This suggests that the stearate effect is unlikely to be inducing selective synthesis of ethyl palmitate and ethyl oleate, but instead it is most likely to be inducing FAEE synthesis from the most abundant FA in the cells.

We have previously studied FA as substrates for FAEE synthesized in HepG2 cells (19). In those experiments, we incubated the cells with 0, 25, 50, or 100 μM supplemental palmitate, oleate, linoleate, or arachidonate in tissue culture medium. The incubation with FA was for 16 h, with an additional 7.5 h with both the FA and 250 mM ethanol. In those studies, in which we used greater amounts of ethanol and very prolonged incubation times relative to the current study, we did not investigate the effect of stearate supplementation in culture medium.

When studying the effect of various concentrations of stearate in preliminary studies, we noted that its effect was present in the physiologic range for stearate as a plasma FFA, and that the effect occurred within 1 h of ethanol exposure, as might be expected with a single alcoholic drink.

We previously demonstrated that there is no preferential uptake by the cells of palmitate vs. oleate (19), and we suspect on this basis that there is unlikely to be preferential

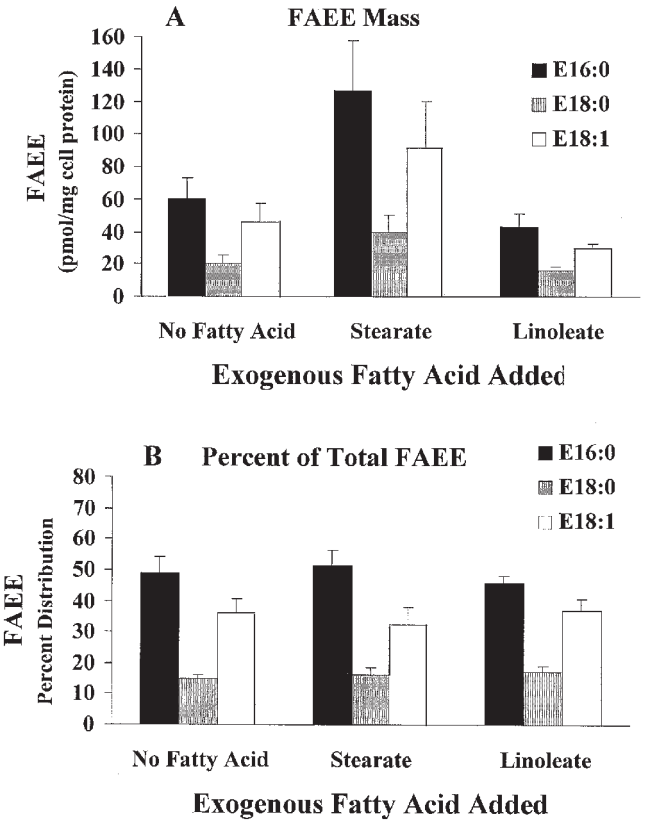

FIG. 2. The mass and percent distribution of FAEE species produced by HepG2 cells after ethanol administration, with stearate, linoleate, or no FA. HepG2 cells were treated with serum-free medium containing 100 mM ethanol, 100 mM ethanol with 25 μM linoleate, or 100 mM ethanol with 25 μM stearate and incubated for 1 h in 35-mm Petri dishes. The cellular lipids were extracted by acetone/hexane. FAEE were isolated by solid phase extraction and quantified by GC–MS as described in the Materials and Methods section. FAEE were expressed as total amount of FAEE in pmol/mg protein (A) and percentage of total FAEE (B). The FAEE species detected were ethyl palmitate, ethyl stearate, and ethyl oleate, and in two samples there were trace amounts of ethyl linoleate. The data are combined from four identical experiments and represent the mean ± SEM, n = 10–12 per bar.

uptake of stearate to explain the increased production of ethyl stearate in our current studies. We have observed that newly incorporated FA are preferred over FA previously incorporated into the cell for FAEE synthesis (27).

It has been shown in platelets that eicosanoid precursor FA (arachidonate, eicosapentaenoate, and dihomo-γ-linolenate) are taken up more rapidly than stearate, linoleate, and palmitate (28). Therefore, it is unlikely that differences in FA uptake explain the disparity between stearate and linoleate in the stimulation of FAEE synthesis in our studies. We have speculated that FA residing in a specific phospholipid or specific membrane are preferred for FAEE synthesis (19).

The compartmentation of the preferred FA substrates (palmitate, stearate, and oleate) in our studies is a plausible explanation for our findings. However, the mechanism by which "low dose" stearate specifically mobilizes these three FA as substrates for FAEE synthesis remains to be determined. It has been demonstrated that arachidonate is made available for eicosanoid production when it originates from phospholipids rather than TG or FFA (29,30).

Further studies to assess the compartmentation of FA substrates for FAEE synthesis within individual phospholipids or TG in HepG2 cells, or in a specific subcellular membrane, should allow us to determine whether this is the basis for preferential use of certain FA for FAEE synthesis and the stimulation of this process by exogenously added stearate.

The addition of 25 μM linoleate did not cause an increase in total cellular FAEE, nor did it stimulate the synthesis of ethyl linoleate. This is consistent with previous work from our laboratory in which no ethyl linoleate was synthesized when HepG2 cells were preincubated with 25 μM linoleate for 16 h or when incubated with 250 mM ethanol for 7.5 h (19).

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Lack of Stereospecificity in Lysophosphatidic Acid Enantiomer-Induced Calcium Mobilization in Human Erythroleukemia Cells

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ABSTRACT: Lysophosphatidic acid (LPA) is a lipid mediator that, among several other cellular responses, can stimulate cells to mobilize calcium (Ca^{2+}). LPA is known to activate at least three different subtypes of G protein-coupled receptors. These receptors can then stimulate different kinds of G proteins. In the present study, LPA and LPA analogs were synthesized from (*R*)- and (*S*)-glycidol and used to characterize the ability to stimulate Ca^{2+} mobilization. The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured in fura-2-acetoxymethylester-loaded human erythroleukemia (HEL) cells. Furthermore, a reverse transcriptase polymerase chain reaction was used to characterize LPA receptor subtypes expressed in HEL cells. The results show that HEL cells mainly express LPA_1 and LPA_2, although LPA_3 might possibly be expressed as well. Moreover, LPA and its analogs concentration-dependently increased $[Ca^{2+}]_i$ in HEL cells. The response involved both influx of extracellular Ca^{2+} and release of Ca^{2+} from intracellular stores. This is the first time the unnatural (*S*)-enantiomer of LPA, (*S*)-3-*O*-oleoyl-1-*O*-phosphoryl-glycerol, has been synthesized and studied according to its ability to activate cells. The results indicate that this group of receptors does not discriminate between (*R*)- and (*S*)-enantiomers of LPA and its analogs. When comparing ether analogs having different hydrocarbon chain lengths, the tetradecyl analog (14 carbons) was found to be the most effective in increasing $[Ca^{2+}]_i$. Pertussis toxin treatment of the HEL cells resulted in an even more efficient Ca^{2+} mobilization stimulated by LPA and its analogs. Furthermore, at repeated incubation with the same ligand no further increase in $[Ca^{2+}]_i$ was obtained. When combining LPA with the ether analogs no suppression of the new Ca^{2+} signal occurred. All these findings may be of significance in the process of searching for specific agonists and antagonists of the LPA receptor subtypes.

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Many lipids play key roles in regulating cellular function. Lysophosphatidic acid (LPA) is an intermediate product in phospholipid biosynthesis and functions as an intercellular lipid messenger (1). LPA can be produced and released from membrane microvesicles in many cells, e.g., from activated platelets, leukocytes, and erythrocytes (2). In microvesicles, phospholipase (PL) C and DAG kinase or PLD hydrolyze different phospholipids to generate high concentrations of PA, which is converted into LPA by PLA_2 (3). LPA can thereafter be released into the extracellular fluid and is suggested to work both as an autocrine and a paracrine mediator (4). Moreover, lysoPLD contributes to the conversion of choline-type phospholipid mediators to lipid phosphate-type mediators (5). Thus, lysoPLD would supply LPA to peripheral tissues (5). Until very recently the molecular identity of lysoPLD had not been determined (6). However, lysoPLD now appears to be identical to autotaxin, a widely expressed transmembrane ectoenzyme (7,8). LPA activates at least three types of G protein-coupled receptors. Isolation and identification of these receptors have been conducted rather recently (9–12). The subtypes were initially named endothelial differentiation gene (Edg)-2, Edg-4, and Edg-7, respectively, but their current names are LPA_1, LPA_2, and LPA_3 (13,14). These subtypes of receptor proteins differ with respect to cell distribution and intracellular signal transduction mechanisms (12). The objective with this study was to synthesize and characterize different LPA analogs by measuring cytosolic calcium concentration ($[Ca^{2+}]_i$) in human erythroleukemia (HEL) cells. The efficacies of the analogs were analyzed with respect to hydrocarbon chain length, and the stereochemical demand of the LPA receptors was investigated. Santos *et al.* (15) have shown a stereochemical discrimination by the LPA receptor, i.e., natural stereochemistry is strongly preferred by the receptor for ethanolamine-based phospholipids (15). In contrast, GTPγS binding, Ca^{2+} mobilization, and inhibition of adenylyl cyclase have been used to show that the (*R*)-enantiomer of *N*-palmitoyl-2-methylenehydroxy ethanolamide phosphoric acid is inactive whereas the (*S*)-enantiomer works as an agonist on the LPA receptor subtypes (16). Moreover, Gueguen *et al.* (17) have shown that LPA receptors lack stereospecificity in platelets. In the present study, not only different LPA analogs but also LPA itself, 3-*O*-oleoyl-1-*O*-phosphoryl-glycerol, were found to be active both in their natural (*R*) and unnatural (*S*) enantiomeric forms. Synthesis of new receptor

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Abbreviations: bp, base pair; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; cDNA, complementary DNA; Edg, endothelial differentiation gene; ee; enantiomeric excess; fura-2-AM, fura-2-acetoxymethylester; HEL cells, human erythroleukemia cells; LPA, lysophosphatidic acid (oleoyl-*sn*-glycero-3-phosphate); PLA_2/C/D, phospholipase A_2/C/D; PTX, pertussis toxin; RT-PCR, reverse transcriptase polymerase chain reaction; TBM, 137 mM NaCl, 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid, 10 mM glucose, 5 mM KCl, 4.2 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4—pH 7.4.

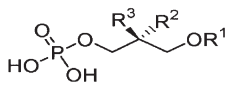
agonists and antagonists will advance our comprehension of both physiological and pathophysiological effects of LPA.

MATERIALS AND METHODS

Materials. Fura-2-acetoxymethylester (fura-2-AM), (*R*)-glycidol [98% enantiomeric excess (ee)], (*S*)-glycidol (98% ee), and L-α-lysophosphatidic acid (oleoyl-*sn*-glycero-3-phosphate) were obtained from Sigma Chemicals (St. Louis, MO). Pertussis toxin was purchased from Calbiochem (La Jolla, CA). All cell culture reagents and the primers were obtained from Invitrogen Ltd. (Paisley, United Kingdom), and cell culture plastics were purchased from Sarstedt (Nümbrecht, Germany). StrataPrep® Total RNA Microprep Kit was purchased from Stratagene (La Jolla, CA), and the Ready-to-go RT-PCR Beads and Ready-to-go PCR Beads were from Amersham Pharmacia Biotech (Uppsala, Sweden). The restriction enzymes were obtained from Promega Corporation (Madison, WI).

Synthesis of LPA enantiomers and analogs. The structures of the LPA enantiomers and analogs are shown in Scheme 1 and will be referred to in the text by their given capital letters as indicated in the table. Analog **L** is, however, not included in the table because of its distinct structure. The LPA enantiomers and analogs **D** and **G–J** (98% ee, purity >95% by NMR) were synthesized according to previously published methods by starting from commercially available (*R*)- or (*S*)-glycidol (18). Analog **K** (98% ee, purity >95% by NMR) was synthesized by using the same procedure. However, to preserve the alkene, deprotection of the phosphate was carried out by using trimethylsilyl bromide followed by freeze-drying from DMSO. Analog **L**, (*R*)-3-*O*-hexadecyl-1-*O*-sulfo-glycerol, was synthesized by debenzylation followed by regiospecific sulfation of (*R*)-1-*O*-benzyl-3-*O*-hexadecyl-glycerol (98% ee, purity >95% by NMR) (19).

Culturing HEL cells. Measurements of $[Ca^{2+}]_i$ were performed using cultured HEL cells. The cells were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and cultured in suspensions. The culturing medium consisted of RPMI-1640 medium supplemented with 3% heat-inactivated (56°C for 30 min) FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. The culture flasks were stored in a humidified incubator at 37°C in an atmosphere of 95% air and 5% CO_2. Fresh medium was added three times every week.



		R^1	R^2	R^3
(*R*)-3-*O*-hexadecyl-1-*O*-phosphoryl-glycerol	**D**	$C_{16}H_{33}$	OH	H
(*R*)-3-*O*-oleoyl-1-*O*-phosphoryl-glycerol	**G**	$C_{17}H_{33}CO$	OH	H
(*S*)-3-*O*-oleoyl-1-*O*-phosphoryl-glycerol	**H**	$C_{17}H_{33}CO$	H	OH
(*S*)-3-*O*-hexadecyl-1-*O*-phosphoryl-glycerol	**I**	$C_{16}H_{33}$	H	OH
(*S*)-1-*O*-phosphoryl-3-*O*-tetradecyl-glycerol	**J**	$C_{14}H_{29}$	H	OH
(*S*)-3-*O*-oleyl-1-*O*-phosphoryl-glycerol	**K**	$C_{18}H_{35}$	H	OH

SCHEME 1

Amplification of LPA receptor subtype complementary DNA (cDNA). Total RNA was isolated using StrataPrep Total RNA Microprep Kit according to the manufacturer´s protocol. RNase-free DNase was used to digest contaminating DNA. Reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using Ready-to-go RT-PCR Beads. A negative control was made by denaturating reverse transcriptase before adding primers and template. cDNA was synthesized by using random hexamer primers during incubation at 42°C for 30 min. The reverse transcriptase was thereafter denaturated at 95°C for 5 min. After the incubation, aliquots of cDNA were transferred to Ready-to-go PCR Beads. Sense and antisense primers used for the PCR-step were the same as used by Motohashi *et al.* (20). The expected sizes of the PCR products were as follows: LPA_1: 398 base pairs (bp), LPA_2: 289 bp, and LPA_3: 256 bp. The reactions were run for 40 cycles consisting of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with an extended denaturation period at the first cycle. The products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining. Restriction digest analyses were used to verify the identity of the PCR products (LPA_1/Ban I, LPA_2/Stu I, LPA_3/Pst I).

Measurement of $[Ca^{2+}]_i$. HEL cells were loaded with 4 µM fura-2-AM in TBM [137 mM NaCl, 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 10 mM glucose, 5 mM KCl, 4.2 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4; pH 7.4], supplemented with 1 mM $CaCl_2$. The incubation was performed for 20 min with gentle agitation at 37°C. Thereafter, cells were centrifuged at 250 × *g* for 5 min and washed with TBM supplemented with 0.1 mM $CaCl_2$. This step was repeated once, and loaded HEL cells were thereafter stored in the dark at room temperature until used. Fluorescence signals were recorded on a Hitachi F-2000 spectrofluorometer especially designed for measurement of $[Ca^{2+}]_i$. The cells were continuously stirred with a magnet during the experiments. Fluorescence emission was registered at 510 nm during alternating excitation at 340 and 380 nm. $[Ca^{2+}]_i$ was calculated by using the general equation described by Grynkiewicz *et al.* (21):

$$[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R)(F_o/F_s) \quad [1]$$

where K_d is the affinity constant of fura-2 (224 nM) and R is the ratio of fluorescence determined at 340 nm excitation over that at 380 nm. Maximal (R_{max}) and minimal fluorescence (R_{min}) ratios were determined by adding 0.1% Triton X-100 and 25 mM EGTA, respectively. F_s and F_o are the fluorescence at 380 nm excitation following cell lysis and Ca^{2+} chelation, respectively. The HEL cells were stimulated with LPA or the analogs, in suspensions containing Ca^{2+} (1 mM), EGTA (500 µM), or Mn^{2+} (100 µM). Cells were incubated at 37°C for 5 min before being stimulated with ligands. For repeated applications, 3 min passed before the second addition. Ca^{2+} ions or EGTA

was added just before each measurement. By removing external Ca^{2+} with EGTA, internal release of Ca^{2+} could be studied. In the experiments where influx of Mn^{2+} was measured, the ions were added 30 s before LPA, and fluorescence emission from the fura-2-loaded HEL cells was registered at 510 nm during excitation at 360 nm. Mn^{2+}-induced quenching of fura-2 fluorescence has been used to study divalent cation entry across the plasma membrane (22). In experiments where pertussis toxin (PTX) was used, cells were incubated with 100 ng/mL PTX for 24 h before measurements. The analogs were soluble in water, and at every series of experiments water was added to one sample at the same time as the analogs. The change in $[Ca^{2+}]_i$ due to a rise of the basal line was thereafter subtracted from every single value obtained with the analogs before data were analyzed.

Data analyses. Data were analyzed with GraphPad Prism™ (GraphPad Software, San Diego, CA). In some figures the results are expressed as means ± SEM; otherwise, sigmoidal dose–response curves are shown using nonlinear regression. The best-fitted EC_{50} values define the concentration causing half-maximal effect. The maximal levels of $[Ca^{2+}]_i$, induced by the enantiomers and analogs, were compared in a 95% confidence interval. Furthermore, the EC_{50} values were also compared in a 95% confidence interval. Statistical significance between PTX-treated cells and their controls was evaluated by use of an unpaired Student's *t*-test. Statistical significance was denoted as $P < 0.05$.

RESULTS

HEL cells express LPA receptor subtypes. To investigate which of the LPA receptor subtypes, LPA_1, LPA_2, or LPA_3, are expressed in HEL cells, total RNA was isolated and cDNA was synthesized from cultured cells. Amplification of the cDNA product was carried out using PCR with primer homologs for the three different receptor subtypes (20). The results can be seen in Figure 1. LPA_1 and LPA_2 receptor subtypes were detected in three independent experiments. LPA_3 was, however, detected with lower intensity and only in one experiment. In the negative control, where the reverse transcriptase was denatured before adding primers and template, no PCR product was obtained, confirming that the product originated from mRNA. A comparison of the sizes of the PCR products by our analysis with that of Motohashi *et al.* (20), as well as by our analysis using a restriction enzyme, shows that HEL cells mainly express the LPA_1 and LPA_2 receptor subtypes.

Characterization of LPA-triggered increases of $[Ca^{2+}]_i$ in HEL cells. Commercially available LPA was first used to characterize if and how LPA can stimulate HEL cells to increase $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ in HEL cells were measured by loading cells with fura-2. LPA was used in concentrations between 1 nM and 10 µM. As can be seen in Figure 2, LPA increased the $[Ca^{2+}]_i$ in a concentration-dependent way. Addition of LPA induced rapid rises in $[Ca^{2+}]_i$ in HEL cells and the Ca^{2+} response reached a peak a few seconds after stimulation. By analyzing the data with nonlinear regression, an EC_{50} value of 20 nM was obtained.

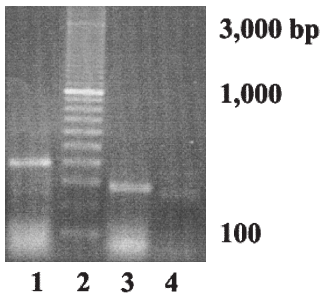


FIG. 1. Expression of lysophosphatidic acid (LPA) receptor mRNA in human erythroleukemia (HEL) cells. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of LPA receptors was carried out with mRNA from cultured cells. Lane 1 contains LPA_1, lane 2: PCR molecular ruler indicated on the right in base pair (bp), lane 3: LPA_2, and lane 4: LPA_3. This is one of three separate experiments.

Changes in $[Ca^{2+}]_i$ may result from release of Ca^{2+} from intracellular stores as well as Ca^{2+} entry across the plasma membrane. In our study internal release was measured by chelating external Ca^{2+} with EGTA. In the presence of EGTA, the $[Ca^{2+}]_i$ changes observed were attributed to intracellular release. Moreover, Mn^{2+} was used as a surrogate for Ca^{2+} influx. Mn^{2+} binds to fura-2 and quenches the fluorescence. A fall in fluorescence at 360 nm at LPA stimulation, when Mn^{2+} was present extracellularly, was due to Mn^{2+} entry and attributed to Ca^{2+} influx. The Ca^{2+} signal initiated by LPA in HEL cells included both an intracellular release of Ca^{2+} and an extracellular influx of Ca^{2+}, as indicated by the measurement in Figure 3, where the cells were stimulated with 10 µM LPA in the presence of either EGTA or Mn^{2+}. Figure 3A shows that LPA induces a transient cytosolic Ca^{2+} increase even in the absence of extracellular Ca^{2+}, i.e., in the presence of 500 µM EGTA. The rise triggered by LPA in Ca^{2+}-free solution was 18 ± 6 nM ($n = 3$), whereas

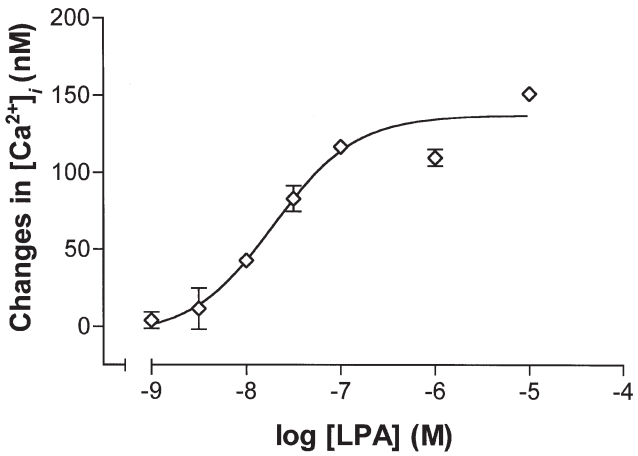


FIG. 2. Effect of LPA on $[Ca^{2+}]_i$ in fura-2-loaded HEL cells. LPA was used at final concentrations between 1 nM and 10 µM. Fluorescence emission was registered at 510 nm during alternating excitation at 340 and 380 nm. The data points represent means ± SEM from three separate experiments. However, at 1 nM and 1 µM, $n = 2$. For abbreviations see Figure 1.

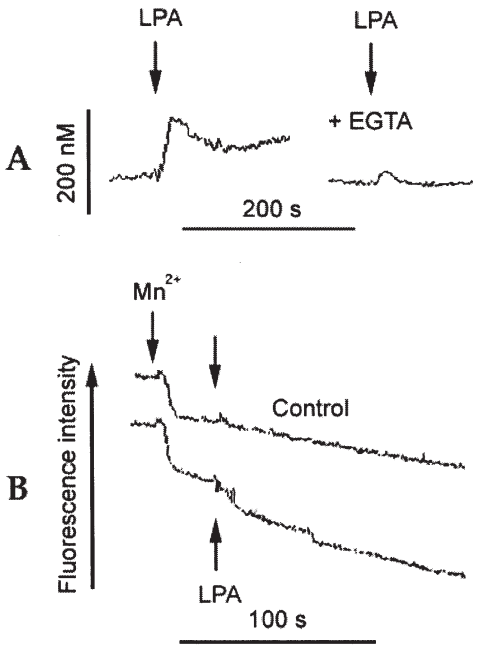


FIG. 3. (A) Effect of LPA on $[Ca^{2+}]_i$ in the absence of external Ca^{2+}. The trace shown is obtained from fura-2-loaded HEL cells stimulated with 10 μM LPA. EGTA (500 μM) was added to the cell suspension just before each measurement. Fluorescence emission was registered at 510 nm during alternating excitation at 340 and 380 nm. The trace illustrated is from a representative experiment; similar results were obtained in three different cell preparations. (B) LPA-induced influx of Mn^{2+}. The trace shown is obtained from fura-2-loaded HEL cells stimulated with 10 μM LPA or vehicle. Mn^{2+} ion (100 μM) was added to the cell suspension 30 s before LPA or vehicle. Quenching of fura-2 fluorescence was compared to the control in the absence of LPA. Fluorescence emission was registered at 510 nm during excitation at 360 nm. The traces illustrated are from representative experiments; similar results were obtained in three different cell preparations. For abbreviations see Figure 1.

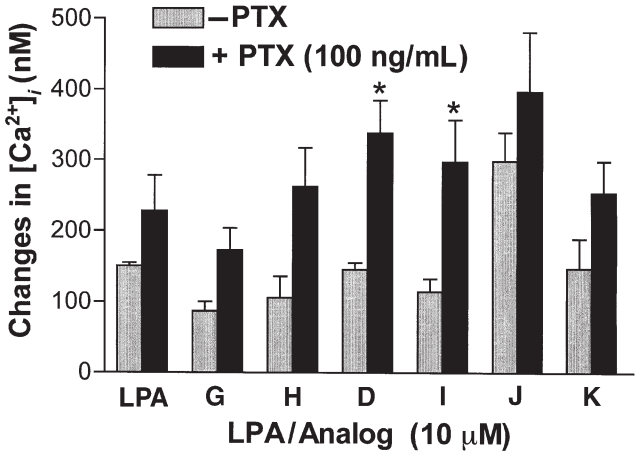


FIG. 4. Effect of pertussis toxin (PTX) on $[Ca^{2+}]_i$ in fura-2-loaded HEL cells. Cells were incubated with 100 ng/mL PTX for 24 h prior to stimulation with 10 μM of LPA or analogs. Fluorescence emission was registered at 510 nm during alternating excitation at 340 and 380 nm. The data represent means ± SEM from three separate experiments. $*P < 0.05$ compared to cells treated with the same ligand but without PTX treatment. For other abbreviations see Figure 1.

it was 119 ± 6 nM ($n = 3$) in the normal TBM buffer. The addition of LPA in the presence of 100 μM Mn^{2+} induced quenching of the fura-2 fluorescence, indicative for divalent cation influx (Fig. 3B). To study whether stimulation by LPA involved a PTX-sensitive G protein in Ca^{2+} mobilization, cells were exposed to 100 ng/mL of the toxin for 24 h before stimulating them with LPA. After this pretreatment, cells still responded with an increase in $[Ca^{2+}]_i$. This increase was even more effective than in the absence of PTX (Figure 4).

The ability of LPA analogs to increase $[Ca^{2+}]_i$ in HEL cells. The ability of the analogs to stimulate HEL cells was compared to commercially purchased LPA. Comparisons were also made between two pairs of enantiomers. The response of all analogs could be fitted to sigmoidal dose–response curves. The synthesized LPA enantiomers **G** and **H** were compared according to their ability to increase $[Ca^{2+}]_i$. These enantiomers did not differ significantly in maximal values and EC_{50} values. The EC_{50} values of **G** and **H** were 73 and 59 nM, respectively (Fig. 5A). When comparing **D** with **I**, the (*R*)-enantiomer was slightly more effective in increasing the maximal value of $[Ca^{2+}]_i$ (Fig. 5B). The EC_{50} values were 65 and 57 nM for **D** and **I**, respectively, and did not differ significantly.

The (*S*)-enantiomers **I**, **J**, and **K** were used to analyze whether the hydrocarbon chain length influences the ability to increase $[Ca^{2+}]_i$ in HEL cells. As can be seen in Figures 5B and 5C, the ability of **J** to increase maximal levels of $[Ca^{2+}]_i$ differed significantly from the other two. Among all analogs that were tested, **J** was the one that increased $[Ca^{2+}]_i$ the most in HEL cells. There were no significant differences in maximal values between **I** and **K**. Furthermore, the EC_{50} values of these three analogs, **I**, **J**, and **K**, were 57, 37, and 42 nM, respectively, and did not differ significantly.

When using **L**, in which the phosphate group is exchanged for a sulfate and the hydrocarbon chain consists of 16 carbons, no increase in $[Ca^{2+}]_i$ appeared. **L** was also used to test whether it could work as an antagonist for LPA, i.e., HEL cells were incubated with **L** for 3 min and then with LPA as well. However, **L** did not antagonize LPA (data not shown).

To study whether stimulation by analogs involved a PTX-sensitive G protein in Ca^{2+} mobilization, cells were exposed to the toxin in the same way as with LPA. After this pretreatment, the cells still responded with an increase in $[Ca^{2+}]_i$. As with the result of LPA, the increases stimulated by analogs were even more effective than without PTX (Fig. 4). The differences observed when comparing analog **D** or **I** with and without PTX treatment were statistically significant ($P < 0.05$).

Repeated application of LPA or the analogs (10 μM) seemed to restrain the Ca^{2+} signal, and no further increase in $[Ca^{2+}]_i$ was obtained. The pattern was the same for all ether analogs and is represented by the measurement for analog **D** and **J** in Figure 6. The same results were obtained when combining an ether analog with another ether analog, e.g., **D** plus **J** or **I** plus

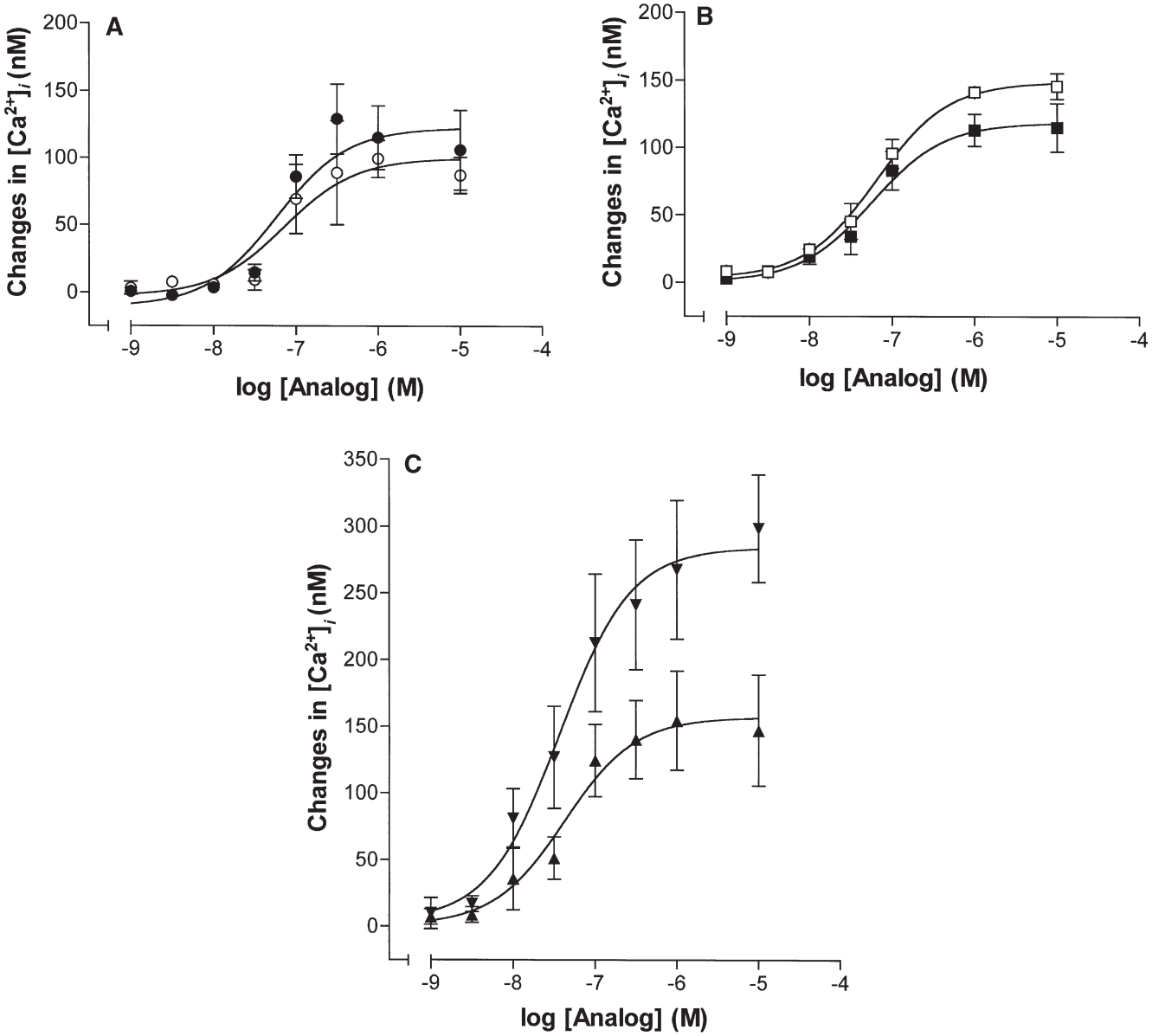


FIG. 5. (A) Effect of LPA (*R*)- and (*S*)-enantiomers on $[Ca^{2+}]_i$ in fura-2-loaded HEL cells. Compounds **G** [(*R*)-3-*O*-oleoyl-1-*O*-phosphoryl-glycerol) (*O*)] and **H** [(*S*)-3-*O*-oleoyl-1-*O*-phosphoryl-glycerol) (●)] were used at final concentrations between 1 nM and 10 μM. Fluorescence emission was registered at 510 nm during alternating excitation at 340 and 380 nm. The data points represent means ± SEM from three separate experiments, except at 0.3 μM $n = 2$. (B) Effect of synthesized LPA analog (*R*)- and (*S*)-enantiomers on $[Ca^{2+}]_i$ in fura-2-loaded HEL cells. **D** [(*R*)-3-*O*-hexadecyl-1-*O*-phosphoryl-glycerol) (□) and **I** [(*S*)-3-*O*-hexadecyl-1-*O*-phosphoryl-glycerol) (■)] were used at final concentrations between 1 nM and 10 μM. Fluorescence emission was registered at 510 nm during alternating excitation at 340 and 380 nm. The data points represent means ± SEM from three separate experiments. (C) Effect of hydrocarbon chain length of synthesized (*S*)-enantiomer LPA analogs on $[Ca^{2+}]_i$ in fura-2-loaded HEL cells. **J** [(*S*)-1-*O*-phosphoryl-3-*O*-tetradecyl-glycerol (▼)] and **K** [(*S*)-3-*O*-oleyl-1-*O*-phosphoryl-glycerol) (▲)] were used at final concentrations between 1 nM and 10 μM. Fluorescence emission was registered at 510 nm during alternating excitation at 340 and 380 nm. The data points represent means ± SEM from three separate experiments. For abbreviations see Figure 1.

J. When studying whether LPA could affect the response of the ether analogs and vice versa, LPA was found not to have an influence on the increases in $[Ca^{2+}]_i$ obtained by the analogs. However, when LPA was added after the ether analogs a major decrease in $[Ca^{2+}]_i$ occurred. The more effective the analog was in increasing the $[Ca^{2+}]_i$ by itself, the larger was the decrease in $[Ca^{2+}]_i$ upon combination of the ligands. All these experiments will here be represented by the analogs **D** and **J** (Fig. 6).

DISCUSSION

Efforts have been made to identify new LPA receptor agonists and antagonists, and the chemical manipulations of the LPA structure have varied (15,23,24). In this study enantiomerically pure phospholipids have been synthesized by starting from (*R*)- or (*S*)-glycidol. Thereafter, intracellular Ca^{2+} mobilization in HEL cells has been measured to determine the biological

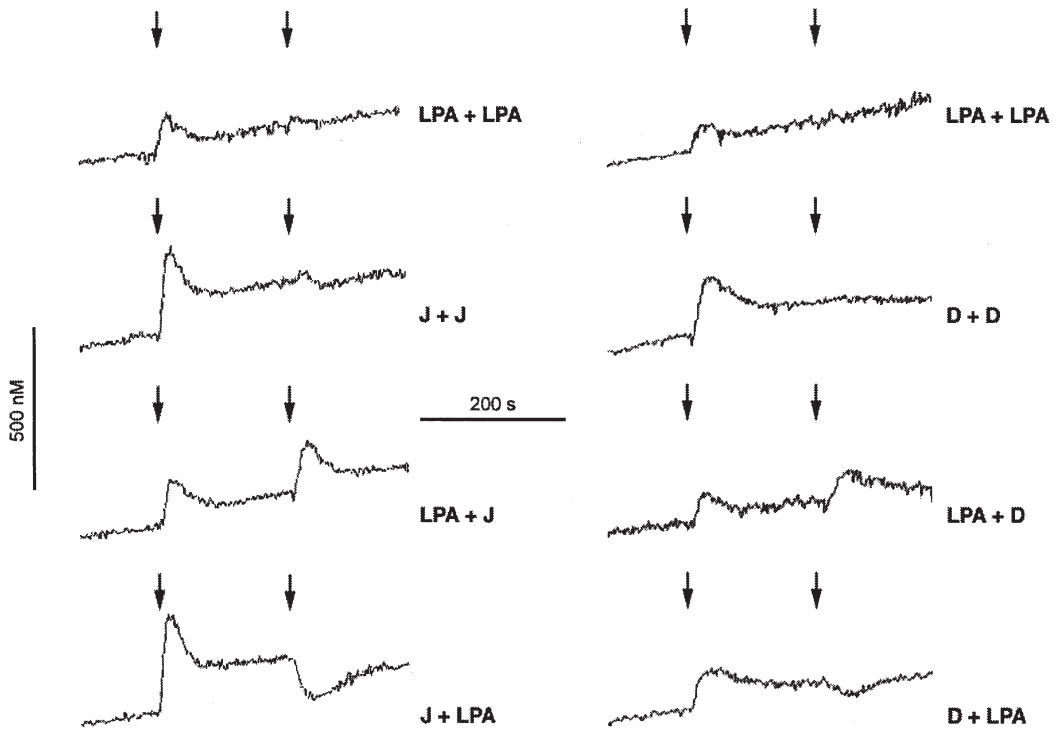


FIG. 6. The influence of different ligand combinations on $[Ca^{2+}]_i$ in fura-2-loaded HEL cells. LPA and analog **D** or **J** were used at concentrations of 10 μM at 3 min apart. Fluorescence emission was registered at 510 nm during alternating excitation at 340 and 380 nm. The traces illustrated are from representative experiments; similar results were obtained in three different cell preparations. For abbreviations see Figures 1 and 5.

activity of the different LPA analogs. The Ca^{2+} mobilization responses involved both influx of extracellular Ca^{2+} and release of Ca^{2+} from intracellular stores. LPA naturally exists in the (*R*) configuration. To our knowledge, this is the first study in which both (*R*)- and (*S*)-enantiomers of LPA, 3-*O*-oleoyl-1-*O*-phosphoryl-glycerol, have been synthesized and analyzed for their ability to stimulate cells. The LPA receptor had earlier been shown to prefer either natural (*R*) stereochemistry of some ligands (15) or unnatural (*S*) stereochemistry of other ligands (16). However, Gueguen *et al.* (17) showed that LPA receptors lack stereospecificity in platelets, which is in accordance with measurements on the analogs in this study not showing marked stereospecificity. When comparing **G** with **H** and **D** with **I**, agonist potency was almost the same for both (*R*)- and (*S*)-enantiomers. This is also in accordance with the results of Simon *et al.* (25), who made measurements on analogs **D** and **I**, which showed that both analogs are active. In their study the two enantiomers showed similar activity in eliciting platelet aggregation. However, their analogs were enzymatically generated and were not analyzed concerning stereochemistry. Our analogs can be compared with those chemically synthesized by Yokoyama *et al.* (26). They have used these analogs to measure DNA synthesis and intracellular Ca^{2+} mobilization in LPA-unresponsive rat hepatoma cells RH7777, i.e., cells not expressing LPA receptors. In contrast to Hooks *et al.* (16), Yokoyama *et al.* (26) needed LPA_1 to be expressed in RH7777 to detect increases in thymidine incorporation.

When comparing the hydrocarbon chain length, our results show that the analog with 14 carbons (**J**), the shortest in this study, had the highest ability to activate cells. The other two analogs used for this purpose (**I** and **K**) had 16 and 18 carbons in the tails, respectively. **I** and **K** stimulated HEL cells in a similar fashion, but to a much lower extent than **J**. In contrast to our results, it has been shown that the biological activity of LPA decreases with shorter chain lengths in human A431 epidermoid carcinoma cells and Sf9 insect cells (27,28). LPA with hydrocarbon chain lengths shorter than 12 carbons have been shown not to function as agonists on LPA_1, LPA_2, and LPA_3 in Sf9 insect cells (28). In addition, it has been proposed that 16:0 and 18:1 are the optimal chain lengths of LPA analogs (15). This is supported by Wang *et al.* (29), who developed a computer model of LPA_1 in which the hydrocarbon chain of LPA occupies a hydrophobic binding pocket. Furthermore, the lipophilic tail of LPA may facilitate the possibility of the phosphate group binding to the appropriate amino acids at the binding site of the receptor (27). According to Xie *et al.* (4), 18:1 LPA can activate all three known receptor subtypes and is generally most efficient in stimulating cells to a variety of responses. Moreover, 16:0, 18:0, and 18:1 are found in all biological samples examined, e.g., human plasma, platelets, and

ascitic fluids (4). The three analogs used in our study to examine chain lengths have an ether group between the chiral center and the lipophilic tail. This can be compared to **G**, **H**, and the commercial LPA, which have an ester linkage. Whether these ether or ester groups are important in the ability of the analogs to increase $[Ca^{2+}]_i$ needs further investigation.

The head group of different phospholipids determines their efficacy to increase DNA synthesis in vascular smooth muscle cells (30). Charge changes and substitutions of head groups, for example to sulfate, produce inactive compounds (15). When using **L**, in which the phosphate group is changed to a sulfate and the hydrocarbon chain consist of 16 carbons, no increase in $[Ca^{2+}]_i$ appeared. **L** did not, however, work as an antagonist to LPA but was only ineffective as an agonist. This suggests that the phosphate group not only is important for the ligand's efficacy but also is necessary for the affinity to the receptor.

Pretreatment with PTX of opossum kidney cells had no effect on LPA-induced Ca^{2+} responses (31). Accordingly, in Sf9 insect cells PTX did not inhibit LPA_3-transduced Ca^{2+} responses, indicating that PTX-insensitive G proteins are coupled to LPA_3 in mediating the Ca^{2+} response (32). Moreover, it was shown by An *et al.* (33) that PTX almost completely blocked LPA-induced Ca^{2+} mobilization by LPA_1 in rat hepatoma cells but only partially blocked that by LPA_2. By comparing PTX-treated HEL cells with nontreated cells, we found that PTX did not inhibit the ability to increase $[Ca^{2+}]_i$. Instead, the increases in $[Ca^{2+}]_i$ stimulated by all ligands were even higher when cells had been incubated with the toxin. This indicates that the PTX treatment blocks a $G_{i/o}$ protein-mediated inhibitory pathway.

To determine whether LPA and the analogs activate the same or distinct receptors, the ligands were added at 3-min intervals. LPA and all ether analogs showed the same response, e.g., at repeated incubation with the same ligand no further increase in $[Ca^{2+}]_i$ was obtained. However, when combining LPA with **J** or **D**, for example, no suppression of the new Ca^{2+} signal occurred. In contrast, Yokoyama *et al.* (26) found a heterologous desensitization between LPA and the two enantiomers of hexadecyl (16-carbon chain) ether analogs. Their experiments were performed in NIH3T3 cells that express LPA_1 and LPA_2 receptors. Furthermore, when starting with **J** or **D** followed by LPA, a major decrease in $[Ca^{2+}]_i$ was obtained. LPA might activate an inhibitory pathway that decreases the $[Ca^{2+}]_i$ after prestimulation with an ether analog. Future work on cells expressing defined receptor subtypes and/or G proteins is necessary to further study the complex Ca^{2+} mobilizing effects.

In conclusion, others have shown that LPA receptors might be unusual since they are not stereospecific. That LPA receptors are not stereospecific has been shown with chemically modified LPA analogs. However, this is the first study to show that both natural (*R*) and unnatural (*S*) LPA, 3-*O*-oleoyl-1-*O*-phosphoryl-glycerol, enantiomers are capable of stimulating cells. Structure–activity analyses are important to understand how LPA activates cells in different ways. These analyses also can help to identify novel lipid mediators that may be useful for the design or discovery of specific antagonists to LPA receptors. Synthesizing LPA receptor agonists/antagonists will increase our knowledge of physiological and pathophysiological significance of the lipids.

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Inhibitory Action of Emulsified Sulfoquinovosyl Acylglycerol on Mammalian DNA Polymerases

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ABSTRACT: We reported previously that sulfoquinovosyl diacylglycerol and sulfoquinovosyl monoacylglycerol (SQDG/SQMG) are potent inhibitors of mammalian DNA polymerases and DNA topoisomerase II, and can be potent immunosuppressive agents and anticancer chemotherapy agents [Matsumoto, Y., Sahara, H., Fujita T., Shimozawa, K., Takenouchi, M., Torigoe, T., Hanashima, S., Yamazaki, T., Takahashi, S., Sugawara, F., *et al.*, An Immunosuppressive Effect by Synthetic Sulfonolipids Deduced from Sulfonoquinovosyl Diacylglycerols of Sea Urchin, *Transplantation 74*, 261–267 (2002); Sahara, H., Hanashima, S., Yamazaki, T., Takahashi, S., Sugawara, F., Ohtani, S., Ishikawa, M., Mizushina, Y., Ohta, K., Shimozawa, K., *et al.*, Anti-tumor Effect of Chemically Synthesized Sulfolipids Based on Sea Urchin's Natural Sulfonoquinovosylmonoacylglycerols, *Jpn. J. Cancer Res. 93*, 85–92 (2002)]. In those experiments, the *in vivo* effectiveness greatly depended on the degree of water solubility of SQDG/SQMG. In the present work, we studied the emulsification of SQDG/SQMG in terms of their use in *in vivo* experiments. Lipid emulsions containing SQDG/SQMG (oil-in-water emulsions) in which the particle size was smaller than 100 nm were designed and synthesized, and then the biochemical modes of emulsified SQDG/SQMG were studied in comparison with those of SQDG/SQMG solubilized by DMSO. Emulsified SQDG/SQMG are also selective mammalian DNA polymerase inhibitors and potent antineoplastic agents but do not inhibit the DNA topoisomerase II activity. The growth inhibition effect of emulsified SQMG to NUGC-3 cancer cells was twofold stronger than DMSO-soluble SQMG (69 and 151 μM, respectively). From these results, the properties of lipid emulsions containing SQDG/SQMG and their possible use in *in vivo* experiments including clinical use are discussed.

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Abbreviations: dNTP, deoxyribonucleotide triphosphate; dTTP, 2′-deoxythymidine 5′-triphosphate; HCO-60, polyoxyethylene hydrogenated castor oil (Cremophor-HR60; BASF Aktiengesellschaft, Ludwigshafen, Germany); HEPC, L-α-phosphatidylcholine from egg yolk; I/E ratio, inhibitor-to-enzyme ratio; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; o/w, oil-in-water; pol, DNA-directed DNA polymerase (EC 2.7.7.7); SQDG, sulfoquinovosyl diacylglycerol = 1,2-di-*O*-acyl-3-*O*-(α-D-sulfoquinovosyl)glyceride with two stearic acid molecules; SQMG, sulfoquinovosyl monoacylglycerol = 1-mono-*O*-acyl-3-*O*-(α-D-sulfoquinovosyl)glycerol with one stearic acid molecule; ssDNA, single-stranded DNA; TAE, Tris-acetate-EDTA; topo, DNA topoisomerase.

Extracted from a fern and an alga, several sulfoglycolipids in the class of sulfoquinovosyl diacylglycerol (SQDG), which has two FA, were found to be DNA polymerase inhibitors (1–4). The sulfoglycolipids were able to potently inhibit the activities of mammalian DNA polymerase α and β (pol α and pol β) in a dose-dependent manner but hardly influenced the activities of prokaryotic DNA polymerases such as *Escherichia coli* DNA polymerase I, and of DNA metabolic enzymes such as terminal deoxynucleotidyl transferase, HIV-1 reverse transcriptase, and deoxyribonuclease I. Sahara *et al.* (5) reported that the other sulfoglycolipid, sulfoquinovosyl monoacylglycerol (SQMG) from sea urchin, which has one FA, showed antitumor activity in an experiment in which human tumors were transplanted into mice. These observations indicated that SQDG/SQMG could be selective inhibitors of mammalian DNA polymerases and potent antitumor agents. Therefore, we tried to synthesize naturally occurring sulfoglycolipids of the class of SQDG and SQMG chemically, because the *in vivo* experiments described above require large quantities of the natural sulfoglycolipids and also because of the interest in developing them as medical agents. We succeeded in the chemical synthesis of SQDG/SQMG and many of their derivatives (6–8). Indeed, many of the newly synthesized SQDG/SQMG may have promise as potent immunosuppressive (SQDG) or anticancer chemotherapy agents (SQMG) (9,10).

In the *in vivo* experiments using mice, SQDG/SQMG compounds, although not insoluble, were hard to solubilize, and the subsequent *in vivo* data were liable to fluctuate with the degree of SQDG/SQMG solubilization. To solve this problem, we focused our attention on oil-in-water (o/w) emulsification methods. Emulsions of o/w stabilized with emulsifiers such as sulfoglycolipids are attractive as drug carriers because they are biodegradable and biocompatible and, unlike liposomes, they can be prepared on an industrial scale and are relatively stable below 25°C for long periods (11). An important parameter regarding the use of emulsion particles as drug carriers is how they leave the vascular space and reach their site of action. The extravascular transfer of particulate carriers largely depends on their size. It is believed that a diameter of approximately 100 nm is the cutoff value for drug carriers to be able to pass through the discontinuous capillary endothelium of tumors

(12). In addition, drug carriers smaller than 100 nm are expected to avoid uptake by the reticulo-endothelial system (RES) more easily and to circulate for longer periods in the blood, as estimated from the finding that small unilamellar liposomes of about 70–100 nm are removed more slowly from the circulation than larger ones of the same composition (13). We designed and synthesized lipid emulsions containing SQDG or SQMG in which the particle size was smaller than 100 nm and investigated their use in *in vivo* experiments.

The purpose of this report is to investigate the biochemical action and the antineoplastic effect of emulsified SQDG/SQMG in comparison with conventional SQDG/SQMG solubilized by DMSO.

MATERIALS AND METHODS

Materials. SQDG [1,2-di-*O*-acyl-3-*O*-(α-D-sulfoquinovosyl)-glyceride with two stearic acid molecules, Scheme 1] and SQMG [1-mono-*O*-acyl-3-*O*-(α-D-sulfoquinovosyl)glycerol with one stearic acid molecule, Scheme 1] were chemically synthesized as described previously (6–8). Polyoxyethylene hydrogenated castor oil 60 (Cremophor-HR60, HCO-60) was supplied by BASF Aktiengesellschaft (Ludwigshafen, Germany). [^{3}H]-dTTP (2′-deoxythymidine 5′-triphosphate) (43 Ci/mmol), nucleotides, and chemically synthesized template-primers such as poly(dA) and oligo(dT)$_{12–18}$ were purchased from Amersham Biosciences (Buckinghamshire, United Kingdom). Supercoiled pUC19 plasmid DNA was obtained from Takara (Tokyo, Japan). All other reagents were of analytical grade and were purchased from Wako Pure Chemical Industries (Tokyo, Japan). NUGC-3, a human gastric cancer cell line (JCRB0822) (14), was supplied by the Health Science Research Resources Bank (Osaka, Japan).

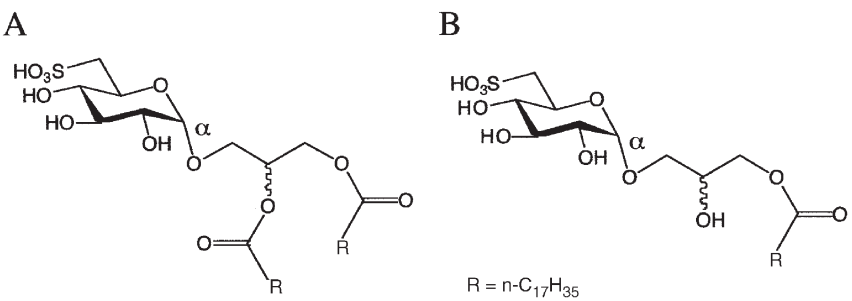
Preparation of SQDG/SQMG solution. Both SQDG and SQMG are water insoluble; therefore, the SQDG and SQMG solutions were made as follows. The chemically synthesized white powders of SQDG and SQMG were added to DMSO at a concentration of 10 mM, and were dissolved using a bath-type sonicator [Branson 1510 (Bransonic), Emerson-Japan, Kanagawa, Japan) for 3 min. The solutions of SQDG and of SQMG were used in enzyme and cell culture assays at a final DMSO concentration of 10 and 1%, respectively.

Preparation of lipid emulsions. The lipid emulsions containing SQDG/SQMG (o/w emulsions) were prepared by a thin-film hydration method combined with a sonication (bath-type sonicator) method (15). The emulsions consisted of soybean oil, water, SQDG or SQMG, hydrogenated L-α-phosphatidylcholine from egg yolk (HEPC), and an appropriate cosurfactant such as HCO-60. The oil (0.43 mL), phospholipid (108 mg), and SQDG/SQMG (50 mg) were dissolved in 10 mL chloroform. Cosurfactant, stored as a 75 mg/mL solution in chloroform, was then added to the solution. The solution was dried by rotary evaporation, followed by vacuum desiccation for 5 h to generate a dry thin-film. The dried film was hydrated using distilled water and then warmed at 55–60°C in a thermostated water bath under N_2. This was followed by swirling/shaking/vortexing to produce coarse lipid emulsions. Small lipid emulsions were prepared by 1 h sonication under N_2 with a bath-type sonicator [Branson 1510 (Bransonic)] that was thermostated at 55–60°C. The sonication was performed by the following procedure: A 3-min sonication and subsequent 2-min cooling were repeated for the first 30 min, and then an 8-min sonication and 2-min cooling were repeated for the next 30 min.

Table 1 shows the formulation of the lipid emulsions with or without cosurfactant. In the formulation, the weight ratio of soybean oil, filtered distilled water, sulfoglycolipid (SQDG or SQMG), HEPC, and cosurfactant (HCO-60) was 7.92:100:1:2.16:3.24, and the cosurfactant weight ratio, defined as cosurfactant/(HEPC + sulfoglycolipid) (w/w), was 1.03.

Particle size measurement. The droplet size of the emulsions was measured by quasi-elastic laser light scattering using a Zeta-Plus apparatus with a particle size option, the BI-MAS (BI-8000AT: Brookhaven Instruments Corp., Ronkonkoma, NY).

Enzymes. DNA pol α was purified from calf thymus by immuno-affinity column chromatography as described previously (16). Recombinant rat pol β was purified from *E. coli* JMpβ5 as described by Date *et al.* (17). DNA polymerase I (plant α-like polymerase) and II (plant β-like polymerase) from a higher plant, cauliflower inflorescence, were purified according to the methods outlined by Sakaguchi *et al.* (18). Purified human placenta DNA topoisomerase I and II (topo I and topo II) (2 units/μL) were purchased from TopoGen, Inc. (Columbus, OH). The Klenow fragment of DNA polymerase I and HIV-1 reverse transcriptase were purchased from Worthington Biochemical Corp.



SCHEME 1

TABLE 1
Formulation and Particle Size of SQDG/SQMG[a]

	SQDG		SQMG	
HEPC	108 mg	108 mg	108 mg	108 mg
Sulfoglycolipid	50 mg	50 mg	50 mg	50 mg
Soybean oil	0.43 mL	0.43 mL	0.43 mL	0.43 mL
Cosurfactant	162 mg	—	162 mg	—
Distilled water	5 mL	5 mL	5 mL	5 mL
Particle size[b] (nm)	92.58 ± 3.47	352.8 ± 12.5	82.84 ± 1.82	336.0 ± 10.9

[a]SQDG, sulfoquinovosyl diacylglycerol, with two stearic acid DG; SQMG, sulfoquinovosyl monoacylglycerol, with one stearic acid side chain; HEPC, hydrogenated L-α-phosphatidylcholine (from egg yolk); cosurfactant, HCO-60 (polyoxyethylene hydrogenated castor oil: Cremophor-HR60; BASF Aktiengesellschaft, Ludwigshafen, Germany).
[b]Data represent mean ± SEM (n = 10 independent experiments).

(Freehold, NJ). Taq DNA polymerase, T4 DNA polymerase, T7 RNA polymerase, T4 polynucleotide kinase, and bovine pancreas deoxyribonuclease I were purchased from Takara (Kyoto, Japan).

DNA polymerase assays. Activities of DNA polymerases were measured by the methods described in previous reports (19,20). The substrates of DNA polymerases used were poly(dA)/oligo(dT)$_{12–18}$ and 2′-deoxythymidine 5′-triphosphate (dTTP) as template-primer DNA and dNTP (deoxyribonucleotide triphosphate) substrate, respectively. The substrates of HIV-1 reverse transcriptase used were poly(rA)/oligo(dT)$_{12–18}$ and dTTP as template-primer and dNTP substrate, respectively. One unit of each DNA polymerase activity was defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of deoxyribonucleotide triphosphate (i.e., dTTP) into the synthetic template-primers [i.e., poly(dA)/oligo(dT)$_{12–18}$, A/T = 2:1] in 60 min at 37°C under the normal reaction conditions for each of the enzymes (19,20).

Topo II assays. The relaxation activity of topo II was determined by detecting the conversion of supercoiled plasmid DNA to its relaxed form (21). The topo II reaction was performed in 20 μL of reaction mixture containing 50 mM Tris-HCl buffer (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT, supercoiled pUC19 DNA (0.25 μg), 2 μL of DMSO-soluble and emulsified SQDG/SQMG solution, and 2 units of topo II. The reaction mixtures were incubated at 37°C for 30 min and terminated by adding 2 μL of loading buffer consisting of 5% Sarkosyl L (*N*-dodecanoyl-*N*-methylglycine), 0.0025% bromophenol blue, and 25% glycerol. The mixtures were subjected to 1% agarose gel electrophoresis in TAE (Tris-acetate-EDTA) running buffer. The agarose gel was stained with ethidium bromide, and DNA was visualized on a UV transilluminator. One unit was defined as the amount of enzyme capable of relaxing 0.25 μg of DNA in 15 min at 37°C.

Other enzyme assays. The activities of T7 RNA polymerase, T4 polynucleotide kinase, and bovine deoxyribonuclease I were measured in each of the standard assays according to Nakayama and Saneyoshi (22), Soltis and Uhlenbeck (23), and Lu and Sakaguchi (24), respectively.

Gel mobility shift assay. The gel mobility shift assay was carried out as described by Casas-Finet *et al.* (25). The binding mixture (a final volume of 20 μL) contained 20 mM Tris-HCl, pH 7.5, 40 mM KCl, 50 μg/mL BSA, 10% DMSO, 2 mM EDTA, 2.2 nmol M13 plasmid DNA, 0.1 nmol of pol β, and various concentrations of DMSO-soluble and emulsified SQDG/SQMG. Plasmid DNA (2.2 nmol; nucleotide, single-strand and singly primed) were added to the binding mixture, followed by incubation at 25°C for 10 min. Samples were run on a 1.0% agarose gel in TAE running buffer, pH 8.3, containing 5 mM EDTA at 50 V for 2 h.

Investigation of cytotoxicity on cultured cells. To investigate the effects of DMSO-soluble and emulsified SQDG/SQMG in cultured cells, a human gastric cancer cell line, NUGC-3, was used. The cells were routinely cultured in modified Eagle's medium supplemented with 10% FCS, 100 μg/mL streptomycin, 100 unit/mL penicillin, and 1.6 μg/mL $NaHCO_3$. The cells were routinely cultured at 37°C in standard medium in a humidified atmosphere of 5% CO_2/95% air. The cytotoxicity of SQDG/SQMG was investigated as follows: High concentrations (10 mM) of the compounds were stocked. Approximately 1×10^4 cells per well were inoculated in 96-well microplates, then the compound stock solution was diluted to various concentrations and applied to each well. After incubation for 48 h, the survival rate was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (26).

Neutral red assay. Individual wells of a 96-well microplate were inoculated with 5×10^3 NUGC-3 cells and incubated for 48 h with DMSO-soluble SQMG and emulsified SQMG. Then the cell viability was measured by neutral red uptake. LD_{50} values of each compound were calculated.

Lactate dehydrogenase (LDH) determination. LDH activity was evaluated by a LDH cytotoxicity kit (Wako, Osaka, Japan), according to the manufacturer's instructions. The combined LDH activity from burst cells treated with 0.1% Triton X-100 in fresh medium was used to define 100% LDH activity. The LDH values in the supernatants from their respective media collected from each well of a 96-well microplate exposed to LD_{50} values of DMSO-soluble SQMG or emulsified SQMG for 48 h were analyzed.

RESULTS AND DISCUSSION

Formation of lipid emulsions containing SQDG/SQMG. The lipid emulsions containing sulfoglycolipids, SQDG, and SQMG were designed based on the following: the particle size

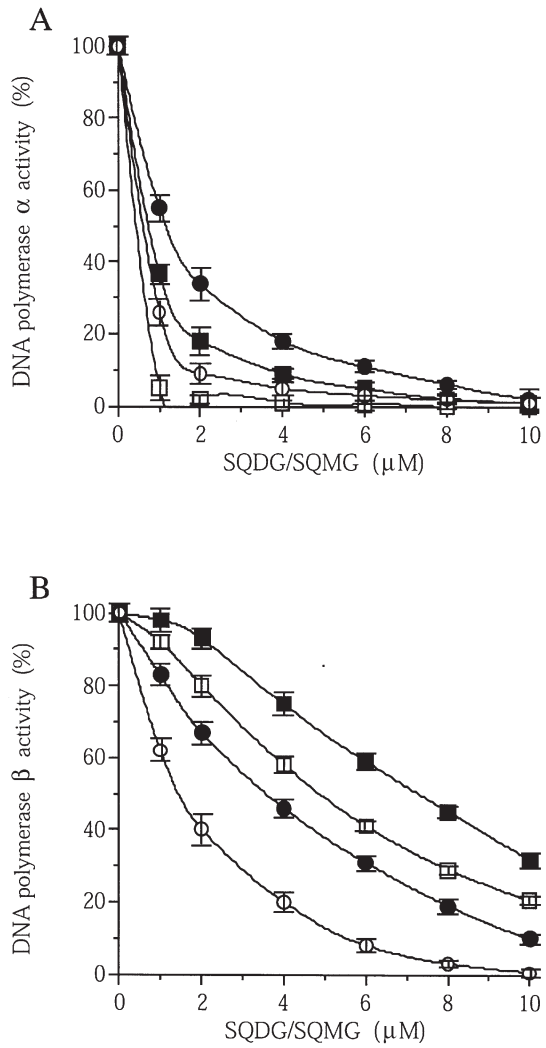


FIG. 1. DNA polymerase inhibition dose–response curves of sulfoquinovosyl diacylglycerol [1,2-di-*O*-acyl-3-*O*-(α-D-sulfoquinovosyl)-glyceride with two stearic acid molecules]/sulfoquinovosyl monoacylglycerol [1-mono-*O*-acyl-3-*O*-(α-D-sulfoquinovosyl)glycerol with one stearic acid molecule] (SQDG/SQMG). The enzymes used (0.05 units of each) were calf DNA polymerase α (A) and rat DNA polymerase β (B). The DNA polymerase activities were measured as described in the Materials and Methods section. DNA polymerase activity in the absence of these compounds was taken as 100%. The compounds tested and symbols used are as follows: DMSO-soluble SQDG (□); emulsified SQDG (■); DMSO-soluble SQMG (○); emulsified SQMG (●). Data are shown as means ± SEM of three independent experiments.

of the emulsions had to be smaller than 100 nm; the sulfoglycolipid content had to be as high as possible; and the surface of the emulsions had to be modified with hydrophilic moieties to allow prolonged circulation in the blood.

The chemically synthesized SQDG/SQMG have two and one hydrophobic tails consisting of a stearyl ester, respectively, that are connected to the hydrophilic sulfoquinovose moiety (Scheme 1). The particle sizes of the lipid emulsions containing SQDG/SQMG and cosurfactant were 92.58 ± 3.47 nm and 82.84 ± 1.82 nm, respectively (Table 1). SQDG/SQMG were expected to be incorporated into the surface of the soybean oil core as a component of the phospholipid monolayer and form an o/w emulsion. Among the many types of commercially available lecithin, HEPC was selected as an emulsifier since it provided better emulsification in a preliminary experiment (15). HCO-60 was used as a cosurfactant in order to build up the stearic hindrance on the emulsion surface and to reduce interfacial tension. As shown in Table 1, since the particle sizes of the emulsions containing SQDG/SQMG without cosurfactant were more than 300 nm, particles that were less than 10 nm in size could form in the presence of HCO-60 emulsions. These particles can be expected to prolong the blood circulation time of the resultant nanoemulsions. The detailed data of the lipid emulsions containing SQDG/SQMG with a reduced particle size will be published elsewhere (Mizushina Y., Maeda, N., Yoshida, H., and Sakaguchi, K., unpublished data).

The sulfoglycolipids emulsified and directly solubilized by DMSO will be referred to as emulsified SQDG/SQMG and DMSO-soluble SQDG/SQMG, respectively, in the rest of this paper for convenience.

Effect of DMSO-soluble and emulsified SQDG/SQMG on the activities of mammalian pol α and pol β. As reported previously, SQDG/SQMG inhibited the activities of mammalian pol α and pol β (1–4, 6–8). At first, we tested how emulsification influences this effect. Figure 1 shows the inhibition dose–response curves of DMSO-soluble and emulsified SQDG/SQMG on pol α and pol β. The inhibition by each of these compounds was dose-dependent, with 50% inhibition of pol α by DMSO-soluble and emulsified SQDG at doses of 0.44 and 0.74 μM, respectively, and DMSO-soluble and emulsified SQMG at 0.61 and 1.14 μM, respectively (Fig. 1A). These data showed that, regardless of the emulsification, SQDG/SQMG were the strongest of all the reported eukaryotic DNA polymerase inhibitors, including aphidicolin (27), dideoxyTTP (28), flavonoids (29–31), steroids (32–35), and FA (19,20). The IC_{50} values of pol β by DMSO-soluble and emulsified SQDG were about 4.86 and 7.25 μM, respectively (Fig. 1B). The inhibitory effect of pol α by SQDG was stronger than that of pol β. DMSO-soluble SQDG/SQMG were slightly stronger inhibitors of pol α and β than emulsified SQDG/SQMG (Figs. 1A,B), and in the *in vitro* experiments, both DMSO-soluble and emulsified SQDG/SQMG showed reproducible values. Therefore, the emulsification was slightly suppressive in the *in vitro* DNA polymerase inhibition experiments.

Inhibition mode of pol α and pol β by DMSO-soluble and emulsified SQMG. Next, to elucidate the mechanism involved in the inhibition of DMSO-soluble and emulsified sulfoglycolipids for pol α and β, the extent of inhibition as a function of DNA template-primer or dNTP substrate concentrations was studied (Table 2). Since the data in Figure 1 show that, with respect to emulsification, there was no difference in the *in vitro* pol α and β inhibition properties between SQDG and SQMG (i.e., for pol α: SQDG > SQMG; for pol β: SQMG > SQDG), the kinetic analysis was performed using one of them, namely, SQMG. In kinetic analysis, poly(dA)/oligo$(dT)_{12–18}$ and dTTP were used as the DNA template-primer and dNTP substrate, respectively, and V_{max} for the DNA template (range: 0–1.5 μM)

TABLE 2
Kinetic Analysis of the Inhibition by DMSO-Soluble or Emulsified SQMG of the Activities of DNA Polymerase α and β, as a Function of the DNA Template-Primer Dose and the dNTP Substrate Concentration

	Enzyme	Substrate (0.05 units)	SQMG (μM)	K_m (μM)	V_{max} (pmol/h)	K_i[a] (μM)	Inhibitory mode
DMSO-soluble SQMG	Pol α	DNA template[b]	0		55.6		
			0.25		46.5		
			0.5		42.5		
			0.75	7.85	36.1	0.26	Noncompetitive
			1		29.6		
			1.5		24.3		
			2		21.2		
	Pol α	dNTP substrate[c]	0		29.2		
			0.25		26.5		
			0.5		23.2		
			0.75	3.60	20.8	0.71	Noncompetitive
			1		18.4		
			1.5		15.7		
			2		13.9		
	Pol β	DNA template[b]	0	14.2			
			0.5	20.0			
			1	29.7			
			1.5	40.3	111	1.49	Competitive
			2	55.6			
			2.5	80.0			
			3	124			
	Pol β	dNTP substrate[c]	0	5.00			
			0.5	7.94			
			1	13.4			
			1.5	18.5	62.5	1.06	Competitive
			2	26.0			
			2.5	39.2			
			3	62.9			
Emulsified SQMG	Pol α	DNA template[b]	0		55.6		
			0.5		50.0		
			1		46.3		
			1.5	7.85	42.5	1.10	Noncompetitive
			2		38.8		
			2.5		36.2		
			3		33.5		
	Pol α	dNTP substrate[c]	0		29.2		
			0.5		27.5		
			1		26.0		
			1.5	3.60	24.1	3.12	Noncompetitive
			2		22.9		
			2.5		21.6		
			3		20.5		
	Pol β	DNA template[b]	0		111		
			1		87.7		
			2		71.4		
			3	14.2	59.8	1.95	Noncompetitive
			4		47.6		
			5		37.2		
			6		28.1		
	Pol β	dNTP substrate[c]	0		62.5		
			1		50.5		
			2		41.3		
			3	5.00	34.5	3.15	Noncompetitive
			4		26.0		
			5		20.1		
			6		15.7		

[a]Inhibition constants.
[b]Poly(dA)/oligo(dT)$_{12–18}$.
[c]dTTP, 2′-deoxythymidine 5′-triphosphate. dNTP, deoxyribonucleotide triphosphate; pol α and pol β, DNA-directed DNA polymerase α and β; for other abbreviations see Table 1.

was measured in the presence of an excess amount of dNTP (40 μM); V_{max} for dNTP (range: 0–3 μM) was measured in the presence of an excess amount of DNA template (20 μM). Lineweaver–Burk plots of the results show that the inhibition of pol α activity by DMSO-soluble SQMG was noncompetitive for both the DNA template primer (the K_m was unchanged at 7.85 μM) and the dNTP substrate (the K_m was unchanged at 3.60 μM). On the other hand, the inhibition of pol β activity by DMSO-soluble SQMG was competitive for both the DNA template-primer (the V_{max} was unchanged at 111 pmol/h) and the dNTP substrate (the V_{max} was unchanged at 62.5 pmol/h).

The inhibition of pol α activity by emulsified SQMG showed the same noncompetitive mode as that by DMSO-soluble SQMG for both the DNA template-primer (the K_m was unchanged at 7.85 μM) and the dNTP substrate (the K_m was unchanged at 3.60 μM), and Dixon plots of the results show that the inhibition constant (K_i) values for the DNA template-primer and dNTP substrate were 1.10 and 3.12 μM, respectively (Table 2). On the other hand, the inhibition mode of emulsified SQMG on pol β differed from that of DMSO-soluble SQMG. Although the inhibition of pol β activity by DMSO-soluble SQMG was competitive, the inhibition by emulsified SQMG was noncompetitive for both the DNA template-primer (the K_m was unchanged at 14.2 μM) and the dNTP substrate (the K_m was unchanged at 5.00 μM), and the K_i values for the DNA template-primer and dNTP substrate were 1.95 and 3.15 μM, respectively (Table 2). These K_i values suggest that the affinity of both DMSO-soluble and emulsified SQMG is stronger at the DNA template-primer binding site than at the dNTP substrate-binding site of both pol α and β.

The inhibition of pol α and β activities by DMSO-soluble and emulsified SQDG showed the same mode as those of SQMG (data not shown).

Inhibition of single-stranded DNA (ssDNA) binding activity of pol β by SQDG/SQMG. To demonstrate the difference in the pol β inhibition mode by sulfoglycolipids shown in Table 2, we tested the inhibition of the ssDNA binding activity of pol β by SQDG/SQMG in more detail. Since pol β could be obtained in measurable amounts by the expression of the recombinant *E. coli*, JMpβ5 (17), we investigated the interaction between pol β and emulsified SQDG/SQMG more precisely in comparison with DMSO-soluble SQDG/SQMG. The ssDNA-binding activity of pol β was analyzed by a gel mobility shift assay. Figure 2 shows the gel mobility shift assay of the M13 ssDNA to the pol β-binding complex. In the binding assay, M13 ssDNA at 2.2 nmol (nucleotide) was added to 0.1 nmol of the enzyme (lanes 2–6 and lanes 8–12 in Figs. 2A, 2B). M13 ssDNA bound to pol β was shifted in the gel (lane 6 and 12 in Figs. 2A, 2B). The molecular ratios of SQDG/SQMG and proteins are shown as the inhibitor-to-enzyme ratios (I/E) in Figure 2. When the I/E ratio was 10 or more and 1 or more, DMSO-soluble SQDG and SQMG interfered with complex formation between M13 ssDNA and pol β, respectively (lanes 2–4 in Figs. 2A, 2B). The inhibition of the ssDNA binding activity of pol β by SQMG was stronger than that by SQDG. At a ratio of 0.1 (lane 5 in Fig. 2B), it completely disappeared, suggesting that one molecule of DMSO-soluble SQMG competes with M13 DNA and subsequently interferes with the binding of ssDNA to one molecule of pol β. Kinetic analysis indicated that DMSO-soluble SQDG/SQMG acted by competing with the DNA template-primer on pol β (Table 2); thus, these compounds directly bind to the ssDNA binding site of pol β. However, no interference in the mobility shift by emulsified SQDG/SQMG occurred (lanes 8–11 in Figs. 2A, 2B). These data coincided well with the fact that the inhibition mode of pol β by emulsified SQDG/SQMG was noncompetitive with the DNA template-primer shown in Table 2. Although the reason the characteristics were changed is unexplainable as yet, the emulsification may disturb the competitive binding of SQMG to the binding sites of the DNA template-primer and dNTP substrate, and subsequently, they may bind to slightly different sites.

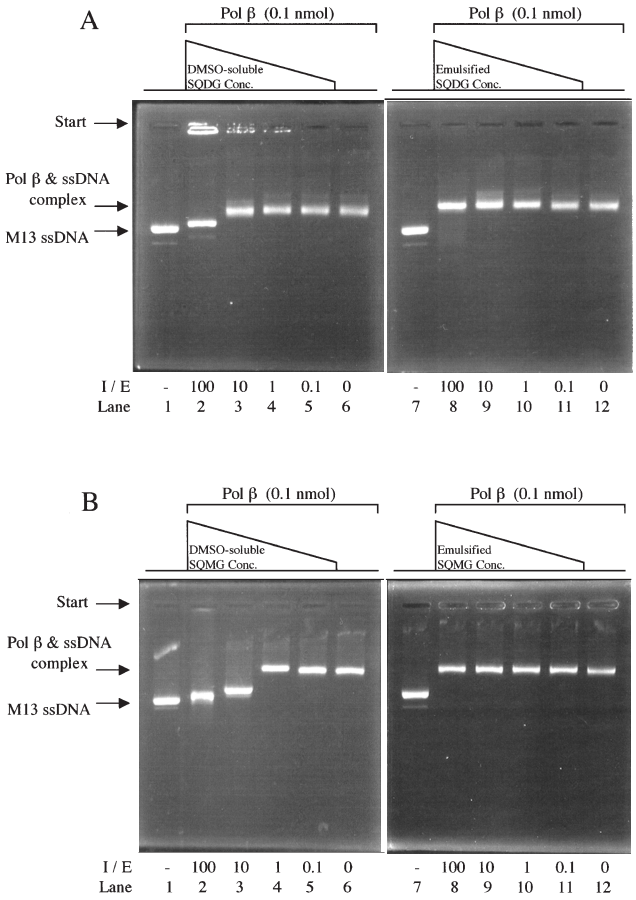


FIG. 2. Gel mobility shift analysis of binding of DNA polymerase β (pol β) to single-stranded DNA (ssDNA). M13 plasmid DNA (2.2 nmol; nucleotide, single strand and singly primed) was mixed with pol β and SQDG/SQMG. (A) DMSO-soluble and emulsified SQDG. (B) DMSO-soluble and emulsified SQMG. Lanes 2–6 and 8–12 contained pol β at a concentration of 0.1 nmol; lanes 1 and 7 contained no enzyme. Lanes 2 and 8, 3 and 9, 4 and 10, 5 and 11, and 6 and 12 contained different concentrations of the compound: 10, 1, 0.1, 0.01 and 0 nmol, respectively. Samples were run on a 1.0% agarose gel in 0.1 M Tris-acetate (pH 8.3), containing 5 mM EDTA at 50 V for 2 h. A photograph of an ethidium bromide-stained gel is shown. 1/E, inhibitor-to-enzyme ratio; for other abbreviations see Figure 1.

TABLE 3
IC_{50} Values of the DMSO-Soluble or Emulsified SQDG/SQMG on the Inhibitory Activities of Various DNA Polymerases and Other DNA Metabolic Enzymes[a]

Enzyme	SQDG (μM)		SQMG (μM)	
	Solution	Emulsion	Solution	Emulsion
Mammalian DNA polymerases				
Calf DNA polymerase α	0.45	0.75	0.60	1.1
Rat DNA polymerase β	4.8	7.2	1.4	3.5
Plant DNA polymerases				
Cauliflower DNA polymerase I (α-like)	>100	>100	>100	>100
Cauliflower DNA polymerase II (β-like)	>100	>100	>100	>100
Prokaryotic DNA polymerases				
E. coli DNA polymerase I (Klenow fragment)	>100	>100	>100	>100
Taq DNA polymerase	>100	>100	>100	>100
T4 DNA polymerase	>100	>100	>100	>100
Other DNA metabolic enzymes				
Calf DNA primase of DNA polymerase α	>100	>100	>100	>100
HIV-1 reverse transcriptase	>100	>100	>100	>100
Human DNA topoisomerase I	>100	>100	>100	>100
Human DNA topoisomerase II	5.0	>100	0.5	>100
T7 RNA polymerase	>100	>100	>100	>100
T4 polynucleotide kinase	>100	>100	>100	>100
Bovine deoxyribonuclease I	>100	>100	>100	>100

[a]The DMSO-soluble or emulsified SQDG/SQMG were incubated with each enzyme (0.05 units). The enzymatic activity was measured as described in the Materials and Methods section. Enzyme activity in the absence of the compound was taken as 100%. For abbreviations see Table 1.

Effects of DMSO-soluble and emulsified SQDG/SQMG on various DNA polymerases and other DNA metabolic enzymes. As shown in Table 3, both DMSO-soluble and emulsified SQDG/SQMG significantly inhibited the activities of both calf pol α and rat pol β. The inhibitory mode of pol β by emulsified SQDG/SQMG differed from that by DMSO-soluble SQDG/SQMG (Table 2); by being emulsified, the inhibitory effect of SQDG/SQMG was weakened (Fig. 1). The emulsification might decrease the chance for approach and collision between the polymerases and SQDG/SQMG. Emulsified SQDG/SQMG noncompetitively inhibited the activities of pol α and β, suggesting that emulsified SQDG/SQMG did not directly bind to either the DNA template primer-binding site or the dNTP substrate-binding site of the DNA polymerases. These compounds had little effect on the activities of a higher plant (cauliflower) DNA polymerase I (α-like polymerase) and II (β-like polymerase), prokaryotic DNA polymerases (such as the Klenow fragment of *E. coli* DNA polymerase I), Taq DNA polymerase and T4 DNA polymerase, and DNA metabolic enzymes such as calf DNA primase of pol α, HIV-1 reverse transcriptase, T7 RNA polymerase, T4 polynucleotide kinase, and bovine deoxyribonuclease I (Table 3). As shown in Figure 3, DMSO-soluble SQDG dose-dependently inhibited topo II relaxation activity, and complete inhibition occurred at 10 μM (lane 2 in Fig. 3A). Similarly, DMSO-soluble SQMG also dose-dependently inhibited topo II relaxation activity, and complete inhibition occurred at 1 μM (lane 3 in Fig. 3B). DMSO-soluble SQDG/SQMG should be referred to as a topo II inhibitor as well as a mammalian DNA polymerase inhibitor. On the other hand, we found for the first time that emulsified SQDG/SQMG did not inhibit the topo II activity at all (lanes 7–10 in Figs. 3A, 3B), indicating that the emulsification caused SQDG/SQMG to lose the topo II inhibition ability *in vitro*. Although the reason is unclear, the emulsification may interfere with the 3-D interaction between topo II and the agent. Emulsified SQDG/SQMG must act as a selective inhibitor of the mammalian DNA polymerases.

Effects of DMSO-soluble and emulsified SQDG/SQMG on cultured mammalian cells. As described previously, SQMG

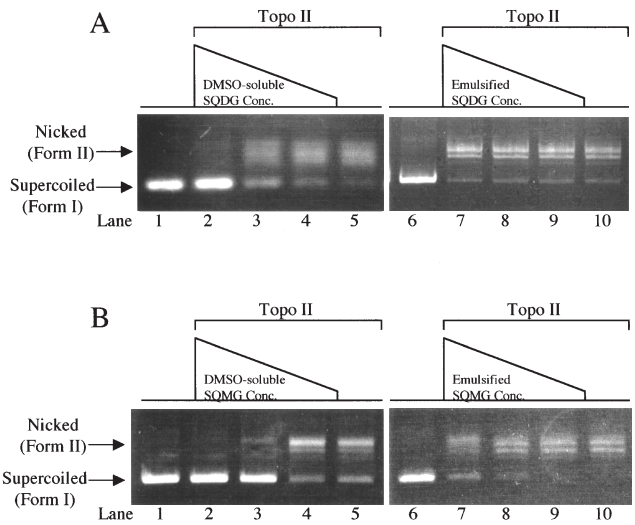


FIG. 3. Inhibitory effects of SQDG/SQMG on human DNA topoisomerase II (topo II). (A) DMSO-soluble and emulsified SQDG. (B) DMSO-soluble and emulsified SQMG. Lanes 1 and 6, 2 and 7, 3 and 8, 4 and 9, and 5 and 10 contained the compound at concentrations of 0, 10, 1, 0.1 and 0 μM, respectively; lanes 2–5 and 7–10, 2 units of topo II; lanes 1 and 6, no enzyme. pUC19 plasmid DNA (0.25 μg) was added to each of the lanes. Photographs of ethidium bromide-stained gels are shown. For abbreviations see Figure 1.

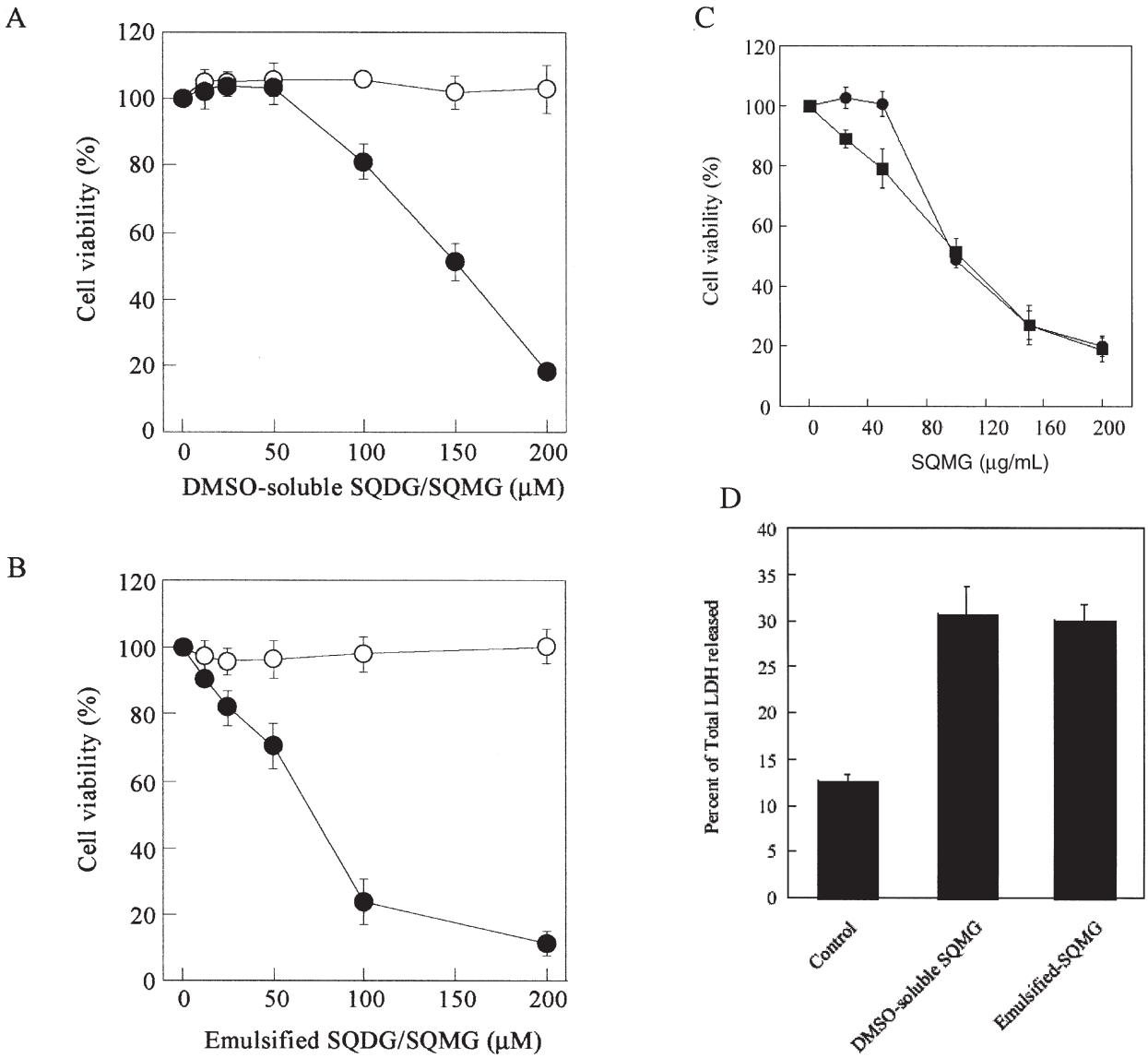


FIG. 4. Effects of SQDG/SQMG on the proliferation of human stomach cancer cells (NUGC-3). (A, B) Dose–response curves of growth inhibition of the cancer cell line NUGC-3. The assays were carried out under the conditions described in the Materials and Methods section with the compound at the indicated concentrations. Survival rate was determined by mitochondrial activity (MTT assay) (26). Data are shown as means ± SEM of three independent experiments. (A) DMSO-soluble SQDG (○) and SQMG (●). (B) Emulsified SQDG (○) and SQMG (●). (C) Effect of SQMG on cell viability by lysosomal membrane stability (neutral red assay). The NUGC-3 cell viability was evaluated after 48 h exposure to various concentrations of DMSO-soluble SQMG (●) and emulsified SQMG (■). The mean value was found as a percentage of the control value. The data are indicated as means ± SEM of four independent experiments. (D) Lactate dehydrogenase (LDH) release in DMSO-soluble SQMG and emulsified SQMG-treated cells. The NUGC-3 cell viability was evaluated by LDH release. A value of 100% LDH was obtained by combining intracellular LDH values (0.1% Triton X-100 extracted) and the corresponding conditioned media values. The data are indicated as means ± SEM of four independent experiments.

showed potent antineoplastic and anticancer chemotherapy activities (5,9). On the other hand, SQDG was not a cell growth inhibitor but did show *in vivo* immunosuppressive effects (6,10,36). The effect seems to result from the inhibition of the pol or topo activities, or both. Since the emulsification changes the inhibitory effects of SQDG/SQMG, the antineoplastic activity of emulsified SQDG/SQMG must also be tested. The results might be useful when considering their use as anticancer chemotherapy agents. We tested the cytotoxic effect of both DMSO-soluble and lipid-emulsified SQDG/SQMG against a human stomach cancer cell line, NUGC-3, *in vitro*. The cytotoxicity shown in Figure 4 was determined by MTT assay, which monitors mitochondrial integrity, and the results indicated that both DMSO-soluble and emulsified SQMG had potent growth-inhibitory effects on this cancer cell line; the LD_{50} values were in the range of 151 and 69 μM, respectively. Neither DMSO-soluble SQDG nor emulsified SQDG showed such an inhibitory effect. The NUGC-3 cell viability after 48 h of exposure to DMSO-soluble SQMG and emulsified SQMG was tested by neutral red assay (Fig. 4C). In this assay, the retention

of neutral red is used as a measure of the lysosomal membrane stability, and a dose-dependent decrease in neutral red uptake was found. The LD_{50} values of each compound were 104 and 99 μM, respectively. Furthermore, the LDH release was determined (Fig. 4D). This assay is based on the principle that dead cells lose the ability to maintain their plasma membrane integrity, permitting intracellular constituents, such as LDH, to leak out of the cell into culture media. After 48 h of exposure to DMSO-soluble SQMG, the released LDH increased to about 30%. An increase in released LDH was observed with emulsified SQMG, as well as with DMSO-soluble SQMG. However, in the MTT assay, emulsified SQMG showed a stronger cytotoxic effect than DMSO-soluble SQMG (Figs. 4A, 4B). Taken together, the cytotoxicity increase after the emulsification was thought to result from the increase in the amount of SQMG that penetrated into NUGC-3 cells. These results suggested that the emulsification makes SQMG more able to penetrate into NUGC-3 cells, and that, after the penetration, the *in vivo* molecular action of SQMG in the cells is probably the same. Interestingly, the cell growth inhibition by emulsified SQMG was stronger than that by DMSO-soluble SQMG, although mammalian DNA polymerase inhibition by emulsified SQMG was weaker than that by DMSO-soluble SQMG, and emulsified SQMG lost the topo II inhibition ability (Fig. 1). Since the IC_{50} values of emulsified SQMG were 1.14 μM for pol α, and 3.55 μM for pol β (Fig. 1), the LD_{50} value was about 60- and 20-fold of the IC_{50} values for pol α and pol β, respectively (Fig. 4). We would like to emphasize in this experiment not only that emulsified SQMG could be an effective antineoplastic agent as well as DMSO-soluble SQMG, but also that the *in vivo* results by emulsified SQMG were very reproducible, unlike those for DMSO-soluble SQMG.

The *in vivo* experiments using DMSO-soluble SQMG/SQDG were relatively difficult to perform because of their solubility. When we use lipid emulsions containing SQMG with high concentrations of SQMG and particle size of 100 nm, this problem can easily be solved. This method should be helpful in the pharmaceutical use of SQDG/SQMG. Indeed, o/w emulsions stabilized with emulsifiers such as phospholipids have been receiving considerable attention as drug delivery systems (13,15). Such lipid emulsions have many appealing properties as drug carriers: For example, they are biodegradable and biocompatible. Nanoparticulate systems including microemulsions have been developed for pharmaceutical use. Their application as carriers of anticancer drugs for cancer therapies has attracted a great deal of attention recently. We succeeded in developing a SQDG/SQMG-particulate drug delivery system in this paper. Since both SQDG/SQMG are thought to be promising immunosuppressive and anticancer agents (9,10), this may be a breakthrough in the clinical use of SQDG/SQMG.

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Lipid Molarity Affects Liquid/Liquid Aroma Partitioning and Its Dynamic Release from Oil/Water Emulsions

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ABSTRACT: Initial dynamic flavor release from oil/water emulsions containing different TAG phases was studied using a computerized apparatus and thermodesorption GC. A significant influence of lipid molarity on liquid/liquid partitioning and release of some flavor compounds was found. The release of the least hydrophobic compounds was not affected by any type of lipid. Hydrophobic compounds showed a positive correlation between their release and decreasing molarity of the lipid phase, that is, with increasing number of lipid molecules; only the most hydrophobic compounds did not show such a correlation. A strong linear correlation between low-melting TAG/water partition coefficients and lipid phase molarity was validated by volatile partition data of C_6, C_{11}, and C_{16} alkane/water systems. Lipid phase transition from the liquid to solid state did not affect flavor partitioning and release. Neither experimental nor theoretical octanol/water partition coefficients agreed with experimental TAG/water and alkane/water partition coefficients.

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Recent studies have investigated the effects of oil/water emulsion properties on flavor partitioning and release. Land (1) found the distribution of a lipid in an aqueous phase to affect flavor release. However, more recently several studies have shown the opposite, that is, flavor release is independent of the interfacial area between the oil and the water phases (2–4). Nonionic emulsifiers, such as Tweens, or surfactants based on MAG and DAG did not serve as barriers at the oil interface nor as solubilizers in terms of micelles (4–6). On the other hand, the oil fraction of an oil/water emulsion had a considerable influence on the partitioning of flavors, which in turn determined their static and dynamic release (7–13). These processes depend on the polarity of the volatiles and are governed by Nernst's law of partitioning (6,13). The parameter commonly used to describe aroma partitioning in lipid/water systems is the log*P* (14). The chemical composition of lipids, particularly the chain length, degree of saturation, and sequence of FA incorporated in a TAG, also determines the affinity of flavors to a lipid phase and their partitioning (15). In contrast, Roberts *et al.* (6) explained their results with the lipophilicity of lipids being generally at such a high level that this parameter does not influence flavor release. Apart from the chemical structure of a lipid, the molarity of the lipid phase was proposed to influence volatile behavior in emulsions (4). Increasing the number of molecules in the lipid phase led to decreased rates of flavor release. The present work attempted to correlate lipid phase properties with the partitioning and dynamic release of flavors from various oil/water emulsions.

MATERIALS AND METHODS

Emulsions consisted of water, different lipid phases, and Tween 80 (Grünau, Illertissen, Germany), which was used for emulsification. The lipids applied were triacetin, tributyrin (Sigma-Aldrich, Steinheim, Germany), Miglyol 812 N (Sasol, Witten, Germany), butter oil (Uelzena, Uelzen, Germany), trimyristin (Dynasan 114; Sasol), and tripalmitin (Dynasan 116; Sasol); they possessed m.p. below or above (the latter two) the ambient temperature. Except for butter oil, all lipids were purified synthetic materials. Olfactory examination ruled out their contamination with flavors.

The emulsions were flavored with a mix of 13 volatile molecules from different chemical classes, resulting in food-like concentrations [calculated log*P* values in parentheses (16)]: diacetyl (–1.33), 0.78 mg L^{-1}; isobutyl acetate (1.71), 0.02 mg L^{-1}; ethyl 2-methylbutyrate (2.26), 0.03 mg L^{-1}; (*Z*)-3-hexenyl acetate (2.61), 0.38 mg L^{-1}; 2,3-dimethylpyrazine (0.64), 1.93 mg L^{-1}; (*Z*)-3-hexenol (1.61), 0.96 mg L^{-1}; 2-isobutylthiazole (2.51), 0.88 mg L^{-1}; furfuryl acetate (1.09), 0.98 mg L^{-1}; linalool (3.28), 0.97 mg L^{-1}; 2-pentylpyridine (3.32), 1.46 mg L^{-1}; D-carvone (3.07), 1.45 mg L^{-1}; β-damascenone (4.21), 1.95 mg L^{-1}; γ-nonalactone (1.85), 4.85 mg L^{-1}. Different concentrations were chosen to obtain well-balanced gas chromatograms. Propylene glycol or Miglyol at 0.5 g per 5 L emulsion served as solubilizers for the flavor mix in water and emulsions, respectively. Flavors, propylene glycol, and triacetin were of analytical grade and were kindly provided by Symrise (Holzminden, Germany). Hexane, undecane, hexadecane, and octanol (Sigma-Aldrich), used for the determination of partition coefficients, were also of analytical grade.

Determination of melting temperatures of trimyristin, tripalmitin, and tristearin. Melting temperatures of pure trimyristin

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Abbreviations: *A*, gas/liquid interface (m^2); c_O, initial flavor concentration in the emulsion; c_{hs}, flavor concentration dynamically released into the headspace; DMR test, Duncan's multiple range test; *k*, mass transfer coefficient ($m\ s^{-1}$); $K_{O/W}$: oil/water partition coefficient; *t*, time (s); PTV, programmable temperature vaporizer; TDS-GC, thermodesorption GC; V_{hs}: volume of the headspace (m^3); V_O, oil volume (mL); V_W, water volume (mL).

and tripalmitin and their melting behavior in emulsions were analyzed by DSC. Analysis was done using a Seiko Thermo Exstar 6200 DSC (Seiko Instruments, Chiba, Japan). Ten milligrams of each sample was sealed into a separate silver pan. An empty pan was used as a reference. The temperature was raised from 25 to 100°C at a rate of 3°C min^{-1}.

Emulsion preparation. For triacetin, tributyrin, Miglyol, and butter oil, Tween 80 at its critical micelle concentration (4.48 g L^{-1} determined for Miglyol/water emulsions; Ref. 4) was dispersed in water (25°C) using an Ultra-Turrax T 50 (IKA®, Staufen, Germany) operated with a dispersing tool (S 50 N–G 45 F; IKA) for fine dispersions at 10,000 rpm (30 s for 1 L of liquid). Flavors were added to the bulk lipid phase, which was subsequently pre-emulsified at 50 mL L^{-1} using the Ultra-Turrax at 10,000 rpm. Afterward, homogenization was performed twice at 40 MPa using a two-pressure piston pilot-plant homogenizer (APV Gaulin, Lübeck, Germany).

For the higher-melting lipids, trimyristin and tripalmitin, a different method of preparation was applied. The emulsifier was dispersed in water that was heated above the m.p. of the lipids. According to DSC measurements, trimyristin and tripalmitin showed endothermic peaks at approximately 56 and 64°C, respectively. The melted lipid was added, pre-emulsified, and homogenized (the homogenizer was preheated). Flavors predissolved in propylene glycol were quickly added to the hot emulsion, which was immediately sealed after a short stirring time to avoid flavor losses.

Because the results of a previous work showed no significant differences for the flavor release from either (i) emulsions prepared according to the method described above or (ii) finished emulsions that were subsequently flavored *via* the aqueous phase (4), flavor losses during emulsion preparation were assumed to be negligible.

Emulsions (5 L) were prepared the day before measurement and stored at least 12 h at room temperature in gas-tight glass bottles to allow for equilibration.

Determination of liquid/liquid partition coefficients. Liquid/liquid partition coefficients of furfuryl acetate, 2-isobutyl thiazole, (*Z*)-3-hexenyl acetate, ethyl 2-methylbutyrate, and D-carvone were determined separately using a shake flask method: For low-melting lipids, alkanes and octanol, respectively, approximately 0.1 (±0.005) g of a compound was transferred into a silanized 70-mL separatory funnel containing 50% (vol/vol) of one of the above lipids. Then 50% (vol/vol) of water was added, followed by vigorous shaking and an equilibration time of approximately 24 h at 22°C. After equilibration, exactly 2 μL of the aqueous phase was transferred into a Tenax trap, which was subsequently thermodesorbed and analyzed by GC-FID. Determination of the partition coefficients of volatiles between high-melting lipids and water followed the method described above, but required a similar pretreatment in the preparation of their corresponding emulsions: The lipid and water at a 1:1 (vol/vol) ratio were heated above the m.p. of the lipid and subsequently poured into a preheated separatory funnel, followed by addition of the flavor compound to be investigated.

Single partition coefficients were determined at least in triplicate and calculated as the (equilibrium) concentration ratio between the lipid phase and the water phase.

Dynamic flavor release measurements. Flavor release was studied using a mouth-simulating device able to measure dynamic short-time flavor release from liquids (17). Operating conditions were the same as in the previous work. The reactor of the apparatus was completely filled with 5 L equilibrated emulsion sample at 22°C. After creation of a defined headspace volume (850 mL) above the emulsion within 3 s, a stirrer was started at 450 rpm to simulate shear stress in the mouth, resulting in a shear rate of approximately 150 s^{-1}. Subsequently, three 1.5-L headspace samples, each representing a 10-s time interval of dynamic flavor release, were sampled on-line in corresponding cascades of high-precision syringes at a volumetric air flow rate through the reactor of 9 L min^{-1}. The flavor-enriched air samples were then drawn off-line through corresponding Tenax traps by means of a low vacuum. A flow rate of 60 to 80 mL min^{-1} ensured quantitative flavor adsorption onto the Tenax traps. The traps were thermodesorbed afterward and analyzed by GC-FID. Independent quantification of volatiles was done by external calibration of each aroma compound. At least three replicates were run for each experiment.

Instrumentation: Thermodesorption (TDS)-GC-FID. Thermodesorption of the Tenax traps was carried out using a thermal desorption device (TDS2; Gerstel, Mülheim an der Ruhr, Germany) mounted on an HP 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a temperature-programmable vaporization inlet [CIS 4 programmable temperature vaporizer (PTV); Gerstel]. The PTV inlet incorporated a Tenax packed liner (glass liners—Tenax TA; Gerstel) and was cooled by liquid nitrogen. Analytical conditions were as follows: thermal desorption: 30 to 260°C at 60°C min^{-1} and held for 8 min; splitless mode; 50 mL min^{-1} desorption gas flow (N_2), PTV: 1°C (cryofocusing temperature) to 260°C at 12°C min^{-1} and held for 10 min; splitless; split mode (1:50) after 1.5 min; gas-saver mode (1:20) after 3 min; column, 30 m × 0.25 mm i.d. × 0.25 μm INNOWAX (J&W Scientific, Folsom, CA), carrier gas flow, 52 cm s^{-1} hydrogen; oven temperature, 40°C (held for 1.5 min) to 130°C at 4°C min^{-1} to 180°C at 8°C min^{-1} to 250°C at 25°C min^{-1} and held for 10 min; detection, FID at 250°C. Chromatograms were evaluated using HP ChemStation Software (Agilent Technologies).

Statistical analysis. ANOVA was performed on release and partition data. Duncan's multiple range (DMR) test and Fisher's LSD test were carried out to determine significant differences among mean values of flavor quantities released after 30 s and partition data, respectively. Significance was defined at $P < 0.05$ for the DMR test and $P < 0.01$ for the LSD test.

RESULTS AND DISCUSSION

Effect of different TAG on dynamic flavor release from lipid/water emulsions. In accordance with recent results (4,13,17), flavor release correlated linearly with time (average

TABLE 1
Comparison of Dynamically Released Quantities of Flavor (μg, after 30 s) from Emulsions Comprising Different Lipid Phases[a]

		Lipids used in emulsions					
		Liquid lipids[b]				Solid lipids[b]	
	Water[c]	Triacetin	Tributyrin	Miglyol[d]	Butter oil[d]	Trimyristin[d]	Tripalmitin
Diacetyl	1.22[a] ± 0.06	2.02[b] ± 0.01	2.28[c] ± 0.14	1.50[d] ± 0.11	5.95[e] ± 0.07	2.26[c] ± 0.17	1.75[f] ± 0.15
Isobutyl acetate	0.81[a] ± 0.01	0.70[b] ± 0.03	0.20[c] ± 0.00	0.25[d] ± 0.00	0.33[e] ± 0.02	0.31[e] ± 0.01	0.31[e] ± 0.03
Ethyl 2-methylbutyrate	1.22[a] ± 0.01	0.91[b] ± 0.04	0.15[c] ± 0.01	0.17[c] ± 0.01	0.25[d] ± 0.01	0.21[c,d] ± 0.01	0.35[e] ± 0.04
(*Z*)-3-Hexenyl acetate	6.22[a] ± 0.22	3.25[b] ± 0.15	0.33[c] ± 0.02	0.48[c] ± 0.01	0.69[d] ± 0.01	0.68[d] ± 0.02	1.48[e] ± 0.11
2,3-Dimethylpyrazine	0.28[a] ± 0.00	0.23[b] ± 0.03	0.30[a,c] ± 0.05	0.27[a,b] ± 0.01	0.31[a,c] ± 0.00	0.34[c] ± 0.01	0.24[b] ± 0.00
(*Z*)-3-Hexenol	0.74[a,b] ± 0.01	0.73[a] ± 0.06	0.58[c] ± 0.07	0.60[c] ± 0.00	0.80[b,d] ± 0.01	0.82[d] ± 0.01	0.61[c] ± 0.01
2-Isobutylthiazole	4.50[a] ± 0.05	2.80[b] ± 0.21	0.58[c] ± 0.05	0.67[c] ± 0.00	0.95[d] ± 0.02	0.93[d] ± 0.03	1.93[e] ± 0.14
Furfuryl acetate	1.23[a] ± 0.07	0.98[b] ± 0.10	0.35[c] ± 0.05	0.52[d] ± 0.01	0.71[e] ± 0.03	0.69[e] ± 0.02	0.79[e] ± 0.05
Linalool	1.98[a] ± 0.10	1.13[b] ± 0.15	0.15[c] ± 0.02	0.18[c] ± 0.00	0.26[c] ± 0.01	0.27[c] ± 0.01	0.42[d] ± 0.01
2-Pentylpyridine	2.36[a] ± 0.14	0.75[b] ± 0.28	0.21[c] ± 0.06	0.24[c] ± 0.09	0.22[c] ± 0.03	0.19[c] ± 0.02	0.30[c] ± 0.04
D-Carvone	1.31[a] ± 0.01	0.64[b] ± 0.13	0.11[c] ± 0.02	0.14[c] ± 0.01	0.17[c] ± 0.01	0.18[c] ± 0.01	0.46[d] ± 0.04
β-Damascenone	4.79[a] ± 0.43	0.89[b] ± 0.28	0.20[c] ± 0.06	0.13[c] ± 0.04	0.11[c] ± 0.01	0.13[c] ± 0.02	0.34[c] ± 004
γ-Nonalactone	0.11[a] ± 0.02	0.07[b] ± 0.03	0.05[b] ± 0.01	0.04[b] ± 0.00	0.05[b] ± 0.00	0.07[b] ± 0.01	0.05[b] ± 0.01
CV[e] (%)	4.2	15.1	13.8	8.3	4.7	6.0	8.0
Log*P*[f]		0.36	3.31	10.7[g]		18.0	20.9
Molarity (mol L^{-1})		0.27	0.17	0.09	0.06	0.06	0.05

[a]Values with different roman letters within a line are significantly different [ANOVA and Duncan's multiple range (DMR) test, $P < 0.05$].
[b]At 22°C.
[c]Adapted from Reference 13.
[d]Adapted from Reference 4.
[e]Average CV.
[f]Calculated with Syracuse Research Corporation's LOGKOW/KOWWIN (Syracuse Research Corp., http://esc.syrres.com/Interkow).
[g]Average between log*P* of tricaprylin and tricaprin.

$R^2 = 0.992$) under the conditions applied for experimentation. Even though foodlike flavor concentrations were applied, mean CV in the range of 4.2 to 15.1%, with an average of 8.6%, showed the reproducibility of the apparatus used (*cf.* Ref. 17).

Table 1 compares quantities of the flavor dynamically released from water or emulsions within the first 30 s. The release of the least hydrophobic diacetyl (log*P* –1.33) was enhanced by the lipid phase. In the case of butter oil, the particularly high release arose from the naturally occurring diacetyl portion in the butter oil, which was not separated prior to experimentation. The release of 2,3-dimethylpyrazine (log*P* 0.64) was barely influenced by the lipids in the emulsions. The same was found for (*Z*)-3-hexenol (log*P* 1.61), but with a broader variation. The quantities of all other compounds released were reduced upon dispersion of a TAG into the water owing to their higher lipophilicity. Figure 1 shows the exponential correlation between the log*P* of the compounds and the reduction of flavor quantities released upon the addition of lipid to the water (effect averaged over all lipids), ranging from factor 1 to 26.

Literature data are in accordance with the present data. Static (2,6,7) and dynamic (8–12) studies have demonstrated that compound polarity and lipid/water partitioning are rate limiting for flavor release, with the most hydrophilic compounds being fairly unaffected upon addition of the lipid, whereas the release of hydrophobic compounds is considerably decreased. As a consequence, a drastic shift in the overall release profile was obtained (18,19).

Influence of liquid lipids on the release of hydrophobic compounds. Triacetin reduced the release of volatile hydrophobic compounds significantly in comparison to water but showed a considerably smaller lowering effect than all other lipids (Table 1). It actually formed no microemulsion but probably a translucent nanoemulsion (20) and served as a solubilizer for the aromas, as in industrial applications (21).

Except for 2-pentylpyridine, β-damascenone, and γ-nonalactone, which showed steady release rates, all other hydrophobic compounds exhibited the lowest release with tributyrin, followed by Miglyol and finally butter oil, and the trend was mostly significant (ANOVA and the DMR test, $P < 0.05$). The results confirm the recently proposed assumption that flavor partitioning and dynamic volatile release in or from oil/water emulsions depends on the molarity of the lipid phase (4). Even though increasing the average carbon number of the FA of TAG increases the nonpolar character of the TAG (*cf.* log*P* values in Table 1) and, as a consequence, should lead to a stronger affinity toward hydrophobic flavors, the opposite was found: Tributyrin, with the highest molarity of the lipids in the 50 mL L^{-1} emulsions showed the highest affinity (Table 1), followed by Miglyol and butter oil.

When comparing the static flavor release from emulsions containing either coconut oil or medium-chain TAG (C_8–C_{10}, 60:40), Roberts *et al.* (6) did not find significant differences for various flavor compounds. However, for most of the hydrophobic compounds a trend that corresponded with the present results was obvious. Flavors showed a higher affinity toward medium-chain TAG possessing a higher molarity than coconut oil, resulting in higher oil/water partition coefficients and lower release. The lack of statistical significance might be attributed to the lower sensitivity (static headspace) and reproducibility (1 min adsorption time) of the solid phase microextraction method.

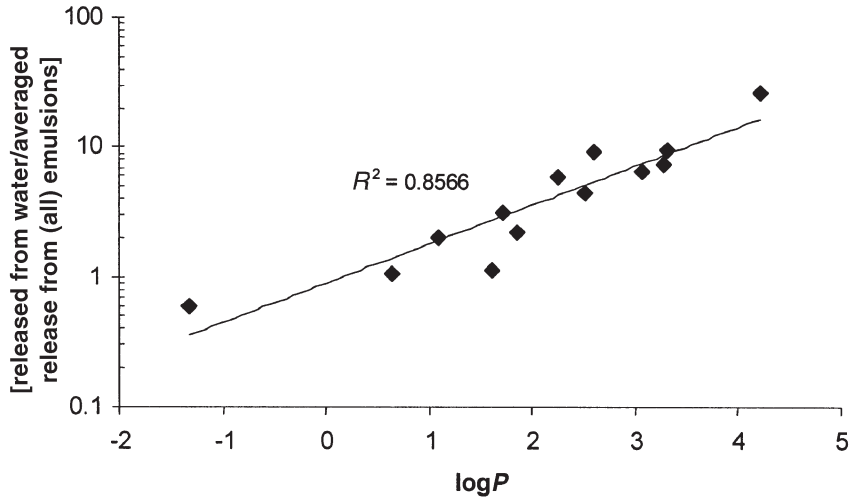

FIG. 1. Correlation between compound log*P* and decreased flavor release upon dispersion of a lipid phase (50 mL L^{-1}) into water. Released fractions of the individual flavor compounds from emulsions were averaged over all lipids.

Influence of high-melting lipids on the release of hydrophobic compounds. Emulsions containing lipids that were solid at ambient temperature were prepared at elevated temperatures to ensure liquid/liquid flavor partitioning before fat crystallization. Fat crystallization was determined by DSC measurements of the corresponding emulsions showing endothermic peaks at the melting temperatures of the lipids (data not shown). Flavor release rates from emulsions containing trimyristin equaled those of butter oil emulsions (Table 1). Tripalmitin showed partly greater quantities of hydrophobic compounds released from its emulsions than butter oil. This is consistent with the results obtained with the liquid lipids; as the molarity of tripalmitin is even smaller than that of the other lipids, according to the above assumption, the corresponding flavor release should be higher (Table 1).

The influence of lipid phase molarity. Figures 2 and 3 illustrate the correlation between the molarity of the lipid phase used in the emulsions and flavor release relative to the initial concentration of the corresponding compound. Molarity, like lipophilicity of the lipids (*cf.* Table 1), barely affected dynamic release of the hydrophilic diacetyl (log*P* –1.33) and 2,3-dimethylpyrazine (log*P* 0.64) (Fig. 2). A positive trend was obtained for furfuryl acetate (log*P* 1.09) and linalool (log*P* 3.28), which represent the majority of flavor compounds with a medium lipophilicity (Fig. 3). The only exception was found with (*Z*)-3-hexenol (log*P* 1.61), which behaved like the most hydrophilic compounds (Fig. 2). Highly nonpolar compounds, such as β-damascenone and 2-pentylpyridine, did not show such an effect, and their release was independent of lipid molarity (Table 1). In conclusion, not only the hydrophobicity of

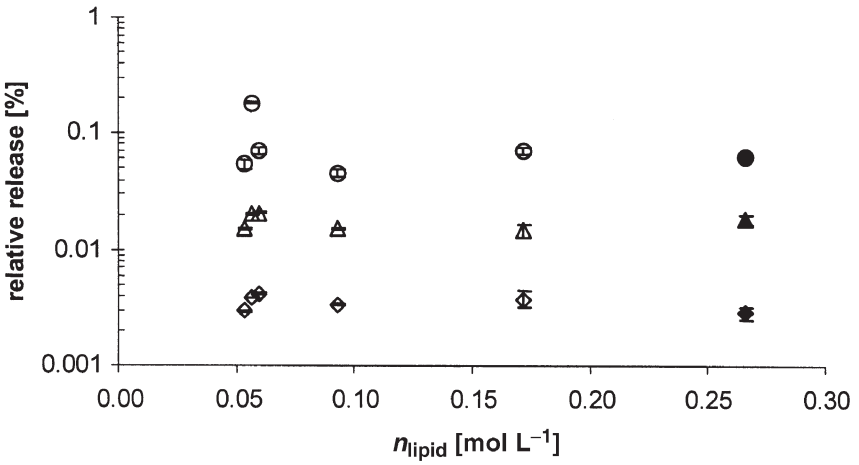

FIG. 2. Influence of lipid phase molarity (see Table 1) on the dynamic release of diacetyl (○), 2,3-dimethylpyrazine (◇), and (*Z*)-3-hexenol (△). Flavor release (within the first 30 s) relative to the initial concentration is compared. Solid symbols represent triacetin. Error bars represent the SD of relative release determined in repeated experiments ($n \geq 3$).

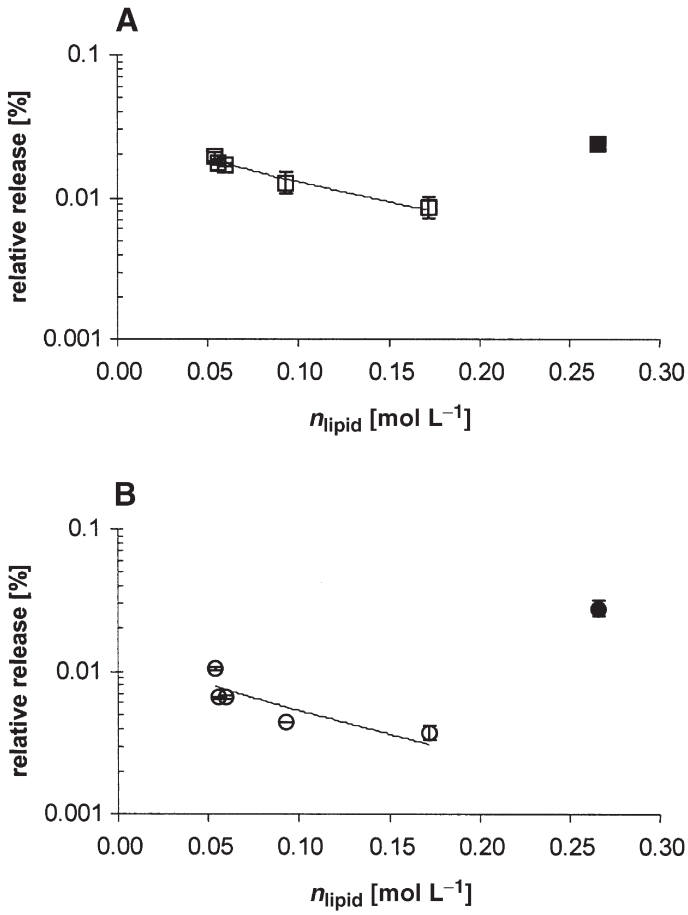

FIG. 3. Influence of lipid phase molarity (see Table 1) on the dynamic release of (A) furfuryl acetate (□) and (B) linalool (○). Flavor release (within the first 30 s) relative to the initial concentration is compared. Solid symbols represent triacetin. Error bars represent the SD of relative release determined in repeated experiments ($n \geq 3$).

the TAG played a role in the affinity of numerous flavors for a lipid phase, but the particle number of the lipid phase also seemed to be important for partitioning and dynamic release processes in or from oil/water emulsions. Consistently, adjustment of the molarity of Miglyol in emulsions (31 mL L^{-1} lipid volume instead of 50 mL L^{-1}) to that of butter oil in emulsions (50 mL L^{-1}) resulted in equal rates of flavor release (4).

Tributyrin comprises approximately three times more lipid molecules per mass unit than the long-chain TAG. Considering, for example, the concentration of furfuryl acetate in the emulsions of approximately 1 mg L^{-1} and its M.W. of 140 g mol^{-1}, its molarity is 7×10^{-6} mol L^{-1}. Consequently, about 8,500 molecules of tripalmitin or butter oil [the average FA carbon number was determined to be 16 (*cf.* Ref. 4)] and 24,000 molecules of tributyrin (*cf.* Table 1 for lipid molarity) faced one molecule of furfuryl acetate. The larger number of lipid particles should lead to a larger number of lipid/flavor interactions. Thus, upon dynamic flavor release, a decreased velocity of movement of flavors in the lipid phase for the dynamic equilibration process during exhaustion of the water phase should result. However, a similar correlation was found under static conditions, as illustrated by Figure 4 for tributyrin, Miglyol, and butter oil (all liquids at room temperature). Accordingly, the larger molarity of the lipid phase led to significantly larger partition coefficients (Table 2; ANOVA and the LSD test, $P < 0.01$), which in turn resulted in decreased flavor release (partly significant, ANOVA and the DMR test, $P < 0.05$; see Table 1) because of the lower flavor concentration partitioned in the aqueous phase of the emulsion (Fig. 5). Figure 6 demonstrates the close correlation of the individual flavor partition coefficients for the different lipids examined and dynamic flavor release from the emulsions containing them. Only triacetin deviated from the general trend.

Comparable to the situation in water, flavors were dissolved in the lipid phase by the formation of lipid molecule shells surrounding each flavor molecule. It is assumed that this solubilization does not depend on the volume of solvent, as would be the case when water is considered on its own (22,23), but on the number of solvent molecules available for shell formation. Owing to the higher molarity and the smaller size of tributyrin compared with the long-chain TAG, flavor/lipid interactions should be numerically and sterically favored. The comparably lower log*P* value of tributyrin (Table 1) might have further improved the affinity of semilipophilic compounds toward tributyrin.

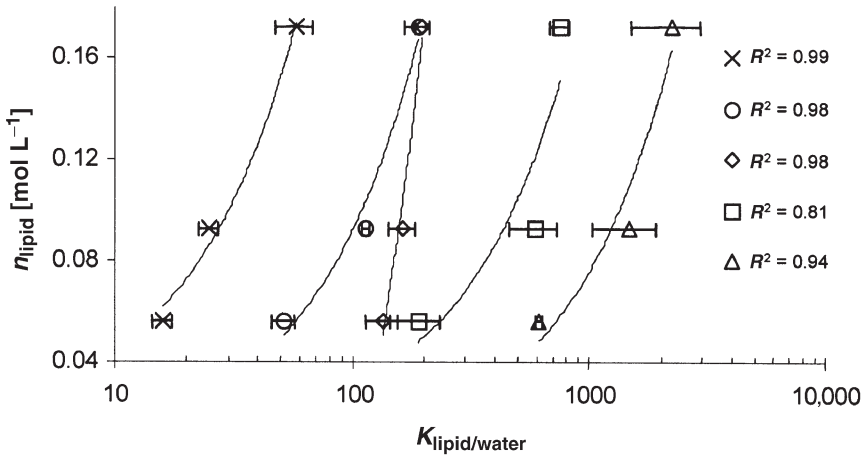

FIG. 4. Correlation between lipid/water partition coefficients (see Table 2) and lipid phase molarity (see Table 1). Tributyrin/water, Miglyol/water, and butter oil/water systems were compared. R^2 represents the linear regression coefficient. (×) Furfuryl acetate; (○) 2-isobutylthiazole; (◇), D-carvone; (□) ethyl 2-methylbutyrate; (△) (*Z*)-3-hexenyl acetate. Error bars represent the SD of lipid/water partition coefficients determined in repeated experiments ($n \geq 3$).

The most hydrophobic compounds, 2-pentylpyridine and β-damascenone, behaved like the solvent in which they were distributed. Consequently, very low quantities of these flavors (on a relative basis) were partitioned into the aqueous phase, independent of the hydrophobicity and nature of the corresponding lipid phase.

Verification of lipid phase molarity influencing flavor partitioning. The influence of lipid phase molarity on the partitioning behavior of flavors was further validated using different alkane/water systems and octanol/water, the common reference system for the characterization of compound lipophilicity (14). Linear correlation coefficients for the alkane/water systems given in Figure 7 (average $R^2 = 0.98$) verified the dependence of volatile partitioning on the molarity of the lipophilic phase. Partition coefficients of furfuryl acetate, (*Z*)-3-hexenyl acetate, D-carvone, and 2-isobutylthiazole in the different alkane/water systems were in general significantly different (see Table 2; ANOVA and the LSD test, $P < 0.01$). Partitioning of ethyl 2-methylbutyrate was insignificant owing to the comparably larger variation within partition data and the corresponding SD (Table 2). Although octanol possessed a molarity between hexane and undecane, octanol/water partition coefficients deviated considerably from those of the alkane/water systems (Fig. 7, Table 2). Coefficients of all flavors were comparably higher, showing that the introduction of an alcoholic group into the hydrocarbons resulted in a stronger affinity of the flavors.

TABLE 2
Lipid/Water Partition Coefficients of Different Flavor Compounds[a]

	Furfuryl acetate	2-Isobutylthiazole	D-Carvone	Ethyl 2-methylbutyrate	(*Z*)-3-Hexenyl acetate
TAG					
Triacetin[b]	$56.2^{a} \pm 2.0$	$97^{a} \pm 0.8$	$131^{a} \pm 27.2$	$363^{a} \pm 173.1$	$359^{a,c} \pm 68.7$
Tributyrin[b]	$57.5^{a} \pm 10.6$	$188^{b} \pm 22.1$	$197^{b} \pm 14.7$	$753^{b} \pm 74.9$	$2225^{b} \pm 721.6$
Miglyol[b]	$24.8^{b} \pm 2.2$	$112^{a} \pm 3.7$	$161^{a,b} \pm 20.6$	$592^{a,b} \pm 138.3$	$1470^{a,b} \pm 439.7$
Butter oil[b]	$15.8^{b,c} \pm 1.5$	$50^{c} \pm 5.6$	$133^{a} \pm 20.9$	$189^{a,c} \pm 43.9$	$614^{a} \pm 18.6$
Trimyristin[c]	$5.8^{c} \pm 0.4$	$18^{d} \pm 1.8$	$19^{c} \pm 1.0$	$20^{c} \pm 4.0$	$46^{c} \pm 7.9$
Tripalmitin[c]	$6.3^{c} \pm 0.5$	$10^{d} \pm 0.2$	$10^{c} \pm 0.7$	$25^{c} \pm 1.6$	$12^{c} \pm 0.7$
Alkanes					
Hexane[b]	$13.2^{x} \pm 0.9$	$156^{x} \pm 4.0$	$125^{x} \pm 3.5$	$608^{x} \pm 256.5$	$578^{x} \pm 27.7$
Undecane[b]	$7.7^{y} \pm 0.2$	$86^{y} \pm 1.2$	$90^{y} \pm 0.6$	$437^{x} \pm 161.8$	$332^{y} \pm 24.6$
Hexadecane[b]	$6.0^{z} \pm 0.1$	$80^{y} \pm 1.6$	$78^{z} \pm 2.0$	$373^{x} \pm 53.9$	$267^{y} \pm 25.4$
Alcohol					
Octanol[b]	29.7 ± 4.7	867 ± 152.5	317 ± 6.0	1467 ± 462.2	978 ± 98.7
$K_{o/w}$[d]	12.0	324	1175	182	407
CV[e] (%)	7.5	5.6	7.5	23.6	13.4

[a]Values with different roman letters within a column are significantly different (ANOVA and LSD test at $P < 0.01$); TAG and alkanes were analyzed separately.
[b]Liquid at ambient temperature.
[c]Solid at ambient temperature.
[d]Calculated octanol/water partition coefficient (*cf.* Ref. 16).
[e]Average CV.

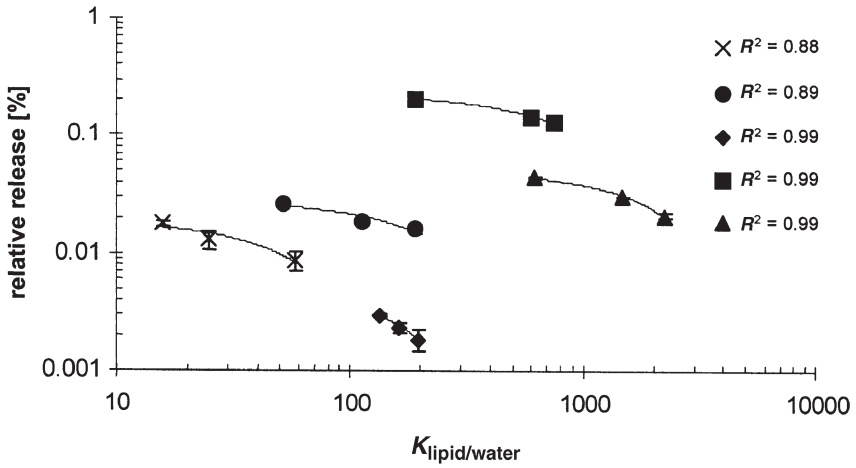

FIG. 5. Correlation between flavor release relative to the initial concentration (Table 1) and lipid/water partition coefficients (see Table 2). Tributyrin/water, Miglyol/water, and butter oil/water systems were compared. R^2 represents the linear regression coefficient. (×) Furfuryl acetate; (●) 2-isobutylthiazole; (◆) D-carvone; (■) ethyl 2-methylbutyrate; (▲) (*Z*)-3-hexenyl acetate. Error bars represent the SD of relative release determined in repeated experiments ($n \geq 3$).

In comparison with the partition coefficients of the TAG and water, the experimental octanol/water partition coefficient fit those of the low-melting lipids in the case of furfuryl acetate and (*Z*)-3-hexenyl acetate (Table 2). Considerable deviations were obtained for 2-isobutylthiazole, D-carvone, and ethyl 2-methylbutyrate. Interactions between the intramolecular functions of the TAG and the volatiles (24) that differed from those between flavors and alkanes or octanol, and different phase molarities could be responsible for these differences. A similar trend was found with the calculated octanol/water partition coefficients, which were generally smaller than those determined experimentally (Table 2). The theoretical coefficient of D-carvone was larger than the experimental coefficient and deviated even more from those of the lipid/water systems. However, by using computer software to calculate octanol/water partition coefficients, i.e., Solaris software version 4.67 [Advanced Chemistry Development (ACD)], that was different from the one used in the previous work (*cf.* Ref. 16), a log*P* value of 2.23 (that is, a partition coefficient of approximately 170) was calculated. This matched the experimental data of the lipid/water systems (low-melting lipids) fairly well. This shows that, despite the congruence of many calculated log*P* values of different flavor compounds, different mathematical approaches might produce erroneous predictions, and theoretical data have to be considered carefully. Owing to the numerous data available in the literature and the possibility of calculating octanol/water partition coefficients, log*P* values are essential for classifying flavors according to their partitioning behavior in lipid/water systems (*cf.*, for example, Refs. 6 and 13). However, the results presented here demonstrate that partition coefficients fail to describe quantitatively the liquid/liquid volatile partitioning in systems containing lipids of different molarity.

Influence of phase transition on flavor release. The phase transition that occurred in liquid trimyristin and tripalmitin in emulsions after falling below the crystallization temperature did not directly affect their initial dynamic flavor release (*cf.* Fig. 3). The partitioning and mass transfer of flavors in emulsions are considered to proceed extremely fast (25,26), presumably during the equilibration time period when the lipids are still melted. Once crystallization started, lipids entrapped and immobilized the volatiles partitioned in the lipid phase. In completely hardened lipid phases or in those with a solidified shell, mass transfer into or from the aqueous phase was extremely decelerated. Similarly, McNulty and Karel (27), Maier (15), and Roberts *et al.* (6) showed a decreasing uptake of added flavor upon increasing the solid fat indexes of the lipid phase used in emulsions; however, Carey *et al.* (2) reported no significant difference in the effects of tricaprylin (liquid at ambient temperature) and trilaurin (solid at ambient temperature) on flavor partitioning in emulsions. In the present case, dynamic flavor release would result in a depletion of the amount of flavor initially partitioned into the aqueous phase, along with a successive increase in the concentration gradient between the lipid phase and water without further equilibration. However, the present study analyzed initial dynamic flavor release, during which depletion should be negligible owing to the very low release rates of volatiles in comparison with the initial flavor concentrations (*cf.* also Ref. 17).

Mathematical modeling. Recently, a mathematical model predicting flavor release from oil/water emulsions was presented and experimentally validated (13). It considered Nerst's laws of partitioning and the flavor concentration initially partitioned into the aqueous phase to be rate limiting for the initial dynamic release process. Equation 1 represents this mathematical model:

$$c_{hs}(t) = \frac{c_O V_W}{V_W + V_O K_{O/W}} \left[1 - e^{-k(A/V_{hs})t} \right] \qquad [1]$$

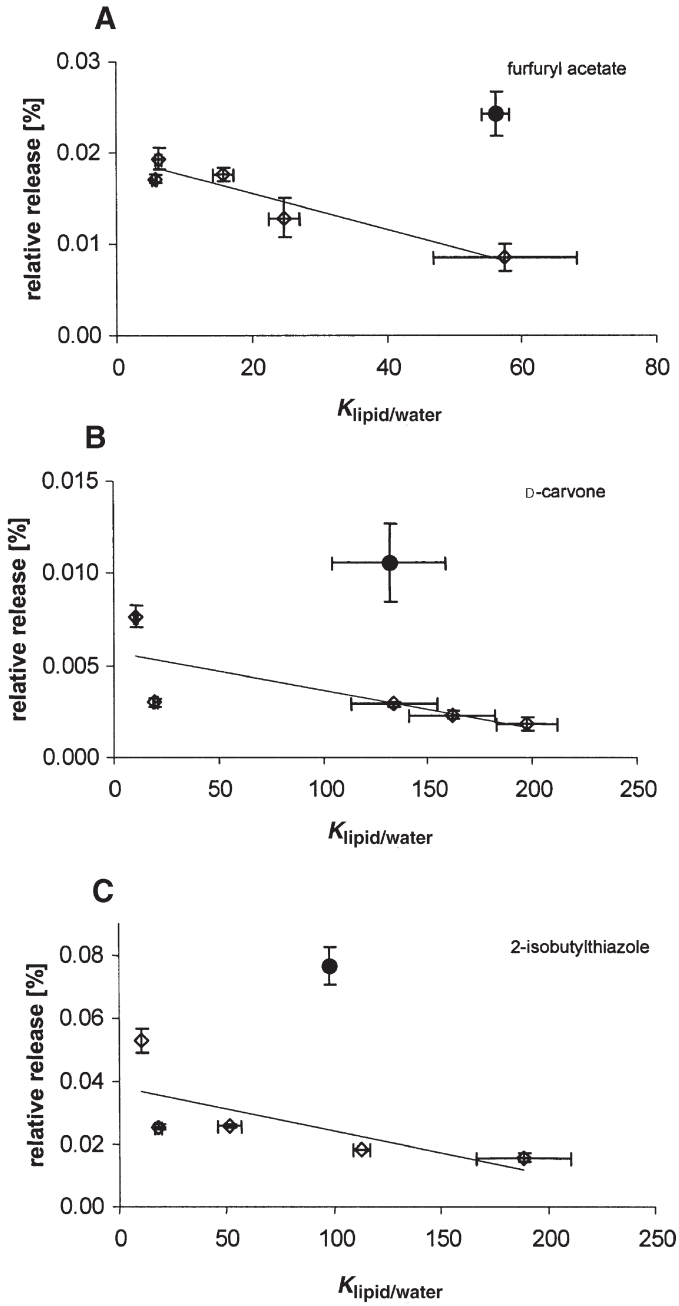

FIG. 6. Influence of lipid/water partition coefficients (see Table 2) on dynamic flavor release. Flavor release (within the 30 s) relative to the initial concentration is compared: (A) Furfuryl acetate; (B) D-carvone; (C) 2-isobutylthiazole. ● represents the system triacetin/water. Error bars represent the SD of relative release and lipid/water partition coefficients determined in repeated experiments ($n \geq 3$).

with $c_{hs}(t)$ being the released flavor concentration with time t, c_O the initial flavor concentration in the emulsion, V_W the volume of the water, V_O the volume of oil, $K_{O/W}$ the oil/water partition coefficient of flavors, k a specific mass transfer coefficient (16), A the interfacial area between the emulsion and the headspace, and V_{hs} the headspace volume.

Equation 1 is valid not only for low-melting lipids that are liquid at room temperature but also for high-melting lipids, provided their melted state continues a sufficient time during liquid/liquid flavor equilibration (*cf.* also explanations above). If the partitioning process occurs between water and a partially crystallized lipid phase, mass transfer into the solidified portions is not likely to occur (6,27). Consequently, depending on the preparation mode of, for example, ice cream, either the solidified fractions would need to be considered in the model equation, i.e., the corresponding volume must be subtracted

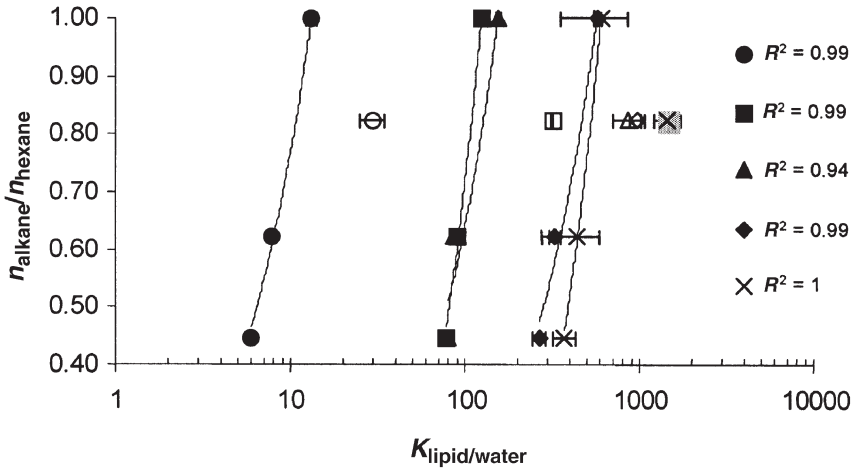

FIG. 7. Correlation between the partition coefficients of furfuryl acetate (●), D-carvone (■), 2-isobutylthiazole (▲), (*Z*)-3-hexenyl acetate (◆), and ethyl 2-methylbutyrate (×) in C_6, C_{11}, and C_{16} alkane/water systems and the corresponding alkane molarity expressed by the alkane molarity normalized to that of hexane. Open symbols and ⊠ represent the system octanol/water. R^2 characterizes a linear correlation. Error bars represent the SD of lipid/water partition coefficients determined in repated experiments ($n \geq 3$).

from V_O, or separate partition coefficients, measured at a solid fat index equal to that in the food product, would have to be used.

Figure 4 shows a linear correlation between lipid molarity and $K_{O/W}$. By having knowledge of the regression equation, including its slope, it is possible to calculate individual partition coefficients for lipids of different molarities, thus enabling initial dynamic flavor release to be predicted from corresponding oil/water emulsions with the help of Equation 1. This can be of interest for compounded foods, especially if one considers the commercial trend for low-calorie products comprising TAG esterified with short- and medium-chain FA (28).

The molarity (particle number) of a lipid phase significantly affected volatile partitioning and release of common flavor compounds (aliphatic esters, heterocycles, terpenols) in oil/water emulsions. Phase transition of lipids from the liquid to the solid state did not influence these processes as a consequence of the extremely fast initial liquid/liquid equilibration of the volatiles. In turn, phase transition from the solid to the liquid state, as would occur upon consumption of ice cream, should result in decreased flavor release owing to the dynamic transfer of flavors partitioned in the water into the liberated fractions of melted fat.

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On the Origin of *cis*-Vaccenic Acid Photodegradation Products in the Marine Environment

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ABSTRACT: The origin of 11-hydroxyoctadec-*trans*-12-enoic and 12-hydroxyoctadec-*trans*-10-enoic acids (photodegradation products of *cis*-vaccenic acid) in the marine environment was investigated. *cis*-Vaccenic acid is commonly used as a bacterial biomarker; however, in heterotrophic bacteria the observed rates of *cis*-vaccenic acid photodegradation are negligible. Here, two hypotheses explaining the source of the photoproducts were tested. According to the first hypothesis, the photoproducts originate from aerobic anoxygenic bacteria, i.e., photoheterotrophic organisms using bacteriochlorophyll-containing reaction centers. Alternatively, the photoproducts come from a heterotrophic bacterial community closely associated with senescent phytoplanktonic cells. *cis*-Vaccenic acid photodegradation was detected in both experimental setups. However, a detailed comparison of the *cis*-vaccenic acid photodegradation patterns with those observed in particulate matter samples of the DYFAMED station (Mediterranean Sea) suggests that photodegradation of heterotrophic bacteria attached to senescent phytoplanktonic cells constitutes the more likely source of *cis*-vaccenic acid oxidation products detected *in situ*.

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Photochemical damage in cells of phototrophic organisms is not exclusively caused by UV radiation (1). In fact, owing to the presence of chlorophylls, which are efficient photosensitizers (2), numerous lipid components of these organisms are being photodegraded during senescence by photosynthetically active radiation (3).

When a chlorophyll molecule absorbs a quantum of light energy, an excited singlet state (^{1}Chl) is formed. In healthy cells the absorbed energy is primarily utilized in photosynthetic reactions (2). However, a small fraction (less than 0.1%) of ^{1}Chl may undergo intersystem crossing to form a longer-living triplet state, ^{3}Chl (4). ^{3}Chl itself not only is potentially damaging in type I (i.e., involving radicals) reactions (4), but also may react with ground-state oxygen ($^3O_2$) to generate reactive oxygen species ($^1O_2$, $HO^\bullet$, H_2O_2, $O_2^{\bullet-}$). The toxic effects of ^{3}Chl and oxygen species in chloroplasts are alleviated by endogenous quenchers or scavengers (carotenoids, tocopherols, ascorbic acid, superoxide dismutase enzyme) (2,5), which constitute the photoprotective system of phototrophic organisms.

In senescent or dead photosynthetic organisms photosynthesis is nonfunctional, which accelerates ^{3}Chl formation (1). In such a situation the formation of ^{3}Chl and toxic oxygen species may exceed the quenching capacity of the photoprotective system, resulting in photodamage (3,6). In phytodetritus, where the organized structure of the thylakoid membranes has been disrupted, pigments tend to associate with other hydrophobic cellular components such as membrane lipids (1). There, the photooxidative effect of chlorophyll sensitization may be strongly amplified because of the longer $^1O_2$ lifetime in such hydrophobic microenvironments compared to water (7). For this reason $^1O_2$ may play an important role in the photodegradation of lipids (3).

Unsaturated FA can be photodegraded quickly in senescent or dead cells of phytoplankton (8), cyanobacteria (Rontani, J.F., unpublished data), and purple sulfur bacteria (9). The detection of significant amounts of photooxidation products of oleic and palmitoleic acids (originating from phytoplankton and/or cyanobacteria) in particulate matter and in recent sediment samples collected in the Mediterranean Sea (10,11) confirmed these laboratory observations. Surprisingly, in these samples significant amounts of photoproducts of *cis*-vaccenic acid also were detected. *cis*-Vaccenic acid is produced by many species of heterotrophic bacteria (12). It also occurs in other marine organisms such as phytoplankton (13,14) but is far less abundant in them than in bacteria (15). Thus, *cis*-vaccenic acid is generally considered as a bacterial biomarker. The origin of these photoproducts is enigmatic since FA photodegradation rates in heterotrophs are expected to be very low due to the lack of chlorophyll photosensitizer.

These puzzling observations could be explained by the presence of aerobic anoxygenic phototrophs (AAP) in the areas investigated. These organisms are a relatively recently discovered bacterial group (16). Kolber *et al.* (17) demonstrated that anoxygenic phototrophy is widespread in the open ocean. AAP are taxonomically classified into two major marine genera, *Erythrobacter* and *Roseobacter* (18,19). They perform photoheterotrophic metabolism, requiring organic carbon for growth, but they can use photosynthesis as an auxiliary source of energy (20). Another explanation for the presence of *cis*-vaccenic

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Abbreviations: AAP, aerobic anoxygenic phototrophs; Bchl *a*, bacteriochlorophyll *a*; BSTFA, bis(trimethylsilyl)trifluoroacetamide; ^{1}Chl, excited chlorophyll, singlet state; ^{3}Chl, excited chlorophyll, triplet state; DMDS, dimethyldisulfide; $^1O_2$, singlet state oxygen; $^3O_2$, ground state oxygen; SIM, selected ion monitoring.

acid photoproducts in the sediments could be the induction of photoprocesses in attached heterotrophic bacteria during the senescence of phytoplankton. It is well known that bacteria colonize marine aggregates and notably phytoplankton-derived particles (21,22). Such an aggregate constitutes a hydrophobic microenvironment where the lifetime and the potential diffusive distance of $^1O_2$ could be long enough to induce type II (i.e., involving $^1O_2$) photoprocesses.

The aim of the present work was to test these two hypotheses. Hence, we studied the photooxidation of the lipid components of two strains of aerobic photoheterotrophic bacteria, *Erythrobacter* sp. strain NAP1 and *Roseobacter*-related isolate COL2P, and we compared the results obtained after irradiation of axenic and nonaxenic cultures of the diatom *Skeletonema costatum*.

EXPERIMENTAL PROCEDURES

Axenic *S. costatum* strain CS-181 was obtained from the CSIRO (Australia) collection of microalgae. The heterotrophic bacterial community used for contamination experiments was isolated from marine sediments on acetate according to a previously described procedure (23). *Erythrobacter* sp. strain NAP1 was isolated from the North Atlantic Ocean (24) and COL2P from the French Mediterranean coast (Koblížek, M., and Kolber, Z.S., unpublished results).

S. costatum was grown in f/2 medium (25) at 20°C using two 30-W fluorescent lamps (Osram fluora) and a 12 h light/12 h dark cycle. The basic growth medium used for the culture of heterotrophic bacteria consisted of autoclaved artificial seawater (26) supplemented with $FeSO_4$ (0.1 mM), K_2HPO_4 (0.33 mM), NH_4Cl (55 mM), and sodium acetate (24 mM) as the source of carbon and energy. Cultures were maintained in 250-mL Erlenmeyer flasks containing 50 mL of medium and agitated at 20°C on a reciprocal shaker at 96 rpm. *Erythrobacter* sp. strain NAP1 and strain COL2P were cultivated on organic medium as described previously (24). After growth, some cultures of *S. costatum* were contaminated with a 1-mL suspension of heterotrophic bacteria and incubated for 1 d prior to the experiments.

Scanning electron microscopy. Cells were fixed with 1% glutaraldehyde in the culture medium for 2 h and filtered on a 0.2 μm Nucleopore filter. They were then post-fixed on the filter in 2% osmium tetroxide and dehydrated in ethanol. After critical-point drying, they were sputter-coated with gold-palladium and observed with a Hitachi S570 electron microscope.

Photodegradation experiments. The grown cultures were distributed into 250-mL Erlenmeyer flasks containing 1 mL of a saturated solution of mercuric chloride (to induce senescence). The stirred cells were then exposed to light at 20°C. The light was provided by two 30-W fluorescent lamps (Osram fluora) in the case of phytoplanktonic cultures and a mix of 30-W fluorescent and 60-W tungsten lamps in the case of aerobic photoheterotrophic bacteria. This mix provided a spectral distribution of light between 380 and 780 nm, well suited for photobiological processes involving bacteriochlorophyll *a*. (Bchl *a*). Irradiance (as photosynthetically active radiation) was measured using the Licor LI 1000 data logger equipped with the LI 1935A spherical quantum sensor. Dark controls were carried out in parallel.

Particulate matter sampling. The DYFAMED station is located in the Ligurian Sea (northwestern Mediterranean Sea, 43°25′ N, 7°52′ E). Particulate matter was collected using the automated sediment traps (type PPS5; Technicap, La Turbie, France) at programmed intervals with a 7-d resolution. The traps were deployed at depths of 200 and 1000 m. To avoid bacterial decomposition, the sample cups were filled with filtered (0.2 μm) seawater containing 5% formaldehyde and 1.1 g $Na_2B_4O_7$ L^{-1}. After elimination of swimmers (zooplankters), sediment trap samples were homogenized and lyophilized.

Treatment. The samples (flasks incubated or particulate matter) were saponified directly or after reduction.

Reduction of hydroperoxides to alcohols. After addition of methanol (50 mL), hydroperoxides were reduced to the corresponding alcohols by excess $NaBH_4$ or $NaBD_4$ (20 mg) and stirred for 15 min at 0°C (27). During this treatment ketones were also reduced, and the possibility of some ester cleavage cannot be totally excluded.

Alkaline hydrolysis. After reduction, 2.8 g of KOH and 50 mL of water were added, and the mixture was directly saponified by refluxing for 2 h. An additional 50 mL methanol was added before saponification of nonreduced samples. The mixtures were then acidified with hydrochloric acid (pH 1) after cooling and were subsequently extracted three times with dichloromethane. The combined dichloromethane extracts were dried over anhydrous Na_2SO_4, filtered, and concentrated to give the total lipid fraction.

Derivatization. After solvent evaporation, the residues were taken up in 300 μL of a mixture of pyridine and bis(trimethylsilyl)trifluoroacetamide (BSTFA; Supelco, St Quentin Fallavier, France) (2:1, vol/vol) and silylated for 1 h at 50°C. After evaporation to dryness under nitrogen, the residues were taken up in a mixture of ethyl acetate and BSTFA and analyzed by GC/EIMS.

Determination of double bond positions [dimethyldisulfide (DMDS) treatment]. Following the procedure of Vincenti *et al.* (28), the extract was dissolved in 250 μL of hexane, 250 μL of DMDS, and 125 μL of an iodine solution (60 mg of iodine in 1 mL of diethyl ether). The reaction mixture was held at 50°C for 24 h and diluted with hexane. The reaction was quenched with 2 mL of an aqueous solution of $Na_2S_2O_3$ (5%), and the hexane layer was pipetted off. The solution was extracted twice with hexane, the hexane extracts were combined, dried on Na_2SO_4, and filtered, and the solvent was evaporated. The residue was then silylated as described above and analyzed by GC/EIMS.

Identification of FA and their oxidation products. Components were identified by comparison of retention times and mass spectra with those of standards and quantified (calibration with external standards) by GC/EIMS. For low concentrations or in the case of coelution, quantification was assessed by selected ion monitoring (SIM). Coeluting derivatives of regioisomeric hydroxyacids usually can be distinguished by their EI mass spectra, since the main fragmentation is the α-cleavage relative to the functional group. Thus, the isomers can be detected and quanti-

fied from the ion currents of the specific fragment ions. Characterization of *cis* and *trans* allylic hydroxyacids was based on comparison of their retention times with those of standard compounds.

GC/EIMS analyses were carried out with a Hewlett-Packard HP 5890 series II plus gas chromatograph connected to an HP 5972 mass spectrometer. The following conditions were used: 30 m × 0.25 mm (i.d.) fused-silica column coated with Solgel-1 (SGE, Courtaboeuf, France; film thickness, 0.25 μm); oven temperature programmed from 60 to 130°C at 30°C min^{-1} and then from 130 to 300°C at 4°C min^{-1}; carrier gas (He) kept at 1.04 bar until the end of the temperature program and then programmed to 1.5 bar at rate of 0.04 bar min^{-1}; injector (on column) temperature, 50°C; electron energy, 70 eV; source temperature, 170°C; cycle time, 1.5 s.

Standards. Palmitoleic and *cis*- and *trans*-vaccenic acids were purchased from Sigma. Hydroperoxides were produced after photosensitized oxidation [in pyridine in the presence of hematoporphyrin as sensitizer (29)] or Fe^{2+}/ascorbate-induced autooxidation (30) of the corresponding FA. The hydroxyacids were obtained by reduction of corresponding hydroperoxides in methanol with excess $NaBH_4$.

RESULTS AND DISCUSSION

Photodegradation in dead cells of AAP. The total lipid fraction obtained after alkaline hydrolysis of living cells of *Erythrobacter* sp. strain NAP1 contained very small amounts of phytol arising from the hydrolysis of Bchl *a* (Table 1). The FA composition of this strain had been determined previously (24). It is characterized by the dominance of 18:1n-7 acid and the presence of significant amounts of PUFA (notably of 6,9-octadecadienoic acid). It is interesting to note that small amounts of 2-hydroxyacids ranging from C_{14} to C_{16} were also present (Table 1). Comparison with authentic standards proved that the 18:1n-7 component was *cis*-vaccenic acid.

To induce photodegradation, the dead NAP1 cells were exposed to visible light. We failed to detect significant amounts of 6,10,14-trimethylpentadecan-2-one and 3-methylidene-7,11,15-trimethylhexadecan-1,2-diol, the typical photoproducts of phytol (31), in irradiated cells of NAP1. The proportion of 2-hydroxyacids increased significantly during irradiation (Table 1). A group of unresolved peaks corresponding to isomeric allylic monohydroxyoctadecenoic acids also was detected after the treatment (Table 1). These compounds, which were absent in dark controls, have been characterized by GC/EIMS on the basis of their retention times and mass spectra (cleavage at the carbon bearing the $–OSiMe_3$ group) (10). This group of hydroxyacids, arising from the oxidation of *cis*-vaccenic acid, was composed mainly of 11-hydroxyoctadec-*trans*-12-enoic, 12-hydroxyoctadec-*trans*-10-enoic, 13-hydroxy-octadec-*trans*-11-enoic, 13-hydroxyoctadec-*cis*-11-enoic, 10-hydroxy-octadec-*trans*-11-enoic and 10-hydroxyoctadec-*cis*-11-enoic acids (Fig. 1A). The amounts of allylic hydroxyacids increased considerably when

TABLE 1
Lipid Composition (% total) of *Erythrobacter* sp. Strain NAP1 and Strain COL2P Before and After Irradiation

	Erythrobacter sp. strain NAP1		COL2P	
	Healthy cells	Irradiated killed cells[a]	Healthy cells	Irradiated killed cells[b]
Dodecanoic acid (lauric acid)	0.2	0.1	0.8	2.8
Tridecanoic acid	Tr[c]	0.1	Tr	Tr
3-Hydroxydodecanoic acid	ND[d]	ND	3.1	1.7
Tetradecanoic acid (myristic acid)	1.4	2.4	1.3	2.7
1,10-Decanedioic acid (sebacic acid)	ND	0.5	0.1	1.1
Pentadecanoic acid	1.0	1.7	0.8	1.8
1,11-Undecanedioic acid	ND	0.3	Tr	1.0
2-Hydroxytetradecanoic acid	Tr	9.2	ND	ND
Hexadec-*cis*-9-enoic acid	2.5	1.8	2.8	5.0
Hexadecanoic acid (palmitic acid)	8.2	9.6	15.5	20.4
2-Hydroxypentadecanoic acid	Tr	6.1	Tr	Tr
Heptadecenoic acids[e]	5.3	5.1	0.4	1.4
Heptadecanoic acid (margaric acid)	1.4	1.8	0.6	1.0
Phytol	0.6	0.1	0.5	0.2
Octadeca-6,9-dienoic acid	12.0	4.5	ND	ND
Octadeca-9,12-dienoic acid (linoleic acid)	0.9	1.9	1.1	1.4
Octadec-*cis*-9-enoic acid (oleic acid)	1.8	1.8	2.1	3.9
Octadec-*cis*-11-enoic acid (*cis*-vaccenic acid)	60.4	35.4	58.0	25.1
2-Hydroxyhexadecanoic acid	Tr	0.9	4.0	3.2
Octadecanoic acid (stearic acid)	2.3	4.4	5.5	6.5
Isomeric allylic hydroxyoctadecenoic acids[e]	ND	9.6	ND	15.8
11,12-Epoxyoctadecanoic acid[f]	ND	1.2	ND	0.7
11,12-*cis*-Methyleneoctadecanoic acid	2.0	1.5	3.4	4.3

[a]302 moles of photons per square meter.
[b]85 moles of photons per square meter.
[c]Traces (<0.1%).
[d]ND, not detected.
[e]Mixtures of isomers.
[f]Estimated by taking into account amounts of the main degradation products of epoxides formed during the treatment (10).

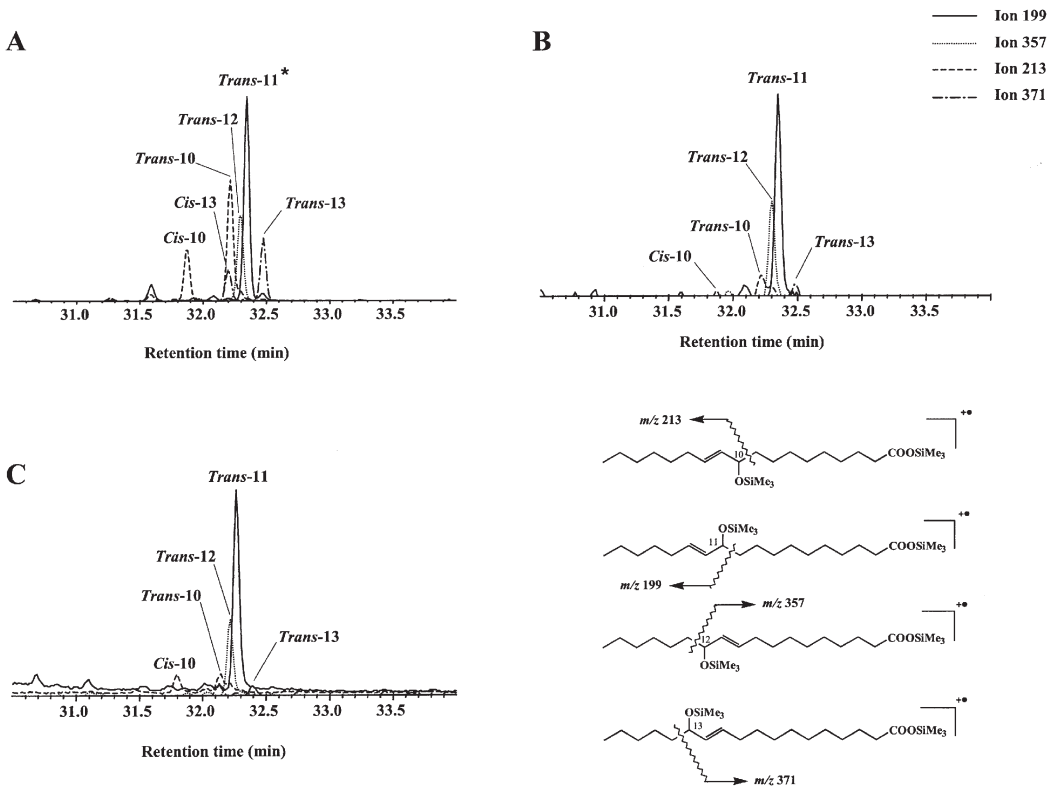

FIG. 1. Mass chromatograms of *m/z* 199, 357, 213, and 371 revealing the presence of hydroxyacids deriving from octadec-11-enoic acid. Isomeric hydroxy acids in silylated $NaBH_4$-reduced lipid extracts of (A) *Erythrobacter* sp. strain NAP1 exposed to 302 mol of photons·m^{-2}, (B) heterotrophic bacteria exposed to 85 mol of photons·m^{-2} in the presence of dead cells of *Skeletonema costatum* strain CS-181, and (C) a particulate matter sample collected between August 3 and 10, 1998, at the DYFAMED station (1000 m depth). The asterisk (*) indicates that the numbers refer to the oxygenated carbon atoms and not to the olefinic centers whose configuration is indicated.

the samples were reduced with $NaBH_4$ before the alkaline hydrolysis. Some attempts at reduction with $NaBD_4$ instead of $NaBH_4$ were carried out to determine whether this additional production of hydroxyacids results from the reduction of the corresponding hydroperoxides or ketoacids. The results demonstrated that, before the treatment, *cis*-vaccenic acid oxidation products constituted 95% of the hydroperoxyacids and 5% of the hydroxyacids. Ketoacids, which were present in a relatively high proportion (30–35%) after photodegradation of dead cells of purple sulfur bacteria (9), could not be detected in the lipid extract of *Erythrobacter* sp. strain NAP1. Also, small amounts of α,ω-dicarboxylic acids were detected after irradiation (Table 1).

Similarly to *Erythrobacter* sp. strain NAP1, the lipid composition of strain COL2P is dominated by *cis*-vaccenic acid and contains only small amounts of phytol (Table 1). Isomeric allylic monohydroxyoctadecenoic acids resulting from the oxidation of *cis*-vaccenic acid also have been detected in significant amounts after irradiation of dead cells of this strain, whereas photoproducts of phytol were absent (Table 1).

Photodegradation of heterotrophic bacteria attached to phytoplanktonic cells. To test whether photodegradation processes could be induced in attached heterotrophic bacteria during the senescence of phytoplanktonic cells, we irradiated in parallel (i) an axenic culture of *S. costatum* strain CS-181, (ii) a culture of the same diatom contaminated with a heterotrophic bacterial community, and (iii) the bacterial community alone that was used for the contamination.

The FA distribution of *S. costatum* strain CS-181 showed a high concentration of *cis*-16:1n-7 (palmitoleic acid). The absence of *cis*-vaccenic acid (Fig. 2A) confirmed that this strain is truly axenic. In contrast, the main unsaturated FA of the heterotrophic bacterial community were palmitoleic, *trans*-16:1n-7, and *cis*-vaccenic acids (Fig. 2B). Irradiation of dead cells of axenic *S. costatum* strain CS-181 by visible light resulted in the quick photodegradation of its unsaturated FA components, which is consistent with earlier observations in other phytoplanktonic strains (8,31). This degradation produced high amounts of photoproducts of palmitoleic acid (Fig. 2A). After irradiation of the same culture contaminated with heterotrophic bacteria, we detected additional photoproducts of *cis*-vaccenic acid (Fig. 2C). These photoproducts were produced in significant amounts in the presence of *S. costatum* strain CS-181, whereas they were present only in trace amounts after irradiation of the heterotrophic bacterial community alone (Fig. 2B). The increasing proportion of *trans*-16:1n-7 acid observed after irradiation of contaminated *S. costatum* strain CS-181 (Fig. 2C) can be attributed to the fact that the *trans* configuration is known to be sevenfold less reactive toward $^1O_2$ than the *cis* geometry (32).

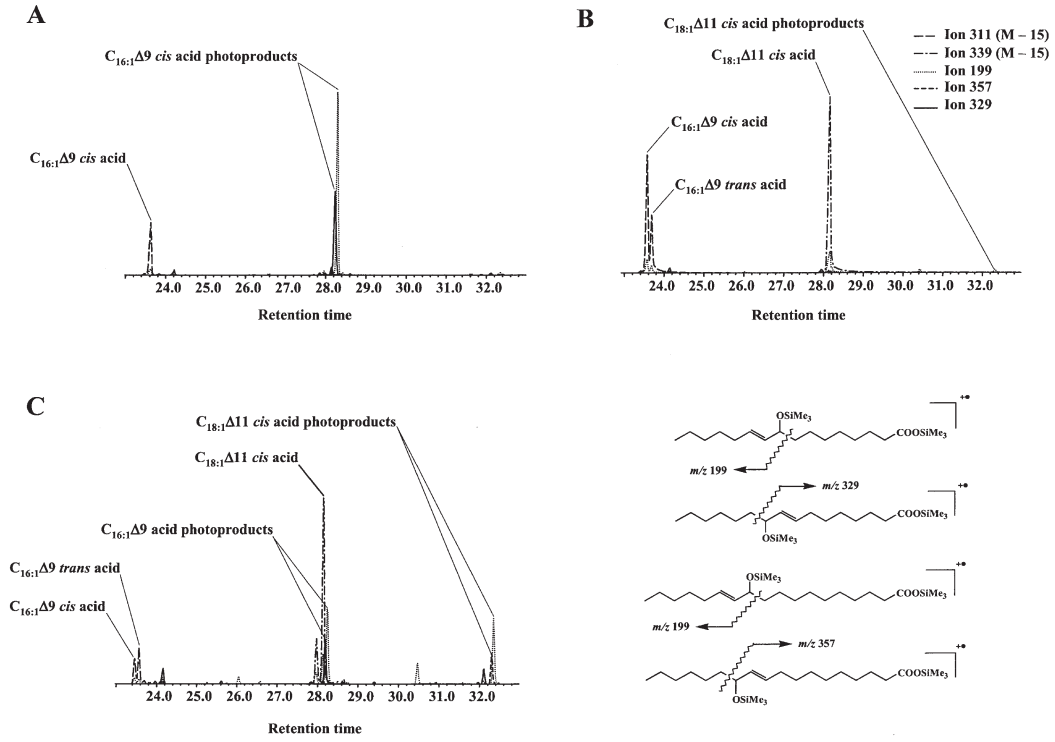

FIG. 2. Mass chromatograms of *m/z* 311, 339, 199, 357, and 329 revealing the presence of hexadec-9-enoic and octadec-11-enoic acids and their oxidation products in silylated $NaBH_4$-reduced total lipid extracts of irradiated (85 mol of photons·m^{-2}): (A) dead cells of axenic *Skeletonema costatum* strain CS-181, (B) heterotrophic bacterial community, and (C) dead cells of *S. costatum* strain CS-181 contaminated with the heterotrophic bacterial community.

Origin of oxidation products of cis*-vaccenic acid detected* in situ. We compared the profiles of oxidation products of *cis*-vaccenic acid obtained after photodegradation of aerobic anoxygenic phototrophs (Fig 1A) and heterotrophic bacteria attached to phytoplanktonic cells (Fig. 1B) with those observed in particulate matter samples of the DYFAMED station (Mediterranean Sea) (Fig. 1C). As in the case of attached heterotrophic bacteria, the compounds detected *in situ* mainly resulted from $^1O_2$-mediated photoprocesses (presence of a high proportion of 11-hydroxyoctadec-*trans*-12-enoic and 12-hydroxyoctadec-*trans*-10-enoic acids) (Fig. 1C). Radical autooxidation products (*cis*-hydroxyacids), which were produced in significant amounts during visible light irradiation of aerobic photoheterotrophic bacteria (Fig. 1A), were present only in trace amounts in the DYFAMED samples (Fig. 1C).

The low amounts of phytol detected in AAP are consistent with the fact that these organisms contain significantly lower amounts of Bchl *a* than anaerobic photosynthetic bacteria (18). Although sensitive to photochemical processes in senescent phytoplankton (31) and purple sulfur bacteria (9), this isoprenoid alcohol is not significantly photodegraded in senescent cells of AAP.

2-Hydroxy and 3-hydroxy FA are often present in polar lipids of many bacteria and notably of some *Erythrobacter* spp. (33). The increasing amounts of 2-hydroxyacids also observed in dark controls suggest that these compounds result from an enzymatic release of polar lipids (probably of sphingolipids) during the senescence of *Erythrobacter* sp. strain NAP1 and not from photochemical processes.

Type II-photosensitized oxidation of monounsaturated FA involves a direct reaction of $^1O_2$ with the carbon-carbon double bond by a concerted "ene" addition (34) and leads to the formation of hydroperoxides at each unsaturated carbon. Thus, *cis*-vaccenic acid produces a mixture of 11- and 12-hydroperoxides with an allylic *trans* double bond (10) (Fig. 3). These two hydroperoxides may undergo highly stereoselective radical allylic rearrangement to 13-*trans* and 10-*trans* hydroperoxides, respectively (Fig. 3) (35).

Radical autooxidation plays a role in destructive biological processes (35) including a significant role during natural senescence of phytoplanktonic cells (36). In case of *cis*-vaccenic acid, these processes yield mainly a mixture of 10-*trans*, 10-*cis*, 11-*trans*, 12-*trans*, 13-*trans,* and 13-*cis* hydroperoxides, with minor amounts of 11-*cis* and 12-*cis* hydroperoxides (Fig. 3) (10). Clearly, a mixture of photooxidation and autooxidation products is present in irradiated AAP. Autoxidative processes can be easily discriminated by the presence of *cis* allylic hydroxyacids, which are specific products of these degradation processes (37) (Fig. 3). The involvement of autoxidative processes in senescent cells of AAP is also confirmed by the presence of several compounds (saturated hydroxyoctadecanoic acids, 11,12-dihydroxyoctadecanoic acid, chlorohydrins,

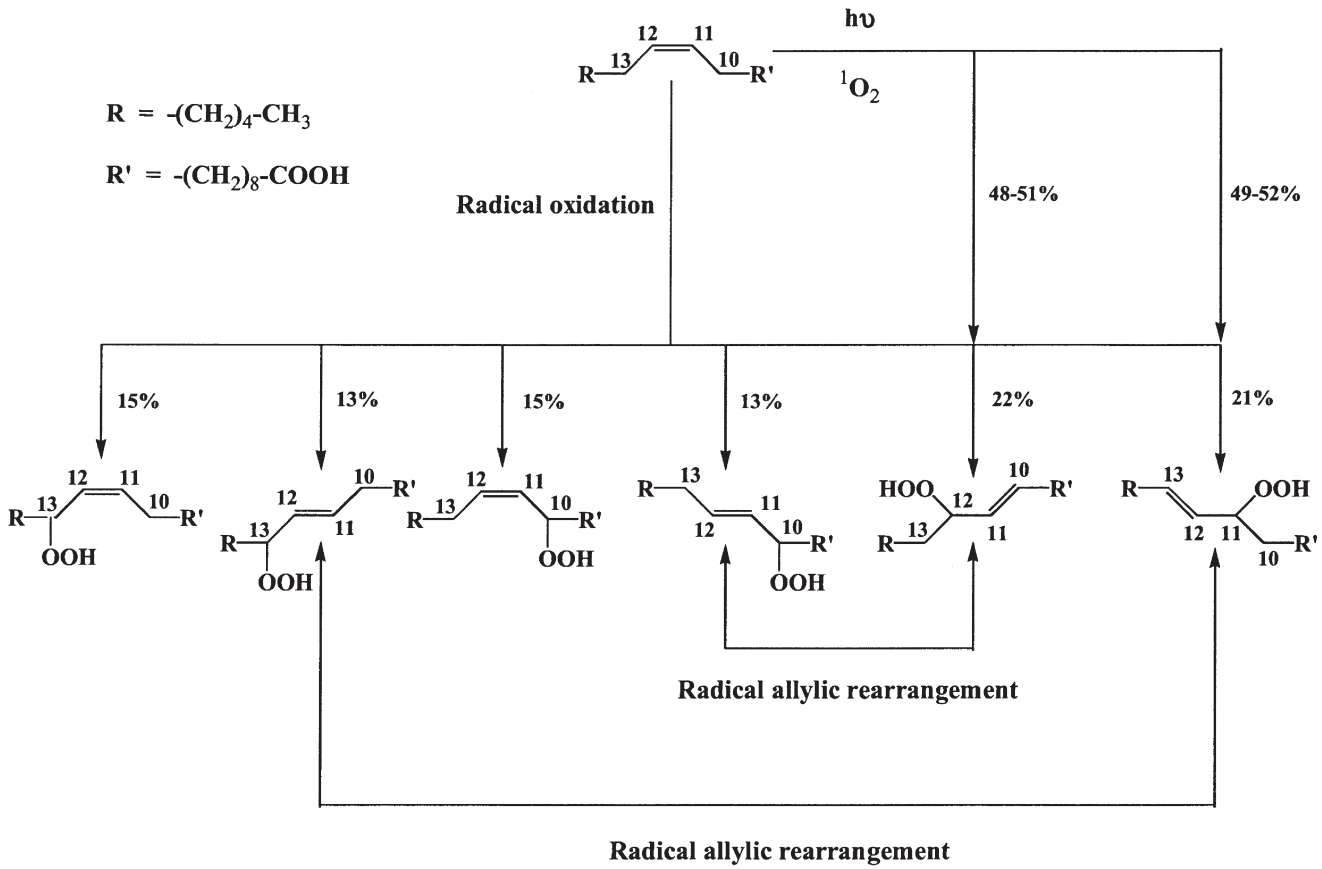

FIG. 3. Hydroperoxides from autoxidation and $^1O_2$-mediated photoprocesses of octadec-11-enoic acid. *Cis*-11- and -12-hydroperoxides (which represent <1% of autoxidation products) have been omitted from this representation to simplify the scheme.

and methoxyhydrins) deriving from the degradation of 11,12-epoxyoctadecanoic acid during the treatment (Table 1) (10). The formation of ω-dicarboxylic acids can be attributed to proton-catalyzed cleavage of allylic hydroperoxyoctadecanoic acids (34) and subsequent oxidation of the ω-oxocarboxylic acids thus formed (10).

Photodegradation of lipids in phytodetritus usually follows first-order kinetics (1,3) and can be described by the equation $\ln(C/C_o) = -KD$ (where C is the concentration at the time of sampling, C_o is the initial concentration, K is the rate constant, and D is the light dose). This is also the case for the photodegradation of *cis*-vaccenic acid in aerobic anoxygenic phototrophs. The photodegradation appeared to be faster in the COL2P strain than in NAP1 (Table 2), probably owing to the higher concentration of polar carotenoids in NAP1 (24). These carotenoids, which are present in all members of the *Erythrobacter* genus (16,24,38), are uncoupled from photosynthetic reaction centers. Such secondary carotenoids may act as inhibitors of photodynamically induced damage in algae (39); our results seem to indicate that nonphotosynthetic carotenoids in cells of aerobic phototrophic bacteria may provide similar protection.

Comparison of calculated first-order rate constants clearly shows that the photodegradation of *cis*-vaccenic acid of heterotrophic bacteria is more than two orders of magnitude faster in the presence of phytoplanktonic cells (Table 2). It is also interesting to note that *cis*-vaccenic acid is photodegraded more quickly in heterotrophic bacteria associated with phytoplankton than in AAP (Table 2). The group of hydroxyacids, arising from the oxidation of *cis*-vaccenic acid FA of absorbed heterotrophic bacteria is mainly composed of 11-hydroxyoctadec-*trans*-12-enoic and 12-hydroxyoctadec-*trans*-10-enoic acids (Fig. 1B). *cis*-Hydroxyacids, which are characteristic of radical autoxidative processes (37) (Fig. 3), are present only in trace amounts. This suggests that photodegradation of this acid involves mainly $^1O_2$-mediated processes. The transfer of $^1O_2$ to bacteria is made possible by close contact between phytoplanktonic cells and bacteria (Fig. 4).

The average lifetime of $^1O_2$ in the cell was estimated to be 10–40 ns, which is <1% of its lifetime in pure water (40). This corresponds to an $^1O_2$ diffusion distance of less then 10 nm, which is less than the thickness of a cell membrane (41). This suggests that $^1O_2$ produced in the cells would have little chance of escaping. However, our results show that this is not the case in phytodetritus. Here, where the $^1O_2$ formation rate probably exceeds the quenching capacity of the photoprotective system, $^1O_2$ migrates outside the chloroplasts and affects the attached bacteria.

TABLE 2
Calculated First-Order Rate Constants for Visible Light-Induced Degradation of Monounsaturated FA at 20°C

Experiment	FA	K (mol of photons^{-1} m^2)
Pyridine/hematoporphyrin[a]	*cis*-Vaccenic acid	3.0×10^{-2}
Pyridine/hematoporphyrin[a]	Palmitoleic acid	3.1×10^{-2}
Killed cells of *Erythrobacter* sp. strain NAP1[b]	*cis*-Vaccenic acid	2.5×10^{-3}
Killed cells of COL2P[b]	*cis*-Vaccenic acid	4.8×10^{-3}
Killed cells of *Skeletonema costatum* (axenic)[b]	Palmitoleic acid	2.5×10^{-2}
Killed cells of (*S. costatum* + heterotrophic bacteria)[b]	*cis*-Vaccenic acid	6.0×10^{-3}
Killed cells of heterotrophic bacteria[b]	*cis*-Vaccenic acid	1.5×10^{-5}

[a]Photodegradation experiments carried out in pyridine and in the presence of hematoporphyrin as $^1O_2$-mediated sensitizer so as to compare the photoreactivity of *cis*-vaccenic and palmitoleic acids.
[b]Photodegradation experiments carried out on killed cells of organisms (aerobic anoxygenic bacteria, phytoplankton and heterotrophic bacteria).

Comparison of the photooxidation pattern of the *cis*-vaccenic acid oxidation products with those observed in particulate matter samples of the DYFAMED station allowed us to conclude that photodegradation of heterotrophic bacteria attached to senescent phytoplanktonic cells constitutes the likely source of oxidation products of *cis*-vaccenic acid detected *in situ*. This conclusion is well supported by the fact that attached bacteria are more likely to sink and become part of the sediment than free-living AAP. This process appears to act intensively in the euphotic layer of the oceans since the amounts of oxidation products detected may constitute up to 30% of the parent *cis*-vaccenic acid (10,11). Photodegradation of attached heterotrophic bacteria also must contribute to the formation of palmitoleic acid photoproducts in the marine environment (10); however, photodegradation of phytoplankton and cyanobacteria constitutes additional sources of these oxidation products.

A large fraction of the particulate matter present in marine systems consists of macroscopic aggregates known as "marine snow." These flocculent aggregates are typically composed of senescent phytoplankton colonized by heterotrophic bacteria (22). It is well established that bacteria cause rapid solubilization and remineralization of the aggregates (42,43). However, the role of phytodetritus in degradation of attached bacteria was totally ignored. Our results show that phytodetritus may induce $^1O_2$-mediated photoprocesses in attached heterotrophic bacteria and thus contribute to the degradation of these organisms in the euphotic layer of oceans.

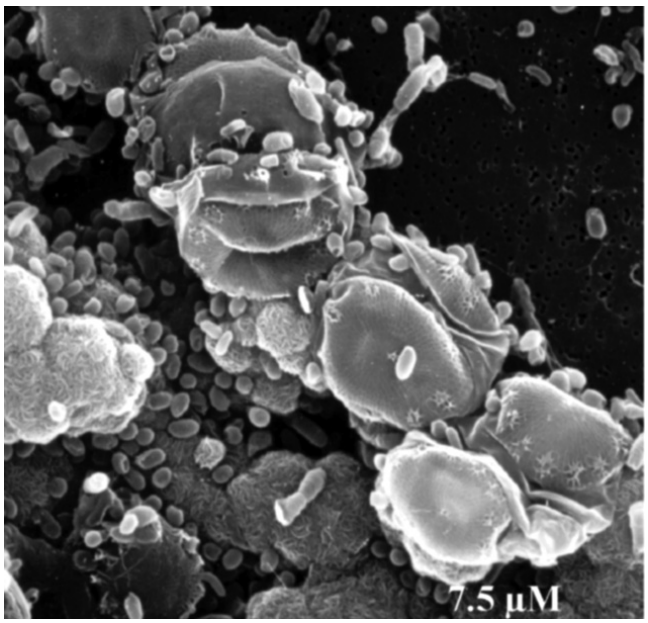

FIG. 4. Scanning electron microscopy image of *Skeletonema costatum* strain CS-181 contaminated with heterotrophic bacteria.

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Fatty Acids of Lipid Fractions in Extracellular Polymeric Substances of Activated Sludge Flocs

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ABSTRACT: Phospholipid (PL), glycolipid (GL), and neutral lipid (NL) FA, and the lipopolysaccharide 2- and 3-hydroxy (LPS 2-OH and 3-OH) FA of activated sludges and extracted extracellular polymeric substances (EPS) were determined on samples collected from two wastewater treatment plants. EPS extracted from sludges by means of sonication and cation exchange contained proteins (43.4%), humic-like substances (11.5%), nucleic acids (10.9%), carbohydrates (9.9%), and lipid-bound FA (1.8%). The lipids associated with EPS were composed of GL, PL, NL, and LPS acids in proportions of 61, 21, 16, and 2%, respectively. The profiles of lipid-bound FA in activated sludges and EPS were similar (around 85 separate FA were identified). The FA signatures observed can be attributed to the likely presence of yeasts, fungi, sulfate-reducing bacteria, gram-positive and gram-negative bacteria, and, in lesser quantities, mycobacteria. Comparison of data from the dates of sampling (January and September) showed that there were more unsaturated PLFA in the EPS extracted from the activated sludges sampled in January. This observation could be partly related to microorganism adaptation to temperature variations. The comparison between two wastewater treatment plants showed that the FA profiles were similar, although differences in microbial community structure were also seen. Most of the FA in sludges had an even number of carbons.

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The activated sludge process, in which microorganisms are aerobically grown in a flocculated form, is a biological process that is widely used in urban wastewater treatment plants. These activated sludge flocs contain a diverse community of microorganisms (1), which are embedded in a matrix of extracellular polymeric substances (EPS). EPS are typically made up of large amounts of proteins, humic substances, carbohydrates, lipids, and nucleic acids (2–5). Some of these polymers can be organized into heteropolymers such as glycoproteins (6).

EPS are involved in the architecture of the aggregates, and they contribute to the physicochemical properties of activated sludge flocs (7). For instance, the cohesiveness of activated sludge aggregates is due to the electrostatic interactions that exist between the multivalent cations (Ca^{2+}, Mg^{2+}) and negatively charged EPS (8–10) as well as to hydrophobic interactions (6,11). Hydrophobic domains represent sorption sites for hydrophobic and amphiphilic organic compounds. The capacity and the importance of these hydrophobic domains have been estimated using techniques that determine (i) the variation in the fluorescence spectrum of the pyrene trapped in the activated sludge (12); (ii) the accumulation of organic molecules in EPS such as pyrene, benzene, toluene (13,14), fluoranthene, phenanthrene (15), and alkylbenzene sulfonates (16); and (iii) the efficiency of capture of hydrophobic bacteria (17). Lipids may play an important role in the hydrophobic properties of activated sludge EPS. These properties have been reported by Jorand *et al.* (6,18).

Lipid profiles could be correlated to the microbial ecology of sludge. For instance, the identification of phospholipid FA (PLFA) allows the characterization of microbial populations in activated sludges (19) as well as in soils (20), drinking water, and biofilms (21). Characterization of the FA also may allow conclusions to be drawn concerning the physiological state (starvation, temperature and stress adaptation) of the microbial communities (20,22,23). Data on other FA, such as the lipopolysaccharide 2-OH- and 3-OH-FA (LPS 2-OH- and 3-OH-FA), also are used to monitor gram-negative species (24).

To complete the relatively scant information available on lipids in activated sludge flocs and EPS, we measured the FA of phospholipids (PL), glycolipids (GL), and neutral lipids (NL), and of LPS 2-OH- and 3-OH-FA. For practical reasons, this study focuses on the FA with 14 to 20 carbons in the PL, GL, and NL, and on the 2-OH- and 3-OH-FA with 9 to 25 carbons in LPS. EPS were recovered by combining floc dispersion techniques (sonication, use of a cation exchange resin and centrifugation). Lipids were extracted (25) and fractionated on a silica column (26), and their FA content was determined by GC–MS with selected ion monitoring (21).

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Abbreviations: a-, anteiso-branched FA; br-, methyl-branched; *cy-*, cyclopropane FA; EPS, extracellular polymeric substances; GL, glycolipid; GLFA, glycolipid FA; i-, iso-branched FA; LPS 2-OH-FA, lipopolysaccharide 2-hydroxy FA; LPS 3-OH-FA, lipopolysaccharide 3-hydroxy FA; 10-Me-16:0, 10-methylhexadecanoic acid; NL, neutral lipid; NLFA, neutral lipid FA; PL, phospholipid, PLFA, phospholipid FA; SIM, selected ion monitoring; TBSA, tuberculostearic acid.

EXPERIMENTAL PROCEDURES

Sludge samples. The activated sludge samples (mixed liquor or whole sludge) were collected directly from the aeration tank of the wastewater treatment plant. Two sludge samples (M_1 and M_2) were collected from Maxéville (site M), one in September 2000 (M_1) and one in January 2001 (M_2), and a sampling was performed in duplicate in Toul (site T) in June 2002: T_1 and $T_{1\ bis}$. Some of the characteristics of the wastewater treatment plants are summarized in Table 1.

Sludge analyses and EPS extractions were carried out no more than 1 h after sampling. The activated sludge samples were characterized by total solids (TS_{Sludge}), volatile solids (VS_{Sludge}), sludge volume index after 30 min of settling (SVI), $pH_{20°C}$, conductivity ($k_{20°C}$), and temperature (27). TS_{Sludge} (g/L of sludge) was measured as the dried weight of sludge (dried for 12 h at 105°C). VS_{Sludge} (g/L of sludge) was calculated by subtracting the weight of sludge dried for 3.5 h at 550°C from the weight of sludge dried for 12 h at 105°C (27). The SVI was determined by dividing the sludge volume after 30 min of settling in a 1-L graduated glass cylinder by the TS_{Sludge} value. These measurements were performed in triplicate (Table 1). The $pH_{20°C}$ and conductivity ($k_{20°C}$) were determined from the supernatant of the sludge after 30 min of settling. Sludge temperature was measured directly in the aeration tank.

EPS extraction. At each step of the EPS extraction procedure, the sludges (samples M_1 and M_2) and the deionized water used for washing were purged with N_2 (Alphagaz N_2, Air Liquide, Nancy, France) to avoid oxidation of the lipids.

Activated sludge samples were washed as follows before experimentation to remove weakly sorbed organic matter: 2 L of whole sludge was settled for 30 min at 20 ± 1°C, and the supernatant (1 L) was replaced by filtered [0.22 µm porosity (Millipore, Milford, MA)], deionized water. After gentle mixing, the sludge was settled again, and 1 L of the supernatant was replaced by deionized water. The washed sludge was dispersed by a combination of sonication (18) and cation exchange (2), as follows. Fifty-milliliter fractions of washed sludge were treated for 20 s at 37 W with a 19-mm ultrasound probe (Labsonic 2000U, Braun Sciencetec) immersed 15 mm into the liquid. The sludge was held in crushed ice for 15 min prior to and throughout the ultrasound treatment. Then cation extraction was performed by mixing a cation exchange resin (Dowex-Na^+ 50 × 8, 44445; Merck, Strasbourg, France) in the amount of 70 g/g of volatile solids into the ultrasound-treated sludge. This mixture was stirred (IKA Labortechnik, Eurostar, Germany) with a plate paddle at 400 rpm at 4°C for 45 min in a baffle beaker, as described by Frølund *et al.* (2). After 5 min of resin settling, the samples were centrifuged at 20,000 × *g* for 45 min at 4°C to separate the EPS from the biomass. The extracted EPS were carefully removed by pipetting the supernatant, and these EPS were kept on crushed ice prior to experimentation. For lipid analysis, samples were freeze-dried and stored under a N_2 atmosphere (atmospheric pressure).

EPS characterization. Values for the total solids (TS_{EPS}) and the volatile solids (VS_{EPS}) in the extracted EPS were determined by the procedure presented above for the activated sludge samples. Both TS_{EPS} and VS_{EPS} were expressed as g/L of extracts.

The protein (i.e., a substance containing two or more peptide bonds) concentrations and humic-like substances were measured using a Folin–Ciocalteu phenol reagent (28) in a two-step process. The first step is a biuret reaction in which the peptide bonds react with copper in an alkaline solution, creating a copper-treated protein. The second step is the reduction of the active phosphomolybdic and phosphotungstic acids by the copper-treated protein. As proposed by Frølund *et al.* (2), the results were corrected for nonspecific color development caused by humic-like substances by measuring absorbance at 750 nm with and without $CuSO_4$. The equations used for calculating the corrected protein and humic-like substance absorbancies were the following: $Abs_{protein} = 1.2 \cdot (Abs_{withCuSO_4} - Abs_{withoutCuSO_4})$ and $Abs_{humic} =$

TABLE 1
Characteristics of Wastewater Treatment Plants (Maxéville and Toul) and Activated Sludge Samples[a] (M_1, M_2, and T_1 and $T_{1\ bis}$)

	Sample M_1	Sample M_2	Samples T_1 and $T_{1\ bis}$
Wastewater treatment plant	Maxéville (average load)		Toul (average load)
Capacity (inhabitant equivalents)	480,000	480,000	25,375
TS_{Sludge} (g/L)[b]	2.04 ± 0.03	2.45 ± 0.02	3.35 ± 0.03
VS_{Sludge} (g/L)[b]	1.49 ± 0.01	1.32 ± 0.04	1.78 ± 0.05
SVI (mL/g of TS_{Sludge})	113 ± 2	66 ± 1	84 ± 2
$pH_{20°C}$	7.3	7.5	7.5
$k_{20°C}$ (µS/cm)	854	649	855
Flow (m^3/d)	53,827	56,310	5,607
Hydraulic retention time (h)	4.0	3.5	4.0
Sludge age (d)	3.5	3.5	13
Oxygen concentration (mg/L)	0.5–3.5	0.5–3.5	0.5–3.5
Temperature (°C)	18	10	22

[a]Sample M_1 was collected at the Maxéville wastewater treatment plant in September 2000; M_2, in January 2001. Samples T_1 and $T_{1\ bis}$ were duplicate samples, collected in June 2002 from the Toul wastewater treatment plant. TS_{Sludge}, total solids of sludges; VS_{Sludge}, volatile solids in sludge; SVI, sludge volume index.
[b]Values represent means ± SD; $n = 3$.

$Abs_{withoutCuSO_4} - 0.2 \cdot Abs_{protein}$. The protein concentration was expressed as mg equivalent BSA (A-7906; Sigma-Aldrich, Steinheim, Germany)/g VS_{EPS} and mg equivalent humic acid (H1 675-2; Sigma-Aldrich)/g VS_{EPS} for the humic-like substance concentrations.

The carbohydrate concentrations were determined using a phenol sulfuric acid method (29). Briefly, sulfuric acid hydrolyzed polysaccharides to monosaccharides during heating at 90°C for 5 min, and the phenol reagent (5%) dehydrated the monosaccharides to furfural derivatives. This reaction developed an orange color, which was measured spectrophotometrically at 492 nm. The carbohydrate concentration was expressed as mg equivalent D-glucose (379.294; Prolabo, Fontenay-sous-bois, France)/g VS_{EPS}.

The nucleic acids were measured using a diphenylamine method (30). Briefly, acetic and sulfuric acids hydrolyzed the DNA at 100°C for 10 min; the hydrolyzed DNA in turn reacted with the diphenylamine reagent. This reaction developed a blue coloration, which was measured spectrophotometrically at 595 nm. The DNA concentration was expressed as mg equivalent DNA of calf thymus (D-1501; Sigma-Aldrich)/g VS_{EPS}.

The EPS characteristics were determined in triplicate, and the average CV on the measures were 4.2, 2.9, 2.9, and 2.2% for proteins, humic-like substances, carbohydrates, and nucleic acids, respectively.

Lipid extraction and analysis from whole activated sludge and extracted EPS. The glassware used in the lipid analyses was heated to 550°C for 6 h prior to use to remove FA from the surface of the glass. Lipid extractions and the storage of lipids at −20°C were performed under nitrogen atmosphere (atmospheric pressure). Duplicate lyophilized EPS extract samples of 43.8 and 71.1 mg ($M_{1\ EPS}$) and of 150.5 and 186.8 mg ($M_{2\ EPS}$); and sludge samples of 1.25 and 1.23 g ($M_{2\ Sludge}$), 1.74 and 1.70 g ($T_{1\ Sludge}$), and of 1.73 and 1.68 g ($T_{1\ bis\ Sludge}$) were extracted with 28.2 mL of a mixture of chloroform/methanol/50 mM phosphate buffer, pH 7.4 (1:2:0.8, by vol) by shaking overnight at a temperature of 21 ± 2°C (20,25,31). At the end of the lipid extractions, diheptadecanoylphosphatidylcholine was added as an internal standard to aid in the quantification of PL, and the samples were then shaken for a further 5 min. After centrifugation (2,000 × *g*), chloroform and a buffer were added to the solvent phase to give a final ratio of chloroform/methanol/phosphate buffer (pH 7.4) of 1:1:0.9 (by vol) (25). Next, the solvent phases were separated and then evaporated to dryness in a centrifugal evaporator. The dry lipid extract was then dissolved in chloroform (4 × 100 µL) and applied at the top of a glass column (height 100 mm, i.d. 6 mm) containing 0.75 g of silicic acid (100–200 mesh size, Unisil; Clarkson Chemical, Williamsport, PA). Prior to use, the silicic acid was activated at 120°C overnight and then washed with chloroform. NL were eluted first from the column with 10 mL of chloroform, GL with 20 mL of acetone, and then PL with 10 mL of methanol (32). The fractions containing NL, GL, and PL were evaporated to dryness in a centrifugal evaporator. After the lipids had been dried, internal standards (tridecanoic and nonadecanoic acid methyl esters) were added to each lipid fraction. FA were then saponified, methylated, and extracted as methyl esters, as described earlier by Jantzen *et al.* (33). Internal standards (3-OH-tridecanoic acid and 2-OH-octadecanoic acid methyl esters) were added to the lipid extraction residues, and LPS 2-OH- and 3-OH-substituted FA were analyzed after mild acid hydrolysis. LPS-OH-FA were methylated in acetylchloride/methanol solution (1.5:10, vol/vol) and extracted with hexane (33,34). The FAME were analyzed by using a model G1800A Hewlett-Packard gas chromatograph equipped with a mass-selective detector, HP7673 automatic sampler, and HP-5 capillary column (30 m × 0.2 mm × 0.11 µm) coated with cross-linked 5% phenyl methyl silicone. Helium (1.0 mL/min) was used as the carrier gas. The injection was splitless, and the injector temperature was 250°C. The detector temperature was 270°C. The oven temperature was programmed to hold at 50°C for 1 min, and then to increase 30°C/min up to 160°C, and thereafter 5°C/min up to 270°C. The FA of PL, GL, and NL fractions were analyzed using either total ion monitoring (for T_1 and $T_{1\ bis}$ only) or selected ion monitoring (SIM) by following the ions *m/z* 74 and 199, except that the latter ion was *m/z* 268 for 16:1 acids, 250 for *cy*-17:0, 298 for 18:0, and i-18:0, 294 for 18:2n-6, 264 for 18:1 acids, 312 for 10-Me-18:0 and 19:0, 278 for *cy*-19:0, and 326 for 20:0, where *cy* = cyclopropane, i = iso-branched, and 10-Me-18:0 = 10-methylhexadecanoic acid.

Calibration standards with a known ratio of bacterial FA relative to the internal standard methyl nonadecanoate (19:0) were used to construct the calibration curves (20,35). The standard contained FA at four or five concentrations ranging from 0.02 to 2 nmol/µL for 16:0 with an internal standard amount of 68 pmol/µL. In the SIM of LPS-OH-FA methyl esters, the ion followed was *m/z* 103 for 3-OH-FA, and *m/z* 90 and M − 59 for 2-OH-FA (36–38). The peaks of FAME were identified by comparing their mass spectra and retention times with those of standards.

Chemicals. The analytical reagents for the lipid analyses were from Merck (Darmstadt, Germany), with the following exceptions: solvents (Rathburn Ltd., Peeblesshire, United Kingdom), 3-hydroxytridecanoic acid methyl ester (Larodan AB, Malmö, Sweden), other FA standards (Sigma, St. Louis, MO), NaOH (FF-Chemicals, Yli-Ii, Finland), HCl (Riedel-de Haën, Seelze, Germany), and acetylchloride (Fluka, Buchs, Switzerland).

Calculations. FA composition was expressed as a percentage of the total peak area. The quantitative amounts of FA were calculated using internal standards, and the total FA content was defined as the sum of the FAME. Amounts of C_{18}/C_{16} FA were calculated as the percentage ratio of 18:0 and 18:1 FA to 16:0 and 16:1 FA. All results are presented as the mean ± SD. The SD for the FA analysis of a pure microbial culture (n = 8) was <0.9% (23). The CV was calculated as the ratio of the SD to the mean value.

RESULTS

Characteristics of the extracted EPS. The EPS extracted from the activated sludges M_1 and M_2 ($M_{1\ EPS}$ and $M_{2\ EPS}$) by

TABLE 2
Proportions (% of VS_{EPS}) of Proteins, Humic-like Substances, Carbohydrates, Nucleic Acids, and FA in the EPS Extracted from Whole Sludges M_1 and M_2 by Sonication and Cation Exchange Resin[a]

Parameters		$M_{1\ EPS}$	$M_{2\ EPS}$	Proportions of polymers (% of VS_{EPS})
Proteins	(mg/g VS_{Sludge})	68.8 ± 0.6	63.2 ± 4.8	
	(mg/g VS_{EPS})	552.6 ± 4.8	315.8 ± 23.8	43.4 ± 16.7
Humic-like substances	(mg/g VS_{Sludge})	15.7 ± 0.3	20.8 ± 0.8	
	(mg/g VS_{EPS})	126.1 ± 2.7	104.0 ± 3.9	11.5 ± 1.6
Carbohydrates	(mg/g VS_{Sludge})	14.9 ± 0.5	15.8 ± 0.4	
	(mg/g VS_{EPS})	119.7 ± 4	79.0 ± 1.9	9.9 ± 2.9
Nucleic acids	(mg/g VS_{Sludge})	ND	21.9 ± 0.5	
	(mg/g VS_{EPS})	ND	109.2 ± 2.4	10.9
FA	(mg/g VS_{Sludge})	2.05 ± 0.96	ND	
	(mg/g VS_{EPS})	17.85 ± 8.34	ND	1.8
VS_{Sludge} (g/L)		1.49 ± 0.01	1.32 ± 0.04	
VS_{EPS} (g/g VS_{Sludge})		0.171 ± 0.01	0.197 ± 0.007	

[a]VS_{EPS}, volatile solids in extracellular polymeric substances (EPS); ND, not determined; for other abbreviations see Table 1. Mean ± SD (n = 3), except for FA extracted from the EPS of sludge M_1 (mean ± SD; n = 2).

sonication and cation exchange resin represented 18.4% of VS_{Sludge}. The EPS contained mainly proteins (43.4% VS_{EPS}), humic-like substances (11.5% VS_{EPS}), nucleic acids (10.9% VS_{EPS}), carbohydrates (9.9% VS_{EPS}), and FA (1.8% VS_{EPS}) (Table 2). Lipids were extracted from the EPS (sample M_1) by a mixture of chloroform, methanol, and phosphate buffer. Lipids extracted were then separated into PL, GL, and NL fractions as a function of their different polarities on the silica column. The FA of these fractions, and the LPS 2-OH-FA and 3-OH-FA of the extraction residue were quantified. The largest amounts of FA were partitioned into GL (61.1%), PL (20.9%), and NL (16.0%) fractions and, to a lesser extent, to LPS 3-OH-FA (1.9%) and 2-OH-FA (0.1%) (Table 3). GC–MS analyses revealed a high diversity of FA: 35 different FA in the PL, GL, and NL (Tables 4–6), 29 FA in the LPS 3-OH-FA (Table 7), and 21 FA in the LPS 2-OH-FA (Table 8). FA were separated into saturated, unsaturated, branched-chain (iso-, anteiso-, and middle-branched) and cyclopropane.

Repeatability and reproducibility of FA analyses. The analytical repeatability (intrinsic measurement variability) in lipid-bound FA extraction, fractionation and derivatization, and GC–MS analysis was estimated from the SD presented in Tables 4–8. The average CV for FA percentages detected in amounts >1% in PLFA, GLFA, NLFA, and LPS 3-OH- and 2-OH-FA was 7.2, 9.0, 14.1, 6.6, and 6.7%, respectively. For the different lipid fractions (PL, GL, NL, LPS 3-OH, and LPS 2-OH), the average of all CV was 18% (range of variation 9–32%).

Two samples ($T_{1\ Sludge}$ and $T_{1\ bis\ Sludge}$) were collected on the same day at the same wastewater treatment plant to estimate overall reproducibility of both sampling and the FA analytical procedure. For the total saturated FA of PL, GL, and NL, and LPS 2-OH and 3-OH (Tables 4–8), the variations between the two samples were 0.4, 0.3, 12.2, 2.2, and 0.8%, respectively. For the total unsaturated FA, the variations were 2.7, 2.8, and 9.7%, respectively, for the PL, GL, and NL. For the total branched-chain FA, the variations were 2.2, 3.1, 2.4, 2.2, and 1.1%, respectively, for the PL, GL, NL, LPS 2-OH-, and LPS 3-OH-FA.

Comparison of FA in whole sludge and extracted EPS. $M_{2\ Sludge}$ was collected to further compare the FA profiles of lipid fractions between sludge ($M_{2\ Sludge}$) and extracted EPS ($M_{2\ EPS}$). As shown in Table 4, the PLFA of both whole sludge and extracted EPS included more unsaturated acids than FA of the other types. The saturated and branched-chain FA were detected in nearly equal proportions of 23.5–28.4%. Comparison of PLFA profiles between sludge and extracted EPS showed differences in the saturated, unsaturated, cyclopropane, iso-, and middle-branched FA (Table 4). The PLFA of both whole sludge and extracted EPS were dominated by 16:0, monoenoic 16- and 18-carbon chains, and i-15:0 FA, except for the br-19:1 acid, which was more important in the PLFA of sludge. These FA represented 70–71% of all PLFA of sludge and extracted EPS. Branched-chain acids were dominated by i-15:0, a-15:0 and br-15 in EPS and sludge. Moreover, the amount of branched-chain acids (i-15:0, i-16:0, i-17:0, i-18:0, and br-17:1) was slightly greater in the EPS than in the sludge. Compounds i-14:0, 10-Me-16:0, br-19:1, and tuberculostearic acid (TBSA) were contained in only small proportions in the PLFA of sludge and EPS, but i-14:0, br-19:1, and TBSA were

TABLE 3
Proportions (% of FA) of Phospholipids (PL), Glycolipids (GL), Neutral Lipids (NL), and 3-Hydroxy and 2-Hydroxy Lipopolysaccharides (LPS 3-OH and LPS 2-OH) in Lipids Extracted from EPS of Sludge M_1[a]

Parameters	mg/g VS_{EPS}	Proportions of polymers (% of FA)
PL	18.95 ± 2.53	20.94 ± 6.78
GL	55.28 ± 28.33	61.09 ± 5.86
NL	14.45 ± 8.03	15.97 ± 1.36
LPS 3-OH	1.72 ± 0.39	1.90 ± 0.41
LPS 2-OH	0.09 ± 0.01	0.10 ± 0.02

[a]For abbreviations see Table 2.

TABLE 4
PL FA Compositions (wt%) in Total Sludges and Extracted EPS[a,b]

Types of FA	$M_{1\ EPS}$	$M_{2\ EPS}$	$M_{2\ Sludge}$	$T_{1\ Sludge}$	$T_{1\ bis\ Sludge}$
Saturated					
14:0	4.13 ± 0.16	4.82 ± 0.03	4.62 ± 0.31	2.41 ± 0.33	2.88
15:0	1.51 ± 0.04	1.40 ± 0.09	1.86 ± 0.14	0.55 ± 0.16	0.65
16:0	19.82 ± 0.90	17.99 ± 0.01	11.90 ± 0.10	16.49 ± 0.68	15.57
18:0	4.34 ± 0.05	3.77 ± 0.05	4.83 ± 0.06	1.49 ± 0.51	2.17
20:0	0.57 ± 0.01	0.43 ± 0.06	0.29 ± 0.04	0.05 ± 0.02	0.13
Total saturated	30.37 ± 0.83	28.41 ± 0.17	23.50 ± 0.33	20.99 ± 0.34	21.39
Unsaturated					
14:1	0.28 ± 0.01	0.43 ± 0.22	0.48 ± 0.04	0.16 ± 0.03	0.23
15:1	ND	0.18 ± 0.04	0.20 ± 0.01	0.08 ± 0.02	0.12
16:1n-5	1.52 ± 0.09	1.68 ± 0.33		2.17 ± 0.11	2.37
16:1n-7*c*	15.10 ± 0.31	14.40 ± 0.06	26.68 ± 3.24[c]	37.37 ± 4.07	31.66
16:1n-7*t*	1.36 ± 0.10	0.33 ± 0.06		ND	ND
16:1n-9	3.65 ± 0.07	4.15 ± 0.03		1.34 ± 0.69	2.49
br-17:1	ND	1.71 ± 0.13	0.29 ± 0.06	0.15 ± 0.03	0.22
18:1n-5	0.41 ± 0.01	0.42 ± 0.02		ND	0.14
18:1n-7*c*	8.55 ± 0.21	15.27 ± 0.10	17.24 ± 1.93[d]	9.85 ± 0.16	9.93
18:1n-7*t*	5.51 ± 0.06	6.86 ± 0.14		ND	ND
18:1n-9	1.08 ± 0.04	2.03 ± 0.12		8.45 ± 0.88	9.09
18:2n-6	0.27 ± 0.38	1.19 ± 0.03	ND	6.98 ± 0.58	7.35
br-19:1	ND	0.26 ± 0.03	7.04 ± 0.20	0.35 ± 0.05	0.58
20:2	ND	ND	ND	0.10 ± 0.03	0.12
20:3	ND	ND	ND	0.08 ± 0.02	0.11
20:4	ND	ND	ND	1.09 ± 0.09	1.12
Total unsaturated	37.72 ± 0.74	48.89 ± 0.24	51.92 ± 1.10	68.17 ± 1.68	65.51
Branched-chain					
i-14:0	0.65 ± 0.04	0.92 ± 0.02	2.57 ± 0.11	0.67 ± 0.13	0.85
i-15:0	16.46 ± 1.10	7.55 ± 0.04	7.03 ± 0.05	5.10 ± 0.42	5.64
i-16:0	4.30 ± 0.37	1.64 ± 0.07	ND	ND	ND
i-17:0	2.40 ± 0.03	1.48 ± 0.37	0.07 ± 0.04	0.41 ± 0.09	0.60
i-18:0	ND	1.15 ± 0.01	ND	ND	ND
a-15:0	3.63 ± 0.11	4.01 ± 0.37	3.95 ± 0.11	2.16 ± 0.27	2.58
a-17:0	1.87 ± 0.14	1.10 ± 0.12	1.11 ± 0.32	0.21 ± 0.07	0.31
br-15	1.19 ± 0.01	3.40 ± 0.22	3.88 ± 0.13	1.52 ± 0.19	1.96
br-17:0	0.19 ± 0.03	0.10 ± 0.01	0.32 ± 0.07	ND	ND
br-17:1	ND	1.71 ± 0.13	0.29 ± 0.06	0.15 ± 0.03	0.22
br-19:1	ND	0.26 ± 0.03	7.04 ± 0.20	0.35 ± 0.05	0.58
10-Me 16:0	1.10 ± 0.09	0.26 ± 0.05	0.19 ± 0.01	0.04 ± 0.01	0.07
TBSA	0.12 ± 0.07	0.02 ± 0.01	0.21 ± 0.05	0.05 ± 0.02	0.08
Total branched-chain	31.91 ± 1.57	23.61 ± 0.12	26.66 ± 0.74	10.66 ± 1.29	12.89
Cyclopropane					
cy-17:0	ND	0.75 ± 0.11	5.02 ± 0.25	0.53 ± 0.10	0.78
cy-19:0	ND	0.07 ± 0.04	0.23 ± 0.03	0.16 ± 0.04	0.23
Total cyclopropane	ND	0.82 ± 0.06	5.24 ± 0.28	0.69 ± 0.14	1.01

[a]$M_{1\ EPS}$ and $M_{2\ EPS}$, EPS from samples M_1 and M_2; $M_{2\ Sludge}$, sludge collected on second sampling date at site M; $T_{1\ Sludge}$ and $T_{1\ bis\ Sludge}$, duplicate sludge samples collected at site T; br, branched; i, iso; a, anteiso; TBSA, tuberculostearic acid; *cy*, cyclopropane. For other abbreviations see Tables 1 and 2.
[b]Data are presented as mean ± SD.
[c]Sum of $C_{16:1}$ FA.
[d]Sum of $C_{18:1}$ FA.

found in greater proportions in the sludge. The unsaturated PLFA contained a greater proportion of FA, with 16- and 18-carbon FA found in different positions on the double bond, including 16:1n-9, 16:1n-7*c*, 18:1n-7*c*, and 18:1n-7*t*. The total amount of 16:1 FA in the PLFA of the sludge (26.7%) was greater than in those of the EPS (20.6%). The cyclopropane FA percentage was greater in the sludge PLFA. The total amount of 18:1 FA in the PLFA of the sludge (17.2%) was less than in those of the EPS (24.6%). Consequently, the ratio of C_{18} to C_{16} FA in the sludge PLFA (0.58 ± 0.10%) was less than in the same fraction of EPS (0.77 ± 0.01%).

For GL and NL, both the EPS and the sludge contained saturated and unsaturated FA in particular (Tables 5 and 6), but their distribution was different from that obtained for PLFA (Table 4). The GLFA and NLFA of the sludge and extracted EPS were dominated by 14:0, 16:0, 18:0, and monoenoic 16- and 18-carbon acids, representing 79–87% of all acids. In the sludge, the GLFA contained threefold, and

TABLE 5
GL FA Compositions (wt%) of Total Sludges and Extracted EPS[a,b]

Types of FA	$M_{1\ EPS}$	$M_{2\ EPS}$	$M_{2\ Sludge}$	$T_{1\ Sludge}$	$T_{1\ bis\ Sludge}$
Saturated					
14:0	4.93 ± 0.06	5.88 ± 1.23	3.63 ± 0.22	4.76 ± 0.48	5.16
15:0	1.61 ± 0.02	1.35 ± 0.45	1.17 ± 0.08	1.23 ± 0.20	1.20
16:0	23.56 ± 0.25	21.01 ± 1.14	23.59 ± 1.21	27.66 ± 3.99	26.71
17:0	1.16 ± 0.01	Tr	Tr	0.74 ± 0.14	0.69
18:0	9.76 ± 0.35	9.55 ± 0.52	11.65 ± 0.01	9.72 ± 0.68	10.63
20:0	1.61 ± 0.04	0.57 ± 0.19	2.79 ± 0.37	ND	ND
Total saturated	42.62 ± 0.24	38.36 ± 1.24	42.83 ± 1.15	44.11 ± 4.81	44.39
Unsaturated					
14:1	0.35 ± 0.03	0.41 ± 0.07	0.25 ± 0.16	0.24 ± 0.02	0.21
15:1	ND	0.16 ± 0.04	0.16 ± 0.01	0.08 ± 0.02	0.07
16:1n-5	1.76 ± 0.08	0.90 ± 0.40	0.47 ± 0.03	1.51 ± 0.23	1.24
16:1n-7*c*	13.62 ± 0.05	9.54 ± 0.86	9.82 ± 0.05	9.13 ± 1.01	10.29
16:1n-7*t*	1.27 ± 0.08	Tr	0.21 ± 0.02	ND	ND
16:1n-9	4.35 ± 0.16	6.58 ± 2.30	1.43 ± 0.13	1.13 ± 0.15	0.80
br-17:1	ND	0.11 ± 0.05	0.79 ± 0.07	0.28 ± 0.11	0.22
18:1n-5	0.76 ± 0.02		1.38 ± 0.32	2.60 ± 0.48	2.97
18:1n-7*c*	14.72 ± 0.03	33.81 ± 5.05[c]	11.72 ± 0.02	5.37 ± 0.25	5.17
18:1n-7*t*	4.69 ± 0.14		5.37 ± 0.25	ND	ND
18:1n-9	2.07 ± 0.02		1.34 ± 0.07	13.91 ± 0.16	14.87
18:2n-6	0.35 ± 0.07	Tr	Tr	7.99 ± 0.36	9.27
br-19:1	0.58 ± 0.19	0.41 ± 0.26	4.34 ± 0.88	0.28 ± 0.14	0.16
20:2	ND	ND	ND	0.09 ± 0.00	0.10
20:3	ND	ND	ND	0.13 ± 0.01	0.04
20:4	ND	ND	ND	0.35 ± 0.04	0.43
Total unsaturated	44.53 ± 0.26	52.52 ± 3.55	37.40 ± 1.51	43.08 ± 1.55	45.84
Branched-chain					
i-14:0	0.48 ± 0.01	0.48 ± 0.19	0.92 ± 0.10	0.63 ± 0.15	0.56
i-15:0	6.03 ± 0.37	2.30 ± 0.60	5.03 ± 0.30	4.91 ± 1.45	3.76
i-16:0	1.84 ± 0.09	0.98 ± 0.48	1.95 ± 0.07	0.82 ± 0.19	0.35
i-17:0	1.18 ± 0.01	0.64 ± 0.19	0.93 ± 0.00	0.70 ± 0.19	0.55
i-18:0	ND	Tr	0.50 ± 0.07	ND	ND
a-15:0	1.74 ± 0.03	1.70 ± 0.22	5.77 ± 0.32	2.61 ± 0.68	2.00
a-17:0	1.02 ± 0.03	0.63 ± 0.19	1.13 ± 0.05	0.57 ± 0.12	0.36
br-15	ND	0.77 ± 0.16	2.34 ± 0.02	1.20 ± 0.32	0.94
br-17:0	0.12 ± 0.01	0.11 ± 0.06	Tr	ND	ND
br-17:1	ND	0.11 ± 0.05	0.79 ± 0.07	0.28 ± 0.11	0.22
br-19:1	0.58 ± 0.19	0.41 ± 0.26	4.34 ± 0.88	0.28 ± 0.14	0.16
10-Me 16:0	0.23 ± 0.02	0.13 ± 0.04	Tr	0.35 ± 0.11	0.29
TBSA	0.21 ± 0.01	Tr	ND	0.44 ± 0.01	0.52
Total branched-chain	13.43 ± 0.68	8.32 ± 2.36	23.78 ± 0.16	12.79 ± 3.47	9.69
Cyclopropane					
cy-17:0	ND	1.09 ± 0.31	0.93 ± 0.03	0.49 ± 0.05	0.39
cy-19:0	ND	0.18 ± 0.05	ND	0.09 ± 0.01	0.07
Total cyclopropane	ND	1.26 ± 0.35	0.93 ± 0.03	0.58 ± 0.06	0.46

[a]Tr, trace (<0.01%); for other abbreviations see Tables 1–4.
[b]Data are presented as mean ± SD, $n = 2$.
[c]Sum of $C_{18:1}$ FA.

NLFA 1.8-fold, more branched-chain FA than the same fractions of EPS. The most common acids were i-14:0, i-15:0, i-17:0, a-15:0, a-17:0, br-15, and br-19:1. The total amount of 16:1 FA in the NLFA of the sludge was greater than in those of extracted EPS, whereas the values obtained for sludge GLFA were lower than those of extracted EPS. The total amount of 18:1 FA in the GLFA and NLFA of the sludge was less than in the GLFA and NLFA of extracted EPS. Consequently, the ratio of C_{18} to C_{16} FA in sludge GLFA (0.89 ± 0.05) and NLFA (0.61 ± 0.02) was less than in EPS GLFA (1.14 ± 0.14) and NLFA EPS (1.09 ± 0.05). The GLFA and NLFA of both sludge and extracted EPS contained a few cyclopropane FA (Tables 5 and 6).

The percentage of saturated, straight-chain 3-OH-FA was greater in the LPS of extracted EPS (74.2%) than of sludge (60.9%) (Table 7), mainly owing to the large amount of FA with an even number of carbons. The dominating saturated and straight-chain 3-OH-FA of EPS and sludge were 3-OH-10:0, 3-OH-12:0, 3-OH-14:0, and 3-OH-16:0. The sum of iso- and anteiso-branched 3-OH-FA was greater in the sludge than in extracted EPS. The major iso-branched FA contained 9 to 17 carbons, whereas the most common anteiso-branched acids

TABLE 6
NL FA Compositions (wt%) of Total Sludges and Extracted EPS[a]

Types of FA	$M_{1\ EPS}$	$M_{2\ EPS}$	$M_{2\ Sludge}$	$T_{1\ Sludge}$	$T_{1\ bis\ Sludge}$
Saturated					
14:0	4.65 ± 0.31	9.08 ± 0.19	7.07 ± 0.47	5.06 ± 0.66	5.53
15:0	1.26 ± 0.27	1.49 ± 0.04	2.09 ± 0.24	0.68 ± 0.18	1.00
16:0	25.25 ± 1.96	23.40 ± 0.89	19.47 ± 0.52	37.70 ± 5.26	24.90
17:0	1.26 ± 0.03	Tr	Tr	0.45 ± 0.03	0.48
18:0	10.48 ± 1.73	9.18 ± 0.36	10.54 ± 0.65	10.14 ± 0.73	9.36
20:0	0.78 ± 0.04	0.49 ± 0.06	0.95 ± 0.07	0.73 ± 0.24	1.24
Total saturated	43.68 ± 3.10	43.65 ± 1.15	40.12 ± 1.95	54.76 ± 4.89	42.52
Unsaturated					
14:1	0.57 ± 0.07	0.79 ± 0.14	1.89 ± 0.29	0.24 ± 0.16	0.45
15:1	ND	0.15 ± 0.01	0.28 ± 0.09	0.14 ± 0.06	0.24
16:1n-5	0.95 ± 0.19	0.72 ± 0.16		0.91 ± 0.05	1.22
16:1n-7*c*	7.75 ± 1.30	6.34 ± 0.05	26.13 ± 1.26[b]	8.73 ± 1.49	12.12
16:1n-7*t*	0.75 ± 0.11	0.19 ± 0.10		ND	ND
16:1n-9	5.04 ± 0.40	7.22 ± 0.17		0.38 ± 0.54	0.38
br-17:1	ND	0.39 ± 0.20	0.18 ± 0.03	ND	0.09
18:1n-5	0.64 ± 0.16	0.70 ± 0.48		1.19 ± 1.42	2.67
18:1n-7*c*	21.34 ± 0.22	25.92 ± 0.74	15.97 ± 0.13[c]	5.45 ± 0.03	5.43
18:1n-7*t*	5.65 ± 0.09	3.11 ± 0.50		ND	ND
18:1n-9	2.72 ± 0.01	0.24 ± 0.11		14.37 ± 0.89	13.45
18:2n-6	0.62 ± 0.12	2.26 ± 0.16	Tr	8.91 ± 2.19	13.63
br-19:1	3.35 ± 0.28	0.61 ± 0.06	1.25 ± 0.06	ND	0.04
20:2	ND	ND	ND	0.29 ± 0.04	0.32
20:3	ND	ND	ND	0.03 ± 0.02	0.06
20:4	ND	ND	ND	0.47 ± 0.08	0.74
Total unsaturated	49.39 ± 2.20	48.64 ± 1.21	47.04 ± 3.52	41.16 ± 3.92	50.86
Branched-chain					
i-14:0	0.34 ± 0.08	0.38 ± 0.02	1.30 ± 0.21	0.19 ± 0.13	0.44
i-15:0	2.47 ± 0.38	1.46 ± 0.06	3.96 ± 0.61	1.53 ± 0.25	2.50
i-16:0	0.77 ± 0.13	0.59 ± 0.05	ND	ND	ND
i-17:0	1.04 ± 0.09	0.49 ± 0.01	0.64 ± 0.02	0.25 ± 0.01	0.44
i-18:0	ND	ND	Tr	ND	ND
a-15:0	1.12 ± 0.14	1.31 ± 0.10	3.05 ± 0.54	0.86 ± 0.18	1.56
a-17:0	0.64 ± 0.03	0.52 ± 0.01	0.69 ± 0.01	0.32 ± 0.04	0.40
br-15	ND	0.64 ± 0.14	2.05 ± 0.35	0.32 ± 0.34	0.32
br-17:0	0.11 ± 0.02	0.53 ± 0.07	0.12 ± 0.10	ND	ND
br-17:1	ND	0.39 ± 0.20	0.18 ± 0.03	Tr	0.09
br-19:1	3.35 ± 0.28	0.61 ± 0.06	1.25 ± 0.06	ND	0.05
10-Me 16:0	0.38 ± 0.04	0.46 ± 0.25	0.11 ± 0.03	Tr	0.06
TBSA	0.08 ± 0.00	0.28 ± 0.02	Tr	0.32 ± 0.04	0.45
Total branched-chain	10.28 ± 1.18	7.65 ± 0.30	13.36 ± 1.53	3.85 ± 0.97	6.30
Cyclopropane					
cy-17:0	ND	1.01 ± 0.01	0.86 ± 0.03	0.28 ± 0.06	0.43
cy-19:0	ND	0.05 ± 0.02	0.05 ± 0.05	ND	0.04
Total cyclopropane	ND	1.06 ± 0.03	0.91 ± 0.08	0.28 ± 0.06	0.46

[a]Data are presented as mean ± SD, $n = 2$. For abbreviations see Tables 1, 2, and 5.
[b]Sum of $C_{16:1}$ FA.
[c]Sum of $C_{18:1}$ FA.

were 3-OH-a-15:0 and 3-OH-a-17:0. There were fewer saturated, straight-chain LPS 2-OH-FA in the EPS (75.6%) than in the sludge (90.4%) (Table 8), so the proportion of short-chain acids 2-OH-10:0, 2-OH-11:0, 2-OH-12:0, and 2-OH-15:0 was greater in the EPS than in the sludge, contrary to the case of the long-chain acids 2-OH-22:0, 2-OH-23:0, 2-OH-24:0, and 2-OH-25:0 that dominated in the sludge. Instead, the proportion of branched-chain LPS 2-OH acids was greater in the EPS (24.4%) than in the sludge (9.6%), mainly because of the large amount of 2-OH-i-15:0 present in the EPS.

Comparison of FA in EPS extracted from sludges collected in September and January. Two EPS extracts ($M_{1\ EPS}$ and $M_{2\ EPS}$) were analyzed to compare the seasonal effect on the FA composition of lipid fractions. Results presented in Tables 4–8 show differences in the FA profiles of the EPS extracted. The amount of unsaturated FA in the PL and GL of the January sample, $M_{2\ EPS}$, was greater than in the respective fractions of the September sample, $M_{1\ EPS}$ (Tables 4 and 5), whereas that in NL was nearly equal in both samples (Table 6). The proportion of 16:1n-9, br-17:1, monoenoic C_{18}

TABLE 7
LPS 3-OH-FA Compositions (wt%) in Total Sludges and Extracted EPS[a]

Types of FA	$M_{1\ EPS}$	$M_{2\ EPS}$	$M_{2\ Sludge}$	$T_{1\ Sludge}$	$T_{1\ bis\ Sludge}$
Saturated					
–OH-8:0	1.00 ± 0.22	1.57 ± 0.03	1.99 ± 0.06	1.15 ± 0.03	1.11 ± 0.09
–OH-9:0	0.21 ± 0.01	0.39 ± 0.02	0.08 ± 0.01	ND	ND
–OH-10:0	14.67 ± 3.06	14.74 ± 0.77	13.35 ± 0.63	13.96 ± 0.33	13.23 ± 0.15
–OH-11:0	0.41 ± 0.04	0.65 ± 0.03	0.24 ± 0.04	0.23 ± 0.02	0.25 ± 0.01
–OH-12:0	17.71 ± 0.12	23.68 ± 0.18	13.12 ± 0.24	12.18 ± 0.14	11.73 ± 0.02
–OH-14:0	33.63 ± 3.43	16.87 ± 0.38	11.68 ± 0.79	17.26 ± 0.22	16.80 ± 0.28
–OH-15:0	1.30 ± 0.04	1.21 ± 0.06	1.47 ± 0.08	1.46 ± 0.03	1.39 ± 0.08
–OH-16:0	10.54 ± 0.36	11.33 ± 0.06	14.44 ± 0.73	16.00 ± 0.24	16.47 ± 0.14
–OH-17:0	1.44 ± 0.12	1.42 ± 0.03	1.48 ± 0.15	1.30 ± 0.01	1.26 ± 0.06
–OH-18:0	2.70 ± 0.43	2.18 ± 0.03	2.72 ± 0.39	2.01 ± 0.04	1.96 ± 0.04
–OH-19:0	0.16 ± 0.01	0.07 ± 0.01	0.08 ± 0.03	ND	ND
–OH-20:0	0.18 ± 0.01	0.11 ± 0.04	0.21 ± 0.04	0.12 ± 0.01	0.12 ± 0.01
Total saturated	83.95 ± 0.96	74.21 ± 0.08	60.87 ± 1.92	65.67 ± 0.21	64.87 ± 0.01
Unsaturated					
–OH-14:1	0.15 ± 0.21	0.95 ± 0.06	0.52 ± 0.01	0.28 ± 0.06	0.30 ± 0.03
–OH-18:1	0.07 ± 0.03	0.10 ± 0.03	0.24 ± 0.03	0.30 ± 0.16	0.54 ± 0.05
Total unsaturated	0.22 ± 0.23	1.05 ± 0.10	0.76 ± 0.04	0.59 ± 0.21	0.84 ± 0.11
Branched-chain					
–OH-i-9:0	Tr	Tr	3.80 ± 2.52	ND	ND
–OH-i-10:0	0.02 ± 0.01	0.02 ± 0.01	0.29 ± 0.01	ND	ND
–OH-i-11:0	0.82 ± 0.12	1.53 ± 0.16	2.21 ± 0.16	ND	ND
–OH-i-12:0	1.15 ± 0.09	2.80 ± 0.05	2.85 ± 0.15	1.04 ± 0.08	1.00 ± 0.04
–OH-i-13:0	0.56 ± 0.02	0.62 ± 0.01	1.14 ± 0.10	0.75 ± 0.05	0.72 ± 0.05
–OH-i-14:0	0.69 ± 0.08	0.81 ± 0.01	1.04 ± 0.11	0.58 ± 0.02	0.51 ± 0.01
–OH-i-15:0	3.90 ± 0.14	5.45 ± 0.19	9.90 ± 0.30	10.28 ± 0.52	10.65 ± 0.20
–OH-i-16:0	0.58 ± 0.03	1.06 ± 0.02	1.98 ± 0.24	1.80 ± 0.02	1.74 ± 0.08
–OH-i-17:0	6.52 ± 0.04	9.92 ± 0.03	10.96 ± 0.21	13.49 ± 1.11	14.29 ± 0.17
–OH-i-18:0	0.20 ± 0.01	0.15 ± 0.01	0.27 ± 0.01	0.40 ± 0.01	0.46 ± 0.03
–OH-a-9:0	Tr	Tr	0.02 ± 0.01	ND	ND
–OH-a-11:0	0.02 ± 0.01	0.08 ± 0.01	0.04 ± 0.02	ND	ND
–OH-a-13:0	0.07 ± 0.02	0.10 ± 0.01	0.16 ± 0.01	0.20 ± 0.01	0.19 ± 0.02
–OH-a-15:0	0.59 ± 0.01	0.98 ± 0.02	1.66 ± 0.22	1.92 ± 0.36	1.99 ± 0.08
–OH-a-17:0	0.70 ± 0.19	1.21 ± 0.01	2.06 ± 0.04	3.30 ± 0.11	3.27 ± 0.12
Total branched chain	15.83 ± 0.72	24.72 ± 0.01	38.37 ± 1.96	33.74 ± 0.01	34.82 ± 1.13

[a]Data are presented as mean ± SD (n = 2). For abbreviations see Tables 1–5.

acids, and 18:2n-6 in the PLFA of $M_{2\ EPS}$ was higher than in those of $M_{1\ EPS}$ (Table 4). Cyclopropane FA were detected only in the January sample, $M_{2\ EPS}$. The percentage of saturated C_{15} to C_{20} FA in the PL, GL, and NL of $M_{1\ EPS}$ was equal to or slightly higher than that in $M_{2\ EPS}$, which was compensated in particular by the increase in the short-chain 14:0 in $M_{2\ EPS}$ (Tables 4–6). Consequently, the ratio of C_{18} to C_{16} FA was higher in $M_{2\ EPS}$ (0.77 ± 0.01) than in $M_{1\ EPS}$ (0.49 ± 0.01). The PL, GL, and NL of $M_{1\ EPS}$ contained more branched-chain FA than the same fractions of $M_{2\ EPS}$ because of the greater or equal proportions of i-15:0, i-16:0, i-17:0, anteiso-17:0, and 10-Me-16:0 in $M_{2\ EPS}$. In contrast, the amount of middle-branched (br-15, br-17:1) FAs was greater in the PLFA and GLFA of sample $M_{2\ EPS}$ than in those of sample $M_{1\ EPS}$. For LPS 2-OH-FA and 3-OH-FA, $M_{1\ EPS}$ contained more saturated FA and fewer branched-chain FA than $M_{2\ EPS}$ (Tables 7 and 8). The percentage of 3-OH-14:0 was much greater in $M_{1\ EPS}$ than in $M_{2\ EPS}$, contrary to the case of $M_{1\ EPS}$, in which the proportion of 2-OH-12:0 was smaller. The proportions of all branched-chain 3-OH-FA in $M_{1\ EPS}$ were equal to or smaller than in $M_{2\ EPS}$.

Comparison of FA profiles in whole sludges from different treatment plants. The two activated sludge samples (M_2 and T_1) were collected to compare the FA profiles of sludges originating from two different wastewater treatment plants ($M_{2\ Sludge}$ and $T_{1\ Sludge}$). The even-carbon-number FA such as 16:0 and 18:0, monoenoic C_{16} and C_{18} acids, and i-15:0 and a-15:0 dominated the PL, GL, and NL profiles of sludges collected in both wastewater treatment plants. However, the sum of unsaturated FA in the PL and GL of $T_{1\ Sludge}$ was greater than in those of $M_{2\ Sludge}$, contrary to the case of the NLFA (Tables 4–6). A remarkable amount of PUFA (18:2n-6, 20:2, 20:3, and 20:4) was found in the PLFA, GLFA, and NLFA of $T_{1\ Sludge}$, whereas 18:2n-6 was scarcely detected in lipid fractions of the $M_{2\ Sludge}$. The small amount of PUFA in $M_{2\ Sludge}$, and a similar amount in the $M_{2\ Sludge}$ sample, which was smaller than that in the $T_{1\ Sludge}$, may have led to the undetectability of C_{20} polyunsaturates in the $M_{2\ Sludge}$. The percentage of monoenoic C_{16} acids was higher in the PLFA of $T_{1\ Sludge}$ than in those of the $M_{2\ Sludge}$, nearly equal in the GLFA of both samples, and lower in the NLFA of the $T_{1\ Sludge}$ than in the $M_{2\ Sludge}$. The greatest proportion of cyclopropane acid

TABLE 8
LPS 2-OH-FA Compositions (wt%) in Total Sludges and Extracted EPS[a]

Types of FA	$M_{1\ EPS}$	$M_{2\ EPS}$	$M_{2\ Sludge}$	$T_{1\ Sludge}$	$T_{1\ bis\ Sludge}$
Saturated					
–OH-10:0	ND	4.29 ± 0.15	1.00 ± 0.01	0.54 ± 0.30	0.28 ± 0.01
–OH-11:0	ND	2.26 ± 0.02	0.27 ± 0.02	0.66 ± 0.01	0.51 ± 0.05
–OH-12:0	51.24	9.06 ± 0.75	2.13 ± 0.03	3.70 ± 0.04	2.75 ± 0.20
–OH-13:0	7.39	ND	ND	ND	ND
–OH-14:0	15.30	10.87 ± 0.53	10.60 ± 0.59	7.96 ± 0.31	6.69 ± 0.57
–OH-15:0	ND	1.01 ± 0.01	0.14 ± 0.01	0.79 ± 0.09	0.66 ± 0.05
–OH-16:0	20.26	13.67 ± 0.26	16.15 ± 0.32	15.86 ± 1.20	14.56 ± 1.17
–OH-17:0	0.44	0.99 ± 0.10	0.25 ± 0.01	0.45 ± 0.02	0.41 ± 0.05
–OH-19:0	ND	0.59 ± 0.05	Tr	0.26 ± 0.10	0.18 ± 0.01
–OH-20:0	1.49	6.08 ± 0.55	3.47 ± 0.16	2.57 ± 0.28	2.54 ± 0.04
–OH-21:0	ND	1.37 ± 0.05	0.86 ± 0.07	0.38 ± 0.03	0.43 ± 0.02
–OH-22:0	ND	7.69 ± 0.39	12.37 ± 0.61	11.61 ± 1.43	12.61 ± 0.35
–OH-23:0	ND	2.70 ± 0.45	11.84 ± 0.18	6.20 ± 0.37	7.11 ± 0.06
–OH-24:0	ND	13.68 ± 0.73	26.63 ± 0.49	21.10 ± 1.06	24.96 ± 2.07
–OH-25:0	ND	1.01 ± 0.03	4.49 ± 0.15	2.20 ± 0.01	2.44 ± 0.01
–OH-26:0	ND	ND	ND	2.47 ± 0.17	2.82 ± 0.16
Total saturated	96.10	75.25 ± 0.63	90.39 ± 0.56	76.75 ± 2.51	78.95 ± 0.21
Branched-chain					
–OH-i-15:0	ND	21.44 ± 0.58	2.80 ± 0.08	17.24 ± 3.52	15.73 ± 0.01
–OH-i-16:0	ND	0.35 ± 0.01	0.21 ± 0.04	0.81 ± 0.03	0.68 ± 0.06
–OH-i-17:0	3.33	1.70 ± 0.09	ND	1.85 ± 0.30	1.40 ± 0.05
–OH-a-15:0	ND	1.00 ± 0.14	6.61 ± 0.44	2.47 ± 0.97	1.98 ± 0.65
–OH-a-17:0	0.57	0.27 ± 0.02	ND	0.87 ± 0.38	1.26 ± 0.87
Total branched chain	3.90	24.75 ± 0.83	9.61 ± 0.56	23.24 ± 2.51	21.05 ± 0.23

[a]Data are presented as mean ± SD (n = 2). For abbreviations see Tables 1–5.

cy-17:0 was detected in the PLFA of the $M_{2\ Sludge}$. The amount of saturated PLFA and GLFA was nearly the same for the two samples, whereas in the NL more saturated FA were detected in the $T_{1\ Sludge}$. However, the ratio of C_{18} to C_{16} acids was almost the same in the $T_{1\ Sludge}$ PLFA (0.47 ± 0.06) as in the $M_{2\ Sludge}$ PLFA (0.58 ± 0.10). The PLFA, GLFA, and NLFA of the $T_{1\ Sludge}$ contained fewer branched-chain FA than the same fractions of the $M_{2\ Sludge}$ as a result of the equal or lower proportions of nearly all iso- and anteiso-branched acids, and acids with unknown methyl branch positions (Tables 4–6). In the LPS, the sum of branched-chain 3-OH acids was lower in the $T_{1\ Sludge}$ than in the $M_{2\ Sludge}$ (Table 7).

DISCUSSION

The EPS extracted from activated sludges was composed of proteins, carbohydrates, nucleic acids, and humic-like substances, in agreement with the literature (5,9,18,39,40), and

TABLE 9
Presumptive Origin of Phospholipid FA (PLFA) Found in Sludge and EPS[a] (sample M_2)

FA signatures found in EPS and sludge	Presumptive origin	References
15:0, br-15:0, 15:1, 17:0, br-17:0, 17:1, br-17:1, and br-19:1	Bacteria	69
16:0, 16:1, 18:1, *cy*-17:0, and *cy*-19:0	Gram-negative bacteria	70
a-15:0, i-15:0	Sulfate-reducing bacteria	65
br-17:0, br-17:1, and middle-chain monoenoic	Anaerobic *Desulfovibrio*-type sulfate-reducing bacteria	67
10-Me-16:0	*Desulfobacter*-type sulfate-reducing bacteria	62
a-15:0, i-15:0, and 17:0	Gram-positive bacteria	53
TBSA	*Mycobacterium,* Actinomycetes	59
16:1n-7*c*, 16:1n-5*t*, 18:1n-7, and 18:1n-8*c*	Anaerobic bacteria, methanotrophs	71
18:2n-6, 20:2, 20:3, and 20:4	Yeast, fungi, and aerobic microeukaryotes	19, 61

[a]For abbreviations see Tables 1, 2, and 4.

proteins were the main polymers recovered. Although the EPS extraction efficiency is unknown, the combination of sonication and cation exchange is considered to be one of the methods of choice for extracting the extracellular polymers to the extent that cell lysis is limited (18,41). The real amount of this EPS fraction (representing 18% of VS_{Sludge}) associated with the cells may then be underestimated.

The EPS extracted contained 1.8% FA, a value similar to that in Dignac *et al*. (42), where neutral lipids and phospholipids represented less than 1% of the total organic carbon of the extracted EPS. However, 22% of the uncharacterized EPS could contain a large amount of lipids that were not taken into account by the FA analysis, such as the non-FA moieties of lipid molecules, poly-β-hydroxyalkanoates (43), and steroids containing no FA (44).

Origin of lipid-bound FA in activated sludge. PL, GL, NL, and LPS are major components of lipids and could originate from three sources: (i) direct sorption by activated sludge of lipids (45,46) and as other wastewater compounds (47, 48); (ii) cell lysis; and (iii) metabolism of microorganisms (49–51). The FA distribution was relatively complex, possibly because of these three sources, as well as strong microbial and eukaryotic diversity (1,52) combined with environmental parameters, including oxygen, pH, and temperature, which may exert an influence on the FA composition of microorganisms (32,53). Indeed, 14:0, 16:0, 18:0, 16:1n-9, 16:1n-7, 18:1n-7*c*, and 18:1n-7*t* FA, measured in both EPS and sludge, have been found in seawater (54,55) and wastewater (46,56,57). These could originate from vegetable fats, animal fats (58), and microorganisms (53,59–61), unlike LPS, which are likely to originate from the cell walls of gram-negative bacteria (59,62–64).

Microbial relevance of FA signatures. PLFA could be a marker of the viable microbial community, as they are quickly degraded by exogenous and endogenous phospholipases within minutes to hours once cells die (31). Qualitatively, the FA profiles of total sludge and extracted EPS were similar for the PL, GL, NL, and LPS. Terminally branched FA, such as a-15:0 and the i-15:0 detected in the PLFA of both EPS and sludges, are characteristic of many gram-positive bacteria (53), but are also found in some gram-negative bacteria such as the sulfate-reducing bacteria (65). The PLFA br-17:0, br-17:1, and 10-Me-16:0, found in small amounts in both EPS and sludge, are common in sulfate-reducing bacteria of the genera *Desulfobacter* and *Desulfovibrio* (66,67). TBSA, which generally indicates the presence of mycobacteria and actinomycetes (59), was not observed in large amounts in EPS or sludge PLFA, GLFA, or NLFA. In EPS and sludge, monoenoic 16- and 18-carbon PLFA are indicative of prokaryotes, and, in particular, 18:1n-7 is considered to result from anaerobic prokaryotic biosynthesis (62,68). The 18:2n-6, 20:2, 20:3, and 20:4 FA found in small amounts indicated the presence of microeukaryotes including yeasts and fungi (61). Thus, these sludges were made up of a very diverse microbial community that included gram-positive and gram-negative bacteria, the *Desulfobacter* and *Desulfovibrio* genera of sulfate-reducing bacteria, actinomycetes, and yeast and fungi. Some relations between the FA signatures in EPS and sludge and the presumptive bacteria are summarized in Table 9.

The percentages of monounsaturated FA were higher in the PL and GL of EPS than in the same fractions of the sludge. The proportions of 3-OH-12:0 and 3-OH-14:0, common for the LPS of gram-negative bacteria, were also greater in the EPS than in the sludge. Indeed, gram-negative bacteria are known to excrete LPS extracellularly (62). In contrast, the proportion of methyl-branched acids in sludge GLFA was higher than in EPS GLFA. Iso- and anteiso-branched acids are commonly related to gram-positive bacteria and GL to gram-positive actinomycetes (59). However, the small amount of TBSA indicates that actinomycetes of the genus *Mycobacterium* were not very common in the activated sludges.

The differences in the PLFA profiles of EPS and sludge may also be indicative of differences in the physiological states of the microbes in the samples. The amount of *cy*-17:0 was greater in the sludge PLFA than in those of the EPS. These cyclopropane FA are synthesized in particular during the stationary growth phase or under stress conditions such as low pH, high incubation temperature, and high ion concentration (72). In addition, the ratio of C_{18} to C_{16} FA in sludge PLFA was smaller than in those of EPS. The lower ratio of C_{18} to C_{16} FA has been found in free-living bacteria as compared to adherent bacteria (73). The amount of long-chain 2-OH-FA with 22 to 25 carbons was also greater in the LPS of sludge than in that of EPS. Long-chain hydroxy FA are found in anaerobic lake sediments (36).

Seasonal effects on EPS lipids. In EPS extracted from sludges collected from the same treatment plant in September and January, few differences were observed in the FA of lipid fractions that could reflect a microbial response and that could be attributed at least partly to bacterial community compositional changes and/or microbial physiological responses. The wastewater treatment plant was not controlled, so these differences could also be attributed to a multiparametric effect (Table 1). The EPS extracted from the sample collected in January contained more unsaturated PLFA (16:1n-9, 18:1n-9, 18:1n-7*c*, 18:1n-7*t*, 18:2n-6) than that collected in September. This increase could be a typical phenomenon of temperature adaptation in membrane lipids (22,74,75), which could not be seen in the NLFA. In addition, the higher proportion of cyclopropane FA and ratio of C_{18}/C_{16} acids in the EPS PLFA of the January sample could be related to an adaptation to stress, such as a temperature decrease, and consequently slower microbial growth (72,73). In winter conditions, the bacterial communities seemed to contain more bacteria in a physiologically stressed state. By contrast, differences in the community structure of gram-negative bacteria with respect to monoenes (62) also can be assumed to be related to differences in the LPS OH-FA profiles of the EPS between January and September. The proportion of gram-positive bacteria, suggested by the iso- and anteiso-branched signatures (59), was elevated in the August EPS sample compared to that collected in January. The *Desulfobacter* spp. were found in a

higher proportion in September, and the *Desulfovibrio* spp. in January, as suggested by the 10-Me-16:0 and br-17:1 signatures (65,66).

Lipids in whole sludges from two different wastewater treatment plants. Comparison of the lipid profiles from two wastewater treatment plants showed differences in microbial community structures. The PLFA of $T_{1\ Sludge}$ from the Toul and $M_{2\ Sludge}$ from the Maxéville wastewater treatment plants indicated that both had gram-negative bacteria with monoenes (62,74) among the dominating microbial groups. However, the $M_{2\ Sludge}$ contained methyl-branched FA more typical for gram-positive bacteria (59) than $T_{1\ Sludge}$, whereas the latter was characterized by eukaryotes having 18:2n-6, 20:2, 20:3, and 20:4 (19,61). The $T_{1\ Sludge}$ contained fewer bacteria in a stressed physiological state, as suggested by the low values of the cyclopropane FA signatures and ratio of C_{18}/C_{16} acids. The wastewater treatment plants were not controlled, and these differences could be attributed to a multiparametric effect such as the wastewater composition, different processes in the two wastewater treatment plants, sludge age, and seasonal effect.

In conclusion, we analyzed the lipid-bound FA associated with EPS extracted from activated sludge. The fractionation of PL, GL, NL, and LPS by this method has apparently not been used before for sludge and EPS analysis. This method showed good repeatability, except for the FA present in very low proportions. Lipid-bound FA accounted for 1.8% of extracted EPS and were distributed as a function of their polarities between GL, PL, NL, and, to a lesser extent, LPS. Despite their low proportion, lipids could impart important properties to EPS, such as hydrophobicity. Lipid-bound FA were made up of methyl-branched, saturated, unsaturated, and cyclopropane FA. The distribution of FA was variable in the PL, GL, NL, and LPS fractions. The source of lipids associated with EPS remains uncertain. However, on the basis of differences in PLFA between EPS and sludge, it is tempting to assume that the EPS extraction selectively co-extracted a group of microorganisms. The activated sludge contained a diverse community of microorganisms, such as gram-negative and gram-positive bacteria, actinomycetes, and eukaryotes. Moreover, the unsaturated PLFA showed an increase in numbers in winter, and changes in cyclopropane FA signatures and the ratio of C_{18}/C_{16} could suggest a more stressed microbial physiological status. The comparison between the two wastewater treatment plants showed that whole activated sludges were dominated by similar types of FA. Nevertheless, some signatures were different, indicating that one plant contained fewer gram-positive bacteria (as suggested by i-15:0, i-17:0, a-15:0, and a-17:0 signatures) and more microeukaryotes including yeasts and fungi (as suggested by 18:2n-6 and C_{20} polyunsaturates) than the other plant, gram-negative bacteria being common to both plants.

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Digalactosyldiacylglycerol Is a Major Glycolipid in Floral Organs of *Petunia hybrida*

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ABSTRACT: In higher plants, glycolipids such as monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are major components of chloroplast membranes in leaves. A recent study identified an isoform of MGDG synthase that is expressed specifically in floral organs, suggesting a novel function for glycolipids in flowers. To elucidate the localization and developmental changes of glycolipids and their biosynthetic activities in flowers, we carried out a series of analytical studies with *Petunia hybrida*. The results showed that the biosynthetic activities of galactolipid synthesis, particularly for DGDG, increased during flower development. Among the floral organs, the pistil had the highest galactolipid synthetic activity. Its specific activity for incorporation of UDP-galactose to yield galactolipids was estimated to be more than twice that of leaves, which are the major site of galactolipid synthesis in plant tissues. Analysis of lipid contents of pistils revealed that they contained higher amounts of galactolipids than other floral organs. Moreover, DGDG was more abundant than MGDG in both pistils and petals. These results show that DGDG is a major glycolipid in floral organs and that DGDG biosynthetic activity is highly upregulated in the pistils and petals of *Petunia* flowers.

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Photosynthetic membranes are characterized by their unusually high composition of glycolipids, such as monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) (1). These galactolipids constitute up to 80% of the thylakoid membrane in chloroplasts, suggesting that they play an important role in the formation of photosynthetic membranes and are thus critical for photosynthetic function.

MGDG is synthesized in the plastid envelope by UDP-galactose:1,2-*sn*-DAG 3-β-galactosyltransferase (MGDG synthase, EC 2.4.1.46) (2,3). This enzyme catalyzes the transfer of a galactose moiety from UDP-galactose to DAG. On the other hand, DGDG is thought to be synthesized mainly from two molecules of MGDG by DGDG synthase (4,5). However, recent studies of DGDG synthases have shown that another type of enzyme, which transfers the galactose moiety not from MGDG but from UDP-galactose, contributes mainly to DGDG biosynthesis (6,7).

A recent study identified three genes of MGDG synthase from *Arabidopsis thaliana*: *atMGD1*, *atMGD2,* and *atMGD3* (8). Whereas *atMGD1* is predominantly expressed in all the organs examined, the other two isogenes are expressed specifically in nonphotosynthetic organs. In particular, *atMGD2* shows floral-organ-specific expression. This result suggests potential roles for galactolipids in floral organs. However, limited information on galactolipid contents (9,10) and their biosynthetic activities (11) is available for flowers or specific floral organs. Furthermore, simultaneous analyses of galactolipid content and biosynthetic activities in floral organs have not yet been conducted, primarily because galactolipids have been considered to function mainly in photosynthetic membranes, although nongreen plastids also exist in nonphotosynthetic tissues.

In this report, we carried out analytical studies with *Petunia hybrida* to characterize the glycolipid content and biosynthesis of floral organs. On the basis of detailed analytical data, we conclude that DGDG is a major glycolipid in floral organs.

EXPERIMENTAL PROCEDURES

Plant material. Petunia hybrida was grown in soil. Flowers were collected during the following three developmental stages: Stage 1, light green buds; Stage 2, flowering buds; and Stage 3, completely opened and pollinated flowers.

Extraction and analysis of lipids. Total lipids were extracted according to the standard procedure of Bligh and Dyer (12). Samples were dissolved in chloroform/methanol (2:1, vol/vol) and stored at –20° C until use. These lipids were then separated by 2-D TLC using chloroform/methanol/7 N ammonia (120:80:8, by vol) in the first dimension and chloroform/methanol/acetic acid/water (170:20:15:3, by vol) in the second dimension. Each spot was identified with 0.01% primuline (in 80% acetone) under UV light and classified as phospholipid, glycolipid, or sterylglycoside (SG) with the Dittmer reagent (phospholipid-specific indicator) (13), the anthrone reagent (glycolipid-specific indicator) (14), or the Zatkis reagent (indicator for cholesterol and other sterol lipids) (15).

Each lipid was collected separately from TLC plates. To obtain FAME derived from each lipid, the extracted lipids were hydrolyzed and methyl-esterified together with 100 μL

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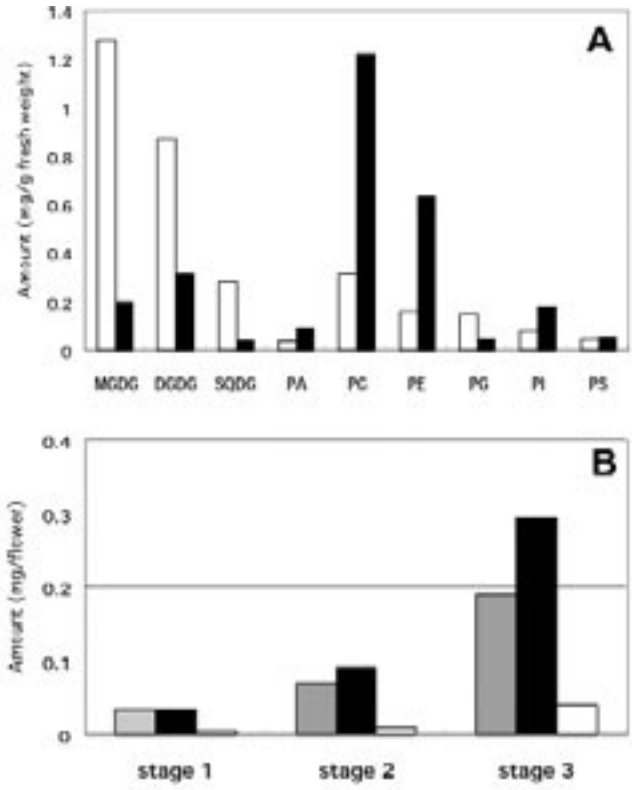
Abbreviations: 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; *atMGD, Arabidopsis thaliana* MGDG synthase; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; SG, sterylglycoside; SQDG, sulfoquinovosyldiacylglycerol; TGDG, trigalactosyldiacylglycerol.

of 1 mM pentadecanoic acid (internal standard) by 5% HCl in methanol at 85°C for 150 min. Finally, FAME were analyzed with GC (GC14 gas chromatograph; Shimadzu, Kyoto, Japan; ULBON HR-SS-10 capillary column, 25 m × 0.25 mm; Shinwa Chemical Industries, Kyoto, Japan) to determine the FA composition and calculate the amount of each lipid.

The amount of SG was determined spectrophotochemically according to the anthrone–sulfuric acid method of Radin *et al.* (14). NMR spectroscopic analysis for SG was carried out by the method of Tian *et al.* (16).

Glycosyltransferase activity assay. The crude enzyme (30 µL) was extracted from *P. hybrida* with MSPB buffer [50 mM MOPS–NaOH (pH 7.9), 10 mM DTT, 800 mM sodium acetate, 1 mM PMSF, 20% glycerol, 0.02% NaN_3, and 10 µM leupeptin] and mixed with 110 µL of MOD buffer [100 mM MOPS–NaOH (pH 7.8) and 3 mM DTT] and 50 µL of DAG [1,2-dioleoyl-*sn*-glycerol (200 µg; dispersed in 0.01% (wt/vol) Tween-20] or 0.01% (wt/vol) Tween-20. A reaction mixture with 10 µL of 8 mM UDP-[^{14}C]galactose (308.3 Bq/nmol) or UDP-[^{14}C]glucose (308.3 Bq/nmol) (200 µL in final volume) was incubated at 30°C for 30 min. The reaction was stopped by vigorous vortexing with 1 mL of ethyl acetate, and the mixture was then centrifuged twice at 1500 × *g* for 5 min with 0.5 mL of 0.45% (wt/vol) NaCl. The upper layer (900 µL) was dried and dissolved in 100 µL of chloroform/methanol (2:1, vol/vol). The product solutions were analyzed by 1-D TLC using chloroform/methanol/water (65:20:2, by vol), and radioactive spots were detected by Image Plate (Fuji Photofilm, Tokyo, Japan) and Image Analyzer (Storm; Amersham Biosciences, Piscataway, NJ). Total protein content in the crude enzyme solution was quantified by the method of Bensadoun and Weinstein (17) with BSA as a standard.



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	Stage 1	Stage 2	Stage 3
Fresh weight (g)	0.10	0.33	0.81
MGDG/f.w.	0.33	0.21	0.23
DGDG/f.w.	0.33	0.27	0.36
DGDG/MGDG	1.00	1.29	1.57

FIG. 1. Lipid analysis of leaves and flowers of *Petunia hybrida*. (A) The amount of total polar lipids per gram fresh weight was compared between leaves and flowers. Total lipids were extracted as described in the Experimental Procedures section. The extract was separated with 2-D TLC, and each lipid was hydrolyzed to detach the acyl moiety. The amount of each lipid was calculated by the amount of FAME as analyzed with GC. White bars, lipids from leaves; black bars, lipids from flowers. (B) Changes in glycolipid contents during flower development. Gray bars, MGDG; black bars, DGDG; white bars, SQDG. (C) Ratios of MGDG to fresh weight, DGDG to fresh weight, and DGDG to MGDG (by weight) in each developmental stage. Results are representative of four independent experiments. f.w., fresh weight; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol.

RESULTS

Lipid analysis of flowers and their developmental changes. Lipid analysis of both fully opened flowers (Stage 3, see the Experimental Procedures section) and leaves of *P. hybrida* was carried out. As shown in Figure 1A, leaves contained a large amount of glycolipids, with MGDG as the most abundant membrane lipid and DGDG as the second-largest component in leaves. In flowers, by contrast, the contents of three plastid glycolipids, MGDG, DGDG, and sulfoquinovosyldiacylglycerol (SQDG), were low whereas the contents of phospholipids, especially PC and PE, were higher. Since these phospholipids are major components of the endoplasmic reticulum and cytoplasmic membrane, the low amount of glycolipids is likely due to the decreased number of chloroplasts in flowers. However, the amount of DGDG was relatively high and exceeded that of MGDG in flowers. Since the ratio of MGDG to DGDG is generally around 2:1 (18), this galactolipid content was distinct.

To elucidate how this unique glycolipid composition was established, the changes in glycolipid content during flower development were analyzed. As flowers developed, the total amount of glycolipids per flower increased with a concomitant increase in the fresh weight of the flower. In particular, the amount of DGDG increased so much as to exceed that of MGDG in later developmental stages (Fig. 1B). Moreover, the ratio of DGDG to fresh weight increased during development while that of MGDG to fresh weight decreased, resulting in an increase in the ratio of DGDG to MGDG (Fig. 1C).

We next analyzed the glycolipid content of petals, stamens, and pistils of flowers in Stage 3 (Fig. 2). Except for stamens, DGDG was the major glycolipid in floral organs. When the size and fresh weight of each floral organ in the developing

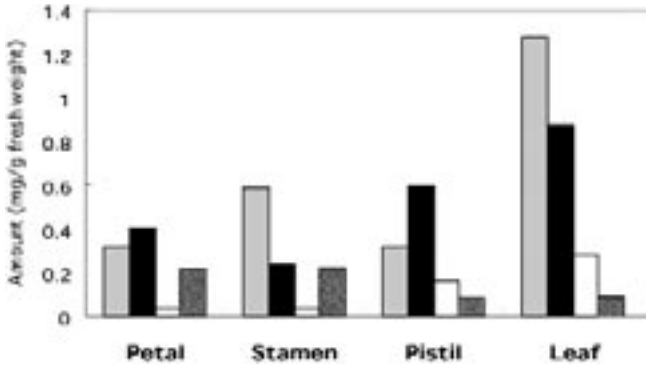

FIG. 2. Comparison of the lipid composition in four organs of *P. hybrida*. The amount of each lipid was quantitatively calculated as described in Figure 1. Results are representative of four independent experiments. Gray bars, MGDG; black bars, DGDG; white bars, SQDG; dark gray bars, SG. SG, sterylglycoside; for other abbreviations see Figure 1.

flowers were taken into account, the increase in DGDG during flower development was mainly attributed to the DGDG content in the petals. It should be noted that, among the three organs, the highest amount of glycolipid per gram fresh weight was found in the pistils. In pistils, the amounts of DGDG and SQDG were much closer to those in leaves, whereas the amount of MGDG was lower.

Comparison of FA composition of lipids from leaf and floral organs. We next compared the FA composition of major glycolipids in leaves and flowers (Table 1). In leaves, MGDG and DGDG contained mostly linolenic acid (18:3), whereas SQDG contained palmitic acid (16:0) as a major FA component. In flowers, however, the mole percentage of 18:3 was lower, and those of 16:0 and linoleic acid (18:2) were higher in comparison with leaves. Furthermore, during flower development, the mole percentage of 18:3 decreased, whereas those of 18:2 and 16:0 increased in all glycolipids.

TABLE 1
Developmental Change of FA Composition (mol%) of Galactolipids from *Petunia hybrida*

FA		16:0	16:1	18:0	18:1	18:2	18:3
MGDG							
Leaf		4.3	1.1	1.0	0.5	9.2	83.9
Flower	Stage 1	10.7	0.7	3.8	2.3	11.0	71.5
	Stage 2	14.8	1.2	5.3	3.8	18.9	56.0
	Stage 3	18.0	1.1	5.0	2.7	25.6	47.6
DGDG							
Leaf		19.6	1.8	3.3	0.9	8.6	65.8
Flower	Stage 1	20.0	1.1	7.8	2.3	9.0	59.8
	Stage 2	23.8	1.1	8.6	2.7	16.7	47.1
	Stage 3	26.3	0.7	8.9	2.1	25.0	37.0
SQDG							
Leaf		49.7	2.2	3.5	1.7	5.8	36.2
Flower	Stage 1	36.4	1.6	12.7	3.8	7.1	38.4
	Stage 2	37.5	4.7	12.1	5.5	8.8	31.4
	Stage 3	41.8	1.5	11.6	3.6	12.3	29.2

[a]MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol.

The FA composition of each glycolipid in the three floral organs was analyzed further (Table 2). Petals showed a glycolipid composition very similar to that of whole flowers at Stage 3, supporting the assumption that the petal is the major contributor of lipid contents in flowers. In stamens and pistils, DGDG and SQDG both showed compositions similar to those in leaves. Concerning MGDG, however, stamens showed a FA composition quite distinct from other organs, containing only 1.7% of 18:3 in contrast to 66.3% of 16:0 and 18.7% of oleic acid (18:1).

Glycolipid synthetic activity in floral organs. The glycolipid biosynthetic activity was assayed to investigate the developmental changes and organ specificity of glycolipid synthesis in flowers. During flower development, production of MGDG and DGDG increased transiently and reached the maximum at Stage 2 (Fig. 3). In addition, the biosynthetic activity of SG, a sterol-containing glycolipid that is known to be rich in nonphotosynthetic organs, also increased.

Among floral organs, the highest MGDG synthetic activity was observed in pistils. The specific activity of the labeled UDP-galactose incorporated into MGDG was estimated to be more than twice that of leaves, which are the major galactolipid-synthesizing tissues in plants (Fig. 4). This activity was observed even without an exogenous supply of DAG as substrate. This indicates the existence of a DAG pool in pistils, since this activity was not significant in other organs. The assay further revealed that the galactose moiety was incorporated to yield DGDG in petals and pistils. It occurred without exogenous addition of DAG, as seen in its incorporation into MGDG in pistils. Furthermore, in petals, incorporation of the galactose moiety into DGDG was higher than that of MGDG without adding DAG. This incorporation of galactose into DGDG did not increase with the addition of DAG, although MGDG synthesis was significantly enhanced under the same condition. These results suggest that in petals and pistils, the pathway to produce radiolabeled DGDG did not depend on MGDG synthesis.

TABLE 2
FA Composition (mol%) of Lipids from Leaves, Petals, Stamens, and Pistils of *P. hybrida*[a]

FA	16:0	16:1	18:0	18:1	18:2	18:3
MGDG						
Leaf	4.3	1.1	1.0	0.5	9.2	83.9
Petal	12.8	ND	5.9	2.3	22.6	56.5
Stamen	66.3	2.2	7.8	18.7	3.2	1.7
Pistil	9.8	ND	5.4	3.0	18.5	63.3
DGDG						
Leaf	19.6	1.8	3.3	0.9	8.6	65.8
Petal	27.9	ND	10.7	2.4	21.9	36.9
Stamen	32.5	ND	10.7	3.7	21.1	32.0
Pistil	18.4	ND	12.5	3.1	14.5	51.6
SQDG						
Leaf	49.7	2.2	3.5	1.7	5.8	36.2
Petal	40.4	ND	13.5	0.6	21.4	24.1
Stamen	48.3	ND	15.0	ND	12.7	24.1
Pistil	48.4	ND	16.4	3.8	12.7	18.8

[a]ND, not detected; for other abbreviations see Table 1.

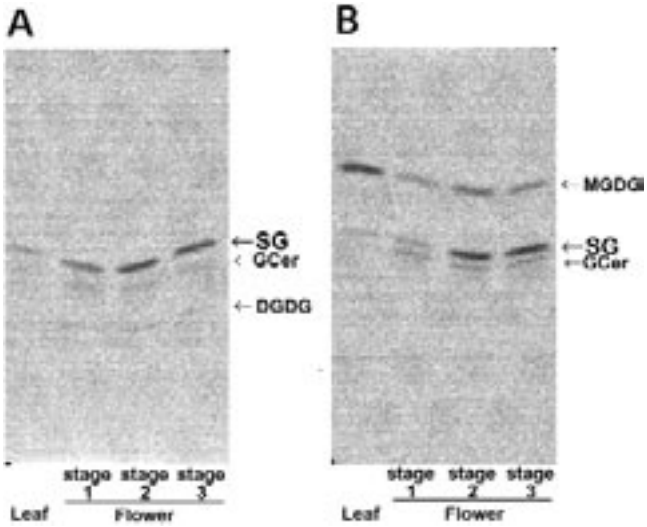

FIG. 3. Developmental changes in galactose incorporation activity in flowers. Total protein was extracted from each sample as described in the Experimental Procedures section. The extracts were incubated with [^{14}C]UDP-galactose for 30 min. Reaction mixtures were analyzed with TLC and radioactive products were detected with autoradiography. (A) Reaction products without exogenous addition of DAG. (B) Reaction products with exogenous addition of DAG. Results are representative of four independent experiments. GCer, glycosylceramide; for other abbreviations see Figures 1 and 2.

The biosynthetic activity of SG synthesis was highest in pistils and second-highest in petals, whereas the amount was high in petals and stamens. This is probably because the sterol glucosyltransferase is located differently from its activated sugar substrate *in vivo*. As in the case of MGDG and DGDG, radiolabeled galactose was also highly incorporated into SG in the enzyme assay. However, when UDP-glucose was added to the reaction mixture instead of UDP-galactose, the incorporation of radioactivity into SG became much higher, whereas incorporation into MGDG and DGDG was quite low (Fig. 5). Indeed, the chemical structure for SG from the petal of *P. hybrida* was determined with NMR to be fucosterol-3-*O*-β-glucopyranoside (data not shown). Therefore, it is possible that UDP-glucose 4-epimerase was active in the enzyme extract, converting UDP-galactose to UDP-glucose during the reaction. These results, together with the report of UDP-glucose:sterol glucosyltransferase in SG synthesis (19), indicate that glucose is used mainly as a sugar moiety in synthesizing the SG detected in the petals of *P. hybrida*.

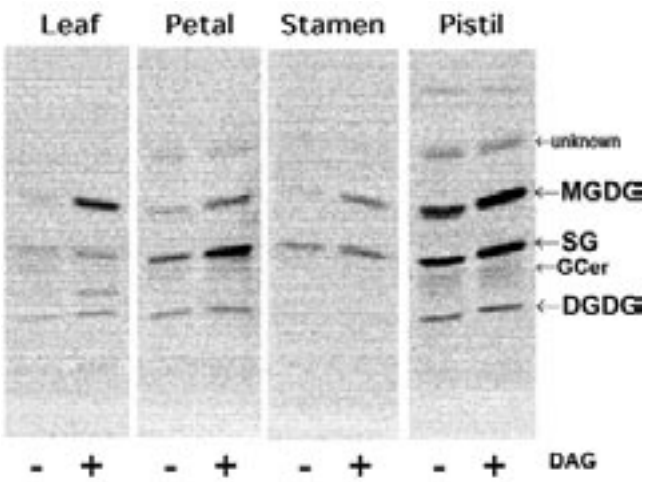

FIG. 4. Comparison of galactose incorporation activity among four organs: leaves, petals, stamens, and pistils. (–) Reaction products incubated without exogenous addition of DAG; (+) reaction products incubated with exogenous addition of DAG. Results are representative of four independent experiments. For abbreviations see Figures 1–3.

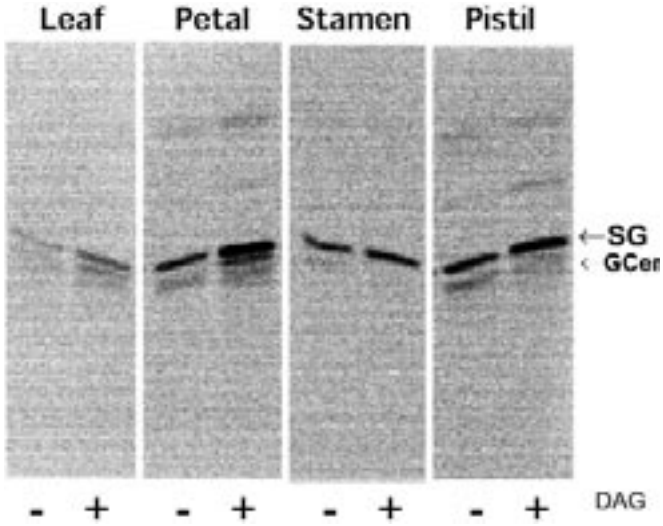

FIG. 5. Comparison of glucose incorporation activity among four organs; leaves, petals, stamens, and pistils. See Figures 2 and 3 for abbreviations used in the figure. Results are representative of four independent experiments.

DISCUSSION

MGDG and DGDG are major components of photosynthetic membranes, and one molecule of MGDG binds to the reaction center of the cyanobacterial photosystem I complex (20). The *mgd1* mutant, which has reduced expression of *atMGD1*, revealed a severe deficiency in chloroplast development (21), supporting the report that *atMGD1* plays a major role in photosynthetic organs (3,8). Furthermore, the mutant of *DGD1*, which carries a defect in the enzyme for bulk synthesis of DGDG, also shows an abnormal phenotype in chloroplast development (6,22). Thus, these galactolipids are considered to be important for the functional assembly of photosynthetic membranes. The present results show that the amounts and biosynthetic activities of MGDG and DGDG in flowers are increased during flower development. This suggests a novel function of these galactolipids, not only in photosynthetic tissues but also in nonphotosynthetic tissues.

A series of analyses of three floral organs showed that pistils and petals had distinct contents and biosynthetic activities of glycolipids, particularly DGDG. The highest glycolipid biosynthetic activity was detected in pistils. Interestingly, the

specific activity of glycolipid biosynthesis in pistils was even higher than that of leaves, a major site for galactolipid biosynthesis. In fact, pistils contained higher amounts of galactolipids than any other floral organ, and the amount of DGDG exceeded that of MGDG. Galactose incorporation assays using UDP-galactose revealed that MGDG was a major galactosylation product in leaves, since MGDG synthesis is a primary step in galactolipid synthesis and its activity is higher than that for DGDG in this organ. In petals, however, DGDG synthetic activity was higher than that of leaves in the presence of exogenous DAG. Furthermore, the activity was detectable even without an exogenous supply of DAG in petals. Like pistils, the amount of DGDG also exceeded that of MGDG in petals. To explain such high DGDG-producing activity in these organs, there must be activation of DGDG synthase(s). As mentioned in the introduction, two pathways for DGDG synthesis have been reported thus far—UDP-galactose-dependent and UDP-galactose-independent (23,24). In the UDP-galactose-independent pathway, two molecules of MGDG are used to synthesize DGDG. If the enzyme for this pathway is activated, there should be accumulation of trigalactosyldiacylglycerol (TGDG), because this enzyme has been shown to be a processive galactosyltransferase that can transfer galactose moieties to DGDG as well as MGDG, yielding oligogalactosyldiacylglycerols such as TGDG (24). In all organs analyzed in this paper, however, we did not observe the accumulation of TGDG from either the radioisotope-labeling experiment or total lipid extraction, suggesting activation of the other type of DGDG synthase, i.e., that requiring UDP-galactose. Activation of a UDP-galactose-dependent DGDG synthase fits our results better for the following reasons: First, in Arabidopsis, this type of DGDG synthase gene is reported to be induced by phosphate deprivation (7). This condition also induces the *atMGD2* that is specifically expressed in floral organs (8). Second, the unique FA composition of DGDG that accumulates in phosphate-limiting conditions (25) was very similar to that observed in floral organs of *P. hybrida*. DGDG was enriched in 16:0 and deficient in 18:3 when compared with that from leaves. Therefore, it is likely that a UDP-galactose-dependent type of DGDG synthase is also expressed in floral organs of *P. hybrida,* and cooperates with an *atMGD2*-like MGDG synthase to activate a galactolipid synthesis pathway similar to that induced during phosphate deprivation. However, it remains to be clarified whether UDP-galactose-dependent DGDG synthase is activated in the floral organs.

Regarding the subcellular site of DGDG accumulation, biophysical analysis suggests that DGDG is capable of forming a lipid bilayer by itself, whereas MGDG is not (18). This report predicts that an approximate ratio of MGDG/DGDG of 2:1 is necessary to maintain the membrane and its function. Consistent with this point, overexpression of MGD in recombinant *Escherichia coli* resulted in abnormal accumulation of MGDG that changed cell morphology, such as cell elongation and deficiency in cell division (26). In flowers, the plastid is differentiated as a chromoplast, which has lipid contents similar to those in the chloroplast envelope membrane (9). Therefore, the abundance of chromoplasts can be corroborated by the amount of MGDG and SQDG, because these glycolipids are known to exist only in plastids. The present results showed that the amount of MGDG and SQDG in flowers was lower than that in leaves, suggesting that the abundance of chromoplasts in flowers is lower than that in leaves. Nevertheless, an excess amount of DGDG was detected in pistils and petals. Recently, extraplastidic accumulation of DGDG was detected in *A. thaliana* under phosphate-limiting conditions (25). DGDG also can replace most of the phospholipids in oats and become a major constituent of the plasma membrane (27). This suggests that extraplastidic accumulation of DGDG might occur in pistils and petals during flower development of *P. hybrida*.

Plants must develop floral organs and seeds for the next generation even during phosphate limitation. For this purpose, plants may conserve phosphates by utilizing galactolipids to construct their membrane systems in reproductive tissues. Alternatively, DGDG may have distinct functions for flower development. A more detailed analysis of galactolipid synthesis in floral organs is required for further elucidation of their potential roles.

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Which of the n-3 PUFA Should Be Called Essential?

Sir:

We refer to the letter published in the August 2003 issue of *Lipids* by Lauritzen and Hansen (1). The letter raises important issues discussed by many in this field, but we believe the scope of the letter goes beyond EFA. We can see the merits in their definition of an essential nutrient ("A food component that directly, or *via* conversion, serves an essential biological function and which is not produced endogenously or in large enough amounts to cover the requirements"). Lauritzen and Hansen suggest that "the essentiality of n-3 PUFA seems to be due to a specific function of 22:6n-3 in membranes and neuronal tissue" (1).

We would like to raise the issue of whether 22:6n-3 is the only n-3 PUFA that has biological functions in mammals. We recognize that the literature on the role of 22:6n-3 in various neural functions is abundant (for a review, see Ref. 2) and that few studies address specific roles of the other n-3 PUFA. However, readers' attention should be drawn to several papers that show that 18:4n-3, 20:4n-3, 20:5n-3, and 22:5n-3 can influence eicosanoid synthesis (3,4), endothelial cell function (5), cytokine production (6), platelet aggregation (7), and platelet function (8). Insofar as it is possible to say the effects are due to the administered n-3 PUFA, it would seem we should not ignore these effects and the possibility that the "other" n-3 PUFA might have essential, unique roles in mammals. Perhaps of greater interest is whether 18:3n-3 itself has any specific functions in mammals other than that of being a precursor of 22:6n-3. There is much discussion about the putative role of 18:3n-3 as a source of carbon for brain cholesterol and brain saturated and monounsaturated FA (9); however, this property appears to be shared with other PUFA (10). It is thought that 18:3n-3 rarely accumulates in mammalian tissues, but we have shown in guinea pigs that high levels of 18:3n-3 do accumulate if the diet contains 18:3n-3, with >250 times more 18:3n-3 being distributed throughout the body (all tissues) than all the other n-3 PUFA together, including 22:6n-3 (11).

Finally, we suggest that researchers should take heed of the plant world, where 18:3n-3 is involved in a number of important processes, including (i) being the precursor of jasmonic acid, a plant growth regulator (12), (ii) being a lipoxygenase substrate producing hydroperoxy FA that mediate defense responses against injury or infection (13), (iii) being the substrate for phytoprostanes (dinor isoprostanes) found in high concentration during drying and storage of plants and in pollen (14), (iv) being the precursor of a stress-induced α-ketol derivative of 18:3n-3 that has strong flower-inducing activity (15), and (v) being a potent inhibitor of a wound-induced mitogen-activated protein kinase (16). Since several different lipoxygenases are found in various mammalian tissues (17), we should not discount 18:3n-3 as a lipoxygenase substrate, with the possibility that the hydroxy-FA product(s) might have significant biological properties in mammals (18).

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Serum and Aortic Levels of Phytosterols in Rabbits Fed Sitosterol or Sitostanol Ester Preparations

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ABSTRACT: Campesterol is present in all the phytosterol-containing dietary hypocholesterolemic agents in current use. Campesterol is absorbed more efficiently than sitosterol, and the question of its possible atherogenicity has been raised. To test this possibility, rabbits were fed either a semipurified, cholesterol-free diet that has been shown to be atherogenic for this species or the same diet augmented with 0.5 g of phytosterol-rich diet preparations (spreads) containing either sitosterol or sitostanol. The diets contained 295 mg phytosterol per 100 g. After 60 d, serum cholesterol levels in the two phytosterol groups were 78 ± 4 mg/dL (sitosterol) and 76 ± 4 mg/dL (sitostanol), respectively. The serum cholesterol level of rabbits fed the control diet was 105 ± 8 mg/dL. Serum campesterol (μg/mL) levels were higher than sitosterol or sitostanol levels in all groups. Aortic phytosterols were present in nanogram quantities compared to cholesterol, which was present in microgram quantities. The ratio of campesterol/sitosterol/sitostanol in the aortas was: control, 1.00:0.43:0.02; sitosterol, 1:00:0.32:0.01; sitostanol, 1:00:0.34:0.11. Aortic campesterol was present at 4% the concentration of aortic cholesterol, sitosterol at 1.4%, and sitostanol at 0.14%. Aortic lesions were not present in any of the animals.

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Treatment of hypercholesterolemia by administration of sitosterol was achieved in animals in 1951 (1) and in humans in 1953 (2). The following years saw the establishment of plant sterols as a treatment for hypercholesterolemia in humans. That era has been described fully (3). The principal modality of treatment was a commercial preparation of plant sterols that had been rigorously tested for safety (4).

The early preparations were known to contain phytosterols other than β-sitosterol, principally campesterol, and concern was expressed regarding the possible effects of absorption of these companions of β-sitosterol (5,6). Lees and Lees (5) characterized sitosterol as fulfilling the criteria of an ideal hypocholesterolemic drug (free of subjective side effects and of objective toxicity), but they expressed unease regarding the possible atherogenic effects of campesterol and other plant sterols.

New and effective hypocholesterolemic preparations of sitosterol (7) and sitostanol (8) esters still contain appreciable amounts of other phytosterols, and the question of their possible atherogenicity has persisted. We fed preparations of phytosterol esters to normocholesterolemic rabbits and analyzed their arteries for evidence of phytosterol deposition. Our findings are the basis of this report.

MATERIALS AND METHODS

Twenty-four male New Zealand White rabbits were randomized into three groups of eight rabbits each. The average starting weight of each group was 2,586 g. The animals were individually caged in stainless steel cages and allowed free access to food and water. They were maintained in a temperature- and humidity-controlled room with 12-h on–off light cycles and fed pelleted diets as described in Table 1. The diets were prepared to our specifications by Dyets, Inc. (Bethlehem, PA). The phytosterol ester preparations were provided by the nutritional group of McNeil Consumer Healthcare (Fort Washington, PA). They were products of interesterification of the specific phytosterols with rapeseed oil; their characteristics are described in Table 2. The amount of ester product added to the diet (0.50 g/100 g diet) provided equal amounts of phytosterol (0.295 g sterol/100 g diet). The rabbits ingested a 90–100 g diet daily; thus, they were provided with 0.266–0.295 g of sterol daily. All animal procedures were approved by the Wistar Institutional Animal Care Use Committee (IACUC).

After 60 d, rabbits were bled and then euthanized with ketamine/xylazine (10:3). Serum cholesterol was determined using a commercial kit (Sigma, St. Louis, MO). The aortas were dissected, cleaned, and examined visually for atherosclerotic involvement (9). Aortas were pooled, minced, and

TABLE 1
Semipurified Diet[a]

	g/100 g	% Calories
Casein	25.00	26.0
DL-Methionine	0.20	
Sucrose	19.68	20.5
Starch	20.00	20.8
Coconut oil	13.00	30.4
Corn oil	1.00	2.3
Cellulose	15.00	
Mineral mix	5.00	
Vitamin mix	1.00	
Choline bitartrate	0.12	
	100.00	100.00

[a]Phytosterol preparations (0.5 g/100 g) added at the expense of sucrose increase total calories by 0.65%.

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TABLE 2
Composition of Phytosterol-Rich Fats[a]

	%
Sterol ester	
Free sterols and stanols	0.70
Total sterols and stanols	58.60
Esterified rapeseed oil FA	40.63
FFA	0.04
Moisture	0.03
Stanol ester	
Free sterols and stanols	0.40
Total stanols	59.20
Total sterols	0.70
Stanes/stenes	0.02
Esterified rapeseed oil FA	39.70
FFA	0.02
Moisture	0.00

[a]Provider's analysis.

extracted with chloroform/methanol (2:1) (10). The organic layer was dried over anhydrous Na_2SO_4 and saponified in 10% alcoholic KOH. The sterol fraction was extracted with chloroform. The extracts were then dried over anhydrous Na_2SO_4 and taken to dryness under a stream of nitrogen. The sera and the aortic nonsaponifiables were analyzed for sterols by GC–MS using the following procedure.

Fifty microliters of serum was added to a test tube. Next, 1.0 mL of a freshly prepared mixture consisting of 6 mL of 33% aqueous KOH and 94 mL of 96% ethanol was added. The sample was mixed and immediately incubated at 56°C for 15 min. The sample was allowed to cool to room temperature, then 1 mL of water and 2 mL of hexane were added for extraction. The internal standard of 5α-cholestane was added to the hexane layer for 30 s, then centrifuged for 5 min at 3200 × *g*. The hexane layer was removed under N_2, and trimethylsilyl (TMS) ethers were prepared by adding Tri-Sil and heating at 65°C for 30 min. Once derivatized, the sample was dried under N_2 and reconstituted in hexane for analysis by GC–MS. The dried aortic nonsaponifiable fractions were dissolved in 0.5 mL hexane. Fifty microliters of the hexane solution was derivatized and analyzed by GC–MS.

GC–MS conditions. Chromatographic separation was achieved on a DB-1 fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific Inc., Folsom, CA) using helium as the carrier gas (flow rate approximately 2 mL/min), with a temperature program of 250–310°C at increments of 10°C/min. Selective ion monitoring GC–MS of specific and characteristic ions in the electron ionization (70 eV) spectra of the TMS ethers of each compound permitted highly sensitive and specific quantification. The following ions were monitored: *m/z* 357 (5α-cholestane and sitosterol) and 383 (sitostanol).

Statistical analysis. ANOVA was determined using a general linear models procedure (SAS PROC GLM-SAS Software, Cary, NC). The Student–Newman–Keuls multiple comparisons test was used to determine which of the treatments significantly affected the dependent variables.

RESULTS

Necropsy results are summarized in Table 3. Rabbits fed the control diet gained more weight than the test groups, but the differences were not significant. The two phytosterol treatments yielded virtually identical serum cholesterol levels, 78 ± 4 mg/dL for the sitosterol group and 76 ± 4 mg/dL for the sitostanol group. Both values were significantly lower than the control. No visible lesions were present in the aortas of any of the animals.

The amounts of cholesterol and phytosterol recovered from the plasma samples are shown in Table 4. The values are presented as micrograms of sterol per deciliter of serum to emphasize the small amounts of phytosterols present in the plasma. Levels of sitosterol and campesterol were significantly elevated in the sera of the sitosterol-fed animals, whereas the sera of the sitostanol-fed animals contained more sitostanol than did the sera of the other two groups.

The average weights of the pooled aortas were similar. The values obtained upon chromatography were corrected for the aliquot taken for chromatography (0.05 of 0.50 mL) and divided by 8 to give the phytosterol content per aorta. By a very large margin, the major sterol recovered from the aortas was cholesterol (Fig. 1). The amounts of phytosterol recovered from the arterial extracts (Fig. 2) are given in Table 5 and reflect the dietary regimens. The phytosterol areas are magnified in Figure 2, and the large cholesterol peak is deleted. These chromatograms (A, control; B, sitosterol group; C, sitostanol group) clearly illustrate the differences among the phytosterols. Cholestanol was present in the arteries of all three groups. The ratios of cholesterol/cholestanol were: control rabbits, 18.0; sitosterol-fed rabbits, 23.0; and sitostanol-fed rabbits, 23.4.

It is noteworthy that sitosterol, campesterol, and sitostanol persisted in the sera and aortas of the control rabbits, which had access to the pelleted, semipurified diet for 60 d prior to necropsy. The control diet contained no phytosterols, so the amounts recovered from the sera and aortas presumably reflected a very slow turnover time.

DISCUSSION

The question of possible atherogenicity of campesterol has been articulated (5,6). This study was designed to test the

TABLE 3
Necropsy Data: Rabbits (8/group) Fed Sitosterol or Sitostanol Esters for 60 d[a]

	Regimen			
	Control	Sitosterol ester	Sitostanol ester	ANOVA *P*-value
Weight gain (g)	284 ± 88	141 ± 67	130 ± 58	NS
Serum cholesterol (mg/dL)	105 ± 8[a,b]	78 ± 4[a]	76 ± 4[b]	0.005
Aortic nonsaponifiables (mg)	0.65 ± 0.02	0.61 ± 0.03	0.64 ± 0.04	NS
Aortic plaques	0/8	0/8	0/8	NS

[a]Data ± SEM. Values in the horizontal row bearing the same superscript letter are significantly different. *Post hoc* multiple comparisons were conducted using the Student–Newman–Keuls multiple range test. NS, not significant.

TABLE 4
Serum Phytosterol Content of Rabbits (8/group) Fed Sitosterol or Sitostanol Esters[a]

	Group			ANOVA
	Control	Sitosterol ester	Sitostanol ester	*P* value
Sitosterol (μg/dL)	9.49 ± 1.30[a]	16.17 ± 2.45[a,b]	6.65 ± 0.79[b]	0.0019
Campesterol (μg/dL)	15.56 ± 1.98[a]	45.19 ± 6.56[a,b]	15.07 ± 2.33[b]	0.0001
Sitostanol (μg/dL)	0.41 ± 0.05[a]	0.29 ± 0.06[b]	1.13 ± 0.07[a,b]	0.0001

[a]Data ± SEM. Values in horizontal rows bearing the same letter are significantly different. *Post hoc* multiple comparisons were conducted using the Student–Newman–Keuls multiple range test.

possibility that an appreciable amount of dietary phytosterols might be deposited in arterial tissue. We chose as the vehicle a cholesterol-free diet that has been shown to be atherogenic for rabbits (11,12). As a source of phytosterols, we used preparations rich in sitosterol or sitostanol esters that resembled commercial margarines available for their hypocholesterolemic properties. This permitted us to examine the effects of phytosterols in a cholesterol-free regimen. Addition of these preparations to an atherogenic, cholesterol-rich diet would be pointless since phytosterols have been shown to inhibit significantly the deposition of atherosclerotic plaques in the aortas of rabbits (13) and chickens (14). Further resolution of this question may have to wait until pure (>90%) campesterol is available. Our data showed that diets containing sitosterol or sitostanol had identical hypocholesterolemic effects in rabbits fed a cholesterol-free, saturated fat-rich, semipurified diet. The two phytosterols were absorbed to a small extent, with sitosterol being absorbed to a greater extent than sitostanol. The accompanying campesterol was also absorbed.

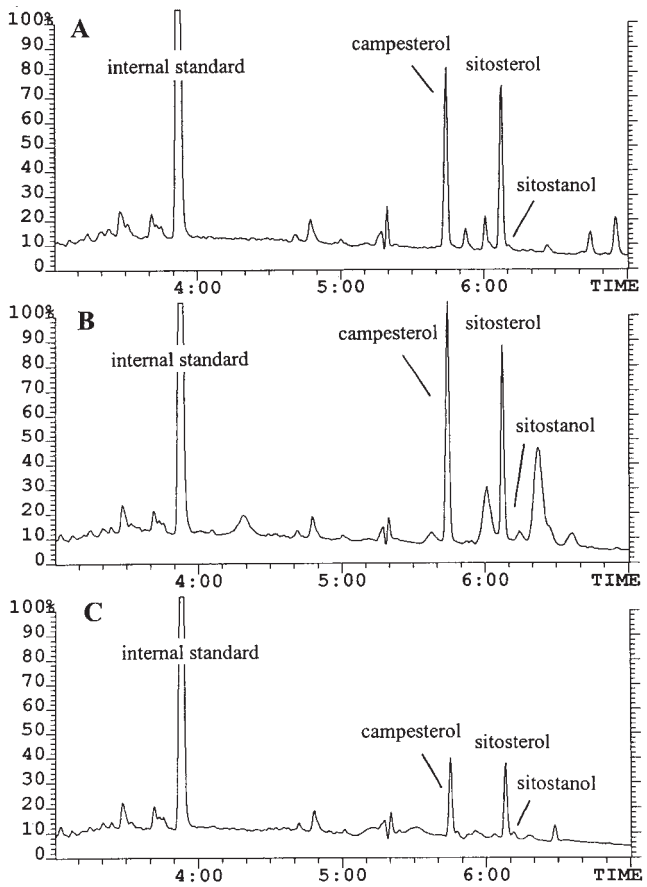

FIG. 1. GC profile of trimethylsilyl (TMS) ethers of sterols extracted from aortas of rabbits fed control, sterol ester-rich, or stanol ester-rich diets.

By several orders of magnitude, the principal aortic sterol was cholesterol, which was present in microgram quantities. Nanogram amounts of sitosterol, sitostanol, and campesterol also were present in the aortas, although no areas of atherosclerotic involvement were found in the aortas of any of the control or test animals.

Sterols other than cholesterol have been found in human aortic tissue. Hardegger *et al*. (15) analyzed a large mass of aortic tissue and found mostly cholesterol plus some oxidized derivatives of cholesterol. Their study was carried out before more sophisticated methods of analysis, such as GC, were available. Brooks *et al*. (16) reported recovery of 26-hydroxycholesterol, 7β- and 7α-hydroxycholesterol, and 24-hydroxycholesterol in human aortas, and Hodis *et al*. (17) also found cholesterol oxidation products in human aortas. Vaya *et al*. (18) found, in decreasing order of concentration, 27-hydroxycholesterol, 7β-hydroxycholesterol, β-epoxycholesterol, 7-ketocholesterol, α-epoxycholesterol, and 7α-hydroxycholesterol in human atherosclerotic lesions.

Cook *et al*. (19) fed rabbits (2–3 animals per group) 1% cholesterol, 7-dehydrocholesterol, lathosterol (Δ^7-cholestenol), or cholestanol for 13–25 d. Cholesterol and cholestanol feeding resulted in well-defined arterial plaques, whereas rabbits fed lathosterol or 7-dehydrocholesterol exhibited barely visible plaques in their arteries. We previously reported a study (20) in which rabbits were fed normal or atherogenic diets, half of which contained 0.2% triparanol. Triparanol inhibits cholesterol synthesis at one of the final steps in the synthetic cycle and results in accumulation of desmosterol (24-dehydrocholesterol). Desmosterol was recovered from normal aortic tissue of rabbits fed the control diet. When rabbits were fed an atherogenic diet, both normal and atherosclerotic aortas contained

TABLE 5
Aortic Phytosterol (ng/aorta) in Rabbits (8/group) Fed Sitosterol or Sitostanol for 60 d[a]

	Group		
	Control	Sitosterol ester	Sitostanol ester
Sitosterol	1319	1394	634
Campesterol	3088	4361	1849
Sitostanol	63	50	211

[a]Aortas pooled for analysis. Cholesterol content of aortas (μg): control, 94; sitosterol ester-fed, 69; sitostanol ester-fed, 71.

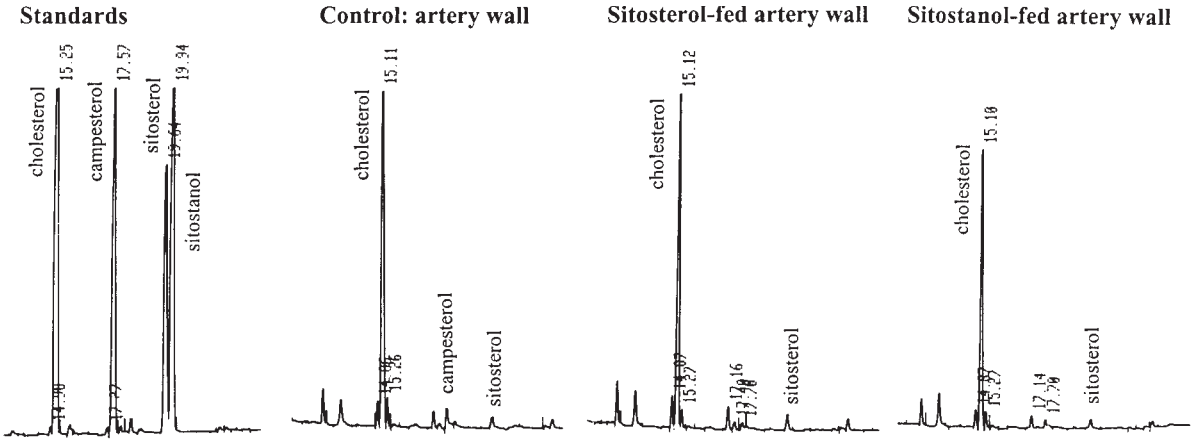

FIG. 2. GC profile of TMS ethers of phytosterols extracted from aortas of rabbits fed a control diet (A), sitosterol ester-rich diet (B), or sitostanol ester-rich diet (C). For abbreviation see Figure 1.

desmosterol. The ratio of cholesterol to desmosterol was 14:1 in normal aortas and 24:1 in involved tissue. Small quantities of cholestanol and coprostanol also were recovered from both normal and atherosclerotic aortas. No 25- or 26-hydroxycholesterol was recovered. Apparently, an unsubstituted 8-carbon side chain is necessary for incorporation of sterols into the arterial wall. Except for very special cases, our findings should dispel concerns regarding the atherogenicity of phytosterols.

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Comparison of the Effects of Dietary Sunflower Oil and Virgin Olive Oil on Rat Exocrine Pancreatic Secretion *in vivo*

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ABSTRACT: The aim of this study was to investigate the functional consequences *in vivo* of adapting the rat exocrine pancreas to different dietary fats. Weanling rats were fed diets containing 10 wt% virgin olive oil or sunflower oil for 8 wk. We then examined resting and cholecystokinin-octapeptide (CCK-8)-stimulated pancreatic secretion in the anesthetized animals. To confirm a direct influence of the type of fat upon the gland, the FA composition of pancreatic membranes as well as tissue protein and amylase content were determined in separate rats. The membrane FA profile was profoundly altered by the diets, reflecting the type of dietary fat given, although this was not paralleled by variations in the pancreatic content of protein or amylase. Nevertheless, dietary intake of oils evoked different effects on *in vivo* secretory activity. Resting flow rate and amylase output were significantly ($P < 0.05$) enhanced by sunflower oil feeding. Time course changes in response to CCK-8 infusion also showed a different pattern in each group. Secretion of fluid, protein, and amylase increased markedly in all animals, reaching a maximum within 20–40 min of infusion that was followed by a dramatic decline in both groups. In the sunflower oil group, this resulted in values reaching the resting level as soon as 60 min after CCK-8 infusion was begun. However, after the initial decline, olive oil group values showed a prolonged plateau elevation above the baseline ($P < 0.05$) that was maintained for at least the infusion time. In addition, a positive correlation between flow rate and both protein concentration and amylase activity existed in the olive oil group, but not in the sunflower oil group. The precise mechanism by which these effects are produced remains to be elucidated.

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Since the findings of Pavlov in early 1900, many authors have investigated the adaptation in various species of the exocrine pancreas to the type of food available. The consensus of these studies is that, for dietary components such as carbohydrates, protein, and lipids, there is a positive relationship between their level in the diet and the tissue content of pancreatic enzymes necessary for their breakdown (1–5). Concerning lipids, both the amount (1–4,6,7) and type (1–4,8–10) seem to have an influence, although a controversy over the effects of the FA composition of dietary fat on the adaptive process of pancreatic enzymes is unresolved (1–4,8–10). On the other hand, not only the enzyme content of the gland but also the overall secretory activity of the organ is susceptible to influence by the type of dietary fat, as we were able to confirm in earlier investigations (10,11) conducted in conscious dogs after long-term (8-mon) intake of diets that differed only in the fat source (olive oil or sunflower oil).

The mechanisms of the pancreatic adaptation to dietary fat are unclear. A first option involves the existence of hormonal mediators. In this respect, the gastrointestinal peptides secretin and cholecystokinin (CCK) have been considered, among others, as candidates based on the ability of ingested fat or fat hydrolysis products to stimulate their release (12–14) and because of their major role in the regulation of exocrine pancreatic secretion (12,15). A second possibility is that dietary fat composition may change the responsiveness of the pancreas to circulating secretagogues. In different tissues, there is evidence that the lipid profile of the diet can influence the FA composition of cell membranes, this being associated with a modification of cell function (16–18). Regarding the exocrine pancreas, information on this topic is very limited (9,19), although it supports the above view. In addition, the results of our recent study of pancreatic acinar cells isolated from rats fed for 8 wk with diets containing either virgin olive oil or sunflower oil suggest that the type of dietary fat can modulate not only the composition of pancreatic membranes but also *in vitro* amylase release and signal transduction evoked by CKK-octapeptide (CCK-8) (20).

The contribution of dietary lipids to human health is actively debated at present. For this reason, and because dietary fat intake is higher than recommended in many countries, it was pertinent to determine whether different types of lipids could affect exocrine pancreatic secretion differently in the anesthetized rat. It should be mentioned here that, although most adaptation studies have been conducted in this species, the most frequent methodological approach has consisted of analyzing of the enzyme content of the gland. To our knowledge, no one but Beaudoin *et al*. (8) has examined the effects of feeding different dietary fats on pancreatic responses in the rat by means of whole-animal preparation. The fats discussed by these authors are rarely used for cooking purposes in our geographical area, so we chose to compare the effects of virgin olive oil, a typical component of the Mediterranean diet and a good source of monounsaturated FA (MUFA), with those of sunflower oil, which is rich in PUFA. Both oils compete in

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Abbreviations: CCK, cholecystokinin; CCK-8, cholecystokinin-octapeptide; MUFA, monounsaturated FA; PYY, peptide YY; SFA, saturated FA.

southern European markets for consumers' preference. To achieve our objectives, two separate groups of weanling rats were fed the experimental diets for 8 wk. We then examined resting and CCK-8-stimulated exocrine pancreatic secretion in the anesthetized animal. The protein and amylase contents of the pancreas were also analyzed. Furthermore, to confirm a direct effect of the type of dietary fat, the FA composition of pancreatic membranes was determined after feeding the respective diets.

TABLE 1
FA Composition[a] of the Experimental Diets

FA	Virgin olive oil	Sunflower oil
16:0	11.44	7.31
16:1n-7	0.85	0.19
18:0	4.38	4.59
18:1n-9	74.88	32.62
18:2n-6	7.72	55.17
18:3n-3	0.62	0.10
SFA	15.82	11.90
MUFA	75.84	32.83
PUFA	8.34	55.27

[a]Data are given in percentages of total FA content (mean values of four replicates). SFA, saturated FA; MUFA, monounsaturated FA.

EXPERIMENTAL PROCEDURES

Animals and diets. Male weanling Wistar rats were obtained from the Animal Farm at the University of Granada in Spain. Upon arrival in the laboratory, they were weighed and randomized into two dietary groups of 24 animals each. The rats were fed over an 8-wk period with two semipurified, isoenergetic and isonitrogenous diets that were essentially AIN-93G diets (21) except that the total fat content was increased from 7 to 10 wt% at the expense of carbohydrate. The composition of the diets (wt%) was as follows: casein, 20; cornstarch, 36.7; dextrinized cornstarch, 13.2; sucrose, 10; cellulose, 5; fat, 10; L-cystine, 0.3; choline bitartrate, 0.25; AIN-93G mineral mixture, 3.5; AIN-93G vitamin mixture, 1.0. The two diets differed only in the nature of the fat source: virgin olive oil or sunflower oil (Table 1). Commercial edible oils were obtained locally (Fedeoliva, S.A., Jaén, Spain; Koipesol®, Koipe, S.A., Andújar, Jaén, Spain). The diets were prepared at the Nutrition Unit of the Animal Farm (University of Granada), packed in plastic bags, sealed, and sent to our laboratory, where they were stored at 4°C in the dark. During the feeding period, the animals were housed individually in a temperature-controlled room (22 ± 1°C) kept on a 12-h light/dark cycle and given free access to water and food; body weight was recorded weekly. Food intake was monitored daily in a subset of rats from both groups (n = 12 each). This was done by providing each rat with a weighed quantity of food and subtracting the amount of uneaten food 24 h later. All rats received fresh food daily. Experimental protocols were approved by the Ethics Committees of the University of Granada and the Spanish Ministry of Science. The animals were handled according to the guidelines for care and use of laboratory animals of the Spanish Society for Laboratory Animal Sciences and were killed humanely. All experiments were done at the end of the 8-wk feeding period.

Chemicals. All chemicals, solvents, and drugs, of the highest quality available, were obtained from Sigma (St. Louis, MO) and Merck (Darmstadt, Germany).

Surgical preparation. The procedure was described in detail elsewhere (22). Briefly, overnight-fasted rats (12 from each dietary group) were anesthetized with intraperitoneal urethane (1.3–2.0 g/kg body weight). After tracheal intubation, the jugular vein was catheterized for infusions. Following midline laparotomy, the stomach pylorus was ligated and the bile pancreatic duct exposed and cannulated at its entrance to the duodenum. The hepatic end of the bile duct was also cannulated, and bile was diverted to collect pure pancreatic juice. The animals were maintained under normothermic conditions throughout the study. Each animal was subjected to only one experiment.

Experimental design. Experiments were started in each rat after 45 min of flow stabilization. Pancreatic juice was collected on ice in preweighed capillary tubes at 20-min intervals for a total of 200 min. The first two 20-min samples were taken as resting values. This was followed by continuous infusion of CCK-8 ([Tyr(SO_3H)^{27}]-CCK fragment 26–33 amide; Sigma) for 100 (150 pmol/kg·h) min. Five 20-min samples were thus collected during the infusion. The experiment continued for an additional 60 min, allowing collection of three postinfusion samples. CCK-8 was freshly prepared in saline solution (NaCl 0.9 wt%) and infused at 2.0 mL/h by means of a syringe pump (B. Braun-Melsungen, Melsungen, Germany).

Measurement of pancreatic juice flow, total protein, and amylase output. The pancreatic flow rate was estimated by reweighing preweighed capillary tubes, and the volume of juice was calculated by assuming a density equal to water. All samples for total protein and amylase determinations were frozen immediately following collection, stored at −80°C, and assayed within a few weeks. Amylase activity in the juice was determined by hydrolysis of the starch substrate and measurement of the maltose released, according to the technique of Noelting and Bernfield (23), as modified by Hickson (24). The results were expressed in units of activity as defined by the latter (24). Protein concentration was measured by means of the Bio-Rad protein assay, based on the Bradford dye-binding procedure (25) and expressed relative to a standard of BSA. Secretory rates (outputs) for both protein and amylase were calculated by multiplying the concentration (or activity) by the corresponding flow rate, and the values were expressed, respectively, as μg/min and mU/min.

Assays in pancreatic tissue. After an overnight fast, 12 rats from each group were killed by cervical dislocation and the pancreases were removed through a midline incision as quickly as possible. The glands were then defatted, deveined, and washed in cold physiological saline solution. The cleaned pancreases were blotted and weighed. A fraction was frozen in liquid nitrogen and stored at −80°C for further determinations of amylase activity and protein content, and the rest was used for the immediate isolation of cell membranes.

Cell membrane collection, lipid extraction, and analysis of

the FA composition. Rat pancreatic plasma membrane fractions were prepared from glandular homogenates by differential and sucrose gradient centrifugation (26). Next, the technique of Lepage and Roy (27) was used; this method combines lipid extraction and FA methylation in a one-step reaction. The analysis of FA esters was done by GLC by using a Hewlett-Packard chromatograph (Model 5890 II; Hewlett- Packard, Palo Alto, CA) equipped with an automatic injector (Hewlett Packard, Model 7673) and a 60-m silica column (i.d., 0.32 mm; particle size, 0.20 μm; SP™ 2330, Supelco Inc., Bellefonte, PA).

Amylase activity and protein content in pancreatic tissue. Homogenates of pancreatic tissue were analyzed for amylase activity and total protein content by the same techniques as those used to analyze the juice (see above).

Statistical analysis. Unless otherwise stated, all data are means and their SE. In the secretion experiments, statistical comparisons within the groups (above resting values) were done by one-way ANOVA followed by the *post hoc* Bonferroni test. Correlations were examined by the method of Pearson. Differences in the secretory parameters between the two dietary groups at the same time points, as well as differences in cell membrane composition, tissue analysis, food intake, and body and pancreas weight were tested for significance with the independent-samples Student's *t*-test. SPSS software was used in all cases (SPSS for Windows, version 11.0.1; SPSS, Inc., Chicago, IL). Only *P* values of less than 0.05 were considered significant.

RESULTS

Food intake, rat weight, and pancreas weight. As shown in Figure 1A, food intake did not vary significantly among groups. Initial values of body weight were similar (48.9 ± 2.3 g, $n = 24$, in olive oil-fed rats and 48.4 ± 2.7 g, $n = 24$, in sunflower oil-fed rats), and feeding diets differing in the fat source did not affect body weight gain during the 8-wk adaptation period (Fig. 1B). Final body weight was 339.5 ± 10.6 g, $n = 24$, in the olive oil group; a slightly, but not significantly, higher value was reached in the sunflower oil group (365.9 ± 16.2 g, $n = 24$). Accordingly, there was no difference between the groups in the weight of pancreases (olive oil group: 1.34 ± 0.069 g, $n = 24$; sunflower oil group: 1.41 ± 0.066 g, $n = 24$).

FA composition of pancreatic cell membranes. Intake of the experimental diets profoundly influenced the FA composition of pancreatic cell membranes (Table 2). Membranes of the olive oil-fed group were characterized by significantly ($P < 0.001$) higher proportions of oleic acid (18:1n-9) and total MUFA as compared with the sunflower oil group. In turn, feeding the sunflower oil diet for 8 wk resulted in significantly ($P < 0.001$) higher levels of total PUFA, particularly the n-6 series in membranes of this group, than in the olive oil-fed animals. The total amount of saturated FA (SFA) was the same in both groups.

Protein content and amylase activity in pancreatic tissue. Regardless of the mode of expression, comparison of pancreatic protein concentrations and amylase activity between rats fed the olive oil and sunflower oil diets revealed a lack of significant differences (Table 3).

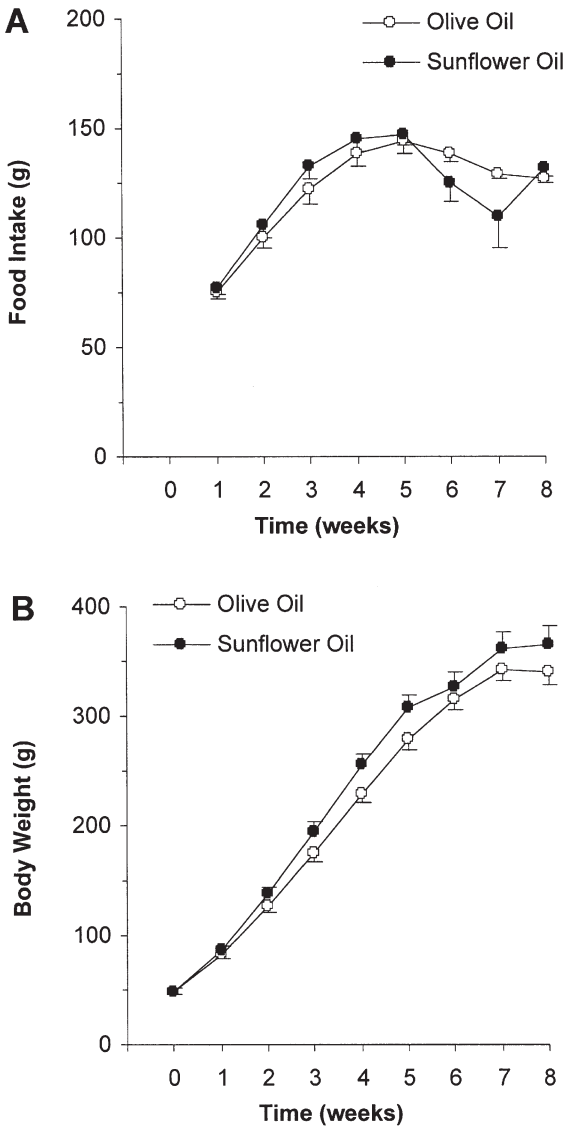

FIG. 1. Evolution of body weight and food intake during the period of adaptation to diets containing different dietary fats. Food intake was determined daily for the whole period, but for clarity results are expressed as total grams consumed per week. All data are means ± SE ($n = 24$ animals per group for body weight measurements, and $n = 12$ animals per group for food intake values).

In vivo *study of exocrine pancreatic secretion. (i) Resting conditions*. The basal pancreatic flow rate and amylase output in rats on the sunflower oil diet were significantly ($P < 0.05$) greater than those of rats on the olive oil diet (Table 4). However, protein concentrations, protein output, and amylase activity were similar in both dietary groups, i.e., slightly but not significantly higher in rats fed sunflower oil.

(ii) Response to CCK-8. Continuous intravenous infusion of CCK-8 at a rate of 150 pmol/kg·h caused a prompt and marked rise in pancreatic flow rate that peaked after 20 min of infusion in both experimental groups (Fig. 2). However, time course

TABLE 2
FA Composition[a] of Pancreatic Cell Membranes in Rats Fed Diets Containing Different Dietary Fats

FA	Olive oil group (n = 12)	Sunflower oil group (n = 12)
16:0	25.04 ± 0.50	26.39 ± 1.22
16:1n-7	5.09 ± 0.45	4.17 ± 0.43
18:0	6.87 ± 0.65*	10.21 ± 0.92
18:1n-9	43.09 ± 2.19***	24.90 ± 1.08
18:2n-6	4.58 ± 0.65***	18.58 ± 1.20
18:3n3	0.40 ± 0.05	0.32 ± 0.08
SFA	39.12 ± 2.49	39.66 ± 2.04
MUFA	48.30 ± 2.52***	29.20 ± 1.41
PUFA	12.58 ± 1.49***	31.14 ± 2.04
n-6 PUFA	10.89 ± 1.34***	29.29 ± 1.99
Unsaturation index (UI)	2.43 ± 0.22	3.15 ± 0.30

[a]Results are expressed as percentage of the total FA content. Values are means ± SE. By row, values with asterisks are significantly different vs. sunflower oil group: *$P < 0.05$; ***$P < 0.001$ (Student's t-test). UI was calculated as follows: UI = [sum (percent FA)·(number of double bonds)]/(percent SFA). For abbreviations see Table 1.

changes in the volume secreted showed a different pattern in each group. Thus, sunflower oil group values returned to levels comparable to basal ones as soon as 60 min after starting the CCK-8 infusion (Fig. 2), whereas in olive oil-fed rats the initial decline was followed by a sustained elevation above the baseline ($P < 0.05$) that was maintained practically throughout the whole experiment (infusion plus postinfusion period). The secretory response in terms of total protein output is shown in Figure 3. CCK-8 induced a large and rapid increase in both groups, reaching a maximum after 40 min and then falling dramatically. In a second phase, protein output in rats from the olive oil group showed a plateau during which values remained significantly ($P < 0.05$) enhanced over basal values for the total infusion period plus the first postinfusion sample (a total of 120 min). In contrast, there was no plateau elevation in the sunflower oil group but a declination to basal values at 60 min after the CCK infusion was begun. As far as amylase activity in pancreatic juice was concerned, CCK-8 evoked a significant ($P < 0.05$) and rapid increase in olive oil-fed rats (within 20 min) that remained elevated over basal values for the duration of the infusion (100 min), as illustrated in Figure 4A. In sunflower oil-fed animals, a significant increase was apparent only at 40 min after the CCK-8 infusion was started. Subsequently, a progressive decrease was observed and amylase activity was lowered below basal values, although the difference was not statistically significant. The effects of CCK-8 infusion on the pancreatic secretion of amylase (amylase output) are represented in Figure 4B. The time course of changes induced by CCK-8 was exactly the same as those described above for the output of total protein, again revealing the existence of different patterns among the dietary groups.

The mean (± SE) peaks and net increases for both total protein and amylase secretory parameters in response to CCK-8

TABLE 3
Protein and Amylase Content[a] of Pancreatic Tissue from Rats Fed Diets Containing Different Dietary Fats

	Olive oil group	Sunflower oil group
Total protein		
mg/g pancreas	141.11 ± 14.16	108.39 ± 9.38
mg/pancreas	186.04 ± 13.61	154.88 ± 16.98
Amylase		
U/g pancreas	21.83 ± 1.36	20.51 ± 2.43
U/pancreas	29.11 ± 1.97	29.15 ± 3.93
U/mg protein	0.161 ± 0.013	0.195 ± 0.217

[a]All values are means ± SE, n = 12.

TABLE 4
Resting Pancreatic Secretion[a] in Anesthetized Rats Fed Diets Containing Different Dietary Fats

	Olive oil group	Sunflower oil group
Flow rate (µL/min)	0.373 ± 0.021*	0.638 ± 0.054
Protein concentration (mg/mL)	29.62 ± 2.76	31.58 ± 5.94
Protein output (µg/min)	10.91 ± 1.06	17.47 ± 1.98
Amylase activity (U/mL)	98.57 ± 8.64	113.70 ± 20.63
Amylase output (mU/min)	35.84 ± 3.55*	61.79 ± 6.03

[a]All values are means ± SE, n = 24 (two resting samples per experiment). *$P < 0.05$ vs. sunflower oil group (Student's t-test).

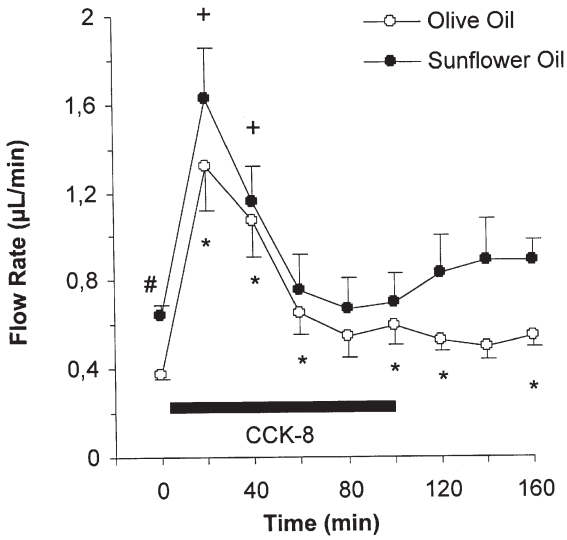

FIG. 2. Time-course changes in cholecystokinin-octapeptide (CCK-8)-evoked pancreatic flow rate in anesthetized rats fed diets containing different dietary fats. After flow stabilization, pancreatic juice was collected continuously in 20-min samples for the duration of the experiment. Collection of two basal (resting) samples was followed by intravenous infusion of CCK-8 (150 pmol/kg·h) for 100 min, as indicated by the black bar. Given that the flow rate was very stable during the basal period, values for the two corresponding samples were averaged. In the chart, time = 0 represents the basal situation, which is immediately followed by the start of CCK-8 infusion. Twelve experiments were conducted in each group. Thus, all data are means ± SE of n = 12, except for basals, where n = 24. For the olive oil group, *$P < 0.05$ as compared with the respective basal value (one-way ANOVA plus *post hoc* Bonferroni test); for the sunflower oil group, $^{+}P < 0.05$ as compared with the respective basal value (one-way ANOVA plus *post hoc* Bonferroni test); $^{\#}P < 0.05$ between the two dietary groups at specific time points (Student's t-test).

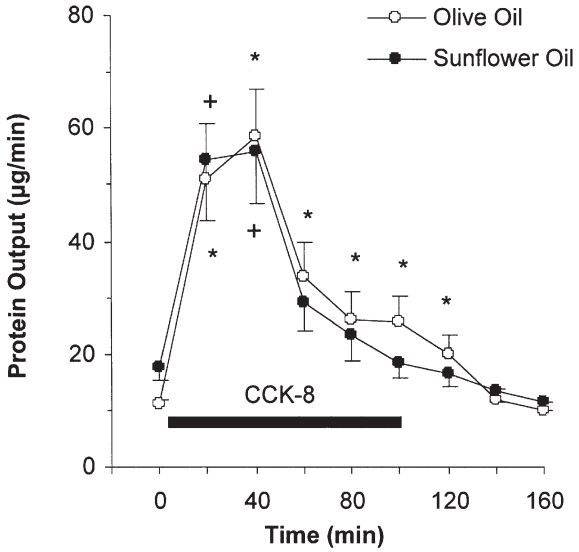

FIG. 3. Time-course changes evoked by CCK-8 on the output of total protein in the pancreatic juice of anesthetized rats fed diets containing different dietary fats. After flow stabilization, pancreatic juice was collected continuously in 20-min samples for the duration of the experiment. Collection of two basal (resting) samples was followed by intravenous infusion of CCK-8 (150 pmol/kg·h) for 100 min as indicated by the black bar. Given that protein output was very stable during the basal period, values for the two corresponding samples were averaged. In the chart, time = 0 represents the basal situation, which is immediately followed by the start of CCK-8 infusion. Twelve experiments were conducted in each group. Thus, all data are means ± SE of $n = 12$, except for basals, where $n = 24$. For the olive oil group, $^{*}P < 0.05$ as compared with the respective basal value (one-way ANOVA plus *post hoc* Bonferroni test); for the sunflower oil group, $^{+}P < 0.05$ as compared with the respective basal value (one-way ANOVA plus *post hoc* Bonferroni test). For abbreviation see Figure 2.

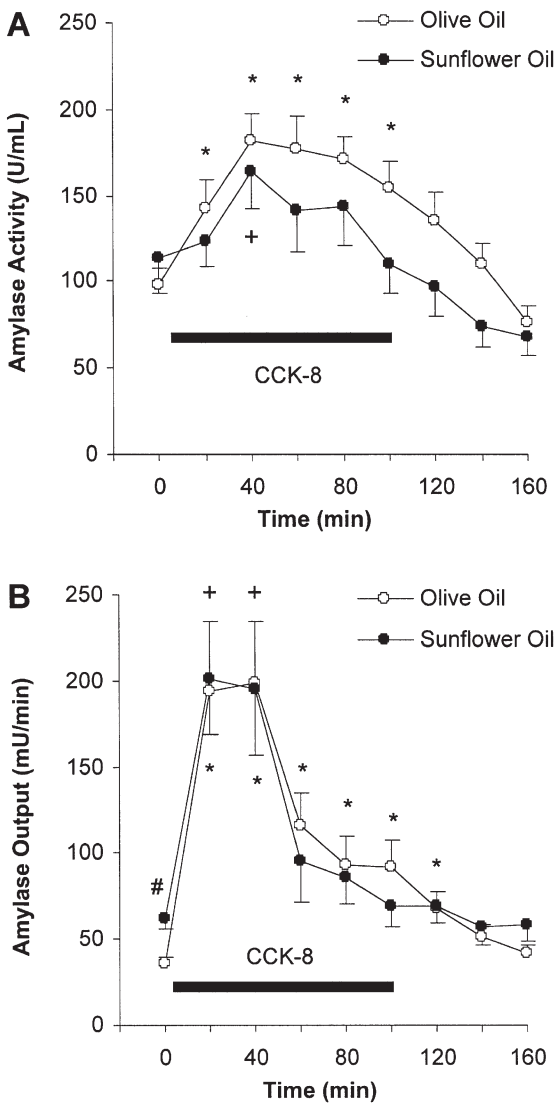

FIG. 4. Time-course changes evoked by CCK-8 on amylase activity (A) and output (B) in pancreatic juice of anesthetized rats fed diets containing different dietary fats. After flow stabilization, pancreatic juice was collected continuously in 20-min samples for the duration of the experiment. Collection of two basal (resting) samples was followed by intravenous infusion of CCK-8 (150 pmol/kg·h) for 100 min as indicated by the black bar. Given that both amylase activity and output were very stable during the basal period, values for the two corresponding samples were averaged. In the chart, time = 0 represents the basal situation, which is immediately followed by the start of CCK-8 infusion. Twelve experiments were conducted in each group. Thus, all data are means ± SE of $n = 12$, except for basals, where $n = 24$. For the olive oil group, $^{*}P < 0.05$ as compared with the respective basal value (one-way ANOVA plus *post hoc* Bonferroni test); for the sunflower oil group, $^{+}P < 0.05$ as compared with the respective basal value (one-way ANOVA plus *post hoc* Bonferroni test); $^{\#}P < 0.05$ between the two dietary groups at specific time points (Student's *t*-test). For abbreviation see Figure 2.

are listed in Table 5. For all parameters, values were slightly, but not significantly, higher in rats fed the virgin olive oil diet. Nevertheless, that the effects of CCK-8 on pancreatic secretion, as measured *in vivo,* were influenced by prior adaptation to different dietary fats is supported by our finding that a significant positive correlation between pancreatic flow rate and protein concentration in the juice ($P = 0.018$, $R = 0.266$) and also between flow rate and amylase activity ($P = 0.022$, $R = 0.258$) was found in the olive oil group but not in the sunflower oil group (flow rate–protein concentration: $P = 0.067$, $R = –0.225$; flow rate–amylase activity: $P = 0.093$, $R = –0.207$).

DISCUSSION

The diets used in the current study did not affect food intake and body weight gain during the 8-wk feeding period, in agreement with previous research on rats fed *ad libitum* with diets varying only in the type of fat added (8,9,19). We also failed to find any difference between the two groups in the weights of pancreases (9).

The analysis of pancreatic cell membranes showed that our dietary protocol was satisfactory, since a direct effect of the type of dietary fat on the pancreatic gland was confirmed. The FA profile of pancreatic cell membranes reflected the composition of the lipid component of the experimental diets: Rats fed the olive oil diet showed higher levels of oleic acid and MUFA, whereas those fed sunflower oil had increased linoleic acid and

TABLE 5
Peaks and Net Increases in Some Pancreatic Secretory Parameters Evoked by Cholecystokinin-octapeptide (CCK-8) Infusion in Anesthetized Rats Fed Diets Containing Different Dietary Fats[a]

	Olive oil group	Sunflower oil group
Protein concentration (mg/mL)		
Peak	54.83 ± 3.94	48.24 ± 4.79
Net increase[b]	25.22 ± 6.38	17.25 ± 6.55
Protein output (μg/min)		
Peak	58.48 ± 8.53	55.78 ± 9.05
Net increase	47.57 ± 8.64	38.27 ± 7.09
Amylase activity (U/mL)		
Peak	181.85 ± 15.38	163.94 ± 21.69
Net increase	83.27 ± 15.50	50.77 ± 20.91
Amylase output (mU/min)		
Peak	197.97 ± 35.91	195.37 ± 38.84
Net increase	162.13 ± 34.13	132.15 ± 32.57

[a]For all parameters, values correspond to the secretory activity observed at 40 min after the CCK-8 infusion was started.
[b]The net increase represents the incremental response above basal values and was calculated as the peak value minus the corresponding basal value. All values are means ± SE, $n = 12$.

PUFA n-6 contents. These results are consistent with those of Begin *et al*. (9) and Soriguer *et al*. (19), who also found an enrichment in the pancreas of those FA most abundant in the fat ingested. The proportion of total SFA was similar in the two groups, supporting the notion that the SFA fraction is the most resistant to dietary-induced alterations (9,17,19). In addition, feeding diets rich in virgin olive oil or sunflower oil did not significantly alter the unsaturation index, suggesting that changes in the proportions of several major FA are associated with some metabolic compensation that keeps plasma membrane fluidity within a certain range of values (17).

We could not find a clear effect of the type of dietary lipids on protein content and amylase activity in pancreatic homogenates after feeding the experimental diets for 8 wk. However, a comparison of the results obtained in ground pancreatic tissue from rats on the olive oil and sunflower oil diets with those from age-matched rats fed from weanling age on a standard chow (Díaz, R.J., Yago, M.D., Martínez-Victoria, E., and Mañas, M., unpublished data) revealed that feeding the chow (Panlab A04; Panlab SA, Barcelona, Spain) was associated with a similar content of total protein but a higher amylase activity in pancreatic tissue (87.5 ± 8.9 U/g pancreas, $n = 8$) compared with rats fed our two experimental diets (see Table 3). Since the body and pancreas weight values of the rats fed the commercial chow were comparable to those in the current study, the above effect can be explained only by the different composition of the chow in relation to that of the experimental diets. The chow was richer in carbohydrates (68.8 wt%) and poorer in fat (3.3 wt%) than our semisynthetic diets (59.9 and 10 wt% for carbohydrates and fat, respectively). It is well established in the rat that tissue amylase content adapts to the amount of its substrates in the diet (1,4), which is in agreement with our finding that when dietary fat increased and carbohydrate decreased (as in the olive oil and sunflower oil diets compared to the chow), a reduction occurred in pancreatic amylase. Interestingly, the study by Deschodt-Lanckman *et al*. (1) showed that the lowering effects of high-fat, low-carbohydrate diets on pancreatic amylase depend on the type of fat, olive oil being the best "repressor." We could not find any such differential effect among rats from the olive oil and sunflower oil groups, but the fact that the high-fat diets used in the study by Deschodt-Lanckman *et al*. (1) had an exaggerated amount of fat (50 wt%) and provided no carbohydrates may account for this discrepancy. Indeed, working in conditions more similar to ours, i.e., feeding the animals for 6 wk with diets comprising 5 wt% of several dietary fats differing in the degree of unsaturation, Beaudoin *et al.* (8) reported a lack of influence of the type of fat upon pancreatic amylase activity in gland homogenates. An exhaustive examination of the levels of digestive enzymes in the pancreatic gland following alteration of the dietary fat source was beyond the goal of this work. However, that tissue amylase activity was not affected in our experimental conditions does not exclude the existence of changes in the levels of other secretory enzymes, a possibility that deserves further investigation.

By using the method of direct cannulation of the pancreatic duct, which collects the actual products of secretion rather than stored enzymes, we were able to confirm in this study the modification of secretory output as a function of the type of dietary fat. Under resting conditions, when the major differences were found, pancreatic flow rate and amylase output were significantly higher in rats on the sunflower oil diet than in those on the olive oil diet. The mechanism underlying this effect is, however, far from clear. In the whole animal, exocrine pancreatic secretion is regulated by a complex integration of hormonal and neural mechanisms, with stimulatory factors being balanced by a variety of inhibitory regulators, including peptide YY (PYY), a distal gut peptide released by the presence of

fat, and fat hydrolysis products in the intestine (28–30). In the pancreas, PYY has been shown to inhibit not only secretion stimulated by secretin and/or CCK, but also basal secretion (28,29,31,32). In previous studies conducted in dogs and humans, we found that a medium- or long-term intake of a diet containing olive oil as the major fat source was accompanied by a significant elevation of plasma PYY in the resting condition as compared with the levels measured after sunflower oil feeding (33,34). Thus, it is tempting to speculate that the diminished exocrine secretion observed in the resting condition in the olive oil group of the present study might reflect an augmentation of the resting PYY levels, but our present data do not allow confirmation of this point.

Changes in exocrine pancreatic secretion following intravenous infusion of CCK-8 suggest that the two groups responded differently to this secretagogue. CCK-8-evoked peaks and net increases in protein content, protein output, amylase activity, and amylase secretion were only slightly, but not significantly, higher in the animals given the virgin olive oil diet. However, the time course of changes in the secretory parameters during and after the infusion of CCK showed a different pattern in each group. The existence in the olive oil group, but not in the sunflower oil group, of a positive direct correlation between flow rate and both protein concentration and amylase activity lends further support to an influence of the type of dietary lipids on the secretory activity of the exocrine pancreas.

At the present time, no definite explanation for this behavior of the gland exists. Clearly, the differences observed in pancreatic secretion from anesthetized rats are not the consequence of a variation in tissue content of protein or amylase. Feeding weanling rats on diets containing 10 wt% of two different fats affected the exocrine pancreas directly, as shown by the changes in membrane FA composition. Thus, the possibility exists that the current *in vivo* data are reflecting a distinct modulating effect of the type of dietary fat on the secretory activity at the cellular level. This notion is reinforced by the results of recent investigations (20) in viable pancreatic acinar cells isolated from rats kept on a dietary protocol identical to the one in this study, where a higher amylase release in basal conditions and reduced amylase responses to CCK-8 were observed in cells from rats fed the sunflower oil diet compared with those from rats fed the olive oil diet (20). Notably, these differences in secretion were associated with changes in signal transduction evoked by CCK-8 (20). Experiments are in progress to better characterize these cellular effects (20), which in part could be related to the change in membrane FA, given the role of these molecules as structural components of membrane lipids and as intracellular messengers themselves. On the other hand, as acknowledged in this article, the existence of hormone-mediated influences in this investigation should not be rejected. Recent reports by Covasa and colleagues (35,36) indicate that chronic exposure of the rat to high levels of dietary fat results in reduced potency of exogenous CCK in such effects as satiation or inhibition of gastric emptying. The authors suggested that dietary-induced elevation of plasma CCK may have contributed to the reduced sensitivity to exogenous CCK (35). Whether the endogenous level of CCK was affected in this study by the type of fat is unknown, but if this were confirmed, both the current results in anesthetized rats and those in isolated pancreatic acinar cells (20) should also be considered to implicate downregulation of CCK receptors (37), since CCK-8 was used in all cases as the stimulus.

In conclusion, the present investigation shows that chronic intake of diets differing only in the type of fat added (olive oil or sunflower oil) influences exocrine pancreatic secretion in anesthetized rats. The major differences between the dietary treatments concerned (i) the magnitude of the secretion of fluid and amylase in the resting conditions and (ii) the time course of changes in all major secretory parameters evoked by a continuous intravenous infusion of CCK-8. Undoubtedly, much work is required to determine the mechanisms by which changes in the composition of dietary fat modulate exocrine pancreatic secretion in the whole animal. The results of this and other studies indicate that the relationship between cellular responsiveness, hormonal regulators of exocrine function, and dietary adaptation needs to be examined further.

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Hyperphagia Modifies FA Profiles of Plasma Phospholipids, Plasma FFA, and Adipose Tissue TAG

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ABSTRACT: Hyperphagia was achieved by continuous intracerebroventricular infusion of a melanocortin receptor antagonist (HS024; Neosystem, Strasbourg, France) in rats. The effects of hyperphagia on FA composition and concentration of plasma phospholipids (PL), plasma FFA, and adipose tissue TAG were studied in rats for 8 d [short-term hyperphagia (STH); $n = 8$], or 28 d [long-term hyperphagia (LTH); $n = 9$]. The control rats were treated with artificial cerebrospinal fluid for 8 d ($n = 8$) or 28 d ($n = 10$). The rats were fed the same regular diet. In STH rats the plasma PL and fasting plasma FFA contained higher concentrations of saturated FA (SFA) and monounsaturated FA (MUFA), and plasma FFA contained lower n-6 PUFA than in the control rats. In LTH rats the plasma PL contained higher concentrations of SFA, MUFA, and n-3 PUFA and higher proportions of 16:1n-7 and 18:1n-9 at the expense of 18:2n-6 than in the control rats. In LTH rats the abundant dietary intake of 18:2n-6 did not enrich 18:2n-6 of the plasma PL or adipose tissue TAG. In LTH rats the fasting plasma FFA contained more than twofold higher concentrations of SFA and MUFA, and higher proportions of 16:1n-7 and 18:1n-9 at the expense of 18:2n-6 than in the control rats. This animal obesity model shows that LTH affects the FA composition and concentration of plasma PL, plasma FFA, and adipose tissue TAG, a result consistent with changes associated with increased risk of various diseases in humans. These results also demonstrate that LTH alters the FA composition of plasma PL and adipose tissue TAG in a way that does not reflect the FA composition of dietary fat.

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In a normal dietary state, the FA composition of plasma phospholipids (PL) and adipose tissue TAG reflects that of dietary fat (1–4). At times of positive energy balance, the abundant FA originating from dietary fat are transported into adipose tissue and converted to TAG. On the other hand, the abundant dietary carbohydrates stimulate hepatic *de novo* FA synthesis (5–7), which involves the conversion of carbohydrate, *via* the acetyl-CoA intermediate, to the saturated FA (SFA) 16:0, which then undergoes chain elongation to 18:0 and/or desaturation to the monounsaturated FA (MUFA) 16:1n-7, 18:1n-7, and 18:1n-9. Moreover, high *de novo* FA synthesis is correlated with a great increase in the concentration of plasma TAG (5,7,8), which is recognized as an independent risk factor for cardiovascular disease (9). Overnight fasting represents a state of ongoing TAG lipolysis in fat cells, which results in increased plasma FFA, an energy source from adipose tissue (10,11). Plasma FFA in high concentrations have been associated with increased risk of cardiovascular disease; in particular, the circulating SFA are known to have prothrombotic and arrhythmogenic actions (12–15).

The central melanocortin (MC) system plays an important role in the control of feeding and body weight (16,17). Disruption of MC receptors of subtype 4 causes rodents to overeat and turn obese (18), whereas stimulation of the MC receptors reduces food intake and at the same time increases metabolism, resulting in lower body weight (19). MC receptor agonists and antagonists are therefore excellent tools for studying various aspects of nutrient metabolism in animals at different nutritional stages. In the present study, an overweight condition was developed in rats by intracerebroventricular (i.c.v.) infusion of an MC receptor antagonist. The purpose of this study was to investigate the effect of hyperphagia on the FA composition and concentration of adipose TAG and fasting plasma FFA as well as on the FA composition of plasma PL in rats that were in a positive energy balance for a short and a prolonged period of time.

MATERIALS AND METHODS

Animals and diets. Male Wistar rats ($n = 35$) were obtained from Taconic M&B (Ry, Denmark) and housed under standard conditions at 22 ± 1°C and at 50 ± 5% RH in a reversed 12-h/12-h light/dark cycle (lights off at 10:00 h, on at 22:00 h). After more than 2 wk of acclimatization, the rats, weighing 292–408 g, were housed individually in plastic cages for 1 wk before the drugs were administered. All rats were fed the same standard commercial laboratory diet (Rat and Mouse Maintenance Diet No. 1; Special Diets Services, Witham Essex, United Kingdom) and water *ad libitum*. The diet contained 9.0 energy% fat (SFA: 16:0, 1.6; 18:0, 0.1; MUFA: 18:1n-9, 1.2; 18:1n-7, 0.1; n-6 PUFA: 18:2n-6, 5.2; n-3 PUFA: 18:3n-3, 0.5), 18.8 energy% protein, and 72.3 energy% digestible carbohydrate (simple: 9.0; complex: 63.3).

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Abbreviations: ACSF, artificial cerebrospinal fluid; i.c.v., intracerebroventricular; LTH, long-term hyperphagia; MC, melanocortin; MUFA, total monounsaturated FA; PL, phospholipid; SFA, total saturated FA; STH, short-term hyperphagia.

Implantation of minipump. After a 1-wk control period, osmotic minipumps (Alzet®, model 2004; Alza Corp., Palo Alto, CA) were implanted subcutaneously in the midscapular region for i.c.v. infusion. The rats were anesthetized with methohexital sodium (50 mg/kg i.p.; Brietal®; Lilly, Indianapolis, IN) and randomly assigned to one of four groups. Two experimental groups received an i.c.v. infusion of 0.1 nmol/h HS024 peptide (Neosystem, Strasbourg, France) for 8 d [the short-term hyperphagia (STH) rats; $n = 8$] or for 28 d [the long-term hyperphagia (LTH) rats; $n = 9$]. Selection of the drug infusion rate was based on previous experience (20,21). Two control groups received an i.c.v. infusion of artificial cerebrospinal fluid (ACSF of NaCl, 138 mM; KCl, 3.37 mM; $CaCl_2$, 1.50 mM; $MgCl_2$, 1.0 mM; NaH_2PO_4, 1.45 mM; Na_2HPO_4, 4.85 mM; pH 7.4) for 8 d (the STH control rats; $n = 8$) or 28 d (the LTH control rats; $n = 10$). The HS024 was dissolved in ACSF, and a stability investigation of the dissolved peptide was performed weekly by HPLC and MS methods. The infusion started immediately upon implantation of the minipumps, which were connected to a brain infusion kit (Alzet®; Alza Corp.) with the cannula opening into the right lateral cerebral ventricle (1.0 mm posterior, 1.5 mm lateral to the bregma). A dental glass ionophore (ChemFil® Superior; Dentsupply DeTray GmbH, Konstanz, Germany) and a stainless steel anchor screw were used to secure the infusion cannula in position. The animals were inspected during the last 2 h of the light phase every morning but were otherwise left undisturbed. Animal experimental procedures were carried out in accordance with the guidelines of the European Union, and with local legislation and policies.

Plasma and adipose tissue samples. At the end of the 8- and 28-d treatment periods and an overnight fast (12–14 h), the rats were anesthetized in the same way as for the implantation of the osmotic pumps. The femoral vein was catheterized, the rats were heparinized (330 IU/kg heparin; Leo Pharma A/S, Ballerup, Denmark), and blood samples were collected. Plasma was prepared immediately, frozen, and stored at –30°C until analyzed for FA composition of plasma PL and FFA. The anesthetized animals were then decapitated, and the retroperitoneal fat pads (adipose tissue) were excised, frozen, and stored at –30°C until analyzed for FA composition of TAG.

Lipid extraction and FA analysis. Total lipids of plasma were extracted with chloroform/methanol (2:1, by vol) by the method of Sperry and Brand (22), as modified by Nelson (23). Adipose tissue total lipids were extracted with chloroform/methanol (1:2, vol/vol) by the method of Bligh and Dyer (24). The antioxidant BHT (5 mg/100 mL) was added to the extraction medium. Plasma PL and FFA, on the one hand, and adipose tissue TAG, on the other hand, were separated by TLC (Adsorbosil H; Alltech, Deerfield, IL) with petroleum ether/diethyl ether/acetic acid (80:20:1, by vol) as the developing solvent. The FA of plasma PL and FFA were transmethylated for 45 min at 110°C using 14% boron trichloride/methanol (Sigma Chemical Co., St. Louis, MO). The adipose tissue TAG were saponified by adding 0.5 M NaOH and heating for 15 min at 90°C, and the TAG FA were then transmethylated for 15 min at 110°C using 14% boron trifluoride/methanol. The FAME were analyzed using a gas chromatograph (Hewlett-Packard 5890A; Hewlett-Packard, Palo Alto, CA) equipped with a Chrompack CP-Wax 52 CB polyethylene glycol column (25 m × 0.25 mm i.d.; Varian B.V., Bergen op Zoom, The Netherlands). The oven temperature was programmed to have an initial temperature of 90°C for 2 min, then rising at 30°C/min to 165°C and at 3°C/min to 225°C. The injector and detector temperatures were maintained at 230 and 250°C, respectively. Hydrogen was used as the carrier gas. The FAME peaks were identified and calibrated against those of commercial standards (Sigma Chemical Co.; Nu-Chek-Prep, Elysian, MN). PC, dipentadecanoyl, and pentadecanoic acid (Sigma Chemical Co.) were used as internal standards for plasma PL and plasma FFA, respectively.

Plasma TAG analytical procedure. Fasting plasma TAG concentration was determined by the Vitros dry chemistry method (Ortho-Clinical Diagnostics Inc., Rochester, NY) based on an enzymatic method described by Spayd *et al.* (25). The sensitivity of the assay was 0.5 ng/mL. Both the intra- and interassay CV were less than 10%.

Statistical analysis. The effects of 8- or 28-d hyperphagia on FA composition of plasma PL, FFA, adipose tissue TAG, and concentration of plasma TAG and FFA were analyzed by comparing data from each group with its corresponding control group by means of Student's *t*-test. A $P < 0.05$ was considered significant in the statistical tests.

RESULTS

Energy intake. As compared with the respective control rats, the cumulative energy intake was 40% higher in the STH rats and 60% higher in the LTH rats, 3795 ± 157 vs. 2732 ± 120 kJ, and 16,280 ± 1293 vs. 10,261 ± 195 kJ, $P < 0.01$, respectively. The increased energy intake led, on average, to 10 ± 1% (32 ± 4 g) more weight gain ($P < 0.05$) in the STH rats and 27 ± 3% (94 ± 17 g) more weight gain ($P < 0.05$) in the LTH rats than in their respective controls.

Plasma PL FA. The concentrations of SFA, MUFA, n-6 PUFA, and n-3 PUFA in plasma PL of the STH and LTH rats are shown in Figure 1. The increased cumulative energy intake was reflected by elevation ($P < 0.05$) in concentrations of SFA, MUFA, and n-3 PUFA in plasma PL of the LTH rats compared with the control rats (Fig. 1B). Increased ($P < 0.05$) concentrations of SFA and MUFA were observed in plasma PL of the STH rats, whereas no changes were found in n-6 and n-3 PUFA compared with the control rats (Fig. 1A). Table 1 shows the proportions of FA in plasma PL of the LTH rats and the respective control rats. The plasma PL of LTH rats contained similar proportions of SFA and higher ($P < 0.01$) proportions of the FA 16:1n-7 and 18:1n-9 than that of the control rats. The most abundant EFA in the diet, 18:2n-6, did not enrich the 18:2n-6 of plasma PL in the LTH rats; the proportion of this FA was lower ($P < 0.01$) in LTH rats compared with the control rats.

Adipose tissue TAG FA. The FA composition of adipose tissue TAG of the STH rats did not differ compared with the control rats (data not shown). The amount (μg) of SFA, MUFA, n-6 PUFA, and n-3 PUFA in adipose tissue TAG per

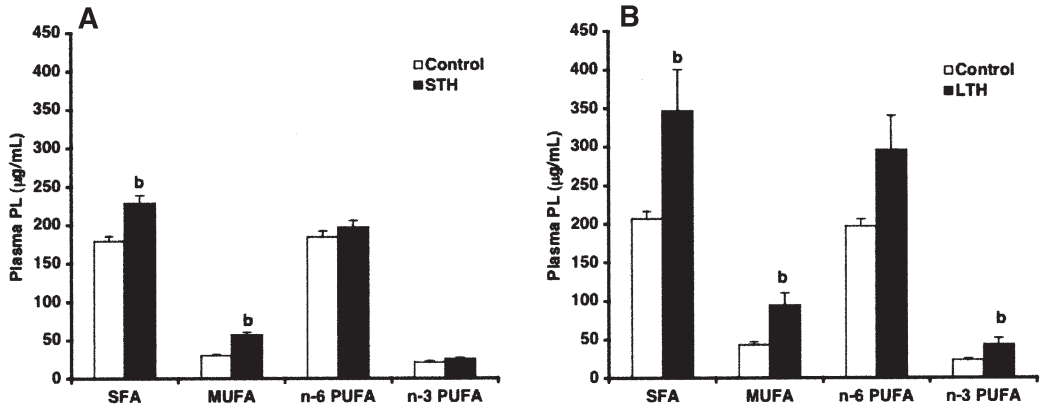

FIG. 1. Effect of short-term hyperphagia (STH) (A) and long-term hyperphagia (LTH) (B) on concentrations (µg/mL) of total saturated FA (SFA), monounsaturated FA (MUFA), n-6 PUFA, and n-3 PUFA in plasma phospholipids (PL). Rats (black bars) were treated with melanocortin (MC) receptor antagonist (HS024; Neosystem, Strasbourg, France) for (A) 8 d (STH rats; $n = 8$) or (B) 28 d (LTH rats; $n = 9$). Control rats (open bars) were treated with artificial cerebrospinal fluid (ACSF) for (A) 8 d ($n = 8$) or (B) 28 d ($n = 10$). Solid bars labeled with a superscript letter were significantly different from open bars (control rats), $P < 0.05$ (*t*-test). Error bars represent SEM.

milligram wet weight adipose tissue in the LTH rats is shown in Figure 2. In adipose tissue TAG, the amount of MUFA was higher ($P < 0.05$), and the amounts of n-6 and n-3 PUFA were lower ($P < 0.01$) in the LTH rats compared with the control rats. Table 1 shows the proportions of FA in adipose tissue TAG of the LTH rats and the respective control rats. In the adipose tissue TAG of LTH rats, the proportions of the FA 16:0, 18:0, 16:1n-7, and 18:1n-9 increased at the expense of the FA 18:2n-6 and 18:3n-3 compared with the adipose tissue of control rats. The lower ($P < 0.01$) proportion of 18:2n-6 found in the adipose tissue of the LTH rats compared with that in the control rats did not reflect the higher cumulative energy intake derived from 18:2n-6 in the LTH rat diet (844 ± 67 vs. 532 ± 10 kJ, $P < 0.01$, respectively).

Plasma FFA. In STH rats, no differences were found in concentrations of overnight fasting plasma FFA compared with

TABLE 1
Effect of Long-Term Hyperphagia (LTH) on FA Composition[a] in Plasma Phospholipids (PL), Adipose Tissue TAG, and Plasma FFA

	Plasma PL		Adipose tissue TAG		Plasma FFA	
FA	Control rats ($n = 9$)	LTH rats ($n = 9$)	Control rats ($n = 10$)	LTH rats ($n = 9$)	Control rats ($n = 10$)	LTH rats ($n = 9$)
14:0	0.12 ± 0.09	0.24 ± 0.08	1.54 ± 0.04	1.36 ± 0.06[b]	1.49 ± 0.42	1.70 ± 0.61
16:0	24.07 ± 0.53	22.18 ± 0.84	26.16 ± 0.29	28.09 ± 0.41[c]	25.62 ± 1.93	29.99 ± 0.88
18:0	17.40 ± 0.49	18.99 ± 0.67	2.90 ± 0.08	5.53 ± 0.49[c]	7.65 ± 0.76	7.85 ± 0.96
24:0	1.19 ± 0.05	1.13 ± 0.06	ND	ND	ND	ND
16:1n-9	ND	ND	0.34 ± 0.01	0.40 ± 0.02[b]	ND	ND
16:1n-7	0.64 ± 0.10	1.39 ± 0.10[c]	5.88 ± 0.42	7.21 ± 0.28[b]	4.72 ± 0.35	8.71 ± 0.50[c]
18:1n-9	3.23 ± 0.25	5.37 ± 0.35[c]	20.74 ± 0.39	28.62 ± 1.09[c]	16.57 ± 0.53	23.17 ± 0.98[c]
18:1n-7	4.30 ± 0.25	3.67 ± 0.34	4.75 ± 0.13	4.05 ± 0.16[c]	5.34 ± 0.23	4.44 ± 0.28[b]
20:1n-9	ND	ND	0.33 ± 0.01	0.26 ± 0.01[c]	1.25 ± 0.31	0.39 ± 0.04[b]
20:1n-7	ND	ND	0.59 ± 0.03	0.29 ± 0.04[c]	ND	ND
24:1n-9	0.68 ± 0.13	0.95 ± 0.15	ND	ND	ND	ND
18:2n-6	14.76 ± 0.34	11.47 ± 0.56[c]	30.27 ± 0.85	19.36 ± 1.04[c]	24.04 ± 1.50	13.26 ± 1.32[c]
20:2n-6	ND	ND	0.21 ± 0.02	0.15 ± 0.01[c]	ND	ND
20:3n-6	0.51 ± 0.14	1.87 ± 0.27[c]	0.13 ± 0.01	0.11 ± 0.01[b]	ND	0.28 ± 0.10
20:4n-6	25.36 ± 0.46	23.14 ± 0.56[c]	0.59 ± 0.02	0.32 ± 0.02[c]	5.85 ± 0.45	3.59 ± 0.30[c]
22:4n-6	ND	ND	0.15 ± 0.01	0.10 ± 0.01[c]	ND	ND
18:3n-3	ND	ND	2.24 ± 0.07	1.64 ± 0.06[c]	ND	ND
20:5n-3	0.13 ± 0.06	0.20 ± 0.07	0.02 ± 0.01	0.01 ± 0.01	0.45 ± 0.20	0.41 ± 0.14
22:5n-3	0.85 ± 0.11	1.20 ± 0.10[b]	0.16 ± 0.01	0.10 ± 0.01[c]	0.12 ± 0.12	0.54 ± 0.16
22:6n-3	3.78 ± 0.20	3.79 ± 0.14	0.22 ± 0.01	0.14 ± 0.02[c]	1.39 ± 0.42	1.25 ± 0.21

[a]Data are expressed as wt% of total FA. Values are mean ± SEM. LTH rats were treated with melanocortin receptor antagonist (HS024; Neosystem, Strasbourg, France) for 28 d. Control rats were treated with artificial cerebrospinal fluid for 28 d. ND, not detected. [b]$P < 0.05$, [c]$P < 0.01$, compared with control rats (*t*-test).

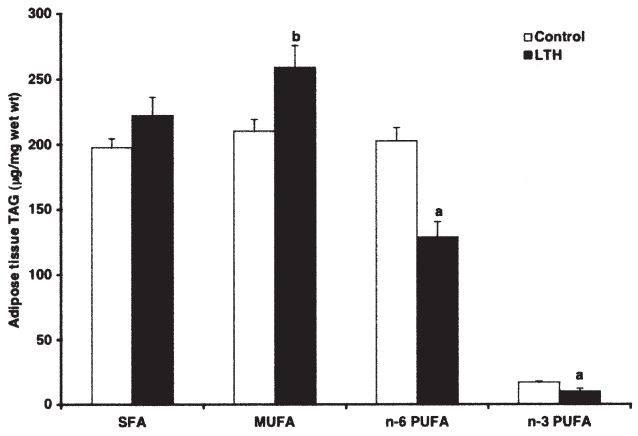

FIG. 2. Effect of LTH on amount (µg) of SFA, MUFA, n-6 PUFA, and n-3 PUFA in adipose tissue TAG per milligram wet weight adipose tissue. LTH rats (black bars; $n = 9$) were treated with MC antagonist (HS024) for 28 d. Control rats (open bars; $n = 10$) were treated with ACSF for 28 d. Significantly different from control rats, $^{a}P < 0.01$, $^{b}P < 0.05$ (*t*-test). Error bars represent SEM. For abbreviations and supplier of HSO24 see Figure 1.

that in the control rats (0.232 ± 0.028 vs. 0.228 ± 0.018 mg/mL, respectively), whereas higher concentrations of FFA were found in the plasma of LTH rats compared with the control rats (0.473 ± 0.059 vs. 0.268 ± 0.033 mg/mL, $P < 0.01$, respectively). Concentrations of SFA, MUFA, n-6 PUFA, and n-3 PUFA of fasting plasma FFA in STH and LTH rats and their respective control rats are shown in Figure 3. In the STH rats, no differences were found in the concentrations of SFA or n-3 PUFA in plasma FFA, whereas the concentration of MUFA was higher ($P < 0.05$) and that of n-6 PUFA was lower ($P < 0.01$) than in the control rats (Fig. 3A). In the LTH rats, the concentration of SFA was 143% higher ($P < 0.01$), and that of MUFA was 160% higher ($P < 0.01$) in plasma FFA, whereas no differences were found in the concentrations of n-6 and n-3 PUFA compared with the control rats (Fig. 3B). Table 1 shows the proportions of FA in plasma FFA. No differences in the proportion of SFA were found in the LTH rats compared with the control rats, whereas the proportions of 16:1n-7 and 18:1n-9 in plasma FFA of the LTH rats were higher ($P < 0.01$), and the proportions of 18:2n-6 and 20:4n-6 were lower ($P < 0.01$) than in the control rats.

Plasma TAG concentrations. In STH rats, no differences were found in concentrations of overnight fasting plasma TAG compared with those in the control rats (0.45 ± 0.05 vs. 0.40 ± 0.03 mmol/L, respectively), whereas higher concentrations were found in the LTH rats compared with the control rats (0.82 ± 0.11 vs. 0.41 ± 0.04 mmol/mL, $P < 0.01$, respectively).

DISCUSSION

In the present study, where all the rats were fed the same diet, which was low in energy% SFA, MUFA, and n-3 PUFA and high in n-6 PUFA, the FA composition of plasma PL and adipose tissue TAG did not reflect the kind of FA fed during LTH. Furthermore, the LTH was characterized by high concentrations of SFA and MUFA in plasma PL, adipose tissue TAG, and fasting plasma FFA.

It is known that the central MC system can potentially have various effects on lipid metabolism *via* its different efferent links (26,27). In this study, hyperphagia was induced by blocking central MC receptors; thus, it is possible that the observed effects are at least in part the result of such modulation but not secondary to the hyperphagia *per se*. However, such implications are highly speculative.

Results from human studies indicate that dietary n-6 PUFA are moderately reflected, and dietary SFA and MUFA poorly reflected, in plasma PL (28,29). In the present study, the elevated concentrations of SFA, MUFA, and n-3 PUFA observed

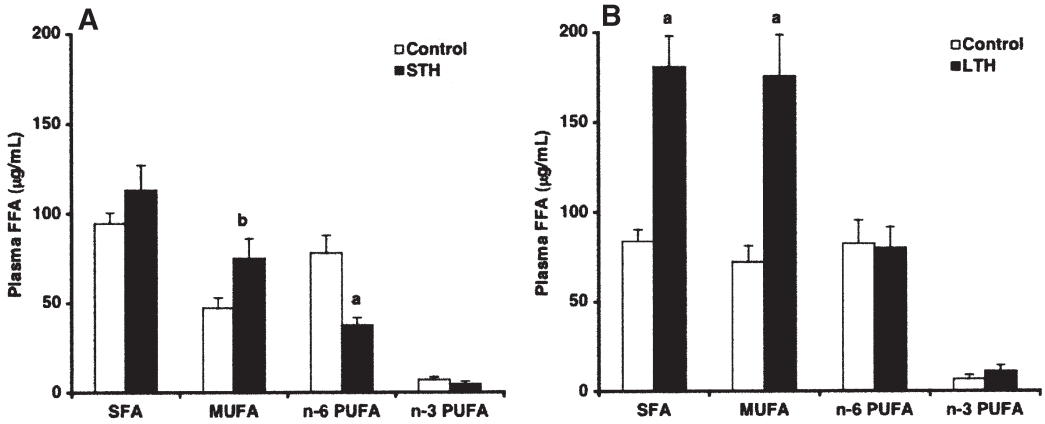

FIG. 3. Effect of STH (A) and LTH (B) on concentrations (µg/mL) of SFA, MUFA, n-6 PUFA, and n-3 PUFA in fasting plasma FFA. Rats (black bars) were treated with MC antagonist (HS024) for (A) 8 d (STH rats; $n = 8$) or (B) 28 d (LTH rats; $n = 9$). Control rats (open bars) were treated with ACSF for (A) 8 d ($n = 8$) or (B) 28 d ($n = 10$). Significantly different from control rats, $^{a}P < 0.01$, $^{b}P < 0.05$ (*t*-test). Error bars represent SEM. For abbreviations and supplier of HSO24 see Figure 1.

in plasma PL after LTH probably reflect the high cumulative dietary energy intake. Moreover, extra energy% derived from carbohydrate in the diet may not be neutral with respect to FA composition of plasma PL. The main products of *de novo* FA synthesis in liver are 18:0, 16:1n-7, and 18:1n-9, the FA whose proportions were increased in plasma PL at the expense of 18:2n-6 after LTH (Table 1). The high energy% of 18:2n-6 in the diet was not reflected in plasma PL 18:2n-6 after LTH, probably due to β-oxidation of 18:2n-6 (30).

The twofold increase in fasting plasma TAG concentration observed after LTH is consistent with human studies, which have shown that consumption of a high-energy, low-fat, high-carbohydrate diet for shorter or longer periods of time increases the fasting plasma TAG concentration (7,8,31) owing to stimulation of *de novo* TAG synthesis in the liver (5,6,32). Furthermore, the concentrations of MUFA, n-6 PUFA, and n-3 PUFA in adipose tissue TAG after LTH probably reflect an ongoing *de novo* FA and TAG synthesis in the liver and adipose tissue.

In animals fed diets containing a high energy% of fat, a positive correlation was observed between the amount of FA consumed in the diet and their deposition into adipose tissue TAG (3). In the present study, the relatively low cumulative energy intake derived from fat did not affect the FA composition of the adipose tissue TAG after STH (data not shown). On the other hand, following LTH adipose tissue TAG were characterized by higher proportions of SFA and MUFA and lower proportions of n-6 PUFA and n-3 PUFA than in the normal dietary state (Table 1). The high amount of dietary 18:2n-6 consumed was not incorporated into adipose tissue TAG, probably owing to β-oxidation of 18:2n-6 (30).

Hudgins and coworkers (6,33) and Parks and coworkers (34) showed in humans that *de novo* FA synthesis in the liver could be elevated during carbohydrate-induced hypertriacylglycerolemia, when the simple-to-complex ratio of carbohydrate energy from the diet was higher than one. On the other hand, the results of the present study in rats indicate that even when complex carbohydrate energy in the diet is abundant, *de novo* FA synthesis is stimulated in the liver after LTH.

LTH resulted in more than twofold higher concentrations of SFA and MUFA in fasting plasma FFA, and also increased proportions of MUFA at the expense of n-6 PUFA. This may be partly explained by stimulated TAG lipolysis and an increased transport of FA out of adipose tissue cells in the fasting state.

Studies have shown that the FA composition of serum PL may be predictive of myocardial infarction or coronary heart disease (35–38), and that the proportion of 18:2n-6 in serum PL is negatively correlated with hemostatic factors (39) and blood pressure (40). Marked elevation of fasting plasma TAG concentration is one clinical feature of overweight individuals, and is associated with diabetes and a greater risk of coronary heart disease (41–44). Thus, our study indicates that LTH modifies the concentrations and FA proportions of plasma PL, fasting plasma FFA, and adipose tissue TAG in a manner, that is associated with an increased risk of overweight-related diseases in humans.

In summary, this animal model of developed obesity has shown that long-term consumption of an ordinary high-energy diet containing 72 energy% from carbohydrate and 9 energy% from fat can affect the FA composition and concentration in fasting plasma PL, plasma FFA, and adipose tissue TAG, which is consistent with those changes associated with an increased risk of various diseases in humans. The present study has also demonstrated that in rats prolonged excessive energy intake alters the FA composition of plasma PL and adipose tissue TAG in a way that does not reflect the FA composition of dietary fat.

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cis-9,*trans*-11 and *trans*-10,*cis*-12 CLA Affect Lipid Metabolism Differently in Primary White and Brown Adipocytes of Djungarian Hamsters

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ABSTRACT: We explored whether CLA isomers and other C_{18} FA affect (i) lipid content and FA concentrations in total adipocyte lipids, (ii) FA synthesis from glucose in TAG and phospholipids of primary brown (BAT) and white adipocytes (WAT), and (iii) mRNA expression of uncoupling protein 1 (UCP1) in primary brown adipocytes of Djungarian hamsters *(Phodopus sungorus)*. *c*9,*t*11-CLA, oleic, linoleic, and α-linolenic acid increased whereas *t*10,*c*12-CLA decreased lipid accumulation in both adipocyte types. *t*10,*c*12-CLA treatment affected FA composition mainly in BAT cells. CLA incorporation into lipids, in particular *c*9,*t*11-CLA, was higher in BAT. In both cell types, *t*10,*c*12-CLA treatment reduced the incorporation of glucose ^{13}C carbon into FA of TAG and phospholipids, whereas *c*9,*t*11-CLA, linoleic, and α-linolenic acid either did not influence or dose-dependently increased glucose carbon incorporation into FA. UCP1 mRNA expression was inhibited by *t*10,*c*12-CLA but increased by *c*9,*t*11-CLA, linoleic, and α-linolenic acid. It is concluded that *c*9,*t*11-CLA and *t*10,*c*12-CLA have distinctly different effects on lipid metabolism in primary adipocytes. The effects of *c*9,*t*11-CLA are similar to those of other unsaturated C_{18} FA. The opposite effects of *c*9,*t*11-CLA and *t*10,*c*12-CLA are evident in both WAT and BAT cultures; however, brown adipocytes seem to be more susceptible to CLA treatment.

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Various CLA isomers occur naturally in animal foods derived from ruminants, such as milk or beef (1,2). CLA is a collective term referring to all conjugated geometric and positional isomers of octadecadienoic acid (18:2). The most abundant isomers in commercial CLA mixtures are *cis*-9,*trans*-11 (*c*9,*t*11)- and *trans*-10,*cis*-12 (*t*10,*c*12)-octadecadienoic acid although there are some others, albeit to a lesser extent (3–5). In ruminant fat, the predominant CLA isomer is *c*9,*t*11 (1). Because of its abundance in natural foods it has long been assumed that *c*9,*t*11 is the bioactive isomer.

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Abbreviations: AP, atom %; BAT, brown adipose tissue; LPL, lipoprotein lipase; MUFA, monounsaturated FA; PDB, Peedee Belemnite limestone carbonate; PL, phospholipids; PPARγ, peroxisome proliferator-activated receptor γ; SFA, saturated FA; SSC; saline sodium citrate; UCP, uncoupling protein; WAT, white adipose tissue; XTT, sodium 3′[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate.

CLA have a variety of different effects *in vivo* including reduction of body fat accumulation and increase of energy expenditure (6–10), alteration of adipose tissue monounsaturated FA/saturated FA (MUFA/SFA) ratio (3,11), and alteration of lipid metabolism (12) in animal models and humans. Recent comparative studies investigating the effects of the two main CLA isomers demonstrated that *t*10,*c*12-CLA is effective in reducing lipid accumulation and differentiation of white fat cells (13–17). Studies *in vivo* also indicated that *t*10,*c*12-CLA rather than *c*9,*t*11-CLA might be the metabolite responsible for the lipid-lowering and glucose-sensitizing effect of CLA (18–20).

White and brown adipose tissues represent counter actors in energy partitioning, channeling energy either to lipid accumulation in white adipose tissue (WAT) or to oxidation, i.e., dissipation in brown adipose tissue (BAT) (21). The uncoupling protein (UCP1), a mitochondrial protein unique to brown adipocytes, is a FA- and nucleotide-binding protein central to uncoupling of the brown fat mitochondrial respiratory chain, the mechanism of heat production in this tissue. In certain animal models, increased BAT thermogenesis can dissipate excess energy and thus prevent obesity. White and brown adipocytes are recognized as distinct cell types, and, although morphologically very similar, white and brown preadipocytes show distinct patterns of gene expression (22). Recently, opposite effects of the major two CLA isomers, i.e., *c*9,*t*11- and *t*10,*c*12-CLA, on the thermogenic capacity of brown adipocytes have been reported (23). However, whether these two CLA isomers as compared to C_{18} FA other than linoleic acid affect lipid accumulation differently in primary white and brown preadipocytes is not yet known. Further, no information exists on effects of specific CLA isomers on the incorporation of glucose carbon into individual FA of adipocyte phospholipids (PL) and TAG.

We recently developed a model system of parallel primary cultures of white and brown preadipocytes isolated from the stromal-vascular fraction of WAT and BAT, respectively, from Djungarian hamsters (22,24,25). Because all other studies on CLA effects in adipocytes use either immortalized cell lines or white or brown preadipocytes cultures only (13–17, 26–29), this is, to our knowledge, the first report on the direct comparison of CLA isomer effects in white and brown adipocytes of Djungarian hamsters.

Thus, using primary white and brown preadipocytes as the cell model we determined (i) whether the lipid-altering effects of CLA are due to a specific isomer, (ii) whether *c*9,*t*11- and *t*10,*c*12-CLA isomers affect FA concentrations and FA synthesis from glucose in adipocyte TAG and PL differently, (iii) whether there are differential effects in white and brown adipocytes, and (iv) whether UCP1 gene expression in brown adipocytes is altered as a response to CLA and other C_{18} FA.

EXPERIMENTAL PROCEDURES

Cell isolation, culture conditions, and experimental design. Primary cell cultures were obtained from stromal vascular fraction of axillary, suprasternal, interscapular, and dorso-cervical brown fat depots (BAT) and inguinal white fat depots (WAT) of Djungarian hamsters *(Phodopus sungorus)*. Hamsters were bred in the animal facilities of the German Institute of Human Nutrition. Use of animals for experimental purposes was in accordance with European Commission regulations and approved by the local animal welfare committee at the Ministry of Agriculture, Nutrition and Forestry, State Brandenburg, Germany (Permission No. L8-3560-0/3). BAT and WAT of 5–10 hamsters (age 4–6 wk; approximately 1 g WAT and 1 g BAT per hamster) were pooled, for each cell culture. For primary cell culture the stromal vascular fraction containing preadipocytes was obtained by centrifugation after collagenase treatment as described previously (24). Cells were cultured for 10–11 d as described (22). Cells were inoculated in petri dishes at approximately 1500–2000 cells/cm^2. Cells were grown at 37°C in air with 5% CO_2 content and 100% RH in cell culture medium (50% modified Eagle's medium (Gibco/BRL, Eggenstein, Germany) and 50% Ham's F12 medium (Gibco/BRL) supplemented with $NaHCO_3$ (1.2 g/L), biotin (4 mg/L), Ca-pantothenate (2 mg/L), glutamine (5 mM), glucose (4.5 g/L) and Hepes (pH 7.4) 15 mM, penicillin G (6.25 mg/L), and streptomycin (5 mg/L). The medium was supplemented with 10% FCS (BioWhittaker, Verviers, Belgium). Medium was changed at days 1 and 3. To induce differentiation, insulin (17 nM; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and triiodothyronine (1 nM, Sigma-Aldrich) were added, and FCS supplement was reduced to 7% at day 3 of culture. Cultures were harvested on day 10 or 11.

*c*9,*t*11- and *t*10,*c*12-CLA isomers were synthesized stereoselectively as described (30). Stock solutions of 10 mg FA per mL ethanol were diluted to obtain final concentrations of 0, 3.6, 17.9, 35.7, or 71.4 μmol/L (0, 1, 5, 10 or 20 mg/L) in culture medium. The final ethanol concentration in culture medium was 0.1%. FA solutions were added to the differentiation medium on day 3 of culture, and the two CLA isomers were compared with the vehicle control (ethanol only), linoleic acid (18:2, *c*9,*c*12), α-linolenic acid (18:3, *c*9,*c*12,*c*15), and in some cases oleic acid (18:1, *c*9) (all from Sigma-Aldrich). In total 20 different treatments were tested.

Cell numbers and cell viability. Determination of cell numbers was performed as described previously (24,25). Briefly, cells were grown in 12-well dishes, trypsinized, suspended in 200 μL PBS buffer, and counted using a Thoma counting chamber. Results were expressed relative to the cell numbers in the respective control culture (ethanol only).

A colorimetric assay with XTT {sodium 3′[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate} (Roche Diagnostics, GmbH, Mannheim, Germany) was used to determine viability of adipocytes treated with CLA isomers and other FA. The test is based on the conversion of XTT to an orange-colored water-soluble formazan salt by viable cells. The formazan dye is quantified spectrophotometrically by absorbance (A_{450nm} minus A_{600nm}).

On culture day 11, medium was removed from cells grown in 96-well plates. Then 100 μL DMEM (without pH indicator) plus 50 μL XTT solution was added. Plates were incubated at 37°C for 24 h, and then the dye was quantified by using a plate reader.

Lipid content. Lipid content was analyzed by intracellular TAG staining with oil red O. On day 11 of cell culture, lipid content of cells grown in 12-well plates was determined by a modified method of Ramirez-Zacarias *et al*. (31) as described before (25).

Briefly, after washing with PBS, cells were fixed with 0.5 mL glutaraldehyde (3% in PBS) for 1 h at room temperature. After successive washing with water and 60% isopropanol (0.5 mL each), cells were stained with O red oil (0.2% wt/vol in 60% isopropanol) for 2 h (under constant gentle agitation). Cells were then washed again successively with 60% isopropanol (to eliminate excess color solution) and water. Water was allowed to evaporate (37°C overnight), and the fixed cells were destained with 0.5 mL 60% isopropanol twice for 2 h (under constant gentle agitation). The supernatants were pooled, and absorbency was measured at 510 nm. At least four dishes of three cultures each were used to determine the treatment effect ($n = 12$).

FA composition in esterified total lipids. FA composition (in % of total FA) in WAT and BAT cultures treated with FA concentrations of 71.4 μmol/L was determined by GC (HP 6890, Agilent Technologies Deutschland GmbH, Waldbronn, Germany) using FID and split injection (1:10). Cultured adipocytes were rinsed to remove media FA, extracted by hexane, evaporated, and re-extracted by chloroform/methanol (2:1 vol/vol) to obtain the total lipid fraction. FAME were generated by derivatization with potassium methylate in methanol at 60°C for 15 min. Separation of FAME was performed on a CP Sil 88 column (100 m; 0.25 mm i.d.; 0.2 μm film thickness) (Varian Deutschland GmbH, Darmstadt, Germany) using a four-step GC oven program (initial 120°C, held 2 min; 2°C/min to 175°C, held 20 min; 5°C/min to 200°C, held 15 min; 2°C/min to 235°C, held 5 min). Hydrogen was used as carrier gas at a flow rate of 1.4 mL/min. Quantification was performed by internal standard calibration with 17:0 FAME.

De novo *synthesis of TAG and PLFA from glucose carbon.* On day 3 of culture, 0.278 mM (50 mg/L) U-^{13}C-glucose [98 atom% (AP) ^{13}C; Cambridge Isotope Laboratories, Woburn, MA] was added to the differentiation medium in addition to the FA solutions to explore incorporation of glucose carbon

into lipids. As a result of dilution by unlabeled glucose already present in the medium ^{13}C-glucose enrichment was on average 11.6 AP ^{13}C. Cells were grown in plastic dishes (7 cm diameter) according to standard conditions described above. On day 10 total lipids from white and brown adipocytes were extracted by hexane/isopropanol (3:2, vol/vol) containing BHT (10 mg/mL). Sodium sulfate was added to remove residual water. By using aminopropyl-bonded silica extraction columns (CLEAN-UP®, 50 mg packed silica per 1 mL cartridge; Amchro GmbH, Hattersheim, Germany), TAG and PL were isolated. The adipocyte lipid extract was applied to a hexane-conditioned silica column, and the void fraction was collected. The column was washed with chloroform/isopropanol (2:1, vol/vol; 2 mL), and the eluate was combined with the void fraction. The combined fraction containing TAG and cholesterol esters was dried under N_2 at room temperature. PL were eluted with methanol (2 mL). The dried fraction was dissolved in hexane (2 mL) and added to a fresh silica column. TAG was eluted with hexane/diethyl ether/methylene chloride (89:1:10, by vol).

The eluates containing TAG or PL were dried and derivatized. FA (myristic acid, 14:0; palmitic acid, 16:0; palmitoleic acid, 16:1, *c*9; stearic acid, 18:0; and oleic acid, 18:1, *c*9) of TAG and PL of WAT and BAT were derivatized to form FAME by a one-step transesterification procedure with trimethylsulfonium hydroxide as described (32). Briefly, lipids isolated by solid-phase extraction were dissolved in 250 µL chloroform and mixed with 250 µL of internal standard solution (120 mg 17:1Δ9 in 100 mL chloroform) and 250 µL of a trimethylsulfonium hydroxide solution. ^{13}C enrichments of FAME were analyzed by GC–combustion–isotope ratio MS. The system comprised an isotope ratio mass spectrometer (Delta S; Finnigan MAT, Bremen, Germany) coupled online with a 5890 GC (Agilent Technologies) and has been described previously (33). FAME separation was performed using a DB-23 capillary column (60 m, 0.32 mm i.d., 0.25 µm film thickness) (J&W Scientific, Folsom, CA). A volume of 0.5 µL (TAG) or 2.5 µL (PL) was injected in split mode (injector temperature 250°C, split ratio 1:10). Carrier gas was He with a flow rate of 2 mL/min. The following temperature program was used: 50°C held 2 min; 50–175°C, ramp 3°C min^{-1}; held 10 min; 175–220°C, ramp 2°C min^{-1}; held 10 min; 220–240°C, ramp 5°C min^{-1}. Reference CO_2 gas pulses with known isotopic composition were introduced at specific times during the GC run for calibration. Peaks were identified routinely by comparison of retention times with known FAME standards. Data processing was performed by ISODAT vendor-provided software (Finnigan MAT). ^{13}C enrichment of FA is expressed as δ^{13}C value calibrated against the international standard Peedee Belemnite Limestone carbonate (PDB) as follows: $\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 10^3$ (‰), where R is the $^{13}C/^{12}C$ ratio. PDB has a $^{13}C/^{12}C$ ratio of 0.0112372 and has been assigned a δ^{13}C value of 0.0‰. To compare ^{13}C carbon incorporation in FA between treatments, the δ^{13}C value had to be converted to AP ^{13}C (33).

UCP1 mRNA expression. Expression of UCP1 mRNA was assessed by Northern blot using total RNA as described (22). RNA was extracted using the single-step acid phenol–guanidine protocol (34), separated by electrophoresis in a 1.2% agarose gel containing formaldehyde, and blotted by capillary transfer to a nylon membrane (Hybond N; Amersham Biosciences, Freiburg, Germany). The blots were probed with a ^{32}P-labeled UCP1 probe in a hybridization solution containing sodium phosphate (0.5 M), EDTA (1 mM), SDS (7%), and BSA (1%) at 42°C overnight; washed twice with 2×SSC (saline sodium citrate)/0.1%SDS for 10 min at room temperature, twice with 0.1×SSC/0.1%SDS for 20 min at 42°C; and twice with 0.1×SSC/0.1%SDS for 20 min at 68°C. A phosphoimager (BAS2000, Fuji) was used for analysis and quantification of radiolabeled signals. Hamster UCP1 probe was kindly provided by Dr. Martin Klingenspor (University of Marburg, Germany).

Statistical analyses. Effects of FA treatments were analyzed by ANOVA using the GLM procedure of SAS© software (SAS Systems, Release 8.2; SAS Institute Inc., Cary, NC). Main fixed factors were cell type (WAT, BAT), FA treatment (*c*9,*t*11-CLA, *t*10,*c*12-CLA, linoleic acid, α-linolenic acid, oleic acid, vehicle control), and FA concentration (0, 3.6, 17.9, 35.7, and 71.4 µmol/L) and interactions between them. Dependent variables tested were cell number and viability, lipid content, FA concentration, and ^{13}C enrichment of FA in TAG and PL or total lipids of white and brown adipocyte cultures. When the *F*-test of the interaction was significant, a multiple comparison of least square means with Tukey-Kramer adjustment was performed. A significance level of $P \leq 0.05$ was set.

RESULTS

Cell numbers and cell viability. Cell number and viability were determined to exclude possible deleterious effects of FA on the cells. Compared to the respective vehicle control culture (ethanol only), we did not find differences in total cell numbers or in cell viability (data not shown). Cell numbers counted were dependent on the respective culture and ranged from 70,000 to 90,000 in BAT and from 60,000 to 120,000 (cells per dish) in WAT, respectively.

Neutral lipid content. Lipid content of BAT and WAT cell cultures as measured by intracellular lipid staining was significantly affected by FA treatment. As shown in Figure 1, *t*10,*c*12-CLA decreased lipid filling of white and brown adipocytes in a dose-dependent manner but to a different degree. In WAT and BAT cultures, lipid contents were significantly lower at *t*10,*c*12-CLA concentrations of 35.7 and 71.4 µmol/L vs. the 3.6 µmol/L concentration and the control (no FA); however, reduction was about 60% in BAT and only 30% in WAT ($P \geq 0.1$). Doubling the *t*10,*c*12-CLA concentration from 35.7 to 71.4 µmol/L in the culture medium did not lead to a further decrease of lipid content in adipocytes (Fig. 1). At the 71.4 µmol/L concentration, the *c*9,*t*11-CLA treatment resulted in a significantly increased lipid content as compared to the control (no FA, vehicle only) by 80 and 50%

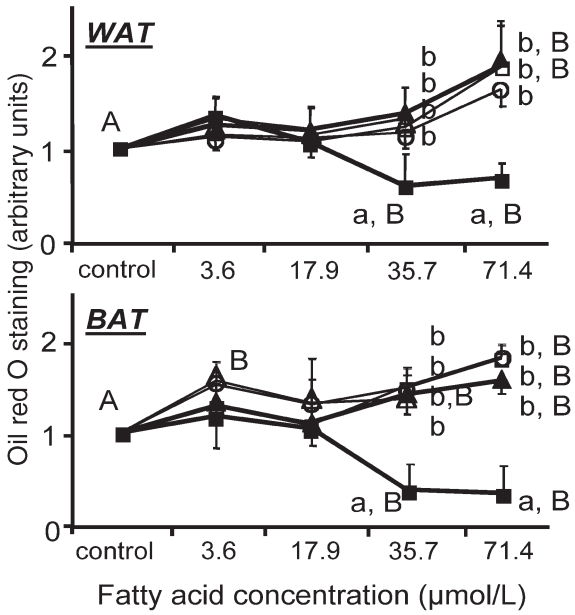

FIG. 1. Oil red O staining in primary white (WAT) and brown (BAT) adipocyte tissue cultures after treatment with *c*9,*t*11-CLA (filled triangles), *t*10,*c*12-CLA (filled squares), linoleic acid (open circles), α-linolenic acid (open squares) and oleic acid (open triangles). Lipid accumulation is expressed as extinction (at 510 nm) relative to the control culture (no FA) (arbitrary units). Within one concentration level, different lowercase letters indicate significant differences between FA treatments ($P \leq 0.01$). Within one specific FA treatment significant differences between concentrations are indicated by uppercase letters.

in WAT and BAT cultures, respectively (Fig. 1) ($P \leq 0.05$). In brown and white adipocytes oleic, linoleic, and α-linolenic acid at 71.4 µmol/L led to a significantly higher lipid content than in the control treatment.

FA composition in esterified total lipids. In untreated white and brown adipocyte cultures, oleic acid (18:1*c*9) was the most abundant FA, followed by palmitic acid (16:0) in BAT and palmitoleic acid (16:1) in WAT (Table 1). Oleic acid concentration was significantly higher in BAT (41 vs. 30% in WAT) whereas palmitoleic acid was higher in WAT (27%) as compared to BAT (20%), where its concentration was not different from palmitic acid (Table 1; $P \leq 0.05$). Only moderate concentrations of 18:0 (stearic acid) and 18:1*c*11 were found (5–8%). We observed low concentrations (1–2%) of myristic acid (14:0). *t*10,*c*12-CLA treatment caused a striking decrease of oleic acid and an increase of palmitoleic acid as compared to the control in BAT and linoleic acid in WAT ($P \leq 0.05$) (Table 1). In BAT, there was a tendency for a decrease in oleic acid after treatment with *c*9,*t*11-CLA. Also α-linolenic acid (18:3) treatment resulted in a decreased oleic acid content.

The incorporation of CLA into lipids, and in particular *c*9,*t*11-CLA, was higher in BAT (Table 1; $P \leq 0.05$). In BAT, concentration differences between *t*10,*c*12-CLA and *c*9,*t*11-CLA treatments were significant for myristic and palmitoleic acid, and total CLA (Table 1).

With the exception of α-linolenic acid- and *t*10,*c*12-CLA-treated cultures in BAT, oleic acid content was always higher

TABLE 1
Mean FA Concentrations (% of total FA in total lipids) in Primary White (WAT) and Brown Adipocyte (BAT) Cultures Treated with CLA Isomers and Other Polyunsaturated C_{18} FA (71.4 µmol/L)

	WAT cultures					BAT cultures				
FA	Control	*c*9,*t*11	*t*10,*c*12	Linoleic	Linolenic	Control	*c*9,*t*11	*t*10,*c*12	Linoleic	Linolenic
14:0	1.6	1.6	1.5	1.2	1.4	1.7	1.8	2.5	1.6	1.5
14:1	0.6	0.6	0.6	0.5	0.6	0.5	0.5	1.0[a,b,c]	0.4[c]	0.3
16:0	21.0	22.0	21.3	17.8	21.2	21.3	26.1	22.2	24.0	31.1[a,b]
16:1	27.0	24.6	28.6[c]	21.5[b]	25.9	19.7[a]	20.3	30.4[b,c]	16.3	16.2[a]
18:0	5.8	5.9	6.0	5.8	5.1	5.9	6.0	5.0[c]	6.6	8.2[a,b]
18:1*t*-9	0.3	0.3	0.3	0.1	0.2	0.6	0.1	0.4	0.2	0.6
18:1*c*-9	30.1	36.3	29.2[c]	39.8[b]	34.0	40.9[a]	34.3	26.6[b,c]	39.4	29.2[b]
18:1*c*-11	7.7	6.7	7.0	8.9	7.4	7.5	5.3	5.8	6.1	3.9
18:1*c*-13	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.1
18:2*c*-9,*c*-12	0.4	0.2	0.9	1.8	0.3	0.4	0.3	1.0	3.3	0.5
20:1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
18:3n-3	0.1	0.0	0.0	0.1	1.7[b]	0.0	0.1	0.1	0.0	6.2[a,b]
CLA	0.1	0.6	0.4	0.2	0.1	0.1	2.0[a,b,c]	0.5	0.1	0.0
22:0	0.2	0.1	0.2	0.1	0.1	1.5	0.1	0.2	0.1	0.1
20:4	1.3	0.5	2.0	1.0	0.6	0.9	0.6	1.7	0.9	0.7
24:0	0.1	0.1	0.1	0.0	0.0	0.4	0.2	0.7	0.2	0.4
20:5n-3	0.0	0.1	0.1	0.0	0.7[b]	0.0	0.1	0.2	0.0	0.2[a]
22:4	0.3	0.1	0.5	0.3	0.1	0.2	0.1	0.4	0.3	0.1
22:6n-3	0.4	0.2	1.0	0.3	0.4	0.5	0.4	1.0	0.3	0.5
SFA	28.7	29.6	29.0	25.0	27.8	30.9	34.2	30.7	32.4[a]	40.8[a,b]
MUFA	66.2	68.7	65.9	71.1	68.3	69.6	60.6[b]	64.4	62.6[a]	50.3[a,b]
PUFA	2.6	1.6	4.8	3.8	3.9	2.2	3.6	4.8	5.0	8.3
MUFA/SFA	2.3	2.3	2.3	2.9	2.5	2.3	1.8	2.1	2.0	1.2
SFA/PUFA	10.9	18.8	8.4	6.6	7.2	11.3	10.2	6.7	7.4	6.0
MUFA/PUFA	25.4	43.7	23.4	18.9	18.0	29.4	18.5	14.4	15.3	7.7

[a]Significantly different from corresponding WAT culture ($P \leq 0.05$).
[b]Significantly different from control treatment of same cell culture type ($P \leq 0.05$).
[c]Significantly different from linoleic acid treatment of same cell culture type ($P \leq 0.05$). SFA, saturated FA; MUFA, monounsaturated FA.

TABLE 2
***De novo* Synthesis of TAG and Phospholipid (PL) FA from Glucose Carbon Given as ^{13}C FA Enrichments Under Control Conditions (no FA treatment) in Adipocytes Derived from BAT or WAT[a]**

	BAT-PL		WAT-PL		BAT-TAG		WAT-TAG	
FA	LSM	SE	LSM	SE	LSM	SE	LSM	SE
14:0	121.0	19.3	164.0	21.2	338.1	17.3	274.2	25.8
16:0	223.3	17.0	229.9	13.3	361.5	17.0	327.5	28.1
16:1	303.5	16.0	296.5	18.8	319.3	17.2	295.9	29.3
18:0	104.6	19.5	132.1	22.3	351.6	17.6	325.8	28.3
18:1	256.7	15.4	242.5	18.8	336.8	16.2	304.5	25.4

[a]^{13}C enrichments in $\delta^{13}C$ ‰; data are least square means (LSM) ± SE. For abbreviations see Table 1.

TABLE 3
UCP1 Gene Expression in Brown Adipocytes After 10 d of Culture with *c*9,*t*11-CLA, *t*10,*c*12-CLA, Linoleic Acid, and α-Linolenic Acid[a]

Treatment	UCP1 mRNA (arbitrary units)	Treatment	UCP1 mRNA (arbitrary units)
Control	1	Linoleic acid	1.92
*c*9,*t*11 CLA	2.68	α-Linolenic acid	1.88
*t*10,*c*12 CLA	0.27		

[a]FA were added at 71.4 µmol/L final concentration. Data are means from two independent cell cultures expressed relative to the control, i.e., ethanol-treated, cells. UCP1, uncoupling protein 1.

than palmitic acid content. With most of the treatments, oleic acid was more highly concentrated than palmitoleic acid except for the control and the *t*10,*c*12-CLA treatment in WAT and the *t*10,*c*12-CLA in BAT (Table 1, $P \leq 0.05$).

As expected, in α-linolenic acid-treated cells, incorporation of this FA was increased compared to the control, with the highest concentrations being detected in BAT. Treatment with α-linolenic acid was furthermore accompanied by higher eicosapentaenoic acid (20:5 n3) contents in WAT. Treatment with linoleic and α-linolenic acids resulted in increased SFA in BAT compared to WAT ($P \leq 0.05$). In α-linolenic acid-treated cultures of BAT, SFA were increased and MUFA were decreased as compared to control (Table 1). In BAT, *c*9,*t*11-CLA caused a reduction of MUFA. No alterations of MUFA/SFA, SFA/PUFA, and MUFA/PUFA ratios were observed.

De novo *synthesis of TAG and PL FA from glucose carbon.* Appearance of ^{13}C-labeled FA in adipocyte lipids after addition of ^{13}C-labeled glucose to the culture medium indicates a *de novo* synthesis of FA and their incorporation in lipids. ^{13}C enrichment of FA was significantly affected by the type of FA treatment (Figs. 2–4). In control cultures (vehicle only) there was an overall tendency for lower ^{13}C enrichments in FA of PL than in TAG in both cell types (Table 2). Enrichments in PL were not different between BAT and WAT control cultures (Table 2). Minor FA in PL, such as 14:0 and 18:0, showed a significantly lower enrichment compared to all other FA, and also compared to 14:0 and 18:0 enrichments in TAG ($P \leq 0.05$). Palmitate (16:0) and stearate (18:0) serve as precursors of the two most common MUFA in animal cells: palmitoleic (16:1) and oleic acid (18:1). Comparison of 16:0 vs. 16:1*c*-9, and 18:0 vs. C18:1*c*-9, respectively, shows that enrichments

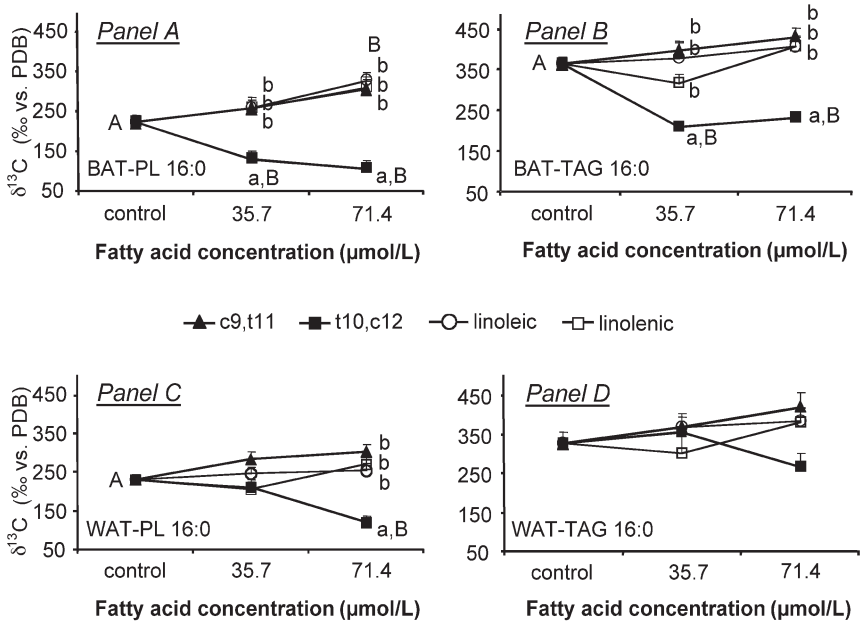

FIG. 2. ^{13}C enrichments (least squares mean ± SE; δ‰ vs. PDB) of palmitic acid in TAG (panels B, D) and PL (panels A, C) of BAT (panels A, B) and WAT (panels C, D) after treatment with *c*9,*t*11-CLA (filled triangles), *t*10,*c*12-CLA (filled squares), linoleic acid (open circles), and α-linolenic acid (open squares) and addition of U-^{13}C-glucose to the culture medium. Within one concentration level, different lowercase letters indicate significant differences between FA treatments ($P \leq 0.01$).Within one specific FA treatment, significant differences between concentrations are indicated by different uppercaseletters ($P \leq 0.01$). PDB, Peedee Belemnite limestone carbonate; for other abbreviations see Figure 1.

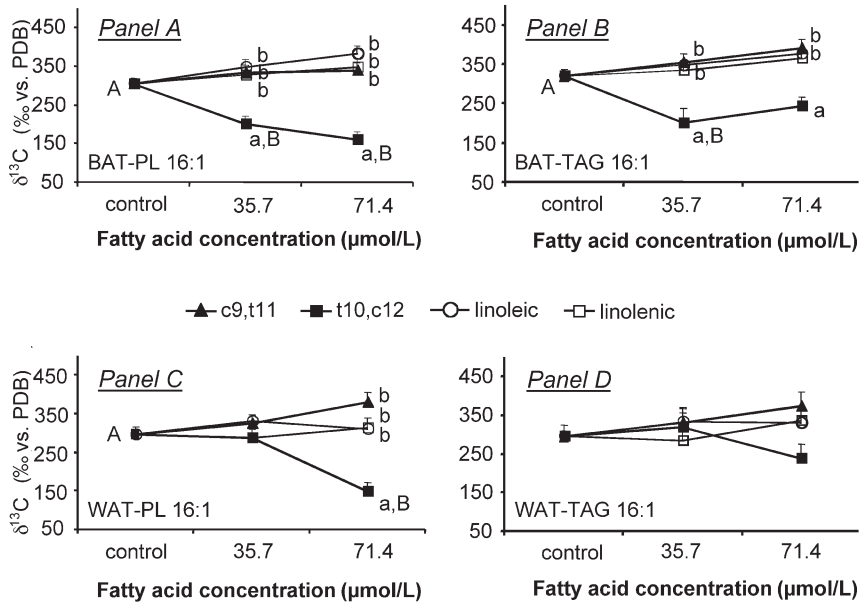

FIG. 3. ^{13}C enrichments (LSM ± SE; δ‰ vs. PDB) of palmitoleic acid in TAG (panels B, D) and PL (panels A, C) of BAT (panels A, B) and WAT (panels C, D) after treatment with *c*9,*t*11-CLA (filled triangles), *t*10,*c*12-CLA (filled squares), linoleic acid (open circles), and α-linolenic acid (open squares) and addition of U-^{13}C-glucose to the culture medium. Within one concentration level, different lowercase letters indicate significant differences between FA treatments ($P \leq 0.01$). Within one specific FA treatment significant differences between concentrations are indicated by different uppercase letters ($P \leq 0.01$). For abbreviations see Figures 1 and 2.

significantly increase from saturated to unsaturated in PL but do not differ in TAG of both cell types (Table 2).

In both cell types, *t*10,*c*12-CLA treatment reduced the incorporation of glucose carbon into all FA measured (Figs. 2–4; stearic and myristic acids not shown). All FA measured showed similar ^{13}C enrichment patterns. With increasing FA concentrations in the culture medium, in general, the treatments with *c*9,*t*11-CLA and linoleic and α-linolenic acids, re-

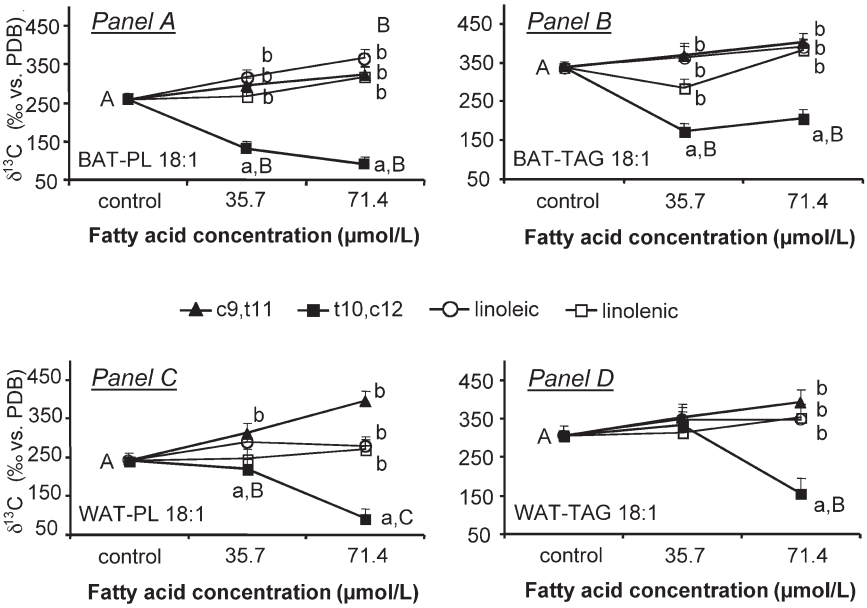

FIG. 4. ^{13}C enrichments (LSM ± SE; δ‰ vs. PDB) of oleic acid in TAG (panels B, D) and PL (panels A, C) of BAT (panels A, B and WAT (panels C, D) after treatment with *c*9,*t*11-CLA (filled triangles), *t*10,*c*12-CLA (filled squares), linoleic acid (open circles), and α-linolenic acid (open squares) and addition of U-^{13}C-glucose to the culture medium. Within one concentration level, different lowercase letters indicate significant differences between FA treatments ($P \leq 0.01$).Within one specific FA treatment significant differences between concentrations are indicated by different uppercase letters ($P \leq 0.01$).

spectively, resulted in a tendency for an increased ^{13}C enrichment in both TAG and PL by a maximum of 0.04–0.16, 0.02–0.12, and 0.02–0.09 AP ^{13}C, respectively, at the 71.4 μmol/L concentration (Figs. 2–4). It appeared as if brown adipocytes were more sensitive to the *t*10,*c*12-CLA treatment than white adipocytes, since significant reductions in glucose incorporation were already apparent at the 35.7 μmol/L concentrations. No differences in ^{13}C enrichments were observed between *c*9,*t*11-CLA, linoleic acid, and α-linolenic acid treatments at the concentrations tested.

UCP1 mRNA expression in brown adipocytes. UCP1 mRNA was increased 1.68-fold by treatment with *c*9,*t*11-CLA (Table 3). Linoleic and α-linolenic acids also increased UCP1 gene expression, though to a lesser extent. The *t*10,*c*12-CLA isomer, on the other hand, substantially reduced UCP1 expression to about one-fourth of the control level.

DISCUSSION

Most studies on specific effects of CLA isomers on cultured adipocytes have been conducted in 3T3-L1 cells, an immortalized preadipocyte cell line derived from segregated mouse embryos (15,17,26–29). Results of gene expression profiling of 3T3-L1 cells, however, indicate that this preadipocyte cell line might not truly reflect adipogenesis *in vivo* (35). Furthermore, most studies have concentrated on WAT, and only very recently have the effects of CLA isomers on BAT functional properties been investigated (23). Therefore, we conducted our experiments in primary white and brown adipocytes in parallel.

Our results show clearly that in primary white and brown adipocytes *t*10,*c*12-CLA is the lipid-lowering isomer. This effect was dose dependent, and the effective dose of *t*10,*c*12-CLA was 35.7 μM (10 mg/L), which agrees with studies in 3T3-L1 preadipocytes (15,16). In contrast, *c*9,*t*11-CLA as well as linoleic acid increased the lipid content at 35.7 and 71.4 μmol/L in BAT and 71.4 μmol/L in WAT, as also shown in primary human and porcine adipocytes (13,36). This is in line with animal studies using *t*10,*c*12-CLA or CLA mixtures particularly rich in this isomer, indicating that this is the active substance responsible for reduction in body fatness, whereas *c*9,*t*11-CLA either has no such effects or slightly increases whole-body lipid contents (12,16,37,38). Interestingly, in WAT at the highest FA concentration tested, the lipid-enhancing effect of *c*9,*t*11-CLA (about 100% increase as compared to control) was more pronounced than the antiadipogenic effect of *t*10,*c*12-CLA, with the latter CLA isomer resulting in a 30% reduction of lipid filling (Fig. 1).

Possible mechanisms responsible for the lipid-lowering and antiadipogenic effects of *t*10,*c*12-CLA in adipocytes include an increase of lipolysis, a decrease of FA and TAG synthesis, an increase in FA oxidation, and a stimulated apoptosis. Lipoprotein lipase (LPL), which is synthesized and secreted by adipocytes, hydrolyzes TAG. LPL is regulated by FA, which increase LPL mRNA but decrease LPL activity (39). The potency to decrease LPL activity increases with chain length; the degree of unsaturation is less important (39). *t*10,*c*12-CLA significantly decreased LPL activity and enhanced TAG release in 3T3-L1 mouse adipocytes, whereas *c*9,*t*11-CLA did not change these parameters (16). Also lower intracellular but higher free glycerol concentrations were found with *t*10,*c*12-CLA, suggesting a lower re-esterification of FA and increased lipolysis (15,16). As we (in this paper) and others (13) have shown, *t*10,*c*12-CLA reduced glucose carbon incorporation in FA, suggesting a reduced *de novo* FA synthesis. Furthermore, *t*10,*c*12-CLA reportedly enhanced oleic acid oxidation in 3T3-L1 adipocytes whereas *c*9,*t*11-CLA reduced CO_2 production from oleic acid by 50% as compared to the control (29).

Peroxisome proliferator-activated receptor γ (PPAR γ) was shown to be required for adipocyte formation both *in vivo* and *in vitro,* PPAR γ2 being the main isoform that mediates FA-induced gene expression in adipose tissue (40). However, results from Evans *et al.* (15) indicate that the lipid-lowering effect of *t*10,*c*12-CLA does not directly depend on a reduction of PPAR γ2 protein expression because acute treatment increases PPAR γ2 expression, whereas chronic treatment decreases it. Stimulated apoptosis might also be a factor explaining the lipid-lowering effect of *t*10,*c*12-CLA. By using CLA mixtures in 3T3-1 adipocyte cultures, Morrison and Farmer (40) showed that CLA- and, in particular, *t*10,*c*12-CLA-treated cultures had more apoptotic cells than the linoleic acid control. This is in agreement with a feeding study in mice in which mixed CLA and *t*10,*c*12-CLA consumption caused apoptosis in WAT (41). Although we did not directly measure apoptosis, the fact that we did not observe any effect of CLA isomers on cell number and viability is not supportive of apoptosis as the major lipid-lowering mechanism. This is in agreement with *in vivo* results in rats (42) and *in vitro* results in 3T3-L1 preadipocytes (43). Taken together, our data indicate that the lipid-lowering effect of *t*10,*c*12-CLA is—at least partly—due to a decreased *de novo* FA synthesis.

In line with earlier reports, we observed that *c*9,*t*11-CLA accumulates to a higher extent than *t*10,*c*12-CLA in adipocyte lipids (Table 1) (29,44). This could be explained by a preferred incorporation or a lower susceptibility of *c*9,*t*11-CLA to oxidation, as previously shown in liver cells (45), and/or more rapid metabolism of the *t*10,*c*12-CLA isomer (44).

We have confirmed the ability of CLA to lower MUFA in neutral lipids, specifically, oleic acid, but not palmitoleic acid, for which we found a paradoxical increase. Our results, based on the simultaneous comparison of WAT and BAT adipocyte cultures, suggest that CLA isomer effects differ according to the adipocyte type (Table 1). Apparently, brown adipocytes were more susceptible to the FA-modulating effects of CLA isomers. We found a tendency for an increase of SFA with *c*9,*t*11-CLA. Reduction of MUFA and increase of SFA levels in adipose tissue and lipids have been reported previously (11,15,46). Whether this was caused by displacement of MUFA by CLA is unclear. However, there is strong evidence that *t*10,*c*12-CLA down-regulates stearoyl-CoA desaturase *in vivo* and *in vitro* at several levels of action (mRNA, protein, enzyme activity). Stearoyl-CoA desaturase is the enzyme re-

sponsible for synthesizing oleate and palmitoleate from oleic and palmitic acid, which are the major FA in adipocytes (almost 60%) (28,47,48).

Choi *et al.* (28) reported a reduced level of arachidonate after treatment of 3T3-L1 adipocytes with *c*9,*t*11-CLA whereas *t*10,*c*12-CLA increased both arachidonate and its precursor linoleate. In contrast, data from Evans *et al.* (15) showed no difference in the levels of arachidonic acid with either CLA isomer. The latter is in line with our findings although we observed a trend toward higher concentrations of linoleate and arachidonate with *t*10,*c*12-CLA treatment (Table 1).

The treatment of WAT and BAT cultures with α-linolenic acid would be expected to result in an enhancement of EPA, which was the case in white adipocytes but not in brown although α-linolenic acid concentration was much higher in BAT. This could possibly be due to a reduced gene expression of Δ6-FA desaturase in brown preadipocytes compared to white preadipocytes in primary cell cultures, which we observed previously (22). Only a few studies have compared FA composition of WAT and BAT. Also, FA composition of BAT is subject to considerable changes mainly related to the activity of BAT (49–51). In BAT we observed a reduction of oleic and palmitoleic acids (Table 1).

In humans, overconsumption of carbohydrates while reducing dietary fat intake can increase lipogenesis (52,53). *In vitro,* glucose and FA act as direct mediators of the transcription of enzymes in the biosynthetic pathway to convert excess carbohydrate into TAG for efficient energy storage (54). Our results on effects of *t*10,*c*12-CLA on glucose carbon incorporation into FA are in contrast to the findings of Satory and Smith (27) and Evans *et al*. (29), who observed an increasing incorporation of glucose carbon into total cellular lipids in adipocytes with treatments of either a CLA mixture (40:40 *t*10,*c*12-CLA/*c*9,*t*11-CLA) or the pure *t*10,*c*12-CLA isomer. However, our finding of a *t*10,*c*12-CLA-induced decrease of glucose carbon incorporation into individual FA of PL and TAG is in line with and further substantiates the studies of Brown *et al.* (13,14) in human primary adipocytes. These authors reported a reduced glucose uptake and oxidation as well as a reduced oleic acid uptake and oxidation (14), presumably resulting in a reduced *de novo* FA synthesis from glucose and a lower incorporation of FA in adipocyte lipids, as found in the present study (Figs. 1, 4). *c*9,*t*11-CLA and linoleic acid caused a significant increase of glucose carbon incorporation into WAT-PL 18:0, and BAT-PL 16:0 and 18:1, respectively, as compared to the control, which again is in agreement with the study of Brown *et al.* (13) but in contrast to other results (29) (Figs. 2–4). This difference might possibly be due to metabolic differences between 3T3-L1 adipocytes and primary adipocytes as used here and in the studies of Brown *et al.* (13,14).

It is interesting to note that *t*10,*c*12-CLA might indeed have cell type-specific effects. It has been shown that in Caco-2 cells this isomer increased cellular *de novo* TAG synthesis (55). In general, it appears as if incorporation of newly synthesized FA was higher in TAG as in PL, and there was a tendency for all FA measured, albeit insignificant, for incorporation to be higher in BAT-TAG than in WAT-TAG.

It has been reported that 75% of the weight-lowering effect of CLA in mice could be attributed to an increase in energy expenditure (10). Members of the mitochondrial uncoupling protein family function to "uncouple" the transfer of electrons and synthesis of ATP in the inner mitochondrial membrane, resulting in thermal dissipation of energy as heat in place of ATP production. Therefore, uncoupling may be a mechanism of increased energy expenditure (56). In brown adipocytes, Rodriguez *et al*. (23) reported an inhibition of UCP1 mRNA expression by *t*10,*c*12-CLA but an increase with *c*9,*t*11-CLA treatment. This is completely in line with our findings. In addition we confirm that linoleic and α-linolenic acids increase UCP1 mRNA, however to a lesser extent than *c*9,*t*11-CLA. In one dietary study using a CLA mixture, UCP1 expression was not altered in BAT, but energy expenditure was increased by 8% (57). In another study dietary CLA was found to decrease UCP1 mRNA levels in BAT (58). The fact that *t*10,*c*12- and *c*9,*t*11-CLA isomers have opposite effects on UCP1 gene expression as shown here and previously (23) may explain why feeding studies using CLA mixtures found only slight effects on energy expenditure in rodents. On the other hand, UCP2 in muscle and WAT and BAT was found to be up-regulated by *c*9,*t*11-CLA and a CLA mixture (9,58,59). Whereas UCP1 expression is clearly linked to brown fat thermogenesis, i.e., energy expenditure in rodents (60), the biological function of UCP2 is not yet clear (61). The finding that the lipid-lowering CLA isomer (*t*10,*c*12-CLA) simultaneously decreases UCP1 expression implies that an activation of BAT thermogenesis is probably not the cause of the weight-reducing effect of CLA. However, it cannot be excluded that *in vivo* CLA may increase energy expenditure through mechanisms not related to BAT thermogenesis.

We have demonstrated that *c*9,*t*11-CLA and *t*10,*c*12-CLA have distinctly different effects on lipid metabolism in adipocytes. *c*9,*t*11-CLA, like other unsaturated C_{18} FA (oleic, linoleic, and α-linolenic acids), increased lipid accumulation in white and brown adipocytes and increased UCP1 expression in brown adipocytes. *t*10,*c*12-CLA, on the other hand, decreased lipid accumulation in both cell types by decreasing glucose incorporation into FA while decreasing UCP1 expression in brown adipocytes. The opposing effects of *c*9,*t*11-CLA and *t*10,*c*12-CLA are similar and evident both in WAT and in BAT cultures; however, brown adipocytes seem to be more susceptible to treatment with *t*10,*c*12-CLA, which might be related to the different function of BAT in energy metabolism. The exact molecular mechanism responsible for the highly specific effects of *t*10,*c*12-CLA remains to be established.

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Trans-18:1 Isomers in Rat Milk Fat as Effective Biomarkers for the Determination of Individual Isomeric *trans*-18:1 Acids in the Dams' Diet

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ABSTRACT: Female rats were fed a diet containing by weight 10% partially hydrogenated sunflower oil, 2% sunflower oil, and 1% rapeseed oil during gestation and lactation. The *trans*-18:1 isomer profile of the fat supplement was (in % of total *trans* 18:1 acids in the fat supplement): Δ4, 0.5; Δ5, 1.0; Δ6–Δ8, 18.0; Δ9 (elaidic), 13.5; Δ10, 22.2; Δ11 (vaccenic), 16.0; Δ12, 11.3; Δ13–Δ14, 12.8; Δ15, 2.5; and Δ16, 2.2 (total *trans* 18:1 acids in the fat supplement: 40.6%). The *cis* 18:1 isomer profile was (in % of total *cis*-18:1 isomers): Δ6–Δ8, 2.1; Δ9 (oleic), 70.9; Δ10, 6.1; Δ11, 8.3; Δ12, 4.0; Δ13, 2.8; Δ14, 4.6, and Δ15, 1.2 (total *cis*-18:1 acids in the fat supplement: 32.6%). Suckling rats from four litters were sacrificed at day 17 or 18 after birth, and their stomach content (milk) was analyzed. The *trans*-18:1 isomer profile of milk was (relative proportions, in % of total): Δ4, 0.3; Δ5, 1.1; Δ6–Δ8, 16.8; Δ9, 15.3; Δ10, 22.0; Δ11, 16.7; Δ12, 11.8; Δ13-14, 11.8; Δ15, 2.5, and Δ16, 1.9 (total *trans* 18:1 acids in milk: %). That of *cis*-18:1 isomers was (proportions in % relative to total *cis*-18:1 isomers): Δ6–Δ8, 4.7; Δ9, 72.5; Δ10, 4.0; Δ11, 8.0; Δ12, 7.1; Δ13, 1.9; Δ14, 1.0, and Δ15, 0.7 (total *cis*-18:1 acids in milk: %). These results demonstrate that all isomeric acids, independent of the geometry and the position of the ethylenic bond, are incorporated into milk lipids. With regard to *trans*-18:1 isomers, the distribution profile in milk is identical to that in the dams' diet, i.e., there is no discrimination against any positional isomer between their ingestion and their deposition into milk lipids. As a consequence, this study indicates that the *trans*-18:1 isomer profile of milk reflects that in the dams' diet and supports our earlier hypothesis that the profile of *trans*-18:1 isomers in milk can be used to deduce the relative contribution of ruminant fats and partially hydrogenated oils in the diet to the total intake of *trans*-18:1 isomers. On the other hand, the *cis*-18:1 isomer profile in milk shows significant differences when compared to that in the dams' diet. Surprisingly, there are no major differences for the *cis*-Δ9 (oleic) and the *cis*-Δ11 (asclepic) isomers, which can be synthesized by the mother. However, there seems to be a significant positive selectivity for the group *cis*-Δ6–Δ8, and for the *cis*-Δ12 isomer, whereas a negative selectivity occurs for the Δ10 and Δ13 to Δ15 *cis* isomers.

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Abbreviations: DCF, 2′,7′-dichlorofluorescein; PHSO, partially hydrogenated sunflower oil; PHVO, partially hydrogenated vegetable oil.

Dr. Robert L. Wolff

Robert Wolff passed away at the age of 53 on the 10th of November, 2002. His know-how in the field of lipids was recognized internationally. He had the ability to lead his research projects in both the animal and vegetal worlds. His scientific achievement, more than 100 publications to his name in the field of trans *fatty acids, made him highly esteemed by his colleagues.*

He was Conference Master at Bordeaux 1 University (France) up until 2001, at which time he joined the Nutritional Lipid Unit in I.N.R.A., Dijon (France). His mission there was to develop a research program on plasmalogens and their role in brain and muscle function, for which his analytical and biochemical skills were a guarantee of success. Unfortunately, his state of health did not allow him to complete this project.

This publication is his final one.

Although many attempts have been made to estimate the total content of *trans*-18:1 isomers in human milk (1), only a few studies have been devoted to the assessment of individual isomeric *trans*-18:1 acids (2–5). In these studies, it was hypothesized that the isomer profile of individual *trans*-18:1 in human milk (2–5) [and in adipose tissue (6,7)] could be used to assess the relative contributions of partially hydrogenated vegetable oils (PHVO) and ruminant fats to the total dietary intake of *trans*-18:1 isomers. This assumption was based on the potential absence of enzymatic discrimination against any positional *trans*-18:1 isomer between their ingestion and their deposition in milk lipids, but this was not experimentally proven. Milk and blood, as well as adipose tissue, are the most easily available human tissues or fluids for such an approach. In the human diet, ruminant fats and PHVO are the predominant sources of *trans*-18:1 acids, the main class of dietary "*trans* fatty acids." In both sources, the double bond in *trans*-18:1 isomers can be found anywhere from position Δ4 to Δ16. However, in considering the quantitative distribution of individual isomers, PHVO differ from ruminant fats. In particular, one of the principal isomers in PHVO is elaidic (*trans*-9 18:1) acid, whereas in ruminant fats it is vaccenic (*trans*-11 18:1) acid (7–9).

There is some evidence indicating that these two isomers have different metabolic fates. Elaidic acid behaves much like a saturated acid in the acylation of phospholipids (10), even if it can be Δ5-desaturated under specific conditions (11), whereas vaccenic acid may be Δ9-desaturated to *cis*-9,*trans*-11 18:2 (rumenic) acid (12), which has potential anticarcinogenic effects (13). Elaidic and vaccenic acids are present in PHVO as well as in ruminant fats, although in different proportions. It is thus important to distinguish between these two isomers. Little is known about the *in vivo* metabolic fate of other positional isomers. By combining TLC on silica gel plates impregnated with silver ions (argentation TLC) with GLC on very long, highly polar capillary columns, practically all individual *trans*- and *cis*-isomers can be separated and reliably quantified (14). In the present study, this combination of procedures has been applied to the study of milk FA from lactating rats fed a diet supplemented with partially hydrogenated sunflower oil (PHSO) containing, among many other geometrical and positional 18:1 acid isomers, both elaidic and vaccenic acids in roughly similar amounts. Our results show that the whole *trans*-18:1 isomer profile in milk lipids reflects that in the dams' diet. One is tempted to speculate that a similar situation would exist in humans, which might justify *a posteriori* the conclusions deduced from studies of human milk FA (2–5). Additional observations were made for *cis*-18:1 isomers, for which no data were available thus far.

TABLE 1
FA Composition of the Experimental Fat Supplement: Partially Hydrogenated Sunflower Oil (10%, w/w), plus Sunflower Oil (2%) and Rapeseed Oil (1%)

FA	Surface area (% of total)[a]	FA	Surface area (% of total)[a]
16:0	6.9	*c*10 18:1	2.0
18:0	7.8	*c*11 18:1	2.7
*t*4 18:1[b]	0.2	*c*12 18:1	1.3
*t*5 18:1	0.4	*c*13 18:1	0.9
*t*6–8 18:1	7.3	*c*14 18:1	1.5
*t*9 18:1	5.5	*c*15 18:1	0.4
*t*10 18:1	9.0	*c*9,*c*12 18:2	8.3
*t*11 18:1	6.5	*c*9,*c*12,*c*15 18:3	1.3
*t*12 18:1	4.6	20:0	0.3
*t*13–14 18:1	5.2	20:1	0.1
*t*15 18:1	1.0	∑*t*18:1[c]	40.7
*t*16 18:1	0.9	∑*c*18:1	32.6
*c*6–8 18:1	0.7	Unidentified[d]	0.7
*c*9 18:1	23.1		

[a]FA composition established by combining analyses of unfractionated and fractionated FAME (see Experimental Procedures section).
[b]*t, trans; c, cis.*
[c]Sum of the corresponding isomers.
[d]Sum of unidentified components.

EXPERIMENTAL PROCEDURES

Animals and diet. Four female Wistar rats aged 10–12 wk were allowed to mate by pairing for 1 wk. After this period, they were housed separately and fed the experimental diet (*vide infra*). At birth, litters were adjusted to eight pups. Dams were kept on the experimental diet until day 17 or 18 after delivery. At that time, four pups (mean weight, 52 ± 2 g) from each litter were sacrificed and their stomachs dissected. The stomach contents of pups from the same dam were pooled, and their lipids were extracted according to the procedure of Folch *et al*. (15). To ensure that suckling rats had not eaten some of the dam's feed prior to sacrifice, the feed was withdrawn and the pups were separated from the dam for 5 h, and then allowed to suck again. During the whole experimental period, animals were kept in an air-conditioned room maintained at 22°C and 55% RH with a 12 h day/12 h night cycle. Fresh food and water were provided every day. The animals were weighed daily.

Female rats were fed a semipurified diet to which was added a blend of PHSO (10 g/100 g), sunflowerseed oil (2 g/100 g), and rapeseed oil (1 g/100 g). The FA composition of the fat supplement is given in Table 1. Other dietary components were (g/100 g diet): cornstarch, 40; granulated sugar, 19; casein, 23; mineral mixture, 5; vitamin mixture, 1; and cellulose, 2.

Preparation of FAME and their fractionation. A convenient aliquot of milk lipids or fat supplement was dissolved in 3 mL hexane to which 300 μL of a 0.5 N solution of sodium methoxide in methanol was added. After thorough mixing and incubation for 10 min at 50°C, water was added, and the mixture was vortexed and centrifuged. The upper hexane phase was withdrawn and evaporated to dryness. For the fat supplement, the residue was taken in a small volume of hexane for further GLC analysis. For milk, the residue was dissolved in a 7% (wt/vol) solution of BF_3 in methanol and incubated at 100°C for 15 min (the purpose was to methylate FFA likely to be present in the stomach lipids). After addition of water, FAME were extracted with hexane. Aliquots of FAME (prepared from either the fat supplement or milk lipids) were fractionated according to the number and the configuration of ethylenic bonds by argentation TLC as described elsewhere (16). The solvent was hexane/diethyl ether (90:10, vol/vol). The bands were revealed by spraying a 0.2% solution of 2′,7′-dichlorofluorescein (DCF) in ethanol and observing under UV light at 366 nm. FAME were extracted from the gel with a biphasic solvent system that allows removal of the DCF (16).

GLC analysis of FAME. Unfractionated as well as fractionated FAME were analyzed on a HP 4890A chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a 100-m CP-Sil 88 capillary column [0.20 mm i.d., 0.25 μm film (cyanopropyl polysiloxane polymer); Chrompack, Middelburg, The Netherlands]. Unless otherwise stated, the oven operating temperatures were: 60°C for 1 min, increase at 20°C/min to 170°C then isothermal for 40 min; second increase at 5°C/min to 190°C, isothermal until the end of the run. The first plateau allowed a satisfactory resolution of isomeric 18:1 acids, whereas the second plateau was aimed at eluting long-chain polyunsaturated FAME, up to 22:6n-3 acid. The injector (splitless) and the FID were maintained at 250 and 280°C, respectively. Hydrogen was the carrier gas,

with a head pressure of 224 kPa. Injections (1 μL) were made manually.

Identification and quantification of FAME. Individual peaks of isomeric 18:1 FAME were identified according to Wolff and Bayard (14), using commercial elaidic acid (*trans*-9 18:1) and the *trans*-monoenoic acid fraction isolated from butter fat to calibrate retention times. Peaks were integrated and quantified with the Diamir software (version 1.5.302.2; JMBS Inc., Newark, NJ). No correction factors were applied, and data are given as area percentages. Calculations of total *trans*- as well as *cis*-18:1 isomers were made through the use of *trans*-11 18:1 acid for *trans*-18:1 isomers, and of *cis*-9 18:1 acid after subtraction, if necessary, of the *trans*-13 plus *trans*-14 critical pair, for *cis*-18:1 isomers.

RESULTS

The chromatographic profiles (18:1 isomer region) of unfractionated as well as those of the *cis*- and *trans*-monoenoate fractions prepared from the PHSO used in this study are given in Figure 1. Similar chromatograms for the whole-fat supplement (including sunflower and rapeseed oils) were obtained, except that the *trans*-13 and *trans*-14 18:1 isomers were not separated as well from the *cis*-9 18:1 peak, which was significantly broadened as compared to PHSO. The former isomers appeared as a shoulder on the leading edge of the oleic acid peak. This is shown in Figure 2, which compares the 18:1 isomer regions of unfractionated FAME prepared from the total fat supplement and from a representative sample of milk. This figure also shows the great similarity between the fat supplement and milk FA in the 18:1 isomer region. Figure 1 as well as Figure 2 also emphasizes the need for silver ion TLC fractionation for accurately quantifying each individual isomer. The critical pair *trans*-Δ13 plus *trans*-Δ14 elutes with the unresolved *cis* Δ6–8 group and may not be fully separated from the *cis*-Δ9 isomer. Isomers *cis*-Δ10 and *trans*-Δ15 on the one hand, and *cis*-Δ14 and *trans*-Δ16 on the other hand, are not resolved at all.

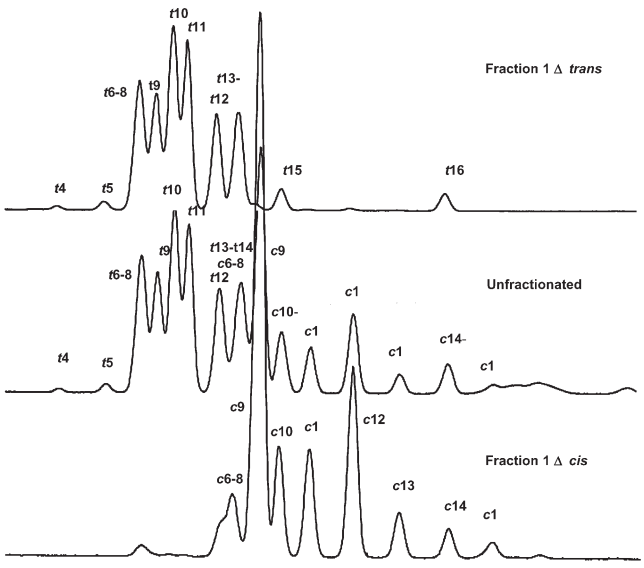

FIG. 1. Partial chromatograms of isomeric 18:1 FAME prepared from partially hydrogenated sunflower oil (part of the experimental dietary fat supplement), either unfractionated (middle) or fractionated by argentation TLC (upper and lower). GC separations performed with a 100-m CP-Sil 88 capillary column (commercial source, characteristics, and operating conditions given in the Experimental Procedures section).

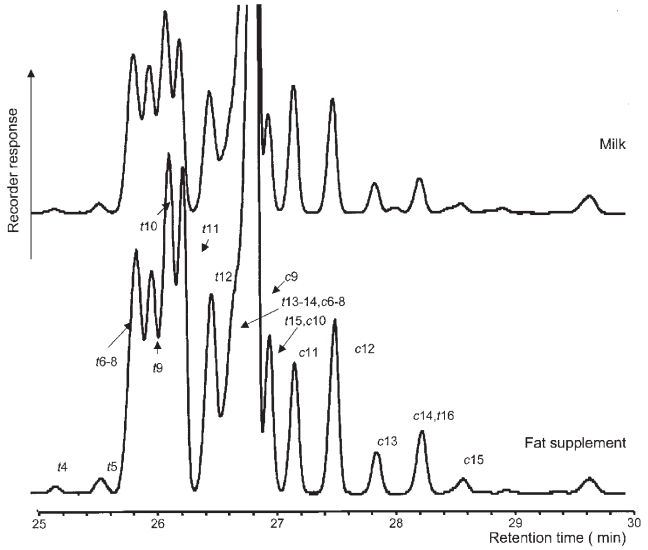

FIG. 2. Partial chromatograms of isomeric 18:1 FAME prepared from rat milk (upper) and from the experimental dietary fat supplement (lower). GC separations performed with a 100-m CP-Sil 88 capillary column (commercial source, characteristics, and operating conditions given in the Experimental Procedures section).

Quantitative data for milk FA compositions are given in Table 2. The presence in the stomach content of suckling rats of chains shorter than 16:0 acid, of 16:0 acid in proportions higher than in the fat supplement, of long-chain PUFA, of (unidentified) isomeric 16:1 acids, and of rumenic (*cis*-9,*trans*-11 18:2) acid, all of which are absent from the experimental blend of oils, clearly indicates that this content was milk. However, as our procedures included several solvent evaporation steps, chains shorter than 10:0 acid, if initially present, were likely lost or their content reduced

Figure 3 compares the distribution profiles of *trans*-18:1 isomers in the experimental fat supplement with that of milk lipids. No significant differences for any individual isomer were noted. From this similarity, one can infer that the *trans*-18:1 isomer profile of milk lipids is a good reflection of the corresponding profile in the dams' diet.

Surprisingly, *cis*-9 and *cis*-11 18:1 acids, which can be synthesized by the dams, are present in almost equal proportions relative to total *cis*-18:1 isomers in milk lipids and in the dietary fat supplement, with, however, a slightly higher proportion of the former isomer in milk (Fig. 4). For other *cis*-18:1 isomers, exclusively of dietary origin, there is a significant positive selectivity for the Δ6–Δ8 isomer group and for the Δ12 isomer, which are present in higher proportions in milk lipids than in the fat supplement. On the contrary, *cis* isomers Δ10, Δ13, Δ14, and Δ15 show an apparent negative

TABLE 2
FA Composition of the Stomach Content[a] (milk) of Rats Suckling at Dams Fed a Diet Supplemented with Partially Hydrogenated Sunflower Oil[b]

FA[c]	Mean	SD	FA[c]	Mean	SD
10:0	1.7	1.22	*t*16 18:1	0.4	0.03
12:0	3.2	0.47	*c*6–8 18:1	0.7	0.27
14:0	3.4	0.73	*c*9 18:1	24.4	1.12
15:0	0.1	0.01	*c*10 18:1	1.9	0.49
16:0	14.3	1.32	*c*11 18:1	3.2	0.17
*t*16:1	0.3	0.04	*c*12 18:1	2.5	0.24
16:1	1.6	0.35	*c*13 18:1	0.8	0.27
17:0	0.1	0.01	*c*14 18:1	0.4	0.17
18:0	5.1	0.14	*c*15 18:1	0.3	0.07
*t*4 18:1	0.1	0.02	*c*9,*c*12 18:2	8.5	0.52
*t*5 18:1	0.2	0.02	20:1	0.9	0.43
t6–8 18:1	3.9	1.09	20:2n-6	0.2	0.18
*t*9 18:1	3.2	0.41	20:4n-6	0.5	0.06
*t*10 18:1	4.7	0.56	22:6n-3	0.1	0.01
*t*11 18:1	4.0	1.00	Others	2.3	0.27
*t*12 18:1	2.5	0.37	∑*t*18:1	23.5	1.01
*t*13–14 18:1	2.6	0.20	∑*c*18:1	34.2	1.43
*t*15 18:1	1.9	0.04			

[a]The stomach contents of four pups within each litter were pooled before fat extraction.
[b]See Table 1 for the detailed composition of the fat supplement.
[c]FA shorter than 10:0 acid likely have been lost during the procedure of FAME preparation, which included several solvent evaporation steps. Percentages of individual isomeric 18:1 acids were established as described in the Experimental Procedures section.

selectivity. As most *cis*-18:1 isomers are baseline-resolved after silver ion TLC, these differences between the fat supplement and milk lipids cannot be attributed to erroneous quantification of individual peaks.

DISCUSSION

The PHSO used in the present experiment as a fat supplement has a *trans*-18:1 profile distinct from that of PHVO used for food processing in France (8). Our comparisons and discussion will be limited to France, one of the few countries for which detailed data on individual *trans*-18:1 isomers have been established for a great number of food samples. Most often, only the total *trans*-18:1 isomers are determined. It is likely that the food industries in France use partially hydrogenated rapeseed oil, this oil being cheaper than sunflower oil. However, we chose PHSO because the elaidic and vaccenic acid (*trans*-Δ9 and -Δ11 isomers) are present in almost similar proportions, 13.5 and 16.0% (relative to total *trans*-18:1 isomers), respectively. In French foods containing PHVO, the corresponding values are 28 and 13% on average (8). Butterfat (or more generally any ruminant fat) would have been a poor model for our purposes, because the content of vaccenic acid (*ca*. 50%) is approximately tenfold that of elaidic acid (8,9).

In our experiments the distribution profile of *trans*-18:1 fats in the experimental fat supplement was not significantly different from that in the milk lipids found in the stomach of rat pups. If one assumes that a similar situation exists in humans, it would validate some earlier studies in France and Germany where the *trans*-18:1 isomer profile of human milk was used to estimate the relative contributions of ruminant fats and PHVO to the mothers' total *trans*-18:1 isomer intake (2,4). Similar estimates were made with data for adipose tissue from French women (7), but here too, experimental bases indicating that dietary *trans*-18:1 isomers are deposited in adipose tissue TAG without selectivity are scarce. For Canada, no trials were made to quantitatively estimate the relative contributions of PHVO and ruminant fats to the total *trans*-18:1 acid intake, but it was concluded, from the study of both milk and adipose tissue FA, that PHVO contributed the "major" part to this intake (5,6). Our present observations also sustain the suggested possibility of predicting the *trans*-18:1 acid profile in human milk, provided the relative contributions of ruminant fats and PHVO to the mothers' diet, and their profile too, are known (8). Wood (17), in dietary experiments with adult rats fed PHVO (prepared with safflower oil), studied the distribution profile of *trans*-18:1 isomers in the TAG fraction from several tissues, including adipose tissue. No gross differences were observed between these profiles and that in the dietary fat supplement. On the other hand, a

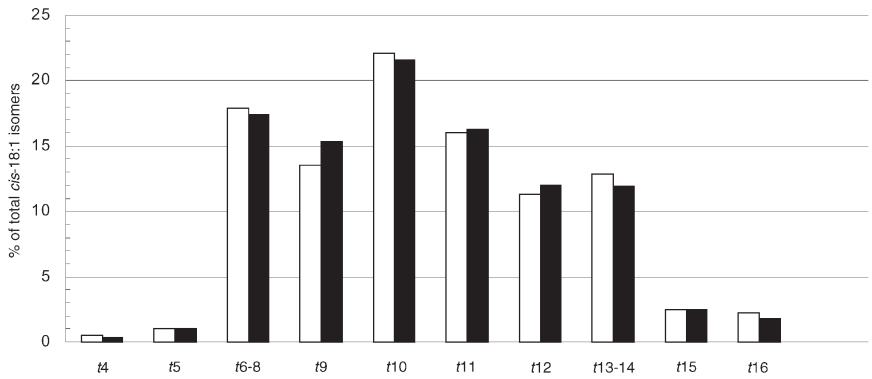

FIG. 3. Comparison of individual *trans*-18:1 isomers present in the experimental fat supplement (open bars) and in milk fat (solid bars; mean ± SD of rats from four litters). The proportions are relative to total *trans*-18:1 isomers and were determined after fractionation by argentation TLC.

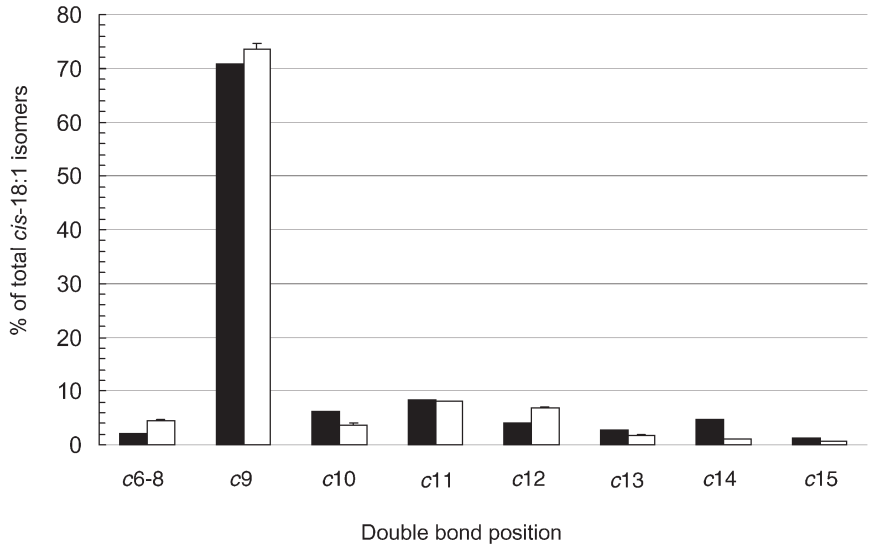

FIG. 4. Comparison of individual *cis*-18:1 isomers present in the experimental fat supplement (open bars) and in milk fat (solid bars; mean ± SD of rats from four litters). The proportions are relative to total *cis*-18:1 isomers and were determined after fractionation by argentation TLC.

considerable negative selectivity against deposition of the *trans*-Δ10 isomer into phospholipids occurred in all organs studied, whereas a large positive selectivity for the *trans*-Δ12 isomer was noted. Similar observations were made with rat liver, heart, and serum PC (18) and liver mitochondria total phospholipids (19). These trends, however, were not detected when considering *trans*-18:1 isomers isolated from total FA of some human organs, supposedly mimicking the average profile of *trans*-18:1 isomers in commercial (U.S.) margarines and cooking oils (20). In these fats, the *trans*-Δ10 isomer was the major isomer, and it also was the prominent *trans*-18:1 acid in FA from all organs, including adipose tissue.

The *cis*-18:1 isomer profile in milk lipids does not faithfully reflect that in the dietary fat supplement. An important discrimination in the deposition of the *cis*-Δ10 18:1 isomer into TAG was observed for several organs of adult rats fed partially hydrogenated safflower oil (17). On the other hand, as in the present study, the proportions of elaidic (*trans*-9) and vaccenic (*trans*-11) acids (relative to total *cis*-18:1 isomers) in the TAG from all organs studied were not very different from their proportions in the PHVO supplement.

FA in milk TAG may have several origins: (i) dietary lipids, (ii) adipose tissue, and (iii) neo-synthesis (liver, mammary gland). Under our experimental conditions, the animals were fed the PHSO supplement during pregnancy, prior to lactation, in order to equilibrate the *trans*-18:1 isomer profile in their adipose tissue with that in the diet. We ignore the respective participation of the dietary lipids and of adipose tissue to the resulting *trans*-18:1 isomer profile in milk lipids. But the similarity between milk *trans*-18:1 isomer profile with that in the diet indicates that the *trans*-18:1 isomer profile in adipose tissue lipids, if mobilized for milk TAG synthesis, should not differ significantly from that in the diet.

To check whether adipose tissue (and plasma) lipids may be as good markers of individual *trans*-18:1 isomer consumption as milk FA, the study of individual *trans*- as well as *cis*-18:1 isomer incorporation into these lipids is mandatory.

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Ligand-Binding Domain of Farnesoid X Receptor (FXR) Had the Highest Sensitivity and Activity Among FXR Variants in a Fluorescence-Based Assay

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ABSTRACT: The farnesoid X receptor (FXR, NR1H4) has been recognized as an attractive therapeutic target because it is a nuclear hormone receptor that controls the expression level of cholesterol-7α-hydroxylase, which in turn regulates bile acid production and cholesterol excretion. To compare receptor activity between each domain and the full-length protein, human FXR cDNA was cloned from a human liver cDNA library. Three human FXR cDNA, designated FXR_{20}, FXR_{33}, and FXR_{53} cDNA, were subcloned and ligated into a pET28a expression vector. Each protein was expressed in *Escherichia coli* (BL21) and purified by nickel-nitrilotriacetic acid column chromatography. Approximately 5 mg of FXR_{33} (1–182 amino acids deleted from FXR, 37 kDa) and 2 mg of FXR_{53} (the full-length protein of FXR, 59 kDa) was purified from 1 L of Luria-Bertani culture, achieving at least 90% purity. The coactivator recruitment assay for FXR activation was carried out with the three variants of the FXR protein by using dissociation-enhanced lanthanide fluoroimmunoassay-europium-N^1-labeled anti-His antibody. From an optimized assay, a saturated hyperbolic fluorescence signal curve was produced when 250 nM of FXR_{33} and 100 nM of steroid receptor coactivator-1 peptide, a coactivator of FXR consisting of 26 amino acids, were used with a concentration dependence on chenodeoxycholic acid (from 0 to 200 μM). The ligand-binding domain of FXR (FXR_{33}) was the most suitable protein for studying the activation of FXR with a fluorescence-based assay, because it showed better structural stability than either the full length of FXR (FXR_{53}) or the DNA-binding domain of FXR (FXR_{20}).

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Bile acid production and cholesterol homeostasis are tightly regulated by complex mechanisms, including the actions of cholesterol-7α-hydroxylase (CYP7A1), the liver X receptor, and the farnesoid X receptor (FXR) on sensitive cellular concentrations of oxysterols and bile acids (1,2). FXR (NR1H4), or bile acid receptor, is a member of the nuclear hormone receptor superfamily (3), which can bind the promoter regions of several target genes, i.e., the ileal-bile acid-binding protein (I-BABP) (4,5), the small heterodimer partner (SHP) (6), the phospholipid transfer protein (PLTP) (7), and apolipoprotein (apo)-E (8), in response to the circulation of excessive bile acid concentrations, especially chenodeoxycholic acid (CDCA) (9). FXR can indirectly repress transcription of CYP7A1 by increasing the transcription of SHP after FXR/retinoid X receptor binding on specific DNA sequences spaced by one nucleotide, referred to as inverted repeat-1 [13 base pairs (bps), GGGTCA-n-TGACCT] (7).

Expressional levels of several genes, i.e., CYP7A1, PLTP, and apoE (8), are affected by activation of FXR, as their activities are closely associated with bile acid metabolism and cholesterol homeostasis. For this reason, FXR has been recognized as a putative pharmaceutical treatment target against hypercholesterolemia (6) and liver disorders of bile acid metabolism (2) because it controls the cholesterol efflux pathway in the liver and the intestine. A few synthetic FXR modulators have been reported, for example, 6-ECDCA (6α-ethyl-CDCA) (10), GW-4064 (11), AGN29 (12), and AGN34 (12). In 2000, GW-4064 was found to be an FXR agonist by *in vivo* testing with Fisher rats, with about 220-fold greater potency than CDCA and a decreasing effect on serum TG concentration (11). Two years later, 6-ECDCA was developed to decrease the liver toxicity of GW4064 with similar agonist activity (10). Very recently, a series of chemicals that were derived from [(tetrahydrotetramethylnaphthalenyl)propenyl] benzoic acid, a synthetic retinoid that can act as a ligand for both FXR and the retinoic acid receptor, were reported as FXR agonists or antagonists, and termed AGN29, AGN31, and AGN34 (12).

On the other hand, Edwards' group (13) identified four isoforms of murine FXR, i.e., FXRα1, FXRα2, FXRβ1, and FXRβ2, and revealed that each isoform has a different expressional level and transcriptional activity depending on its location within specific tissues. Four amino acid residues (M-Y-T-G) were inserted into the middle region of FXRα1 and FXRβ1 and were found to play a critical role in modulating the function of FXR (13). FXRα1 and FXRβ1 showed poor activation ability for murine I-BABP in response to the same ligand treatment when compared to FXRα2 or FXRβ2. With the increasing significance of FXR activity measurement, it is necessary to find the optimal size of the FXR protein to maximize its activity in the assay. This is especially true since FXR is difficult to purify.

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Abbreviations: apo, apolipoprotein; bp(s), base pair(s); CDCA, chenodeoxycholic acid; CYP7A1, cholesterol-7α-hydroxylase; DELFIA, dissociation-enhanced lanthanide fluoroimmunoassay; 6-ECDCA, 6α-ethyl-chenodeoxycholic acid; FXR, farnesoid X receptor; I-BABP, ileal-bile acid-binding protein; Ni-NTA, nickel-nitrilotriacetic acid; PLTP, phospholipid transfer protein; SHP, small heterodimer partner; SRC-1, steroid receptor coactivator-1; TBST, Tris-HCl + NaCl + EDTA + Tween.

To maximize its solubility and activity in the aqueous buffer, Edwards and coworkers (7) successfully purified an FXR fragment (Δ1–110) that is lacking 1–110 amino acids from the N-terminus of FXR.

In this work, a full-length human FXR cDNA (termed FXR_{53}) was cloned, and three kinds of FXR cDNA were subcloned from the FXR_{53} cDNA. Three variant proteins were expressed and purified from *Escherichia coli*. These three proteins were applied to the coactivator recruitment assay by using fluorescence detection to compare the receptor sensitivity in response to CDCA binding.

EXPERIMENTAL PROCEDURES

Materials. Taq DNA polymerase and mixed deoxynucleotide triphosphates (dNTP) were purchased from Neurotics (Daejeon, Korea) and Promega (Madison, WI). For the fluorescence-based assay, dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) Eu-N^1-labeled anti-His antibody and other assay reagents (i.e., assay buffer, wash concentrate, and enhancement solution) were purchased from PerkinElmer Lifesciences (Wallac, Turku, Finland). Nickel-nitrilotriacetic acid (Ni-NTA) column media and plasmid preparation kits were purchased from Qiagen (Valencia, CA). Anti-FXR polyclonal IgG (#sc-1204) and anti-His monoclonal IgG (#sc-8036) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Gene cloning, protein expression, and purification of FXR. Human FXR cDNA was isolated by PCR from a human liver cDNA library (Clontech, Palo Alto, CA) by using the following appropriately designed primers: 5′-ATGGGATCAAAAATGAATCTC-3′ and 5′-TCACTGCACGTCCCAGATTTC-3′. Two subcloned full-length FXR cDNA were ligated into a pGEM-TE vector (Promega), and the sequences were verified by DNA sequencing at the sequencing facility of the Korea Research Institute of Biosciences and Biotechnology (Daejeon, Korea) by using the 3700 model of the Applied Biosystem Institute (Foster City, CA). Two full-length FXR cDNA, termed FXR_{20} cDNA and FXR_{53} cDNA, were cloned by PCR. The FXR_{20} gene was a possible PCR artifact that had a point mutation to terminate amino acid synthesis at the 180th codon. The FXR_{53} cDNA had a mutation that included the insertion of 12 bps into the hinge region of the full-length FXR protein, the same as in the FXRα1 that was reported recently (13). The other FXR gene, FXR_{33} cDNA, which encodes the ligand-binding domain region of native FXR, was generated after *Sal* I digestion in the middle region (at 547 bps) of the FXR_{20} cDNA. Collectively, the three FXR cDNA were generated and ligated individually into pET28a vector (Novagen, Madison, WI) to construct an expression vector. After transformation, a single colony containing each target plasmid was selected and inoculated into fresh Luria-Bertani medium supplemented with 30 μg/mL of kanamycin sulfate (Gibco-BRL, Grand Island, NY), and was incubated at 37°C until cell density reached $OD_{600\,nm}$ = 0.8–0.9. Synthesis of the target proteins was induced by the addition of a final 1 mM of isopropyl-β-D-1-thiogalactopyranoside (Sigma, St. Louis, MO) and a further 4-h incubation under the same conditions. Induced cells were collected and disrupted by sonication under the presence of one tablet of mini-complete-EDTA-free protease inhibitor (Roche, Mannheim, Germany). The lysates were solubilized in 20 mM Tris-HCl/6 M guanidine-HCl, pH 7.9, and were subjected to Ni-NTA column (1.5 × 5 cm) chromatography according to a standard protocol described previously (14). The target proteins were eluted from the column with 20 mM Tris-HCl/1 M imidazole/500 mM NaCl, pH 7.9. After elution of the column, 2 mL of each aliquot of the purified FXR proteins was dialyzed against 25 mM HEPES, pH 7.6, 2 mM $MgCl_2$, 20% glycerol, 1 mM EDTA, 100 mM KCl, 1 mM DTT, 1% Nonidet P-40, 200 μM phenylmethyl sulfonylfluoride, and 1 μg/mL leupeptin to maintain the solubility of the FXR. Each aliquot of the FXR was stored at −70°C until use.

Fluorescence-based FXR assay. Each purified FXR protein, which contained a His^6-tag at the N-terminus (around 4 kDa), was used for the assay. As a coactivator protein, biotinylated human steroid receptor coactivator-1 (SRC-1) peptide was synthesized according to a standard protocol and purified by HPLC at the protein facility of the Korea Research Institute of Biosciences and Biotechnology. The SRC-1 peptide, which consists of 26 amino acids with a LXXLL motif, where L is leucine and X is any amino acid (15), was added as a coactivator for the assay. The assay was carried out following a protocol described previously (16,17), with only slight modifications by using a DELFIA-Eu-N^1-labeled anti-His antibody. Nunc-Maxisorp 384-well black plates (Cat. #460518) were coated with 30 μL of 100-nM NeutrAvidin (Pierce, Rockford, IL) in 50 mM sodium bicarbonate/150 mM NaCl containing 0.02 mg/mL of sodium azide (pH 9.6) by incubation with gentle shaking overnight at 4°C. After applying the coating, 50 μL of 4% BSA in PBS (pH 8.0) was added for 2 h at room temperature; following that, the blocking solution was removed in three washes with 100 μL of TBST (50 mM Tris-HCl/150 mM NaCl/1 mM EDTA/0.05% Tween 20, pH 8.0). After the blocking, 100 nM of the biotinylated SRC-1 peptide in 10 μL of assay buffer (50 mM Tris/50 mM KCl/1 mM DTT/0.1% BSA, pH 7.4), 5 μL of chemical to be tested, and 250 nM of FXR (as final conc.) were added. The mixture was preincubated for 30 min at room temperature to ensure that the putative modulator (an agonist or antagonist) interacted with the FXR for a longer period of time than the CDCA. After the preincubation, 10 μL of CDCA solution in the assay buffer was added to the mixture at final concentrations of 0–100 μM. The mixture was incubated for 2 h at room temperature and then 10 μL/well of a 1-nM DELFIA Eu-N^1-labeled anti-His antibody (Wallac; AD0108) was added to each well, and the plate was further incubated for 1 h at room temperature. The plate was washed three more times by using TBST as previously described in the blocking step. For development and detection, 40 μL of enhancement solution (Wallac; 1244-104) was added and mixed. The fluorescence signal was detected at 340 nm excitation and 615 nm

emission wavelengths with a 400-μs delay time and a 400-μs counting time by using a Victor^2 optical microplate reader (Wallac).

SDS-PAGE, Western blotting, and protein sequencing. Protein purity was determined by SDS-PAGE using Phastprecast gel and a Phast electrophoresis system (Amersham Pharmacia, Uppsala, Sweden) or Criterion precast gel (BioRad, Seoul, Korea). Western blotting was carried out with a PhastTransfer semidry transfer kit (Pharmacia, Uppsala, Sweden), and the blotted bands were detected by using a BM Chemiluminescence blotting substrate (Roche Diagnostics, Indianapolis, IN).

Protein samples for sequencing were electrotransferred onto a polyvinylidene fluoride membrane (Immobilon-P^{SQ}) with a Hoefer TE22 mini-tank transfer unit (Amersham Pharmacia) according to the protocol outlined by Matsudaira (18). After the blotting, the membrane was stained with 1.25% Coomassie Brilliant Blue R-250 and the desired band was cut out with a clean razor. The NH_2-terminal amino acid sequence of the excised band was determined with an Applied Biosystems model 491A sequencer that was located in the Korea Basic Research Institute (Daejeon, Korea).

Miscellaneous methods. Protein concentration was determined according to a Lowry protein assay as modified by Markwell *et al.* (19) or by using the Bradford assay reagent (BioRad) with BSA as a standard.

RESULTS

Gene cloning, vector construction, and protein purification. Two variants of FXR cDNA were cloned from a human liver cDNA library and the sequences were verified as shown in Figure 1. One was termed FXR_{53} (GenBank accession no. AF384555) and contained a full-length sequence of NR1H4 plus 12 bps (-ATG-TAT-ACA-GGC-) that were inserted in the 588th position, similar to the FXR variants (FXRα1 and FXRβ1) reported by Edwards' group (13). This mutation allowed a protein to be produced that consisted of 476 amino acids and four amino acids (M-Y-T-G) that were inserted into the hinge region of the native FXR (the 197th to 200th amino acid positions). The other, referred to as FXR_{20} cDNA, consisted of 1,419 bps and had a sequence identical to NR1H4 (GenBank no. NM_005123) except that a point mutation occurred at the 538-bp position (C was replaced by T) to produce a stop codon at the 180th amino acid. Therefore, FXR_{20} cDNA encoded only the 1st to 179th amino acids of human FXR, which corresponded to the DNA-binding domain of FXR. Although this mutant might have been a possible PCR artifact, the FXR_{20} cDNA was restricted to produce a truncated protein of FXR (FXR_{33}). FXR_{33} cDNA was generated after removing 1–546 bps from the 5′-region of FXR_{20} cDNA, including the stop codon at the 538th bp, by *Sal* I digestion. Thus, FXR_{33} had only a truncated region spanning the 183rd to 472nd amino acids of FXR (Δ1–182 of FXR), which corresponded to the ligand-binding domain of the native FXR, as illustrated in Figure 1.

The subcloned genes were individually ligated into a pET28a expression vector using the *EcoR* I site, and the orientation was verified again by DNA sequencing. After transformation, the expressional level and the pattern of proteins were different depending on each gene. FXR_{33} and FXR_{20}

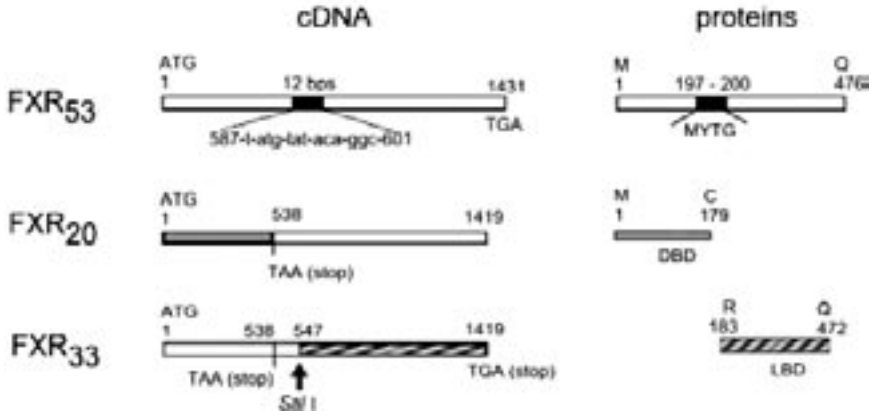

FIG. 1. Schematic illustration of the subcloned farnesoid X receptor (FXR) variant cDNA and expected proteins. A full-length sequence of FXR cDNA was subcloned from a human cDNA library as described in the Experimental Procedures section. FXR_{20} cDNA consists of 1,419 base pairs (bps), and its sequence was identical with NR1H4 (GenBank no. NM_005123, except that a point mutation occurred at the 538th base pair (bp) position (C is replaced by T) to produce a stop codon at the 180th amino acid. Although this mutation might be a PCR artifact, the FXR_{20} protein had a DNA-binding domain (DBD) region only as indicated in gray. The other was termed FXR_{53} cDNA and contained a full-length sequence of NR1H4 plus 12 bps (-ATG-TAT-ACA-GGC-) inserted at the 588th position. The FXR_{53} protein consisted of 476 amino acids, with four amino acids (M-Y-T-G) inserted into the hinge region of the FXR at the 197th to 200th amino acid positions (GenBank no. AF384555). FXR_{33} cDNA was generated by *Sal* I digestion to remove 1–546 bps, including the stop codon at the 538th bp from FXR_{20} cDNA, which allowed encoding of the 183rd to 472nd amino acids of FXR (Δ1–182 of FXR) and corresponded to the ligand-binding domain (LBD) of FXR, as indicated by the cross-hatched line.

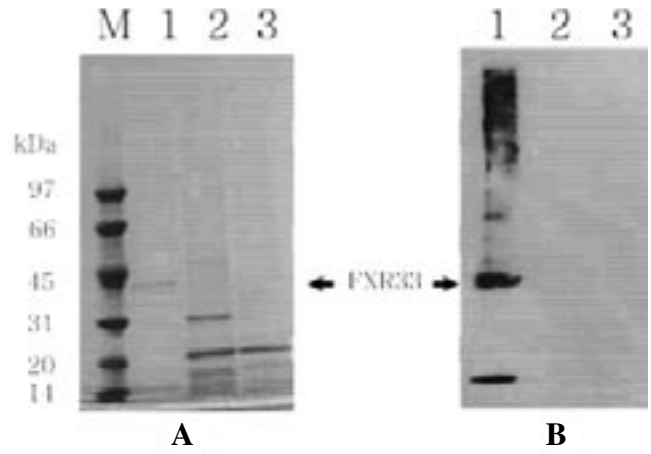

FIG. 2. Purified FXR variants shown by SDS-PAGE (A) and Western blotting (B). In panel A, the FXR_{33} protein showed a 37-kDa band position owing to the presence of the His-tag (4 kDa) on a 4–15% gradient Criterion precast gel (BioRad, Seoul, Korea). Lane M, low M.W. marker (Amersham Pharmacia, Uppsala, Sweden); lane 1, FXR_{33}; lane 2, FXR_{20}; lane 3, FXR_{53}. The FXR_{33} protein also showed a minor 12-kDa fragment at the bottom of the gel, possibly a degraded product. In panel B, the 37 and 12 kDa of FXR_{33} were detected clearly by anti-FXR antibody (#sc-1204; Santa Cruz Biotechnology, Santa Cruz, CA). In contrast, the FXR_{20} and FXR_{53} were not detected at all by the FXR antibody (lanes 2 and 3), which indicates that the carboxyl terminus was more susceptible to proteolysis. For abbreviation see Figure 1.

showed levels of protein expression that gradually increased over 4 h and were dependent on incubation time, whereas FXR_{53} reached the highest level of expression at 3 h (data not shown). As shown in panel A of Figure 2, the purified FXR_{33} protein appeared at about the 37-kDa band position and included the 4-kDa His-tag and a minor 12-kDa band by SDS-PAGE. In the Western blot experiment, both the 37- and 12-kDa bands were detected clearly by the anti-FXR polyclonal antibody. This result suggests that the 12-kDa band was a possible C-terminal fragment, because the FXR antibody recognized only the C-terminal region of the FXR and because the N-terminal region that included the His-tag disappeared during protein purification. A similar pattern of degradation was observed in the purification of FXR_{20} and FXR_{53}, as shown in panel A of Figures 2 and 3. A major band, around the 26- and 20-kDa bands, appeared at the FXR_{20} protein (lane 2) and only a 20-kDa band appeared at the FXR_{53} protein (lane 3); however, the expected sizes of FXR_{20} and FXR_{53} were 26 and 59 kDa, respectively. In panel B of Figure 2, the proteins in lanes 2 and 3 were not detected by the anti-FXR antibody, which suggests that proteolytic degradation occurred from the C-terminus region of the FXR_{53} protein. Indeed, the FXR antibody could not detect the FXR_{20} protein because it was lacking the C-terminal region of the native FXR.

As illustrated in panel B of Figure 3, the Western blot analysis revealed that the FXR_{33} protein was detected as an aggregated form in the upper stacking gel when using either the anti-FXR polyclonal antibody (lane 1, panel B of Fig. 3) or the anti-His monoclonal antibody (lane 1, panel C of Fig. 3). On the other hand, the FXR_{20} protein was detected by using only the anti-His antibody, since it lacked the antigenic epitope region for the anti-FXR antibody that was raised against a peptide of the carboxyl terminus (C-20) of the human FXR.

N-terminal protein sequencing results revealed that major fragments of FXR_{20} or FXR_{53}, around the 20-kDa fragment, started with the sequence G-S-S-H-H-H-H-H-H-S-, which corresponds to the N-terminal region of each protein, including the His-tagged region. This result reconfirmed that proteolysis occurred from the C-terminus region of each protein,

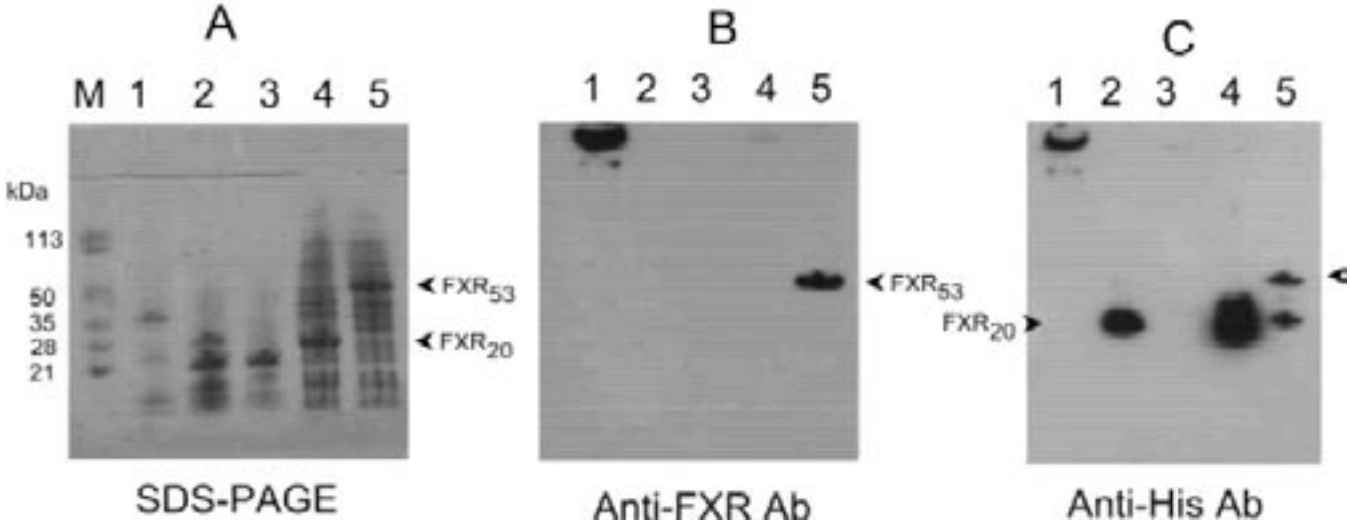

FIG. 3. Electrophoretic patterns of FXR variants expressed by SDS-PAGE (A), Western blotting with anti-FXR antibody (B), and Western blotting with anti-His antibody (C). Panel A, proteins were displayed on 8–25% Phastgradient gel (Amersham Pharmacia). Lane M, low M.W. marker; lane 1, FXR_{33}; lane 2, FXR_{20}; lane 3, FXR_{53}; lane 4, FXR_{20} in *Escherichia coli* cell lysate; lane 5, FXR_{53} in *E. coli* cell lysate. The expected size of FXR_{20} and FXR_{53}, 26 and 59 kDa, respectively, were maintained in the state of cell lysate, just after cell membrane disruption by sonication and before column application. Protein degradation of the intact forms seemed to begin as early as the purification step, despite the presence of a protease inhibitor. Ab, antibody; for other abbrevation see Figure 1; for manufacturer see Figure 2.

and it concurs well with the results of the Western blot experiment as shown in Figure 2.

Fluorescence-based FXR assay. With the three FXR proteins, a FXR assay was tested and optimized on a 384-well plate. A hyperbolic fluorescence signal curve was obtained after several parameters were adjusted, including concentrations of the SRC-1 peptide, the FXR protein, CDCA, and the Eu-N^1-labeled anti-His antibody. As shown in panel A of Figure 4, the FXR_{33} protein gave a saturated fluorescence signal of over 200,000, and the signal increase was dependent on CDCA concentration. As expected, the FXR_{20} protein did not respond to the coactivator recruitment signal, or at a level similar to the buffer-only control, since it lacked the ligand-binding domain. However, the FXR_{53} protein also did not produce the signal at the same level as the buffer-only control, even though it had the ligand-binding domain. The inactivity of the FXR_{53} protein might be attributed to proteolytic degradation at both the amino and the carboxyl termini, since the major band (around 20 kDa, lane 3 of panel A of Figs. 2 and 3) was recognized by neither the anti-FXR antibody nor the anti-His antibody (lane 3 of panels B and C of Fig. 3). This result strongly suggests that the 20-kDa fragment was a proteolytic product that originated from the middle region of the FXR_{53} protein, not from either the amino or the carboxyl terminus, since either of those antibodies can recognize at least one terminus of the FXR_{53} protein. In addition, the reactivity of the FXR_{33} protein in the established assay was not significantly interfered with by the addition of the anti-FXR antibody (final 1 nM) during the incubation, as shown in panel B of Figure 4. However, addition of the anti-His antibody caused a decrease of around 50% of the signal when compared with the control, because the Eu-labeled His antibody could not detect the His-tag region of FXR_{33} once the anti-His antibody had bound to FXR_{33}.

The FXR assay was tested with various solvents to assess their availability as a vehicle for putative modulators. As shown in panel A of Figure 5, ethanol and methanol served as good solvents for the FXR assay, with concentrations of up to 75% of the solvent in 3 μL of added volume, whereas DMSO, acetone, isopropanol, and ethyl acetate disturbed the assay, even in the final 6% of solvent concentration in the assay mixture. This result indicates that ethanol could serve as a good solvent for the assay, since it did not interfere with the normal binding of ligands or with the receptors in the production of the fluorescence signal. Based on this result, the putative candidates as modulators were tested after being dissolved in 50% ethanol. A few cholic acid derivatives and fenofibrate, an agonist of peroxisome proliferator-activated receptor α, were applied to the assay to test its ability to function as a putative modulator. As shown in panel B of Figure 5, only CDCA was able to activate FXR, and cholic acid showed a much weaker activation ability than CDCA. The other chemicals showed no effect on FXR activity.

DISCUSSION

It is well-documented that CYP7A1-mediated bile acid production is the major pathway for cholesterol excretion, regardless of whether it is found as endogenous or exogenous cholesterol in the liver (20). Although it has not been thoroughly elucidated, CYP7A1 activity is modulated by activation of SHP, a negative transcriptional factor for CYP7A1 (6).

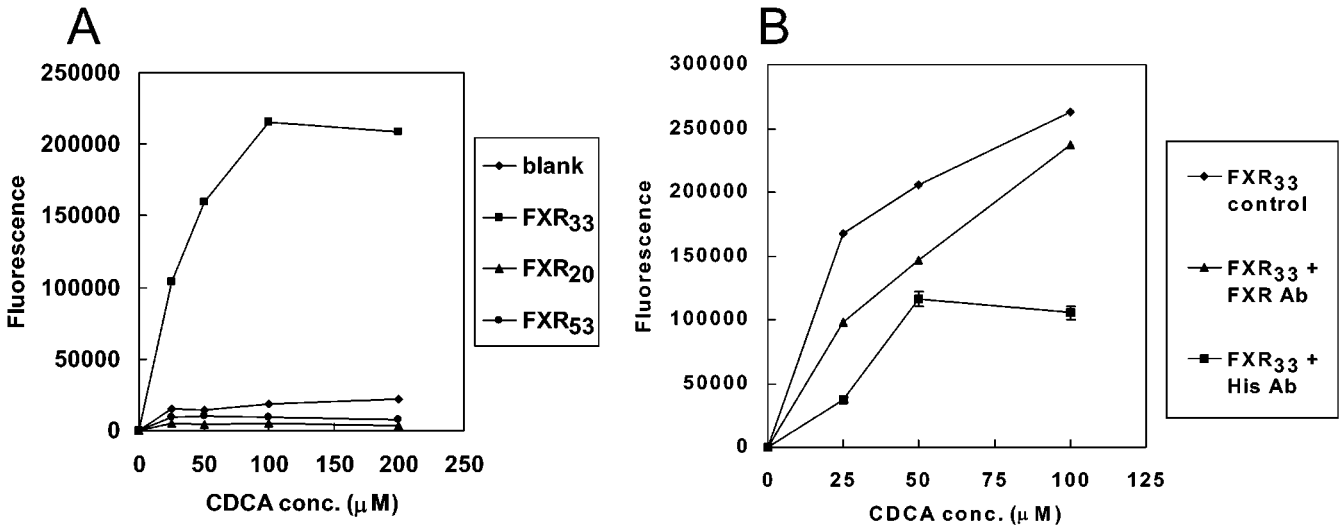

FIG. 4. Fluorescence signal patterns of the steroid receptor coactivator-1 (SRC-1) recruitment assay. A plate-binding assay was carried out in 40 μL of assay mixture that included 250 nM of FXR-His with 100 nM of SRC-1 peptide. Activation of FXR was detected by addition of 1 nM dissociation-enhanced lanthanide fluoroimmunoassay-Eu-N^1-anti-His antibody with an optical microplate reader. (A) Assay with FXR variants. FXR_{33} (■) gave a hyperbolic signal curve in response to chenodeoxycholic acid (CDCA) concentrations up to 200 μM, whereas neither FXR_{20} (▲) nor FXR_{53} (●) gave a signal under the same conditions. (B) Influence of the addition of antibody in the assay. A final 1 nM of anti-FXR antibody (▲) or His antibody (■) was added to the assay reaction of FXR_{33} to test the possibility that the antibody could change the reactivity of the FXR. Treatment with the anti-His antibody decreased the fluorescence signal, since the antibody competed with the Eu-^{1}N anti-His antibody for the His-tagged region of FXR_{33}. For other abbreviations see Figures 1 and 3.

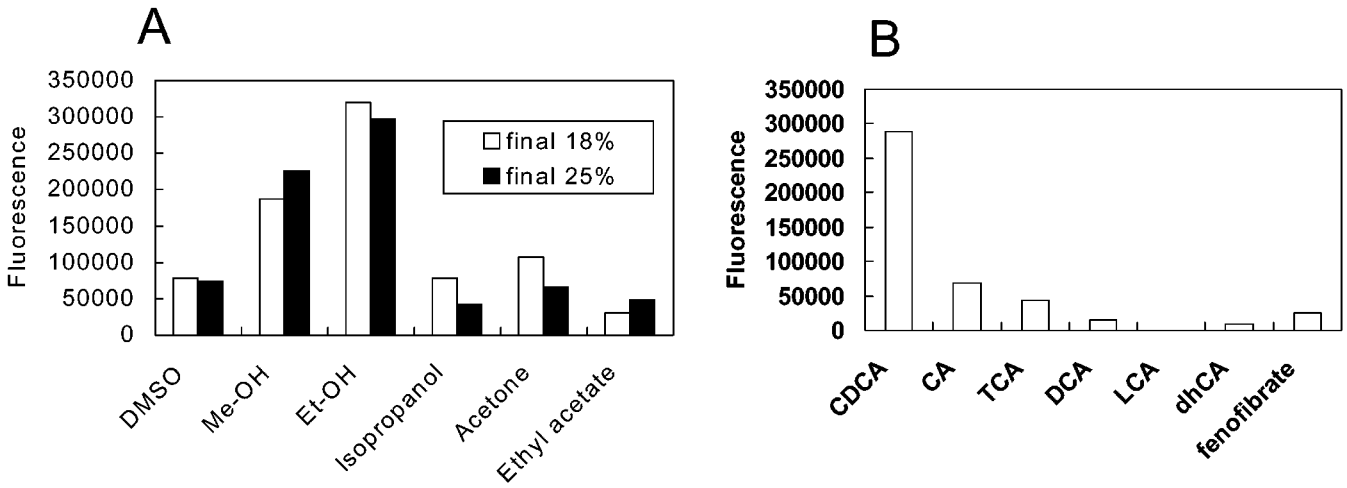

FIG. 5. Effects of organic solvent and bile acid derivatives in the FXR assay. (A) Several organic solvents were tested to investigate the possibility that they could interfere with a normal reaction between the ligand and receptor. Ethanol and methanol did not disturb the assay until a final 25% concentration was added, although ethanol showed a slightly more enhanced signal than methanol. However, the other solvents disturbed the assay reaction at final concentrations of both 17 and 25% (in 10 µL). (B) Cholic acid derivatives and fenofibrate were tested as modulators of the FXR. Each chemical was dissolved in 50% EtOH and treated with a final 100-µM concentration. Data are expressed as mean values from three independent experiments. CA, cholic acid; TCA, taurocholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; dhCA, dehydrocholic acid; for other abbreviations see Figures 1 and 4.

FXR also participates in the activation of several genes that are involved in bile acid homeostasis, including I-BABP (16,21), hepatic bile salt export pump (22), PLTP (7,23), and apoE (8). Interestingly, the expressional level of FXR in mouse liver and kidney showed a marked decrease during the acute phase response caused by lipopolysaccharide treatment (24). In that situation, the response was followed by a decreased expressional level of the FXR target genes apoC-II and SHP. Kim *et al.* (24) suggested that FXR plays a crucial role in changes to cholesterol and lipoprotein metabolism during the acute phase response.

In this report, a variant of the human FXR gene (FXR_{53}) and its cDNA fragment were cloned and expressed in *E. coli*. The proteins were purified and characterized by their application to an SRC-1 binding assay using time-resolved fluorescence. Among the variants, FXR_{33}, the ligand-binding domain of FXR, showed a saturated fluorescence signal only in response to dose-dependent and time-dependent bile acid treatments in the assay.

Like the other hormone receptors, the proteins were highly unstable when exposed to the aqueous buffer during purification. FXR_{53} showed a more severe proteolytic degradation pattern, as illustrated in lane 3, panel A of Figure 3. A major band appeared at around the 20-kDa position from SDS-PAGE, and it was detected in neither the anti-FXR antibody nor the anti-His antibody (lane 3, panel B or C of Fig. 3). However, the proteins in the cell lysates prior to column chromatography showed a more stable and distinct 59-kDa band from both SDS-PAGE and Western blotting. The FXR_{20} protein was expressed well at a 26-kDa band and was detected clearly by the anti-His antibody (lane 4, panels A and C of Fig. 3). The FXR_{53} protein also was expressed clearly as a 59-kDa band (lane 5, panel A), and was detected distinctly when either antibody was used (lane 5, panel B or C). In lane 5 of panel C, FXR_{53} was susceptible to unidentified proteolysis, and degradation occurred even though the protein was in the cell lysate state and in the presence of the complete protease inhibitor. At least two bands, of about 59 and 25 kDa, were detected by using the anti-His monoclonal antibody. These results support the possibility that cleavage of the FXR_{53} protein occurred 25 kDa from the amino-terminus region that contained the His-tag, because the 25-kDa fragment was detected by only the anti-His antibody and not the anti-FXR antibody.

The sequence homology study revealed that the FXR_{53} cDNA (1,431 bps) sequence was identical to that of human FXR mRNA (NR1H4, 1419 bps), except that 12 bps were inserted in the 588th position. Insertion of four amino acids into the hinge region of the FXR_{53} protein was comparable to that reported by Edwards and coworkers (13), who described four murine FXR isoforms, especially for FXRα1 and FXRβ1. Strikingly, the four inserted amino acids of FXR_{53} (M-Y-T-G) were identical to those of FXRα1 and FXRβ1, even though the number of full-length amino acids of each variant differed. Furthermore, FXR_{33}, the ligand-binding domain, revealed a high amino acid sequence homology when compared with murine FXRβ. Otte *et al.* (25) reported similar results, especially in the middle region of FXR following the hinge domain region, as shown in Table 1. The amino acid sequences of human and murine FXR variants were compared on the basis of their deduced amino acid sequence in GenBank (FXR_{53}, AF384555; RIP14-1, U09416; and mFXRβ, AY094586, respectively). Moore's group (26) reported the RIP14-1 clones, which were named mFXR1 by Edwards' group (13). The sequences showed high homology, especially in the middle region of the ligand-binding domain. Another variant, the

TABLE 1
Comparison of Amino Acid Homology in the Ligand-Binding Domain of Human and Murine Farnesoid X Receptor (FXR) Variants[a]

MYTG

$hFXR_{53}$ (AF384555)	^{181}-ECRLRKCKEMGMLAEC ■ LLTEIQCKSKRLRKNVKQHADQTVNED SEGRDLRQVTSTTKSCREKTELT-^{250}
RIP14-1 (U09416)	^{192}- ECRLRKCREMGMLAEC ▒ LLTEIQCKSKRLRKNVKQHADQTVNEDDSEGRDLRQVTSTTKFCREKTELT-^{258}
mFXRβ (AY094586)	^{176}- ECRLKKCKAVGMLAEC LLTEIQCKSKRLRKNFKHGPALYPAIQVEDEGADTK HVSSSTRSGKGVQDNMTL-^{245}
$hFXR_{53}$	^{251}-PDQQTLLHFIMDSYNKQRMPQEITNKILKEEFSAEENFLILTEMATNHVQVLVEFTKKLPGFQTLDHEDQIALLKGSAVE-^{330}
RIP14-1	^{259}-ADQQTLLDYIMDSYNKQRMPQEITNKILKEEFSAEENFLILTEMATSHVQ I LVEFTKKLPGFQTLDHEDQIALLKGSAVE-^{338}
mFXRβ	^{246}-TQEEHRLLNTIVTAHQKSM I PLGETSKLLQEGSNPELSFLRLSEVSVLHIQGLMKFTKGLPGFENLTTEDQAALQKASKTEVMFL-^{330}
$hFXR_{53}$	^{331}-AMFLRSAEIFNKKLPSGHSDLLEERIRNSGISDEYITPMFSFYKS I GELKMTQEEYALLTAIVILSPDRQYIKDREAVEK-^{410}
RIP14-1	^{339}-AMFLRSAEIFNKKLPAGH ADLLEERIRKSGISDEYITPMFSFYKS VGELKMTQEEYALLTAIVILSPDRQYI KDREAVEK-^{418}
mFXRβ	^{331}-HVAQLYGGKDSTSGSTMRPAKPSAGTLEVHNPSADESVHSPENFLKEGYPSAPLTDITKEFIASLSYFYRRMSELHVSDT-^{410}
$hFXR_{53}$	^{411}- LQEP LLDVLQKLCKI HQPENPQHFACLLGRLTELRTFNHH HAEMLMSWRVNDHKFTPLLCEIWDVQ-^{476}
RIP14-1	^{419}- LQEPLLDVLQKLCKMYQPENPQHFACLLGRLTELRTFNHHHAEMLMSWRVNDHKFTPLLCEIWDVQ-^{484}
mFXRβ	^{411}- EYALLTATTV LFSDRPCLKNKQHIENLQEPVLQLLFKFSKMYHPEDPQHFAHLIGRLTELRTLSHSHSEI-^{480}

[a]The sequence of $hFXR_{53}$ and RIP14-1 was identical except that four amino acids (M-Y-T-G) were inserted in the 197th position. Human FXR_{53} and murine FXRβ shared relatively low sequence homology; however, the early part of the sequences (from 176th to 240th) showed higher homology. Insertion of amino acids (M-Y-T-G) into the RIP14-1 is represented by a gray box. The black box indicates the position of amino acids inserted (-M-Y-T-G); amino acid numbers are indicated as superscripts.

mFXRβ, was reported as a functional hormone receptor that could form a heterodimer with RXRα. More interestingly, lanosterol was found to act as its physiological agonist ligand, not CDCA. Although the target gene of mFXRβ was not identified clearly, its physiological role in the mouse model is speculated to be somewhat different from its role in human FXR.

From a pharmaceutical point of view, a number of reports have proved that FXR regulation is a potent approach to treating hyperlipidemia. Indeed, guggulsterone, which has been used for hundreds of years in ayurvedic medicine, was recently identified as an FXR antagonist (27–29). It is well-known that the guggulipid has a strong effect on decreasing blood TG concentrations, as well as on lowering LDL cholesterol levels while increasing HDL cholesterol levels in human subjects (30,31). Those reports agree well with other *in vivo* experiments using the FXR antagonist (11).

In conclusion, from this work two human FXR genes were subcloned, three FXR variants (FXR_{20}, FXR_{33}, FXR_{53}) were expressed, and a coactivator binding assay was established using FXR_{33}, the ligand-binding domain of FXR. Among the three FXR variants, FXR_{33} showed greater protein stability after purification and the strongest fluorescence signal in the assay.

ACKNOWLEDGMENTS

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Genes Regulated by Arachidonic and Oleic Acids in Raji Cells

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ABSTRACT: FA are known to modulate immune function in conditions such as arthritis and lupus erythematosus. The effects of arachidonic (AA) and oleic acids (OA) on function and pleiotropic gene expression of Raji cells were investigated. The following parameters were evaluated: cytotoxicity as assessed by loss of membrane integrity and DNA fragmentation; proliferation as measured by [^{14}C]thymidine incorporation; production of interleukin (IL)-10, interferon (INF)-γ, and tumor necrosis factor (TNF)-α; and expression of pleiotropic genes by a macroarray technique (83 genes in total). AA was more toxic to Raji cells than OA. Both FA promoted an increase in Raji cell proliferation at 75 μM, whereas OA at high concentrations (200 μM) decreased proliferation. AA reduced the production of IL-10, TNF-α, and INF-γ. On the other hand, OA provoked an increase of INF-γ production but did not affect the production of IL-10 and TNF-α. The proportions of genes with altered expression were 27% for AA and 35% for OA. The FA affected the expression of genes clustered as: cytokines, signal transduction pathways, transcription factors, cell cycle, defense and repair, apoptosis, DNA synthesis, cell adhesion, cytoskeleton, and hormone receptors. The most remarkable changes were observed in the genes of signal transduction pathways. These results led us to conclude that the effect of these FA on B-lymphocytes includes regulation of gene expression. Thus, diets enriched with fat containing OA or AA may affect B lymphocyte function *in vivo*.

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Investigations of the effects of FA on the immune system date back many years (1–3). Dietary FA were shown to regulate the occurrence of diseases that are characterized by impaired immune function such as arthritis, Crohn's disease, and lupus erythematosus (4–6). This interest was intensified with the elucidation of the role of eicosanoids (leukotrienes, prostaglandins, and thromboxanes) derived from arachidonic acid (AA; 20:4n-6) in regulating inflammation and immunity (7–9). Oleic acid (OA; 18:1 *cis* 9), however, may be a precursor for leukotrienes but is not a precursor for prostaglandin biosynthesis (10).

Several studies have demonstrated that nonesterified FA modulate lymphocyte proliferation in response to a mitogenic stimulus *in vitro* (11–14). These metabolites stimulate lymphocyte proliferation at low concentration but inhibit it when given at high doses. In general, the greater the degree of unsaturation of the FA, the more potent is its inhibitory effect on proliferation (15).

FA can elicit some of their effects by AA-independent mechanisms, including actions upon intracellular signaling pathways and transcription factor activity (16,17). FA can modulate calcium signaling (18), protein kinase C (19), ceramide production (20), phospholipase C activation and subsequent production of inositol-1,4,5-triphosphate and diacylglycerol (20), and protein phosphorylation (21). PUFA regulate the expression of enzymes related to lipid metabolism (22), and PUFA-rich diets reduce the expression of glucose-6-phosphate dehydrogenase in lymphoid organs (23). OA can increase CD44 and CD45 expression in lymphocytes (24).

FA can alter rates of cytokine production, cell surface receptor, and expression of adhesion molecules (2–25). However, few studies have attempted to correlate the effects of FA on cytokine production, cell proliferation, and cytotoxicity with changes in gene expression. Therefore, we have investigated the effect of AA, a well-known modulator of immune cell function (26), on Raji cells, a B-lymphocyte model established by Epstein *et al.* (27,28). The reported effects were compared with those of OA. The functional parameters evaluated were: cytotoxicity (assessed by flow cytometric analysis of cell membrane integrity and DNA fragmentation); Raji cell proliferation by the measurement of [^{14}C]-thymidine incorporation, production of anti [interleukin-10 (IL-10)]- and pro-inflammatory [tumor necrosis factor (TNF)-α and interferon (INF)-γ] cytokines by ELISA; and expression of pleiotropic genes by macroarray technique.

MATERIALS AND METHODS

Assessment of Raji cell viability by flow cytometric analysis. Raji cells were obtained from the Dunn School of Pathology (Oxford

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Abbreviations: AA, arachidonic acid (20:4n-6); BAX, Bcl-2-associated X protein membrane; Bcl-xL, apoptosis regulator bcl-x; CASP3, caspase 3; CDK10, cyclin-dependent kinase 10; CSAID-binding protein, cytokine-suppressive anti-inflammatory drug-binding protein; Con A, concanavalin A; IL, interleukin; HER3, ErbB3 proto-oncogene; INF, interferon; JAK-1, Janus tyrosine-protein kinase 1; MAPKK 1, 3, 6, mitogen-activated protein kinase kinase 1, 3, 6; MAPKp38, mitogen-activated protein kinase p38; MYBL2, MYB-related protein B; OA, oleic acid (18:1); PI, propidium iodide; PKC-β, protein kinase C-β1; PLA_2, phospholipase A_2; PRKAR2B, cAMP-dependent protein kinase type II β regulatory subunit; RT-PCR, reverse transcriptase PCR; SOD1, cytosolic superoxide dismutase 1; SSC, 0.15 M sodium chloride + 0.15 M sodium citrate; TOP2A, topoisomerase II-α; TNF, tumor necrosis factor.

University, England). The cells were maintained in log phase at 37°C in a humidified 5% CO_2 atmosphere. Cells were grown in RPMI-1640 medium (2 mM glutamine and 2 g/L D-glucose) supplemented with 10% FCS, 20 mM HEPES, 24 mM $NaHCO_3$, 10 U/mL ampicillin, and 10 μg/mL streptomycin.

The cells (2×10^5 per mL) were plated in 24-well plates and treated for 24 h with AA and OA. Addition of these FA to the medium did not result in significant cellular toxicity up to 50 μM (data not shown). Membrane integrity and DNA fragmentation analyses were performed with increasing concentrations of FA, from 50 up to 150 μM for AA and up to 350 μM for OA. The FA were first dissolved in ethanol before emulsification in the serum protein-containing medium. The percentage of ethanol was always lower than 0.05% of the total volume of culture medium. This concentration of ethanol is not toxic for the cells, as also found by Siddiqui *et al.* (29).

At the end of the culture period, 0.5 mL of medium containing cells was used to test the membrane integrity. In this assay, 50 μL of a propidium iodide (PI) solution (100 μg per mL in saline solution) was added to the cells (30). (PI is a highly water-soluble fluorescent compound that cannot pass through intact membranes and is generally excluded from viable cells. It binds to DNA by intercalating between the bases with little or no sequence preference.) After 5 min incubation at room temperature, the cells were evaluated using FACScalibur™ flow cytometer equipment (Becton, Dickinson, Frankin Lakes, NJ) by using the Cell Quest software. The loss of membrane integrity was correlated with an increase in the PI fluorescence (620 nm). Ten thousand events were evaluated per experiment.

The remaining medium containing cells was centrifuged at high speed (12,000 × *g* for 30 s), and the pellet was resuspended in 0.2 mL lysis buffer (0.1% sodium citrate and 0.1% Triton X-100) containing 50 μg per mL of PI. The lysed cells were then incubated in the dark at 4°C for 24 h, and used for the DNA fragmentation analysis. DNA fragmentation is observed by the occurrence of low-intensity fluorescent particles. These particles are cleaved DNA, which, because of the associated high condensation and small size, cannot be intensively bound by PI. These particles are therefore detected as low-intensity fluorescent particles.

Proliferation assay. Raji cells (3.3×10^5 cells per mL) were plated in 96-well microtiter plates and treated for 48 h with AA (6.25, 12.5, 25, 50, and 75 μM) or OA (25, 50, 100, 150, 200, 250, and 300 μM) acids. [^{14}C]Thymidine (1 μCi per mL) was added to the medium at the beginning of the experiment. The plates were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were harvested, and the radioactivity of the [^{14}C]-thymidine incorporated into DNA was determined by using a liquid scintillation counter (Packard TRICARB 2100 TR counters; Downers Grove, IL). The incorporation of [^{14}C]thymidine is expressed as total cpm.

Measurement of cytokines. Raji cells (2×10^5 cells per mL) were plated in 24-well plates and treated for 24 h with AA (25 μM) or OA (50 μM). Cells were then cultured for another 24 h in the presence of 25 μg per mL concanavalin A (Con A). Con A receptors have been described in the surface membrane of B-lymphocytes (31). In fact, Con A can stimulate B-lymphocytes in the presence of humoral factors released by T cells (32). Afterward, cell culture supernatant fluid was harvested to carry out the determinations of secreted cytokines.

The production of IL-10, TNF-α, and INF-γ was evaluated by ELISA using Kit OptEIA™ from Pharmingen (San Diego, CA). The detection limit of both IL-10 and TNF-α was 7.8 pg per mL and of INF-γ was 4.7 pg per mL, according to the manufacturer.

Treatment of Raji cells with the FA to evaluate expression of pleiotropic genes. Cells were resuspended at a density of 2×10^5 cells per mL in 25-cm^3 flask containing RPMI 1640 medium and 10% FCS. Cells were treated for 24 h with AA and OA at 25 and 50 μM concentrations, respectively.

Total RNA extraction. Total RNA was obtained from 0.5–1 $\times 10^7$ cells using Trizol reagent (Life Technologies, Rockville, MD). Briefly, the cells were lysed using 1 mL Trizol reagent (Life Technologies). After 5 min incubation at room temperature, 200 μL chloroform was added to the tubes, and they were centrifuged at 12,000 × *g*. The aqueous phase was transferred to another tube, and the RNA was precipitated by centrifugation (12,000 × *g*) with cold ethanol and dried in air. RNA pellets were redissolved in RNase-free water and treated with DNase I. Afterward, the RNA were stored at −70°C until the time of the experiment. The RNA was quantified by measuring absorbance at 260 nm. The purity of the RNA was assessed by the ratio of the 260/280 nm absorbances and on a 1% agarose gel stained with ethidium bromide at 5 μg per mL (33). These samples were used for macroarray and reverse transcriptase (RT)-PCR analysis.

Synthesis of cDNA probes. The cDNA probes were synthesized using the pure total RNA labeling system Atlas Kit™ according to manufacturer's recommendations (Clontech Laboratories, Palo Alto, CA). Briefly, 10 μg of total RNA was heated at 70°C for 5 min. The temperature was decreased to 50°C, and 13.5 μL of the following reagents were added: 4 μL reaction buffer 5×, 0.5 μL 100 mM DTT, 2 μL 10× deoxynucleotide triphosphate (dNTP) mix (dCTP, dGTP, dTTP), and 5 μL of [α-^{33}P]ATP (at 10 μCi per μL; Amersham Biosciences, San Jose, CA). After 2 min, a CDS primer mix and 2 μL of reverse superscript enzyme (Life Technologies) were added (the CDS primer mix is a mixture of primers specific for each different type of Atlas Array). The reaction was incubated for 25 min at 50°C and stopped by using 2 μL Termination Mix. The ^{33}P-labeled probe was purified from unincorporated nucleotides by passing the reaction mixture through a push column (NucleoSpin Extraction Spin Column, Clontech Laboratories).

Hybridization of macroarrays. All solutions for hybridization were obtained from Clontech Laboratories. The membrane was prehybridized for 30 min at 68°C in Express Hyb containing 50 μg freshly denaturated salmon sperm DNA. Subsequently, the membrane was hybridized for 18 h at 68°C with 2×10^6 cpm per mL ^{33}P-labeled denaturated probe. The membrane was washed twice at 68°C with 1×SSC (0.15 M sodium chloride + 0.15 M sodium citrate), 0.1% SDS; followed by two washings in 1×SSC, 1% SDS. It was then exposed to a phosphor screen for 48 h and

scanned in a Storm 840 (Molecular Dynamics, Sunnyvale, CA). The results were obtained from two membranes.

Analysis of macroarray results. Changes in gene expression induced by the FA were analyzed by comparison with untreated cells using the software Array-Pro® Analyzer, version 4 (Media Cybernetics, Silver Spring, MD). Local ring background was subtracted from the density value of each spot to obtain a "net" value. Spots with a mean intensity of greater than 1.2 times the mean local background intensity were further considered as "measurable spots." Normalization was done by calculating total intensity ratios and by using the housekeeping gene β-actin (the same used for RT-PCR analysis) present in the membrane. Duplicate hybridizations using separate sets of nylon membranes were performed for all conditions. Only signals that differed from the control by at least twofold in the two independent experiments were considered as significant. Yamazaki *et al.* (34) used a similar procedure.

RT-PCR. RT-PCR using specific primers was performed to confirm the differential expression of the mRNA detected with the macroarray analysis. The sequences of the primers were designed using information contained in the public database GeneBank of the National Center for Biotechnology Information (NCBI, Bethesda, MD).

The RT-PCR was performed using parameters described by Innis and Gelfand (35). The number of cycles used was selected to allow linear amplification of the cDNA under study. For semiquantitative PCR analysis, the housekeeping β-actin gene was used as a reference. The primer sequences and their respective PCR fragment lengths are shown in Table 1. Published guidelines were followed to guard against bacterial and nucleic acid contamination (36).

Analysis of the PCR products. The analysis of PCR amplification products was performed in 1.5% agarose gel containing 0.5 μg per mL ethidium bromide; electrophoresis proceeded for 1 h at 100 V. The gels were photographed using a DC120 Zoom Digital Camera System from Kodak (Life Technologies). The images were processed and analyzed in the software Kodak Digital Science 1D Image Analysis (Life Technologies).

PCR band intensities were expressed as OD normalized for β-actin expression. Data are presented as a ratio to the respective controls, which received an arbitrary value of 1 in each experiment.

Statistical analysis. The results of toxicity, the proliferation assay, and measurement of the production of cytokines were expressed as mean ± SEM of six determinations from two or three experiments. Comparisons with the control (ethanol) were performed by ANOVA. Significant differences were found by using the Tukey–Kramer method (INStat; Graph Pad Software, Inc., San Diego, CA).

RESULTS

Assessment of Raji cell viability by flow cytometric analysis. Toxicity was assessed either by loss of cell membrane integrity or by DNA fragmentation using flow cytometry (Fig. 1). The proportion (%) of control cells with integral membrane was 91.2 ± 3.7 and with intact DNA was 90.2 ± 4.7 after treatment. There were remarkable differences in the toxic effect of the AA and OA on Raji cells (Fig. 1). OA did not cause a significant loss of membrane integrity up to 200 μM (93% of viable cells) and did not lead to DNA fragmentation up to 300 μM (18.9% of cells with fragmented DNA). In contrast, AA resulted in loss of membrane integrity at lower concentrations, i.e., 150 μM (67.8% of viable cells). [Previous studies have shown that AA causes cytochrome C release from heart mitochondria (37).] The concentrations of FA then selected to test the effect of the FA on cytokine production and pleiotropic gene expression were 25 μM for AA and 50 μM for OA.

Proliferation assay. [^{14}C]Thymidine incorporation by Raji cells was examined in the presence of FA. AA caused an increase of Raji cell proliferation by 1.6-fold at 6.25 μM and reached a maximal effect of 1.8-fold at 75 μM. Low concentrations of OA increased cell proliferation by 1.9-, 2.0-, 2.1-, and 2.0-fold at 25, 50, 100, and 150 μM, respectively (Fig. 2).

Measurement of cytokines. OA (at 50 μM) did not alter the production of IL-10 and TNF-α but raised the production of

TABLE 1
Standardized Conditions for Reverse Transcription-PCR (RT-PCR) Analysis[a]

Genes	Primer sense	Primer antisense	Annealing temperature (°C)	PCR fragment lengths	Number of cycles
PKC-β	5′-CCATCAAATGCTCCCTCAACCC-3′	5′-TTGCCAAAGCTGCCTTTCCC-3′	57	424 bp	35
JAK-1	5′-TCTGCTACAATGGCGAGATCCCK-3′	5′-AGCTCAACCTTCCCAAAGTGGC-3′	56	318 bp	30
Kruppel-related zinc finger[b]	5′-GCAAAGCATTTAGCCAGCCTTC-3′	5′-TTCTCTCCAGTGTGCATCCTCG-3′	56	313 bp	40
Myc proto-oncogene	5′-TACCCTCTCAACGACAGCAGCT-3′	5′-TCTTGACATTCTCCTCGGTGTCC-3′	60	455 bp	35
CASP3[b]	5′GTCGATGCAGCAAACCTCAGGG-3′	5′–TGTTTCAGCATGGCACAAAGCG-3′	56°C (15 cycles) plus 58°C (20 cycles)	470 bp	35
Bcl-xL	5′-CATGGCAGCAGTAAAGCAAGC-3′	5′-GGTCAGTGTCTGGTCATTTCCG-3′	59	470 bp	45
TOP2A	5′-GCTCAGCTCTTTGGCTCGATTG-3′	5′-GTCTGGGTCCATGTTCTGACGG-3′	59	375 bp	40
β-Actin	5′-GTGGGGCGCCCCAGGCACCA-3′	5′-CTCCTTAATGTCACGCACGATTTC-3′	56	545 bp	25

[a]The sequences of the primers, the PCR fragment lengths, the temperature, and the number of cycles are shown for each gene under study. For all genes 1.5 mM $MgCl_2$ was used.
[b]For Bcl-xL, CASP3, and Kruppel-related zinc finger protein RT-PCR, formamide was used at 0.5, 1.5, and 2.5% concentration. PKC-β, protein kinase C type β I; JAK-1, Janus tyrosine-protein kinase I; CASP3, caspase 3; Bcl-xL, apoptosis regulator bcl-x; TOP2A, DNA topoisomerase II-α.

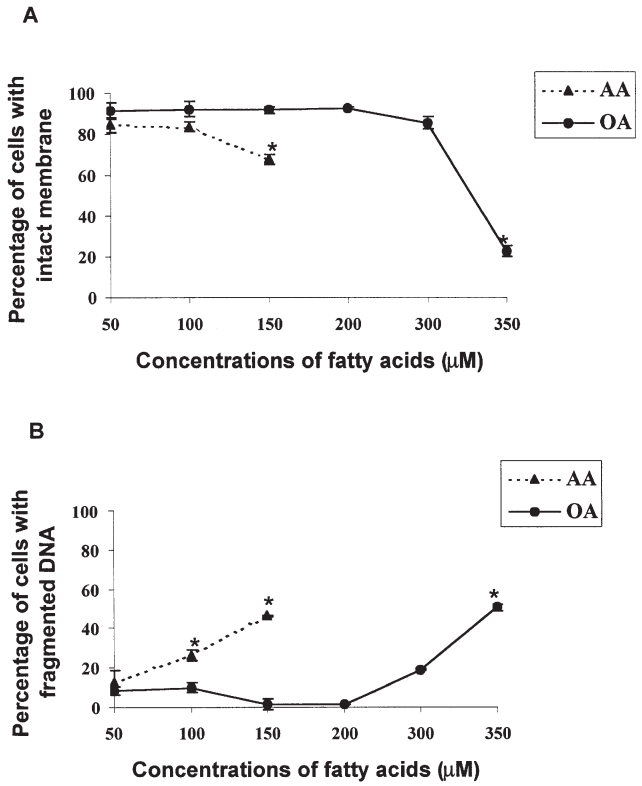

FIG. 1. Determination of the toxicity of arachidonic (AA) and oleic (OA) acids on Raji cells. (A) Percentage of cells with intact membrane after treatment for 24 h with concentrations up to 150 μM for AA and up to 350 μM for OA. (B) Percentage of cells with fragmented DNA after the same treatment. The values are presented as mean of six determinations from two experiments. SEM was always lower than 10% of the mean. *$P < 0.05$ as compared with 50 μM concentration. The values of 50 μM were not different from the corresponding controls (not treated with FA).

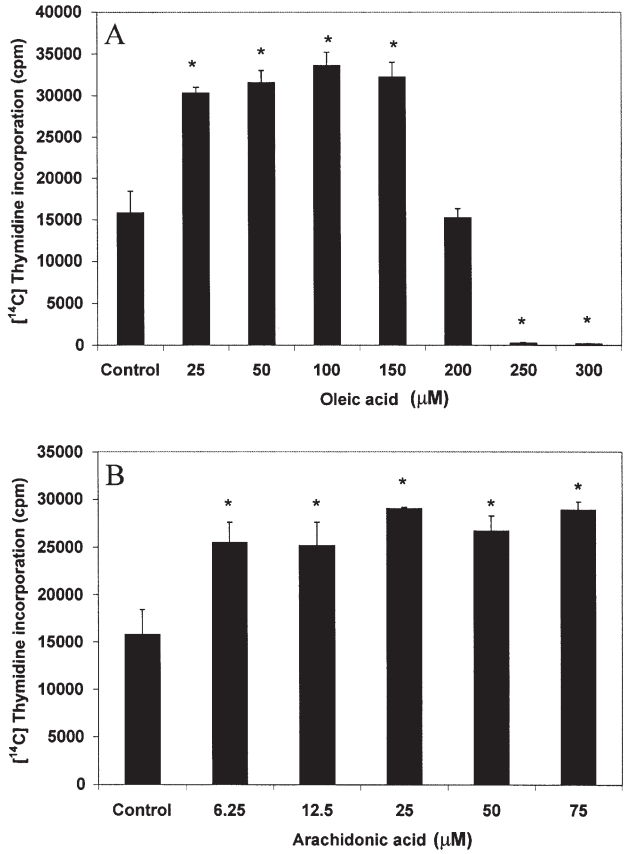

FIG. 2. Effect of AA and OA on Raji cell proliferation. The cells (3.3×10^5 cells per mL) were cultured in various FA concentrations for 48 h. The cells harvested and the radioactivity of the [^{14}C]thymidine incorporated into DNA were determined by using a liquid scintillation counter. The incorporation of [^{14}C]thymidine is expressed as total counts per min. The values are due to the effect of FA presented as mean ± SEM of six determinations from three experiments. (*) indicates $P < 0.05$ as compared with the corresponding controls (no FA added). See Figure 1 for abbreviations.

INF-γ (1.3-fold) (Fig. 3). AA (at 25 μM) caused a significant reduction in the production of IL-10 (62%), TNF-α (39%), and INF-γ (23.5%) (Fig. 3).

Pleiotropic gene expression. The comparative effects of AA (25 μM) and OA (50 μM) on expression of selected genes from B lymphocytes are described in Table 2. To validate the results of the macroarray analysis, seven genes were selected for confirmation by RT-PCR (Fig. 4). Although the magnitude of changes was not identical, the direction of changes induced by the FA was the same for both macroarray and RT-PCR analysis. Therefore, macroarray analysis performed in duplicate using pooled cells from two experiments provided reliable results.

Of the surveyed genes (83 in total), 35 were modified by at least one of the FA tested. The proportions of these genes changed by the FA were 27% for AA and 35% for OA. The treatment with AA caused an increase in the expression of 20 genes and a decrease of 2. On the other hand, OA increased the expression of 29 genes but did not decrease the expression of any of the genes analyzed.

RT-PCR results. The genes selected to perform RT-PCR were those altered by the treatments with AA and OA as indicated by macroarray analysis. AA increased the expression of PKC-β, (protein kinase C-β1), CASP3 (caspase 3), BcL-xL (apoptosis regulator bcl-x), TOP2A (topoisomerase II-α), and Myc-proto-oncogen. OA raised the expression of JAK-1 (Janus-tyrosine-protein kinase 1), Kruppel-related zinc finger, CASP3, BcL-xL, TOP2A, and Myc proto-oncogen (Fig. 4).

DISCUSSION

B-Lymphocytes play a crucial role in inflammatory and immune reactions (38). Several parameters of B-lymphocyte (Raji cells) function were found to be altered by incubation in the presence of nontoxic concentrations of AA or AO for 24 h. Therefore, the changes observed were due to the effect of the FA *per se* and not to apoptosis or necrosis. Cell proliferation was increased in the presence of OA (approximately twofold at 25–150 μM, Fig. 2) and AA (approximately 1.5- to 2-fold at 6.25–75 μM, Fig. 2). AA (25 μM) significantly decreased the production of IL-10, TNF-α, and IFN-γ from Raji B-lymphocytes (Fig. 3). However, OA had no significant effect on IL-10

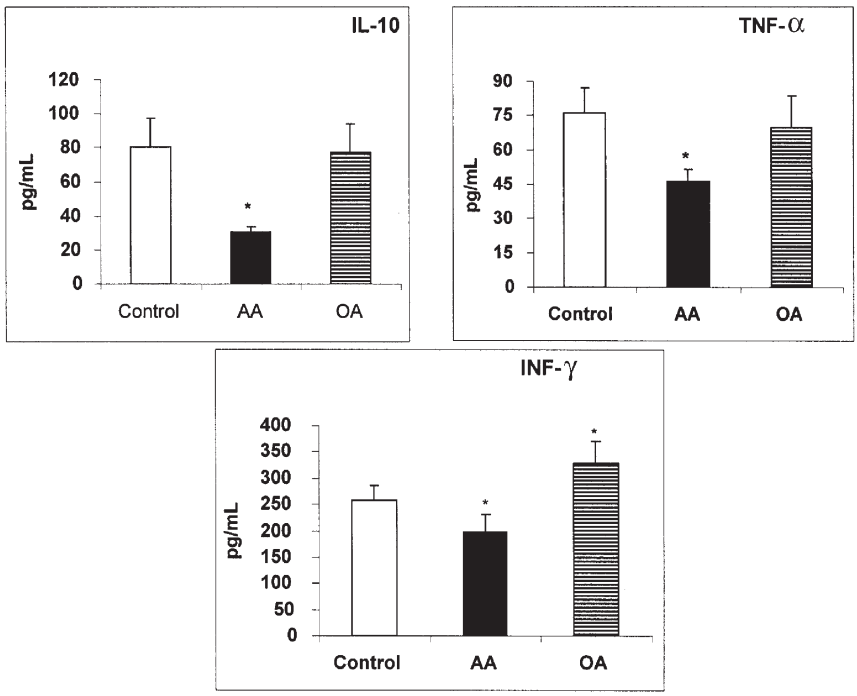

FIG. 3. Effect of FA on cytokine production by Raji cells. Cells were cultured in the presence of AA (25 μM) and OA (50 μM) for 24 h. Cells were then cultured for another period of 24 h in the presence of 25 μg per mL concanavalin A and the FA. Afterward, the supernatant was used for determination of the cytokines (by ELISA) as described in the Materials and Methods section. Values represent mean ± SEM of six determinations from three experiments. (*) indicates $P < 0.05$ as compared with the corresponding controls (no FA added). IL, interleukin; TNF, tumor necrosis factor; INF, interferon; for other abbreviations see Figure 1.

and TNF-α production by Raji B-lymphocytes even at 50 μM (Fig. 3) but significantly increased the production of IFN-γ (Fig. 3).

Expression of specific genes related to signal transduction, survival, cell cycle, apoptosis, and cytokine production was altered by the presence of 25 μM AA or 50 μM OA. The genes selected for macroarray analysis were related to key aspects of B-lymphocyte function. The significant increase in expression of 20 of 83 selected genes and decrease in 2 by AA and an increase in expression of 29 genes by OA argues for selective effects of PUFA and monounsaturated FA on gene transcription.

The effect of FA on gene expression occurs by their binding to transcription factors such as peroxisome proliferator-activated receptors and islet/duodenum homeobox-1 (16,39,40). Evidence is presented that FA are also able to regulate the expression of other transcription factors. Several genes of this group were up-regulated by OA: c-jun proto-oncogene, ErbB3 proto-oncogene (HER3), nuclear factor of κ light polypeptide gene enhancer in B cells 3, Kruppel-related zinc finger protein, Myc proto-oncogene, MYB-related protein B (MYBL2), and v-erbA homolog-like 2. Of these genes, AA did not raise four: c-jun proto-oncogene, Kruppel-related zinc finger protein, v-erbA homolog-like 2, and MYBL2. AA raised the expression of mitogen-activated protein kinase kinase (MAPKK) 1, 3, and 6; PKC-β; cAMP-dependent protein kinase type II β regulatory subunit (PRKAR2B); mitogen-activated protein kinase p38 (MAPKp38); and phospholipase A_2 (PLA_2). Of these genes, OA did not raise MAPKp38, MAPKK6, and PLA_2 but increased JAK-1, MAPKK1, MAPKK3, PKC-β, PRKAR2B, and mitogen-activated protein kinase MAPK/ERK kinase kinase 3.

There is substantial evidence that FA can regulate lymphocyte proliferation (11,12,41). OA and AA up-regulated cyclin-dependent kinase 10 (CDK10) and increased cell division cycle 25 homolog A, whereas G1/S-specific cyclin E was raised by AA and cyclin-dependent protein kinase 2 was down-regulated by AA. Therefore, the changes in lymphocyte proliferation may be related to the effects of FA on expression of genes controlling the cell cycle. In fact, an increase in cell proliferation was observed by treatment with OA up to 300 μM and AA up to 75 μM.

Oxidative stress and lipid peroxidation have been associated with impaired immune function and development of several diseases (42,43). OA presented a stimulating effect on expression of six genes related to defense and repair: cytosolic superoxide dismutase 1 (SOD1), glutathione reductase, glutathione S-transferase pi, glutathione S-transferase A1, growth arrest and DNA damage-inducible protein, and heat shock 70 kDa protein 1. This FA was therefore effective in inducing expression of several antioxidant proteins. In comparison, AA increased SOD1.

TABLE 2
Modification in Raji Cell Gene Expression After Treatment with Oleic (OA) and Arachidonic (AA) Acids[a,b]

Cluster/GAN[c]	Gene name	OA	AA
Cytokines and related receptors			
L08096; S69339	CD27 ligand (CD27LG); CD70 antigen	+4.4	+4.0
Signal transduction pathways			
L36719	Mitogen-activated protein kinase kinase 3 (MAPKK3)	+3.7	+2.9
X06318	Protein kinase C β I (PKC-β)	+2.3	+2.0
M31158	cAMP-dependent protein kinase type II β regulatory subunit (PRKAR2B)	+3.9	+2.5
M35203	Janus tyrosine-protein kinase 1 (JAK-1)	+2.7	
L05624	Mitogen-activated protein kinase kinase 1 (MAPKK1)	+2.9	+2.3
L35253; L35263	Mitogen-activated protein kinase p38 (MAPKp38); cytokine-suppressive anti-inflammatory drug-binding protein (CSAID-binding protein)		+2.7
U39657	Mitogen-activated protein kinase kinase 6 (MAPKK6)		+4.9
U78876	Mitogen-actived protein kinase MAPK/ERK kinase kinase 3 (MEKK3)	+3.6	
M86400	Phospholipase A_2 (PLA_2)		+2.5
Transcription factors and related genes			
J04111	c-jun proto-oncogene	+4.4	
M29366; M34309	ErbB3 proto-oncogene (HER3)	+3.4	+3.5
L19067	Nuclear factor of κ light polypeptide gene enhancer in B cells 3 (NFKB3)	+5.5	+5.5
L11672	Kruppel-related zinc finger protein	+3.3	
V00568	Myc proto-oncogene	+3.7	+2.8
X13293	MYB-related protein B (MYBL2)	+2.8	
X12794	v-erbA homolog-like 2	+3.9	
Cell cycle			
L33264	Cyclin-dependent kinase 10 (CDK10)	+2.9	+2.6
M68520	Cyclin-dependent protein kinase 2 (CDK2)		–3.4
M81933	Cell division cycle 25 homolog A (CDC25A)	+4.7	+4.0
M73812	G1/S-specific cyclin E (CCNE)		+2.3
Defense and repair			
K00065; X02317	Cytosolic superoxide dismutase 1 (SOD1)	+2.9	+2.8
X15722	Glutathione reductase (GR)	+2.9	
X08058	Glutathione S-transferase P1 (GSTP1)	+2.8	
M25627	Glutathione S-transferase A1 (GSTA1)	+2.3	
M60974	Growth arrest and DNA damage-inducible protein (GADD45) +2.0		
M11717	Heat shock 70 kDa protein 1 (HSP70.1)	+3.1	
Apoptosis			
U13737	Caspase 3 (CASP3)	+3.4	+2.6
L22474	Bcl-2-associated X protein membrane (BAX)	+2.8	
Z23115	Apoptosis regulator bcl-x (Bcl-xL)	+4.1	+3.5
X89986; U34584	NBK apoptotic inducer protein; BCL-2 interacting killer protein (BIK)	+6.9	+5.6
M14745	B-Cell leukemia/lymphoma protein 2 (Bcl2)	+13	
DNA synthesis			
M63488	Replication protein A 70-kDa subunit (RPA70)	+4.1	+2.7
J04088	Topoisomerase II (TOP2A)	+3.1	+2.1
Hormones			
M27544	Insulin-like growth factor (IGF1)		–2.3

[a]Results are described as fold-changes in expression.
[b]Raji cells were treated for 24 h with OA (50 μM) or AA (25 μM). Total RNA was isolated, retrotranscribed, ^{33}P-labeled, and hybridized to the cDNA array presenting 83 transcripts of known genes. The signals were then analyzed by Pro-Analysis Software Array-Pro® Analyzer, version 4 (Media Cybernetics, Silver Spring, MD) and expressed as fold of increase or decrease with respect to untreated cells. Data are presented as means of two different experiments. Only signals that differed from the untreated cells by at least twofold were considered as significant. For details of the calculations see the Materials and Methods section. Changes are indicated as (+) increase and (–) decrease as compared with untreated cells.
[c]GAN, GeneBank accession number.

The commitment of lymphocytes to apoptosis is an important aspect of their function (44,45). OA and AA up-regulated CASP3, Bcl-xL, NBK apoptotic inducer proteins, or Bcl-2 interacting killer protein, whereas Bcl-2 associated X protein membrane (BAX) and B-cell leukemia/lymphoma protein 2 (Bcl-2) were increased by OA only. These findings imply a role for OA and AA on regulation of B-lymphocyte apoptosis. The effect of the FA on apoptotic gene expression was observed at concentrations even lower than those causing DNA fragmentation. It is also interesting that the concentrations used (25 μM for AA and 50 μM for OA) are able to stimulate Raji cell proliferation.

The described selective effects of FA on gene expression may be responsible for the observed increase in Raji cell proliferation and the decrease in IL-10, TNF-α, and INF-γ production by AA. Because IL-10 is an inhibitor of macrophage activation, macrophage and associated T-cell-dependent B-lymphocyte activation may be promoted in the presence of AA *in vivo*.

Specific effects of FA on B-lymphocyte function may be identified by the approaches outlined in this paper. Thus,

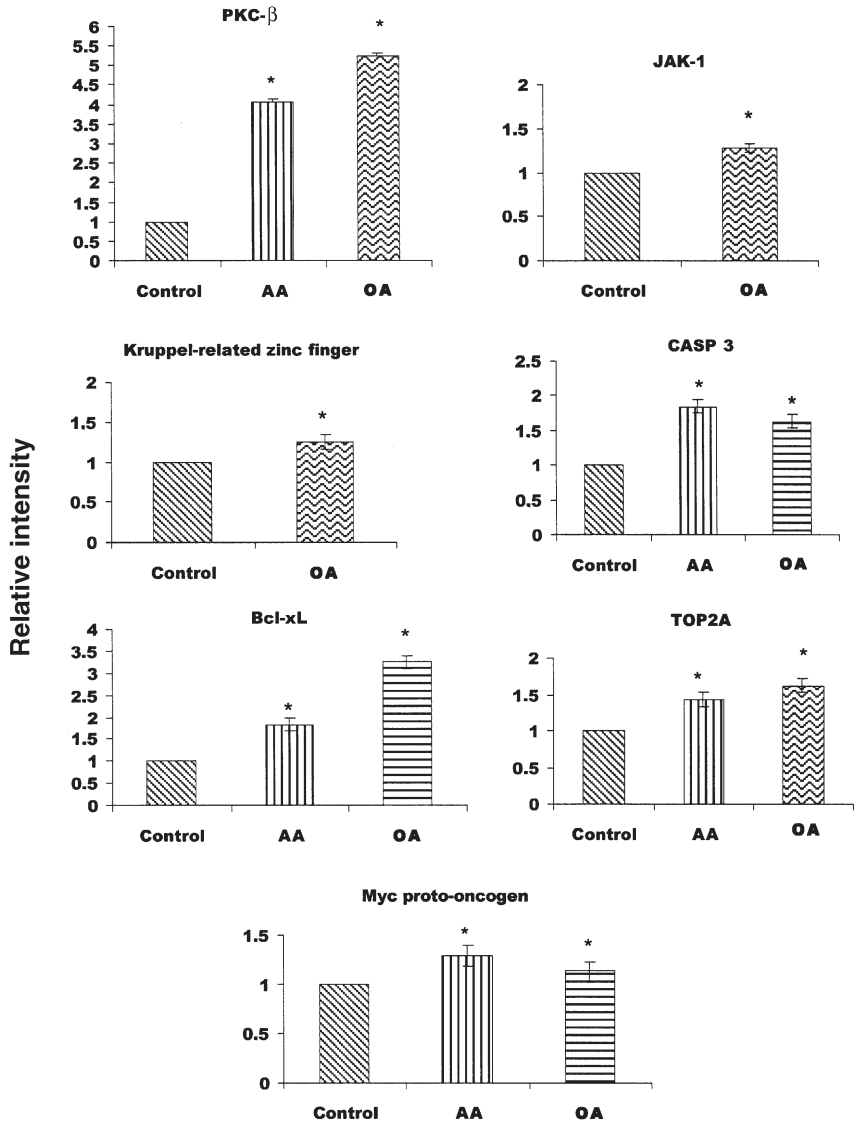

FIG. 4. Confirmation by reverse transcription-PCR (RT-PCR) of the genes detected by macroarray analysis as being modified by FA (AA and OA) after treatment for 24 h (Table 2). Raji cells (10^7 cells/condition) were exposed for 24 h to the following conditions: control (no FA added), AA (25 μM), and OA (50 μM). After this period of time, the cells were harvested, mRNA was extracted, and RT-PCR was performed with the equivalent of 10^7 cells. PCR band intensities were expressed as OD corrected for β-actin expression. The data are presented as the ratio with the respective controls, which received an arbitrary value of 1 in each experiment. The values are presented as mean ± SEM for four experiments. PKC-β, protein kinase C-βI; JAK-1, Janus tyrosine-protein kinase I; Kruppel-related zinc finger protein; CASP3, caspase 3; Bcl-xL, apoptosis regulator bcl-x; TOP2A, topoisomerase II-α. (*) indicates $P < 0.05$ as compared with the corresponding controls.

specific modulation of B-lymphocyte function by FA may be a goal in future studies, based on an understanding of the mechanisms by which FA exert their effects.

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Kinetics of Barley FA Hydroperoxide Lyase Are Modulated by Salts and Detergents

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ABSTRACT: The cDNA from barley coding FA hydroperoxide lyase (HPL) was cloned. A recombinant protein derived from the cDNA was expressed in *Escherichia coli* as an active enzyme. Thus far, there have been no reports on HPL in monocotyledonous plants. The recombinant protein was shown to be most active to linolenic acid 13-hydroperoxide, followed by linoleic acid 13-hydroperoxide. 9-Hydroperoxides of the FA could not be substrates for the recombinant HPL. The activity was dramatically enhanced in the presence of a detergent and/or a salt in the reaction mixture. At the same time, the kinetics of the reaction, including inactivation and the V_{max} value of the HPL, were also greatly modulated, depending on the concentration of a monovalent cation and/or a detergent in the reaction mixture. These results suggest that these effectors induced a conformational change in barley HPL, resulting in an improvement in substrate binding and in enzyme activity.

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Oxylipins are oxygenated derivatives of FA formed either by the action of enzymes, such as lipoxygenases (LOX), α-dioxygenase, and/or cytochrome P-450 enzymes, or by chemical oxidation (1–3). The LOX-derived FA hydroperoxides are subsequently metabolized *via* at least four major pathways: the peroxygenase (4), the FA hydroperoxide lyase (HPL) (5), the allene oxide synthase (AOS) (6), or the divinyl ether synthase (DES) (7) pathway. HPL cleaves the C–C bond adjacent to the hydroperoxy group, leading to the formation of either C6- or C9-short-chain aldehydes and the corresponding C12- or C9-ω-oxo FA. The short-chain aldehydes may be involved in wound healing and disease resistance (8,9). Recently, HPL have been cloned and purified from several kinds of plants. They were found to be heme proteins belonging to the cytochrome P-450 family; thus, they were given the new subfamily name of CYP74B (10–15). HPL are unique, peroxide-specific P-450 enzymes because they need neither molecular oxygen nor a reducing equivalent, which is generally essential for most P-450 enzymes.

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Abbreviations: ADAM, 9-anthryldiazomethane; AOS, allene oxide synthase; DES, divinyl ether synthase; EST, expression sequence tag; HPL, FA hydroperoxide lyase; HPOD, hydroperoxy-octadecadienoic acid; HPOT, hydroperoxy-octadecatrienoic acid; HvHPL, FA hydroperoxide lyase from barley (*Hordeum vulgare*); LOX, lipoxygenase.

The HPL cloned and characterized thus far are limited to those from dicotyledonous plants; monocotyledonous HPL have never been cloned and characterized. We succeeded in cloning an HPL cDNA of barley (*Hordeum vulgare*) and expressing a recombinant protein derived from the cDNA in *Escherichia coli*. In this work, the substrate and product specificities of the recombinant enzyme were determined. Through comprehensive analyses of the properties of the recombinant barley HPL, we found that certain reaction conditions, such as ionic strength and the presence of a detergent, greatly affected its activity. A comparison of the kinetics of reactions in the absence and presence of a salt and/or a detergent showed that these conditions could modify the kinetics of the HPL reaction.

EXPERIMENTAL PROCEDURES

cDNA cloning and enzyme preparation. An expression sequence tag (EST) clone (AL500401) from a barley library prepared from etiolated leaves was shown to have high similarity with *Arabidopsis* HPL. To isolate full-length cDNA encoding barley HPL, a cDNA library with a λZAP II vector was reconstructed with poly (A)-RNA isolated from barley leaf segments treated with 1 M sorbitol for 24 h as described previously (16). The 556-bp fragment from the EST clone was used as a probe for plaque hybridization. Screening and hybridization were performed according to standard techniques (17). The resultant positive phage clones were excised, and the phagemid clone containing the longest insert was sequenced and found to encode a full-length barley HPL gene. For *E. coli* expression, *Bam*HI and *Hind*III sites were introduced at the 5′ and 3′ ends, respectively, by PCR. The primers used for the PCR were 5′-GGATCCATGCTGCCGTCCTTCTCGCCG-3′ (the initiation codon is underlined) as the sense primer, and 5′-AAGCTTTCAGCTGTTGGGCTGCGGCTG-3′ as the antisense primer. The amplified fragment was ligated into the *Bam*HI and *Hind*III sites of the pQE30 vector. *Escherichia coli* SG13009 was transformed with the construct. The cells were grown until mid-log phase at 37°C, then for 24 h at 16°C with Luria-Bertani medium containing isopropyl-1-thio-β-D-galactopyranoside (1 mM). The recombinant *H. vulgare* HPL (HvHPL) was purified to a homogenous state essentially as described previously (18). Briefly, the membrane fraction was collected from the bacterial lysate, and

recombinant HvHPL was solubilized with 0.2% Triton X-100. The resultant supernatant was applied to a 1.6 × 1.5 cm TALON metal affinity column (Clontech Co., Palo Alto, CA). The column was washed with 40 mL of potassium phosphate buffer (50 mM, pH 8.0, containing 1 M KCl and 0.2% Triton X-100) and 200 mL of potassium phosphate buffer (50 mM, pH 6.0, containing 1 M KCl and 0.2% Triton X-100). The enzyme was eluted with 5 mL of potassium phosphate buffer (50 mM, pH 4.0, containing 1 M KCl and 0.2% Triton X-100).

Enzyme activity measurements and product identification. As a typical reaction (1 mL), HPL activity was determined in 10 mM potassium phosphate buffer, pH 8.0, with 4 μL of 10-mM substrate dissolved in ethanol (final concentration of ethanol: 0.4%) or 4 μL of 10-mM substrate dissolved in 0.2% detergent (final concentration of detergent: 0.0008%) by measuring the decrease in absorbance at 234 nm due to cleavage of the substrate. Protein concentration was determined by the method of Bradford (19). The amount of aldehydes formed during incubation was analyzed by HPLC as described previously (3), except that a Mightysil RP-18 column (4.6 × 250 mm; Kanto Kagaku Co., Tokyo, Japan) was used and the solvent was acetonitrile/water/THF (74:25:1, by vol). To analyze the counterpart of the cleavage reaction, the products were extracted with ether. The ether extract was treated with 9-anthryldiazomethane (ADAM) reagent. The ADAM esters of carboxylic acids were separated by HPLC with a Wakosil-II 5C18 RS column (4.6 × 250 mm; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and the solvent was acetonitrile/0.1 M ammonium acetate (75:25, vol/vol). Detection of the resultant ADAM esters of carboxylic acid was performed with reversed-phase HPLC as described previously (3).

RESULTS

Molecular cloning. By BLAST searches on the barley EST database [in the Plant Genome Resources Center of the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany], one clone (accession number: AL500401) having high sequence homology to *Arabidopsis* HPL (CAB39331) could be found. The EST clone had only a partial sequence; thus, we used a cDNA library from barley seedlings treated with 1 M sorbitol (20) because oxylipin pathways in the seedlings treated with this osmoticum are known to have been induced (16). It was screened by the EST clone as the probe. The longest cDNA clone isolated contained 1461 nucleotides, encoding a protein of 487 amino acids (GenBank accession number: AJ318870). There is a stop codon in the 5′-untranslated region at 33 bp upstream of the possible initiation codon, and a polyadenylation signal and a stretch of the poly (A) tail could be found, suggesting that this was almost a full-length cDNA clone. The deduced amino acid sequence had high similarity to alfalfa 13-HPL (AJ249246, 51.6%), guava 9/13-HPL (AF239670, 50.8%), and *Arabidopsis* 13-HPL (AF087932, 50.1%), whereas the similarity to 9/13-barley AOS-1 and -2 was low (AJ250864, 38.4% and AJ251304, 38.8%). The Cys-443 known as a heme ligand of HPL could be also found. The sequence had greater similarity to 13-HPL (CYP74B); and similarities to 9-HPL (CYP74D), AOS (CYP74A), or DES (CYP74C) were lower, which suggested that the barley sequence belonged to the CYP74B subfamily. TargetP (http://www.cbs.dtu.dk/services/TargetP/) prediction on the deduced amino acid sequence indicated that there might be a transit peptide directing the enzyme to plastids in its N-terminal end (Reliability Class; 5). The predicted size of the transit peptide was relatively short, but it was rich in prolines and serines, which is a diagnostic feature of the transit peptide. It has been reported that tomato HPL associates with the outer envelope membrane of chloroplasts, although the size of the predicted transit peptide is also short (21).

Substrate specificity. To determine whether this clone encodes for an HPL, we made transgenic *E. coli* cells harboring the vector pQE30 containing the coding region of the cDNA. In this expression system, recombinant HPL was formed as a His-tagged protein. After purification with a Co^{2+}-affinity column, a protein having M.W. of 55 kDa was obtained (data not shown). The size of the recombinant protein was equivalent to the size expected from the sequence. When the recombinant protein was incubated with (13*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid (13-HPOD) and (13*S*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid (13-HPOT), *n*-hexanal and (3*Z*)-hexenal as the products, respectively, and 12-oxo-(9*Z*)-dodecenoic acid were detected by HPLC (Fig. 1). Extensive HPLC analyses showed that the C6-aldehyde and C12-oxo acid were the major products and that formation of the other products could hardly be detected. In contrast, with (9*S*)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid (9-HPOD) and (9*S*)-hydroperoxy-(10*E*,12*Z*,15*Z*)-octadecatrienoic acid (9-HPOT) as the substrates, neither formation of the corresponding C9-aldehydes nor a decrease in absorbance at 234 nm of the conjugated diene chromophore of the substrate could be observed (Table 1). Thus, it was concluded that this cDNA encoded barley 13-HPL, belonging to the CYP74B subfamily. The pH–activity profile of the purified enzyme was determined by using 13-HPOT as a substrate. The highest activity was found at pH 8.0 with 0.1 M KP_i buffer. Most HPL characterized thus far have optimal pH values in a weakly acidic region ranging from 5.0 to 6.5. The acidic half of the pH–activity curve of the recombinant barley HPL was very similar to the titration curve of 13-HPOT (Fig. 2). In contrast, no activity could be detected with the methyl ester of 13-HPOT, which has no dissociable functional group. These results suggest that barley HPL prefers the carboxylate anion form of a substrate, in contrast with the 13-HPL from *Arabidopsis,* which can act on the methyl ester of 13-HPOT and free 13-HPOT with almost equal efficiency (22).

Effect of addition of salt on activity. While trying to reveal properties of the recombinant HPL, we noticed that the activity was highly dependent on the kind of buffer used for the reaction. Several attempts showed that the concentration of salt in the buffer was a critical parameter modulating the activity. As shown in Figure 3A, activity of purified recombinant barley HPL increased almost linearly as the concentration of potassium chloride in the reaction buffer increased, and activity in

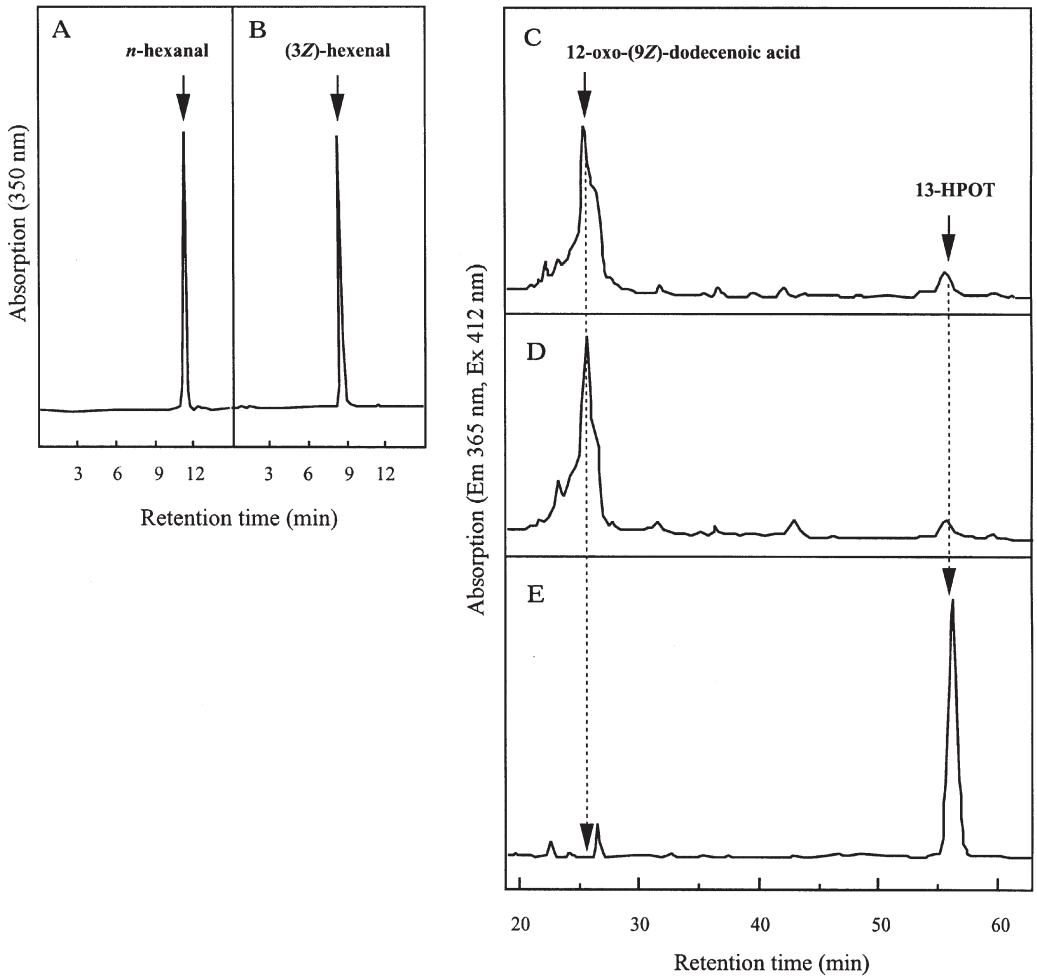

FIG. 1. HPLC detection of the products formed from 13-hydroperoxy-octadecadienoic acid (HPOD) or 13-hydroperoxy-octadecatrienoic acid (HPOT) by recombinant FA hydroperoxide lyase (HPL). C6-aldehydes formed after the reaction of the purified recombinant barley HPL with 13-HPOD (A) or 13-HPOT (B) were resolved by HPLC after being converted into the corresponding hydrazone derivative. 13-HPOT was incubated with the recombinant bell pepper (*Capsicum annuum*) HPL as a positive control (C), barley (*Hordeum vulgare*) HPL (HvHPL) (D), and heat-inactivated (heated at 95°C for 15 min) HvHPL (E). Nonvolatile products were extracted from the reaction mixture, esterified with 9-anthryldiazomethane, and analyzed with HPLC. The trailing shoulder on the main peak of 12-oxo-(9*Z*)-dodecenoic acid may be the 12-oxo-(10*E*)-dodecenoic acid isomer of the primary enzymatic product.

the presence of 2 M potassium chloride was more than five times higher than that in the absence of the salt (Fig. 3A). As shown in Figure 3B, such an effect was also observed with NaCl or with Na_2SO_4. On the other hand, the salts composed of a divalent cation such as calcium or magnesium failed to increase the activity. The addition of calcium chloride especially decreased the activity. Divalent cations are known to associate with the carboxylate anion of FA. Thus, the carboxylate anion needed for catalysis of this HPL may be masked with the divalent cation. This again suggested that the terminal carboxylate anion was important for the HPL to recognize its substrate.

Effect of detergents. A high concentration of salt may affect either the physical state of the FA hydroperoxide in the reaction mixture or the conformation of the HPL, or both. To gain insight into the reason for the enhancement of activity, we tried to use detergents to suspend the hydrophobic substrate. As shown in Figure 4, greater activity could be observed when detergents, instead of ethanol, were used to suspend the substrate. Addition of Triton X-100 and Brij 99 enhanced the activity the most: Almost three times greater activity could be found with each of them. Tween 80 and Nonidet P-40 followed, and Tween 20 showed the lowest ability to enhance the HPL activity. This effect could be seen with either a low-salt buffer or a high-salt buffer with almost the same tendency. Therefore, there seemed to be no apparent correlation between the enhancing effect and the physicochemical nature of each detergent, such as HLB. It must be noticed that the final concentration of detergent used for these analyses was 0.0008% (wt/vol). For example, the CMC of Tween 20 and octylglucoside are 0.006 and 0.7%, respectively; thus, it is plausible to assume that the detergent stays in the buffer as a monomer (or a small aggregate), although the state of the detergent must change depending on the

TABLE 1
Substrate Specificity of Barley FA Hydroperoxide Lyase[a]

Substrate	Lyase activity (μkat/mg)
9-Hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid	ND
9-Hydroperoxy-(10*E*,12*Z*,15*Z*)-octadecatrienoic acid	ND
13-Hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid	23.5 ± 5.9
13-Hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid	341.1 ± 17.6

[a]Enzyme activity was determined by measuring the decrease in absorbance at 234 nm caused by the decomposition of the substrates. Values are means ± SD of three replicates. ND, not detected.

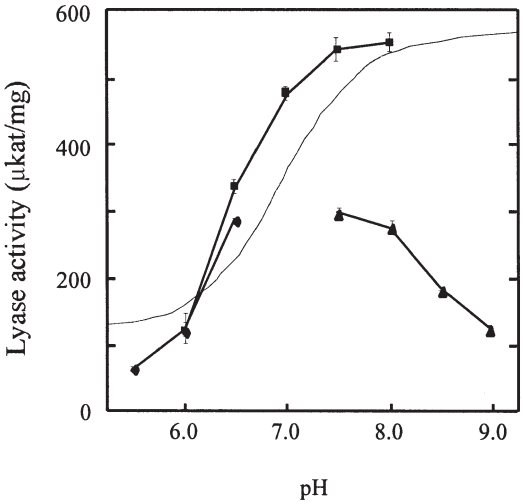

FIG. 2. Effect of pH on recombinant HPL activity. 13-HPOT was used as substrate in 0.1 M MES-KOH (●), 0.1 M KP_i (■), or 0.1 M Tris-HCl (▲). A titration curve of 13-HPOT is shown with a thin line. MES, 2-(*N*-morpholino)ethanesulfonic acid; for other abbreviations see Figure 1.

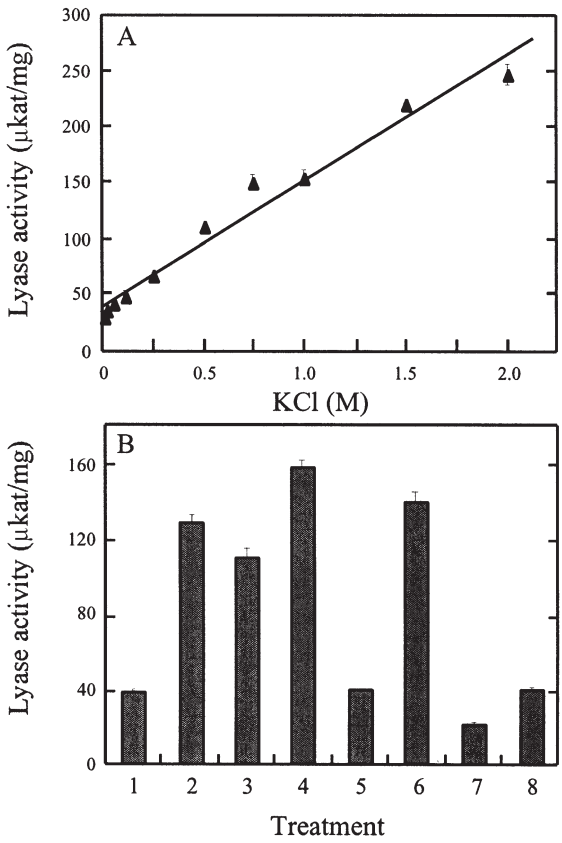

FIG. 3. Effect of addition of salts on recombinant HPL activity. (A) The lyase activity was estimated in the presence of various concentrations of potassium chloride. Error bars represent SD (n = 3). (B) Effect of various salts on lyase activity. (1) Without addition; (2) 1 M KCl; (3) 1 M NaCl; (4) 0.5 M Na_2SO_4; (5) without addition; (6) 1 M KCl; (7) 0.5 M $CaCl_2$; (8) 0.5 M $MgCl_2$. With the reactions for 1 to 4, 10 mM KP_i (pH 8.0) was used as the buffer, whereas 10 mM Tris-HCl (pH 8.0) was used with reactions 5 to 8 to avoid formation of insoluble materials with the divalent cations. Error bars represent SD (n = 3). For abbreviation see Figure 1.

ionic strength and the concentration of substrate. This indicates that formation of micelles was not critical to the effect.

Enzyme kinetics. Kinetic parameters of HPL under various reaction conditions were determined (Table 2). Under the reaction conditions used here, the reaction could fit the normal Michaelis–Menten kinetics. The V_{max} increased approximately three times after addition of 1.5 M potassium chloride, whereas the K_m value essentially was not affected. In the presence of both potassium chloride and Brij 99, the V_{max} value increased further and also lowered the K_m value. This suggests that addition of the salt and the detergent exerted their effect on the enzyme itself. It is well documented that HPL is inactivated in a suicide-inactivation manner. This inactivation is thought to be accomplished by the incidental attack of an active intermediate formed during HPL catalysis on the heme moiety (15,23). As shown in Figure 5, the rate of inactivation of the HPL was higher in the presence of potassium chloride. A slight increase in the inactivation rate could be observed after further addition of Brij 99, but the difference in the rates was not so high.

DISCUSSION

In this work, we isolated a cDNA encoding HvHPL and succeeded in expressing the corresponding recombinant protein with the activity. This is the first report of cloning and characterizing an HPL in a monocotyledonous plant. HvHPL showed the highest activity against 13-HPOT, followed by 13-HPOD. In contrast, 9-HPO(D/T) were not the substrates. This biochemical property and its deduced amino acid sequence indicate that HvHPL belongs to the CYP74B subfamily. HvHPL preferred an alkaline pH for its reaction. The pK_a value of 13-HPOT under the reaction conditions used here was 6.79; thus, the terminal carboxylic acid was mostly dissociated to a carboxylate anion at the

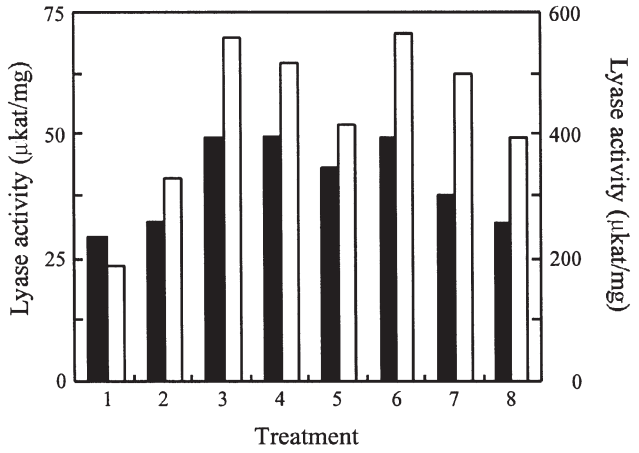

FIG. 4. Effect of detergents on recombinant HPL activity. The reactions were carried out in 10 mM KP_i (pH 8.0) without (closed bars, left vertical line) or with (open bars, right vertical line) 1.5 M KCl. The detergents added were (1) ethanol (control); (2) Tween 20; (3) Tween 80; (4) Triton X-100; (5) Triton X-114; (6) Brij 99; (7) Nonidet P-40, and (8) *n*-octyl-β-D-glucoside. Final concentration of the detergent was 0.0008%. For abbreviation see Figure 1.

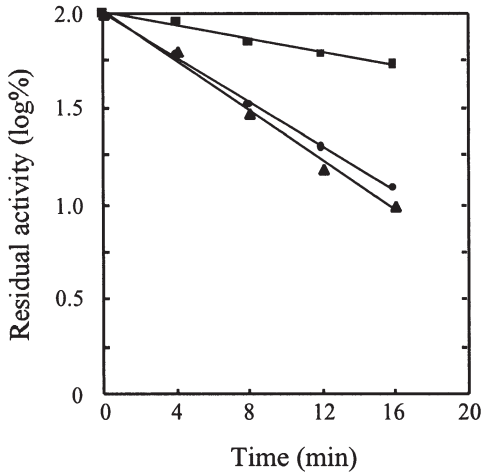

FIG. 5. Inactivation of barley HPL in the presence of a high concentration of salt and/or a detergent. The HPL reaction was carried out with 13-HPOT prepared with ethanol in 10 mM KP_i (pH 8.0, ■) or in 10 mM KP_i (pH 8.0) containing 1.5 M KCl (●). Brij 99 was used to prepare the substrate, and the reaction was carried out in 10 mM KP_i (pH 8.0) containing 1.5 M KCl (▲). For abbreviations see Figure 1.

optimal pH of the HvHPL. Furthermore, no activity could be detected with the methyl ester of 13-HPOT. These results suggest that the carboxylate anion moiety of the substrate is a prerequisite for HvHPL action. This is in contrast to most dicotyledonous HPL characterized thus far. They show optimal activities at a weakly acidic pH where the carboxylic acid form is predominant (15,24,25). 13-HPOT can be a substrate for *A. thaliana* HPL (22), and hydroperoxides of N-acyl(ethanol)amines can be substrates for alfalfa HPL (26). Therefore, the carboxylate anion moiety is not essential to the reaction of these HPL.

A notable finding with HvHPL was that the activity was greatly enhanced in the presence of high concentrations of monovalent cations. This effect also could be observed with recombinant bell pepper HPL (data not shown); thus, it seems not to be specific for only HvHPL. We expected that higher ion strength in the buffer would modulate the electrostatics of the carboxylate anion of the substrate, resulting in a shift of the pK_a value. The pK_a of 13-HPOT shifted from 6.79 to 6.40 by the addition of 1.5 M potassium chloride, which suggests that the abundance of the carboxylate anion form of 13-HPOT is essentially the same in the reaction medium at pH 8.0. Thus, the dissociation of the substrate is not the primary reason for the enhancement of activity. After the addition of 1.5 M potassium chloride, the V_{max} value increased, but the K_m value showed little change, which suggests that the monovalent cation affected the nature of HvHPL. Activities of some enzymes, such as *Aspergillus niger* glucose oxidase, are quite sensitive to such an effect, and its conformation changes in response to NaCl up to 2 M (27). It can be expected that monovalent cations enhance the activity of HvHPL through the global effect on the conformation. This hypothesis also could be supported by the fact that the rate of self-inactivation caused by its own substrate, 13-HPOT, increased in the presence of 1.5 M KCl. It has been shown that self-inactivation proceeds by degradation of the heme essential to the catalytic activity of HPL (15). Therefore, this incident strongly suggests that at least the spatial arrangement surrounding the heme in the HPL was modulated by the salt.

Addition of a detergent to the reaction mixture also dramatically increased the HPL activity. Again, an increase in the V_{max} value was evident, but in this case, the K_m value also was lowered. The latter evidence suggests that substrate binding to the enzyme also was affected. The concentration of detergents was well below their CMC values. The effect of detergent could be seen either in the presence or in the absence of a high concentration of salt. Therefore, the effects of salt and detergent seemed to be independent. Noordermeer *et al*. (28) reported that a detergent, Triton X-100, induced a subtle conformational

TABLE 2
Kinetic Parameters of HvHPL[a] Obtained With or Without Potassium Chloride and 0.0008% Brij 99

	K_m (μM)	V_{max} (μkat/mg)
10 mM KP_i (pH 8.0)	109.1	113.6
10 μM KP_i (pH 8.0) + 1.5 M KCl	127.9	384.6
10 mM KP_i (pH 8.0) + 1.5 M KCl + Brij 99	51.6	1250.0

[a]HvHPL, barley (*Hordeum vulgare*) hydroperoxide lyase.

change in alfalfa HPL, which resulted in an improved spin state of the heme. With many HPL characterized thus far, enhancements of activity after solubilization from cell membranes by a detergent have been reported (11,24). The hydrophobic environment of HPL on the cell membrane must undergo a vigorous change after solubilization. Therefore, these HPL must be sensitive to such a hydrophobic environment surrounding the enzyme. It was reported that higher HPL activity could be observed when the enzyme was extracted by homogenization than when it was isolated by a gentle extraction method through the preparation of protoplasts (29). It is well known that HPL products, C6- and C9-aldehydes, are formed rapidly after homogenization of plant tissues. Such a rapid formation of products might be facilitated by the activation of HPL through conformational changes caused by a hydrophobic environment.

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Isomers of Hexadecenoic and Hexadecadienoic Acids in *Androsace septentrionalis* (Primulaceae) Seed Oil

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ABSTRACT: Seeds of *Androsace septentrionalis* of the genus *Androsace* (tribus Primuleae) from the plant family Primulaceae were studied for their oil content and FA composition. The seed oil of *A. septentrionalis* was found to contain two unusual FA rarely occurring in plants: 11-*cis*-hexadecenoic acid (16:1Δ11*c* or 16:1n-5) and 9-*cis*,12-*cis*-hexadecadienoic acid (16:2Δ9*c*,12*c* or 16:2n-4). It also contained an unusually high amount (21.4%) of 9-*cis*-hexadecenoic acid (palmitoleic acid; 16:1Δ9*c* or 16:1n-7), i.e., at a level higher than that of oleic acid, in addition to common FA. Compared with most plant seed oils, at 3.8% the level of 18:1Δ11*c* (or 18:1n-7) also was elevated. The nonidentity of the *Androsace* 16:2-acid with the 16:2-acid, which is very typical for *Ranunculus* spp., as well as its identity with the 16:2-acid typically found in Asclepiadaceae was established by co-chromatography. The structure and composition of the constituent FA of *A. septentrionalis* were also determined by various chromatographic methods (TLC, Ag^+-TLC, capillary GLC) and spectroscopic methods (IR, GC–MS). The significant deviation of the *Androsace* FA pattern from that of other Primuleae, indicating a separate phylogenetic position of *Androsace*, is discussed.

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Androsace, a genus of about 100 species, is one of the larger genera of the family Primulaceae (1). Some of the species from this plant family are cultivated as ornamentals and medicinals (2). Although many of the species of Primulaceae already have been studied with regard to their physiologically active chemical constituents (3), only a few references appear in the literature that deal with the analysis of seed oils from this plant family.

At the time of our first analyses of *Androsace* spp. (1994–1995), there was no information in the literature on seed oils from the genus *Androsace*. Earlier investigations by our working group, in our project to explore new sources of unusual FA in seed oils, had already been reported, including the results of our studies on seed oils of the genus *Primula* (4). In the meantime, our initial report on the presence of γ-linolenic acid (18:3Δ6*c*,9*c*,12*c* or 18:3n-6) and, in particular, stearidonic acid (18:4Δ6*c*,9*c*,12*c*,15*c* or 18:4n-3) in various *Primula* spp. had prompted Sayanova *et al.* (5) to investigate in more detail the distribution of γ-linolenic acid in the family Primulaceae, including *Androsace*. In accordance with our own data (6), γ-linolenic acid was not found in *Androsace,* but the presence of elevated levels of 16:1 in certain species of *Androsace* was briefly mentioned by Sayanora *et al.* (5) in a different context.

Our studies on the FA composition of seed oils from other representatives of the genus *Androsace*, and from other *Lysimachia* and *Primula* species will be reported separately. However, our data on the FA composition of these species have already been included in the recently published "Seed Oil Fatty Acids Database," which is available on the Internet (6,7). Our present communication deals with the identification and structural determination of unusual C_{16} FA in the seed oil of *A. septentrionalis* growing in Mongolia. This work is part of our project to supplement the literature on plant chemotaxonomy and sources of genes for renewable resources, and to discover new seed oil sources in the plant kingdom for unusual FA.

MATERIALS AND METHODS

Seed materials. Seeds of *A. septentrionalis* were collected during July–August 1994 in the surroundings of Ulaanbaatar, Mongolia. Extracts of seeds of *Ranunculus repens* (Ranunculaceae), *Grevillea robusta* (Proteaceae), and *Asclepias syriaca* (Asclepiadaceae) were used as reference substances for comparison and co-chromatography (see below). They were available in this laboratory from previous investigations (6).

Oil extraction and preparation of FA derivatives.The ground seeds (8 g) were extracted with *n*-hexane in a Soxhlet extractor. FA were first prepared by saponification of the seed oils in 1.0 N KOH/ethanol. After extraction of the unsaponifiable materials, the FA were reacted with 20% BF_3/methanol to form FAME, as described in our previous papers (8,9).

Preparation of dimethyldisulfide (DMDS) adducts and 4,4-dimethyloxazoline (DMOX) derivatives of FA. DMDS adducts were prepared as described by Francis (10). The procedure of Yu *et al.* (11) for the preparation of DMOX derivatives of FA as described earlier (12) was modified as follows: 15 mg of 2-amino-2-methylpropanol was added to 5 mg of the FA mixture in a two-necked flask equipped with a gas inlet, a reflux condenser, and a magnetic stirring bar. The mixture was heated at 190°C under nitrogen for 1.5–2 h. The cooled mixture was triturated with 1 N KOH and extracted three times with hexane (10 mL). The extract was washed with water, dried over

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Abbreviations: DMDS, dimethyldisulfide adducts; DMOX, 4,4-dimethyloxazoline derivatives; PRV, peak recognition value; SOFA, seed oil FA (for SOFA database, go to netlink <www.bagkf.de/SOFA>).

anhydrous sodium sulfate, and evaporated to dryness in a rotary evaporator.

TLC. To obtain information on unusual components in oils, analytical TLC of oils and of FAME was carried out on 0.25-mm layers of Silica gel G (Merck, Darmstadt, Germany) using a solvent system of *n*-hexane/diethyl ether (70:30 or 80:20 vol/vol, respectively). Spots were visualized by spraying with phosphomolybdic acid (5% in ethanol) and heating at 100°C.

Preparative silver ion TLC (Ag^+-TLC). Argentation-TLC of FAME was carried out on 20 × 20 cm plates with 0.25-mm layers of Silica gel G dynamically impregnated (13) with 10% silver nitrate in acetonitrile. The plates with FAME were developed with the solvent system *n*-hexane/diethyl ether/acetic acid (90:9:1, by vol), which resulted in a separation of zones according to degree of unsaturation. When the plates were sprayed with 2,7-dichlorofluorescein, the separated zones were observed under UV light, marked, then removed from the plate separately (Zones I–IV). The FAME were recovered from the silica by extraction with diethyl ether, and each fraction was analyzed by capillary GLC as reported earlier (14).

Capillary GLC. GLC of FAME was performed on a Hewlett-Packard HP 5890 gas chromatograph equipped with an FID and with a fused-silica WCOT capillary column (length 50 m, i.d. 0.25 mm) coated with stationary-phase Silar 5 CP (Chrompack, Middelburg, The Netherlands) as described for our standardized "seed oil FA (SOFA) fingerprints" (8). Chromatographic data were evaluated with an integrator (Chromato-Integrator D2000). Identifications of FAME were confirmed by chromatographic comparison with authentic standards and by calculation of peak recognition values (PRV, similar to equivalent chain lengths; see below). GLC conditions were as follows: The temperature was held at 165°C for 1 min, then programmed from 165 to 205°C at 1°C/min, then held at 205°C for *ca.* 60 min before the column was cooled to 165°C. The injector and detector temperatures were 230 and 260°C, respectively. The temperature gradient used was identical to the one used for our standardized fingerprints (8,15).

Capillary GC–MS. GC–MS analysis was carried out using the electron ionization mode (EI, 70 eV) on a Hewlett-Packard model 5890 Series II/5989 A instrument, equipped with a 0.23-µm Permabond OV-1 fused-silica capillary column (Macherey-Nagel, Düren, Germany), 25 m × 0.32 mm i.d. The carrier gas was helium at a flow rate of 1.5 mL/min.

The column temperature for the DMOX derivatives was held at 170°C for 5 min, then programmed from 170 to 250°C at 2°/min. For the DMDS adducts, the column temperature was held at 100°C for 2 min, then programmed from 100 to 280°C at 10°/min. In both cases the final temperature was held for 5 min. Other operating conditions were: a split/splitless injector (split 1:20, temperature 280°C), an interface temperature of 280°C, and an ion source temperature of 200°C.

IR spectra. IR spectra were determined from oil films on sodium chloride cells using a PerkinElmer 781 Infrared Spectrophotometer (Beaconsfield, England) with a focusing attachment to check for the presence of any unusual (*trans* unsaturated or oxygenated) FA.

RESULTS AND DISCUSSION

From 8.0 g of seed of *A. septentrionalis,* 408 mg of seed oil was obtained (5.1%). The FA composition of the oil, based on GLC analysis of the FAME as well as the PRV, is given in Table 1. [In our standardized SOFA fingerprint chromatograms, PRV are calculated by linear interpolation between adjacent even-numbered saturated FA, under the specified standardized temperature-gradient conditions (8)]. One interesting aspect of the FA analysis was the appearance of unusual components with PRV 16.43 (1.7%) and 16.91 (4.7%) on the Silar 5 CP column. These PRV suggest that the acid moieties are an isomeric 16:1 acid (other than palmitoleic) and an isomeric 16:2 acid, respectively. On the other hand, we already knew (6) *A. septentrionalis* seed oil had a high content (21.4%) of *cis*-hexadec-9-enoic acid (16:1Δ9*c*, palmitoleic) as well as 3.8% of *cis*-octadec-11-enoic (*cis*-vaccenic) acid in addition to linoleic (37.7%) and oleic (19.4%) acids as the predominant FA. The presence of palmitoleic acid at a level higher than that of oleic acid is quite unusual for a seed oil. Figure 1 shows the standardized SOFA fingerprint (8) obtained on a Silar 5 CP column. Since IR spectra of the oil showed no bands in the 960 cm^{-1} region, unsaturation in the oil presumably has the *cis* configuration exclusively. Results of analytical TLC of the seed oil and mixed FAME excluded the presence of any oxygenated FA. Figure 2 illustrates the strong deviation between *Androsace* and *Primula* seed oils by way of a superposition of the fingerprints of *A. lactea* (which is similar to *A. septentrionalis*) and *P. nutans*, obtained during our ongoing study of Primulaceae SOFA patterns (Aitzetmuller, K., and Tsevegsuren, N., unpublished data). Clearly, *Primula* contains peaks not found in *Androsace*, and vice versa.

TABLE 1
FA Composition of Total *Androsace septentrionalis* Seed Oil FAME[a]

FAME	PRV[b]	GLC area%
10:0	10.00	Trace[b]
12:0	12.00	0.1
14:0	14.00	1.2
14:1n-5	14.40	0.1
15:0	14.90	0.1
16:0	16.00	4.4
16:1n-7	16.35	21.4
16:1n-5	16.43	1.7
16:2n-4	16.91	4.7
18:0	18.00	0.4
18:1n-9	18.31	19.4
18:1n-7	18.36	3.8
18:2n-6	18.83	37.7
19:0	18.97	0.1
18:3n-3	19.42	4.0
20:0	20.00	0.1
20:1n-9	20.28	0.2
22:0	22.00	0.1
Others		0.5

[a]Analyzed by capillary GLC on a Silar 5 CP column, using the standardized fingerprint technique (8).
[b]PRV, peak recognition value.
[c]<0.05%.

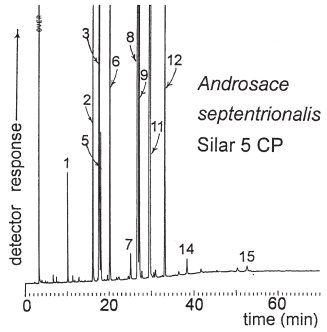

FIG. 1. Standardized "seed oil FA fingerprints" (8) of *Androsace septentrionalis* on a Silar 5-CP GLC column. Peak numbers: **1** = 14:0; **2** = 16:0; **3** = 16:1Δ9*c* (16:1n-7); **5** = 16:1Δ11*c* (16:1n-5); **6** = 16:2Δ9*c*,12*c* (16:2n-4); **7** = 18:0; **8** = 18:1Δ9*c* (18:1n-9); **9** = 18:1Δ11*c* (18:1n-7); **11** = 18:2Δ9*c*,12*c* (18:2n-6); **12** = 18:3Δ9*c*,12*c*,15*c* (18:3n-3); **14** = 20:1Δ11*c* (20:1n-9); **15** = 22:0.

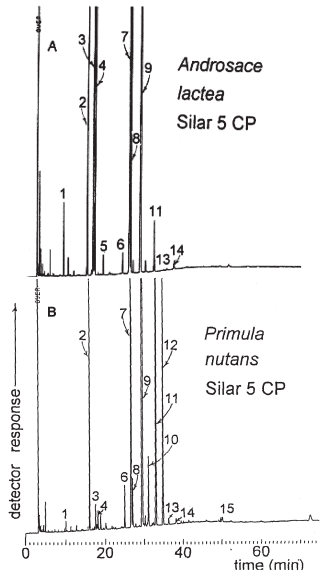

FIG. 2. Superposition of standardized "seed oil FA fingerprints" (8) of *Androsace lactea* (A) and *Primula nutans* (B). The considerable differences between the two may indicate a separate position of *Androsace*. Peak numbers: **1**: 14:0; **2**: 16:0; **3**: 16:1Δ9*c*; **4**: 16:1Δ11*c*; **5**: 16:2Δ9*c*,12*c*; **6**: 18:0; **7**: 18:1Δ9*c*; **8**: 18:1Δ11*c*; **9**: 18:2Δ9*c*,12*c*; **10**: 18:3Δ6*c*,9*c*,12*c* (γ-linolenic acid); **11**: 18:3Δ9*c*,12*c*,15*c*; **12**: 18:4Δ6*c*,9*c*,12*c*,15*c* (stearidonic acid); **13**: 20:0; **14**: 20:1Δ11*c*; **15**: 22:0.

Further evidence regarding the unknown components in *A. septentrionalis* seed oil was obtained by capillary GLC analysis of the four fractions (Zones I–IV), that resulted from preparative Ag^+-TLC of the methyl esters (Table 2). The typical fingerprint (8) of mixed FAME of *A. septentrionalis* seed oil and the fingerprints of the preparative Ag^+-TLC Zones II and III, corresponding to monoenoic and dienoic FAME obtained on a Silar 5 CP column, are superimposed in Figure 3. All three were obtained by GLC under identical conditions. (Additional FA, not seen in Table 1 or Fig. 1, occur in Table 2 and Fig. 3 because of their enrichment by preparative TLC.) Figure 3 illustrates the clear separation of monoenes and dienes on the silver nitrate plate, and it also shows that two different monenes each were found in the 16:1- and 18:1-regions of the GLC chromatogram.

Zone I was the area where saturated FAME were found. They consisted mainly of 16:0 (69.2%), 14:0 (16.6%), and 18:0 (6.7%), with small amounts of other homologs, such as 12:0, 15:0, 17:0, 20:0, 22:0, and 24:0.

Zone II was in the *cis*-monoene region, and GLC indicated the presence of four major peaks: 16:1Δ9*c* (44.1%), 18:1Δ9*c* (44.0%), 18:1Δ11*c* (7.0%), and an unknown 16:1 isomer (3.7%), In addition, there were trace amounts of 14:1Δ9*c*, 17:1Δ10*c*, and 20:1Δ11*c*.

Zone III contained two dienoic acids. One was the common linoleic acid 18:2Δ9*c*,12*c* (87.4%), and the other was an unusual isomer of 16:2 (11.8%).

Trienoic acids were found in the Zone IV, which consisted nearly exclusively of α-linolenic acid (18:3Δ9*c*,12*c*,15*c*).

Isomers of 16:2 have been observed before as constituents of seed oils of a few phylogenetically closely related genera in the plant kingdom (6,16). In the Ranunculaceae, an acid corresponding to the structure 16:2Δ7*c*,10*c* (or 16:2n-6) is commonly observed in seed oils of *Ranunculus* and *Batrachium* spp. (9,17), as well as in *Krapfia* (Aitzetmuller, K., Tsevegsuren, N., and Werner, G., unpublished data), at levels of up to 10%. Another 16:2 isomer (16:2Δ9*c*,12*c*, or 16:2n-4) was previously found in *Asclepias syriaca* seed oil (16,18) and in a few seed oils from other closely related Asclepiadaceae (Aitzetmuller K., Werner, G., and Albers, F., unpublished data). The presence of the same FA was also reported once in a seed oil sample of South African *Acacia giraffae* (19). Therefore, seed oils from *A. syriaca* and *R. repens*, available in our laboratory from earlier investigations,

TABLE 2
FA Composition of Zones I–IV Obtained by Preparative Argentation-TLC (Ag^+-TLC)

Fractions (Ag^+-TLC zones)	FA in Ag^+-TLC zones	GLC area%
Zone I (saturated)	10:0	0.1
	12:0	1.1
	13:0	0.1
	14:0	16.6
	15:0	0.8
	16:0	69.2
	17:0	0.6
	18:0	6.7
	20:0	1.1
	22:0	1.2
	24:0	0.9
Zone II (monoene)	14:1n-5	0.1
	16:1n-7	44.1
	16:1n-5	3.7
	17:1n-7	0.1
	18:1n-9	44.0
	18:1n-7	7.0
	20:1n-9	0.3
Zone III (diene)	16:2n-4	11.8
	18:2n-6	87.4
Zone IV (triene)	18:3n-3	93.9

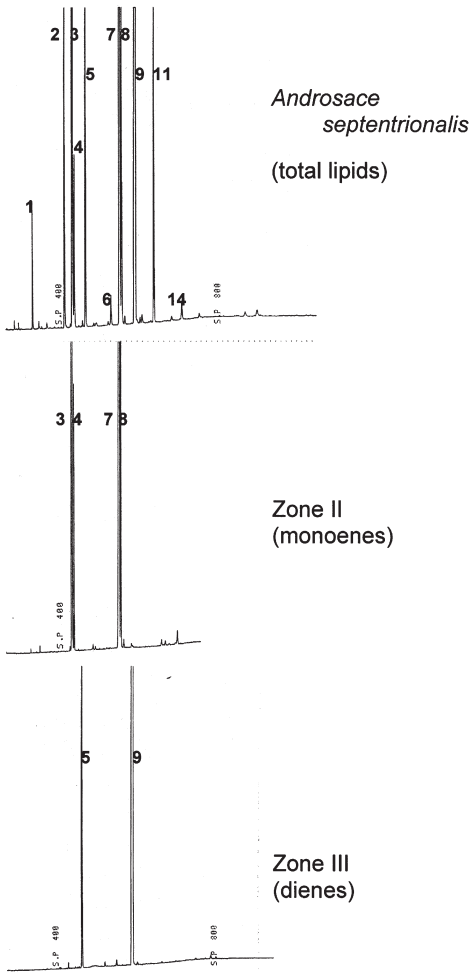

FIG. 3. Superposition of capillary GC fingerprints of total FAME from *Androsace septentrionalis* seed oil (top), and of the Ag^+-TLC Zones II and III, corresponding to monoenoic and dienoic FAME. The *x*-axis is time (total about 60 min), and the *y*-axis is recorder response (large peaks are cut off at the top). For peak numbers see Figure 2.

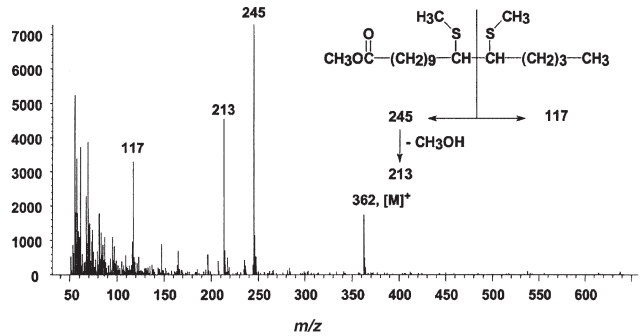

FIG. 4. Mass spectrum of the dimethyldisulfide (DMDS) adduct of *cis*-11-hexadecenoic acid methyl ester from the Ag^+-TLC monoenoic Zone II of *Androsace septentrionalis* seed oil FAME.

were used as reference substances to compare the GC peaks and their PRV values under our two GC conditions. The absence of 16:2Δ7*c*,10*c* in *A. septentrionalis* was established by this comparison. Co-injection of mixed FAME from *A. septentrionalis* seed oil with those from *R. repens* seed oil gave two clearly separated peaks. The unknown from *A. septentrionalis* eluted just after the 16:2Δ7*c*,10*c* from *R. repens*; therefore, the unknown hexadecadienoic acid in *A. septentrionalis* cannot be 16:2Δ7*c*, 10*c*.

The presence of 16:2Δ9*c*,12*c* (16:2n-4) in *A. septentrionalis* was then established in a similar way by comparison with *A. syriaca,* which contains this FA in its seed oil (6,16). Co-chromatography of *A. septentrionalis* seed oil FAME with those from *A. syriaca* on Silar 5 CP resulted in a single peak for the unknown 16:2 FA from *A. septentrionalis* and the known 16:2Δ9*c*,12*c* from *A. syriaca*.

The double-bond positions in the unusual 16:2 FA of *A. septentrionalis* seed oil (which appeared in Zone III; see Fig. 3) were further confirmed by the GC–MS fragmentation pattern of the DMOX derivative. The molecular ion at *m/z* 305 indicates the presence of two double bonds. Their position was easily recognized by applying the empirical rule for double-bond location (20). The mass intervals of 12 *m/z*, instead of the regular 14 *m/z*, occurred between *m/z* 196 (C-9) and 208 (C-10), as well as *m/z* 236 (C-12) and 246 (C-13), indicating a Δ9 and a Δ12 double bond.

Further evidence for the double bond position in the unusual 16:1 FA of *A. septentrionalis* seed oil was given by the GC–MS fragmentation pattern of the DMDS adducts prepared from the monoenoic Zone II of Ag^+-TLC chromatograms (see Fig. 4). In both 16:1 FA, palmitoleic acid and the unknown acid, the molecular ion (M^+) at *m/z* 362 indicated the presence of one double bond in a C_{16} acid. The position of the double bond in the unknown minor FA was easily recognized by the distinctive fragmentation of its DMDS adduct, which gave intense key fragment ions at *m/z* 245 (base peak) and 117, formed by the cleavage between the C-11 and C-12 carbon atoms of the DMDS adduct. Together with the ion at *m/z* 213, which is derived from the ion at *m/z* 245 by the loss of methanol, they clearly establish the structure of the unknown FA as 16:1Δ11*c*, also known as 16:1n-5 (Fig. 4). Again, this is confirmed by the PRV value of the unknown FAME on the Silar 5 CP column, which was identical with the one found for the known 16:1Δ11*c* in *G. robusta* (21) and other Proteaceae, which also was available for comparison from our earlier investigations (6).

On the other hand, the mass spectrum of the DMDS adduct of palmitoleic acid methyl ester—the other isomer predominating in Zone II from the preparative Ag^+-TLC plate—gave the expected characteristic peaks at *m/z* 217 (base peak), 185 (217 – CH_3OH) and 145, in agreement with previously published data (10,22).

During this study, the seed oils of *A. septentrionalis* and *A. lactea* were both found to contain *cis*-11-hexadecenoic and *cis,cis*-9,12-hexadecadienoic acids (6). However, the content of *cis*-11-hexadecenoic acid, at 7.2% of total FA, was much higher in *A. lactea* than in *A. septentrionalis*, where it was only 1.7% of total FA. On the other hand, the content of *cis,cis*-9,12-hexadecadienoic acid, at 4.7% of total FA, was much higher in

A. septentrionalis, whereas this FA was only 0.4% of total FA in the seed oil of *A. lactea* (6). The list of natural sources for these two FA should therefore be extended by including the plant family Primulaceae. So far, *cis*-hexadec-11-enoic acid is known to occur as a seed oil component in some Proteaceae (21,23), and, together with *cis*,*cis*-hexadeca-9,12-dienoic acid, in *A. syriaca* (Asclepiadaceae) (16,18).

One possible interpretation of these data is that *A. septentrionalis* (and *A. lactea*) may contain a fairly active Δ11-desaturase, which may accept both 18:0 and 16:0 as substrates for a Δ11-desaturation. (The formation of 16:1Δ11*c* in a number of Proteaceae would then likely be carried out *via* a different biosynthetic route, e.g., by way of an n-5-desaturase.) This *Androsace* Δ11-desaturase produces noticeable levels of 16:1Δ11*c* as well as an elevated level of 18:1Δ11*c*. *Androsace septentrionalis* and other *Androsace* spp. also may contain both a Δ9- and a Δ12-desaturase of broader specificity, both accepting C_{16} and C_{18} FA as substrates. The Δ9-desaturase may lead to the observed high levels of 16:1Δ9*c* (equal to 16:1n-7) and 18:1Δ9*c* (equal to 18:1n-9), whereas the Δ12-desaturase may accept both oleic and palmitoleic acid as substrates, producing measurable levels of 16:2Δ9*c*,12*c* as well as the usual 18:2Δ9*c*,12*c*.

Evidence for a broader substrate specificity of desaturases was occasionally found in different plant seed oils. The reason for this may be mutations during phylogenetic evolution, leading to small differences in the desaturase amino acid sequences. This has recently been demonstrated experimentally. When a few amino acids were replaced by others in a *Thunbergia* Δ6-desaturase, changes in the substrate specificity of the enzyme and changes in the position of the double bond introduced into the FA carbon chain were observed (24). Moreover, it has recently been found that exchanging the identity of amino acids at four key locations within oleate desaturase can convert a desaturase into a hydroxylase (25). It is quite possible that similar events may have happened by way of natural mutation during the early phylogenetic evolution of *Androsace* and other Primulaceae, and that these should be considered in studies of systematic botany. In this context, it is also of interest that John (26) found significant differences between *Androsace* on the one hand and *Primula* and other members of the Primuleae subfamily (Primulaceae) on the other hand in a serological investigation using seed proteins. The superposition of standardized SOFA fingerprints (8) of *A. lactea* and *P. nutans* in Figure 2 reveals notable differences, which may support a separate position of *Androsace* within the Primuleae. (Note the size of peaks nos. 3, 4, and 8 in *Androsace* vs. peaks nos. 10 and 12 in *Primula*.) Our data regarding the very different FA patterns of *Androsace* vs. *Primula* (see Refs. 4 and 6, and the illustration in Fig. 2 here) can therefore be taken as additional evidence for a separate position of *Androsace* within the Primuleae and seem to support the data of John (26).

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Tentative Identification and Quantification of TAG Core Aldehydes as Dinitrophenylhydrazones in Autoxidized Sunflowerseed Oil Using Reversed-Phase HPLC with Electrospray Ionization MS

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ABSTRACT: The molecular species of TAG core aldehydes (aldehydes still esterified to parent molecules) were detected and quantified in dietary-quality sunflowerseed oil autoxidized for 0–18 d at 60°C in the dark. The analyses were performed by reversed-phase HPLC with UV (358 nm) absorption or light scattering and electrospray ionization-MS (ESI/MS) detection following preparation of the dinitrophenylhydrazone derivatives. Aldehyde production, as estimated by UV and ESI/MS, increased gradually over the 18-d period following a rapid initial destruction of the core aldehydes accumulated during storage of the commercial oil at 10°C for 3 mon. The contents of hydroperoxides and hydroperoxide core aldehyde combinations were estimated to account for about 5% of total TAG, quantified as area in the chromatographic trace, after 18 d of autoxidation as estimated by an evaporative light scattering detector (ELSD). The major species of core aldehydes were tentatively identified as 9-oxononanoyl (70%)-, 12-oxo-9,10-epoxydodecenoyl (10%)-, and 13-oxo-9,11-tridecadienoyl (5%)-containing acylglycerols, plus smaller amounts of simple and mixed chain-length dialdehydes, and hydroxy and epoxy monoaldehyde-containing acylglycerols (15% of total). Quantitatively, the core aldehydes made up 2–12 g/kg of oil by UV detection and 2–9 g/kg of oil by ESI/MS detection, whereas the hydroperoxides measured in the unreduced state by HPLC with ELSD were estimated at 200 g/kg after 18 d of autoxidation. The major hydroperoxides of sunflowerseed oil were as previously identified.

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Much research has been published on the autoxidation of unsaturated vegetable oils, which yield hydroperoxides as primary oxidation products. These primary products are rapidly decomposed to form a variety of secondary products, including aldehydes (ALD), ketones (KET), hydrocarbons, and other products (1,2). The volatile low-M.W. oxidation products, which produce off-flavors and other odors in foods, have been extensively studied, and specific structures have been assigned to them (3). For every mole of volatile ALD, a mole of nonvolatile oxidation residue is produced, which remains bound to the parent acylglycerol molecule as a core ALD (4,5). ALD formation is promoted by metal ions, such as Fe^{2+} and other transition metals (6). Some studies have reported core ALD formation during acylglycerol peroxidation (7–13), but only a few have isolated TAG core ALD and determined their properties (7,13).

The exact nature of the core ALD arising by autoxidation of neutral acylglycerols has not been investigated, but it would be expected to vary with the FA composition of the fat or oil. In the present study we identified and quantified the acylglycerol core ALD produced by autoxidation of sunflowerseed oil, which is a common dietary oil. For this purpose the core ALD were first converted into the dinitrophenylhydrazones, which were resolved and characterized mainly by reversed-phase HPLC (hereafter, HPLC) and HPLC with on-line electrospray ionization-MS (ESI/MS).

MATERIALS AND METHODS

Materials. Commercial sunflowerseed oil (Kultasula) was supplied by Raision Margariini (Oy, Toijala, Finland) and was used without further purification. The oil had been stored 3 mon beyond the "best before" date. Conventional estimates for the PV and *p*-anisidine value are given in Table 1 along with the estimates for core ALD content derived from the present work. The sunflower oil sample contained 16:0 (5.9%), 16:1 (0.1%), 18:0 (4.3%), 18:1 (22.3%), 18:2 (65.9%), 20:0 (0.3%), 18:3 (0.4%), 20:1 (0.2%) and 22:0 (0.6%), in close agreement with previous reports (13,14). Synthetic acylglycerol core ALD were available in the laboratory from previous studies, including the internal standard, 18:0/18:0/9:0 ALD (15).

Autoxidation. Three replicate aliquots of the sunflowerseed oil (10 mL) were kept at 60°C in the dark for 18 d in open bottles. Samples were taken at regular intervals and stored under nitrogen at −18°C until analyzed. The hydroperoxide residues were converted to alcohols to prevent core ALD formation during workup. Next, the three replicate samples of autoxidized sunflowerseed oil were reduced with triphenylphosphine (1 mg/mL benzene) (16). Triphenylphosphine was removed by

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Abbreviations: ALD, aldehyde; DNPH, 2,4-dinitrophenylhydrazone; ELSD, evaporative light-scattering detector; ESI, electrospray ionization; KET, ketone; HPLC, reversed-phase HPLC; TBHP, *tert*-butyl hydroperoxide.

TABLE 1
PV, *p*-Anisidine Values, Estimated Content of Core Aldehydes (ALD) by UV, Estimated Content of Core ALD by LC/ESI/MS, and Estimated Content of Polar TAG by HPLC/ELSD of Sunflowerseed Oil During Autoxidation[a]

	Days of autoxidation							
Method of measurement	0	2	4	6	8	10	12	18
PV	7.3	21.6	51.0	83.4	121	170	267	556
p-Anisidine value	8.6	10.1	10.6	10.5	12.0	17.0	30.3	70.2
Core ALD (g/kg) by UV	3.5	2.9	2.8	2.7	2.2	3.0	5.1	12.6
Core ALD (g/kg) by LC/ESI/MS	12.6	7.3	5.2	5.0	4.9	6.0	8.9	9.9
Polar TAG (g/kg) by HPLC/ELSD	0.8	5.3	7.8	26.0	41.3	60.8	99.5	263.4

[a]ESI, electrospray ionization; HPLC, reversed-phase HPLC; ELSD, evaporative light-scattering detector.

TLC in a neutral lipid solvent system after derivatization of the core aldehydes to 2,4-dinitrophenylhydrazones (DPNH) (17).

p-*Anisidine and PV.* The PV and *p*-anisidine values were determined according to IUPAC method 2.501 (18) and AOCS Official Method Cd 18-90 (19), respectively. Disposable plastic cuvets for visible light were used in the *p*-anisidine determination at 350 nm in a Beckman DU-60 Series spectrophotometer.

TAG hydroperoxides. The amount of total hydroperoxy TAG was estimated by HPLC (Supelcosil LC-18 column, 250 × 4.6 mm i.d.; Supelco, Mississauga, Ontario, Canada) of the underivatized oils using a linear gradient of 20–80% 2-propanol in methanol as the mobile phase and an evaporative light scattering detector (Varex ELSD II; Varex Co., Rockville, MD). Trimyristoylglycerol was used as the internal standard.

TLC analysis. The removal of triphenylphosphine following reduction of the hydroperoxides was accomplished using a TLC plate (Silica gel H) and heptane/isopropyl ether/acetic acid (60:40:4, by vol) as the developing solvent. Synthetic core ALD and hydroperoxides were used as reference standards.

HPLC and LC/ESI/MS of DNPH derivatives. The DNPH derivatives of the TAG core ALD were resolved by HPLC on a Supelcosil LC-18 column (250 × 4.6 mm i.d.; Supelco) by using a linear gradient of 20–80% 2-propanol in methanol (0.85 mL/min) in 30 min. The instrument was a Hewlett-Packard (Palo Alto, CA) Model 1050 liquid chromatograph with UV detector (358 nm). The ALD were identified by LC/MS using a Hewlett-Packard Model 1090 HPLC interfaced with a nebulizer-assisted electrospray source connected to a Hewlett-Packard Model 5989A quadrupole mass spectrometer. The ionization voltage of this instrument was set at 170 V but could be increased to 300 V, to obtain fragment ions from any clearly resolved peaks (pseudo MS/MS) (10,15). The HPLC conditions were the same as described above for HPLC/UV analyses, except that for purposes of improved ionization, ammonia/isopropanol (2%) was added at a postcolumn flow rate of 0.25 mL/min. DNPH derivatives of TAG core ALD were detected as $[M - H]^-$ ions in the negative ion mode. Peak identification was supported by the elution factors observed or calculated from standards (10,15).

FA analyses. The analysis of FAME of sunflowerseed oil was performed following transmethylation with sulfuric acid/methanol (20). The FA were analyzed on a polar capillary column (SP 2380, 15 m × 0.32 mm i.d.; Supelco) installed in a Hewlett-Packard Model 5880 gas chromatograph equipped with an FID.

TAG analysis. TAG and oxo-TAG were resolved by HPLC as described above for the DNPH derivatives (see also Ref. 15).

It should be emphasized that attempts to isolate small amounts of lipid peroxidation products from natural sources should be carried out with great care (21). The ready peroxidation of polyunsaturated lipids on TLC plates and on HPLC columns cannot be fully avoided by working under an inert gas atmosphere, apparently because trace levels of contaminant transition metal ions are present in the supporting gel. To avoid this problem, all aqueous solvents should be Chelex-100-rinsed prior to use, and all columns and filters should be pre-rinsed with diethylenetriamine pentaacetic acid-containing solvents at neutral pH (21).

RESULTS

Resolution and quantification of oxo-TAG. The separation and quantification of native and oxo-TAG were performed by HPLC by using an ELSD and, following preparation of the DNPH derivatives, by UV monitoring. HPLC/ELSD of the oxidation extracts of sunflowerseed oil following 0, 4, 10, and 18 d of autoxidation showed prominent peaks for the native TAG along with gradually increasing proportions of the oxidation products, as the autoxidation progressed (chromatograms not shown). Monohydroperoxides eluted clearly between DAG and TAG, and the dihydroperoxides before DAG. Double peaks are due to regioisomers depending on the position of hydroperoxide group in the TAG. The major monohydroperoxides were 18:2/18:2/18:2 OOH and 18:2/18:2/18:1 OOH, corresponding to the major unsaturated TAG in sunflowerseed oil. The major hydroperoxides were identified by reversed-phase LC/ESI/MS as $[M + NH_4]^+$ ions (Sjövall, O., Kuksis, A., and Kallio, H., unpublished results).

The HPLC/UV elution profiles of the DNPH derivatives of the TAG core ALD obtained for the 0, 4, 10, and 18 d autoxidation samples of sunflower oil in relation to the internal standard are shown in Figure 1. For the purpose of graphical presentation the chromatograms have been compressed. Nevertheless, the total UV-absorbing area due to the DNPH derivatives of the

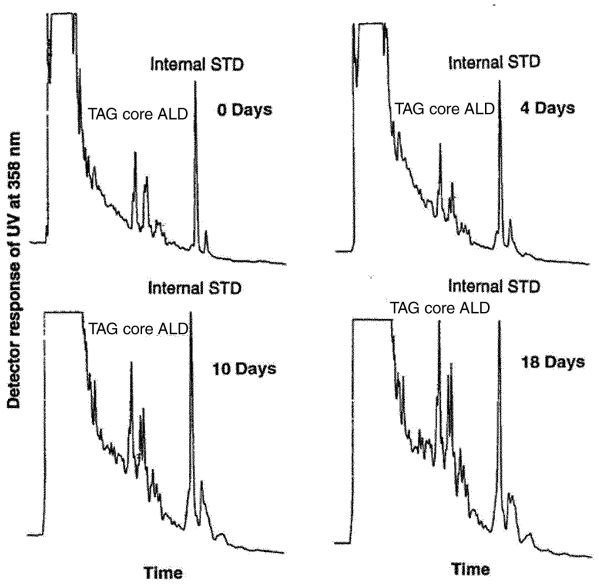

FIG. 1. Comparison of reversed-phase HPLC(HPLC)/UV elution profiles of 2,4-dinitrophenylhydrazone (DNPH) derivatives of TAG core aldehydes (ALD) isolated from autoxidized sunflowerseed oil after 0, 4, 10, and 18 d storage at 60°C in the dark. The internal standard was 18:0/18:0/9:0 ALD. Detection of DNPH was carried out by UV at 358 nm. Conditions of HPLC separation are given in the Materials and Methods section. Solvent gradient: 20–80% 2-propanol in methanol (0.85 mL/min) in 30 min. Abbreviation: STD, standard.

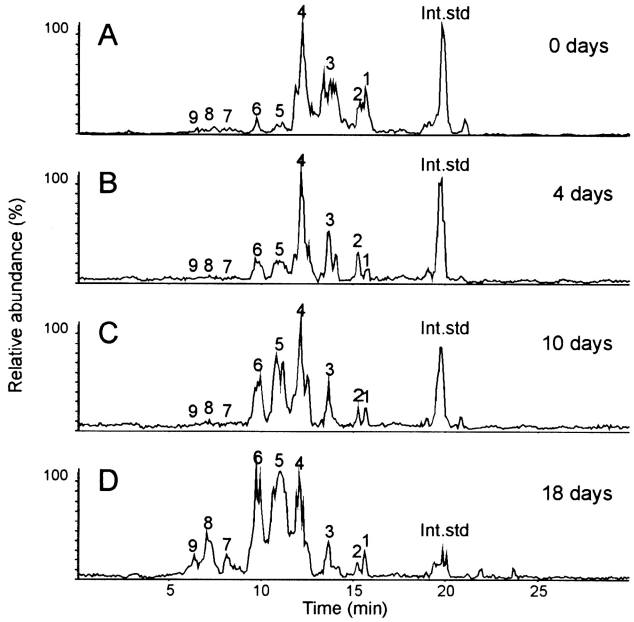

FIG. 2. The total negative ion current electrospray ionization (ESI)/MS of DNPH-derivatized core aldehydes in 0- (A), 4- (B), 10- (C), and 18-d (D) oxidation samples of sunflowerseed oil. The peak clusters numbered 1–9 represent mixtures of core ALD of approximately the same HPLC elution time. [The major species present in the various peaks are indicated in Figs. 3–5 for the 0-, 4-, and 12-d autoxidation samples, respectively.] The core aldehydes were detected as $[M - H]^-$ ions. LC/ESI/MS conditions are given in the Materials and Methods section. The solvent system is described in Figure 1. For other abbreviations see Figure 1.

oxo-TAG can be clearly distinguished from that of the internal standard, the DNPH derivative of the 18:0/18:0/9:0 ALD, and the basis for numbering of the major peak clusters (see Fig. 2) also may be discerned. To be able to absorb at 358 nm, each molecule had to have at least one DNPH group. The DNPH derivatives of volatile short-chain ALD were lost in the solvent front.

Figure 2 shows the total negative ion current profiles recorded for the DNPH derivatives of the TAG core ALD at 0, 4, 10, and 18 d of autoxidation, as obtained by reversed-phase LC/ESI/MS. These profiles closely resemble the UV peak clusters seen in Figure 1 and provide the basis for quantification of the TAG ALD by LC/ESI/MS in Table 1. Unfortunately, we had added to the 18-d sample a partially decomposed internal standard, which affected the quantitative (see Table 1) but not the qualitative assessment of the sample.

Table 1 gives the estimates obtained at the various times of autoxidation for the PV and *p*-anisidine values by conventional methods, the core ALD as the hydrazones estimated by UV absorption and reversed-phase LC/ESI/MS, and for the total polar TAG as estimated by HPLC/ELSD. The PV is seen to increase throughout the incubation time, as does the anisidine value, which for the most part measures the volatile carbonyl content. The core ALD content estimated by the UV absorption of the hydrazones appears to remain at a rather constant low level until the 12th and 18th days of autoxidation, when it rapidly increases. The core ALD as estimated by LC/ESI/MS at the same incubation times gave nearly two times higher values, except for the 18-d sample (ratio of 0.79), to which decomposed internal standard had been added (see above). The estimates for total polar TAG by HPLC/ELSD increased in parallel with the PV. The commercial sunflowerseed oil showed surprisingly high PV, anisidine values, and core ALD values at the starting day. Some of the core ALD initially present in the oil were decomposed and replaced by more stable core ALD. The content of hydroperoxides and hydroperoxide core ALD combinations were estimated to account for about 5% of total acylglycerol area after 18 d of autoxidation as estimated by ELSD.

Identification of TAG core ALD. Figure 3 shows the total negative ion current profile (A) of the 0-d sample as obtained by reversed-phase LC/ESI/MS over the mass range 850 to 1150, along with the full spectrum averaged across the elution range of 5 to 25 min (B). The masses of the major DNPH derivatives of the core ALD range from 946 to 1006 *m/z* as indicated by the labeling of the major masses making up each peak. These ions represent mainly the 9:0 and 12:1 core ALD of the major sunflowerseed oil TAG, which were produced on storage at ambient and below ambient temperatures. The major peak with a calculated neutral mass of 958 is due to the internal standard, 18:0/18:0/9:0 ALD. The complete identification of the core ALD making up the individual HPLC peaks along with the supporting chromatographic and mass spectrometric evidence is given in Tables 2–6. As seen from Tables 3–6, all samples contained minor amounts of mixed-function oxo-TAG,

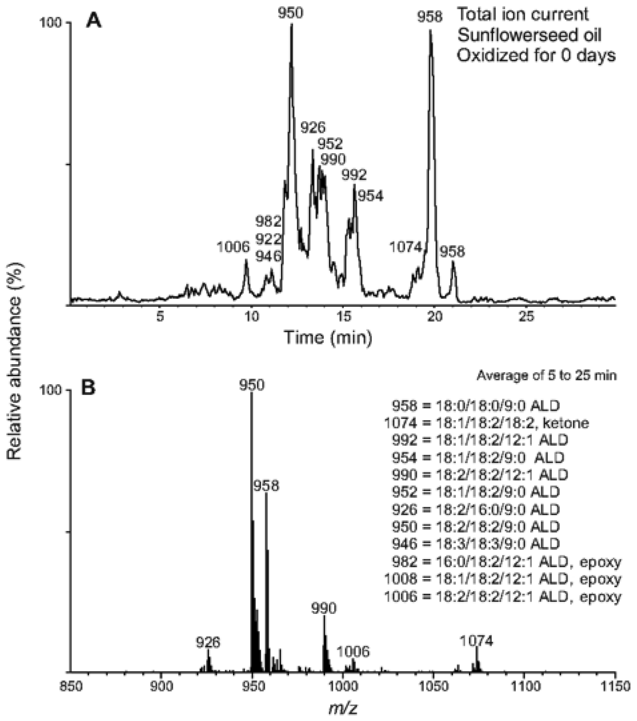

FIG. 3. The total negative ion current ESI/MS of DNPH-derivatized core ALD in 0-d oxidation sample of sunflowerseed oil (panel A), and the full mass spectrum (panel B) averaged over the core ALD elution range (5–25 min). The HPLC peaks are labeled as the major calculated neutral species, and the identities of the masses are given in the figure. The tentative identities of all species are given as nominal mass of [M + DNPH] in Tables 2–6. LC/ESI/MS conditions are given in the Materials and Methods section. The solvent system is described in Figure 1. For abbreviations see Figures 1 and 2.

where the core ALD groups were accompanied by epoxy, keto, hydroxy, or a second ALD group on an adjacent FA chain of the same TAG molecule.

Figure 4 shows the total negative ion current profile (A) of the sample autoxidized for 4 d at 60°C, along with the full mass spectrum (B) recorded in the 850 to 1150 *m/z* range averaged over the elution range from 5 to 25 min. The major masses making up the major HPLC peaks indicate that significant changes have taken place in the qualitative and quantitative composition of the sample as a result of the 4-d autoxidation at the higher temperature. Although 18:2/18:2/9:0 ALD remains the major species, other more saturated and more oxygenated species have now appeared, and some of the unsaturated ALD-containing species present in the starting sample have decreased. The quantitative proportions of the major identified core ALD are given in Tables 2–6.

Figure 5 shows the negative total ion current profile (A) of the sample autoxidized for 12 d at 60°C, along with the full mass spectrum (B) recorded in the 850 to 1150 *m/z* range over the entire elution period. The major masses making up the major HPLC peaks indicate that, compared to the 0- and 4-d samples, further changes have taken place in the qualitative and quantitative composition of the 12-d sample as a result of the autoxidation at 60°C. In relation to the internal standard, the total amount of core ALD has increased and the composition has become more saturated and more oxygenated. The quantitative proportions of the major species, given in Tables 2–6, provide detailed information on the core ALD species and their mixed-function derivatives along with supporting chromatographic and mass spectrometric evidence. The reference compounds with different structures are needed for more accurate quantification. These values can be considered as semiquantitative.

Similar results were obtained when the 2-, 6-, 8-, 10-, and 18-d samples were examined, as seen from the quantitative and qualitative data in Tables 2–6. This similarity indicates the reproducibility of the analytical methodology and the anticipated gradual change in the composition of the oil sample with progressive autoxidation.

Figure 6 shows the resolution of the regioisomers, when present, of selected mono-ALD. The *sn*-2-isomer emerges ahead of the *sn*-1(3)-isomer, whereas the mixed-function core ALD themselves are eluted in order of decreasing polarity, as observed earlier (13,15). Specifically, the 18:2/12:1 ALD/18:2 was identified as the *sn*-2-regioisomer on the basis of the relative retention time of the standard, although the natural regioisomer 12:1/18:2/18:2 was not found. Likewise, only the *sn*-2-regioisomer was found for 18:1/13:2 ALD/18:2. In contrast, 18:2/13:2 ALD/18:2 and 16:0/13:2 ALD/16:0 were found to be represented by both regioisomers in nearly equal proportions. The relative proportions of the regioisomers presumably reflect the regioisomer distribution of the unsaturated FA in the parent TAG.

Figure 7 illustrates the resolution of the TAG core ALD carrying additional epoxy and hydroxy groups. The hydroxy groups represent the hydroperoxy groups, which were reduced by triphenylphosphine to prevent continued core ALD production due to hydroperoxide breakdown during sample workup. As a result, there were no hydroperoxy groups left in our core ALD preparations.

Figure 8 shows the mass spectrum of the DNPH derivatives of the TAG possessing a KET group on one of the FA chains; these were eluted with retention times ranging from 12 to 25 min and included calculated masses for neutral species at 1050 (16:0/18:1/18:2 KET), 1072 (18:2/18:2/18:2 KET), 1074 (18:1/18:2/18:2 KET), 1076 (18:1/1:1/18:2 KET), and 1078 (18:1/18:1/18:1 KET). The elution factor for a KET group was estimated experimentally (from synthetic 18:0/18:1 KET/18:1) to be in the same range as for an aldehyde group (15). The values were −7 for an epoxy group and −4 for a DNPH-derivatized epoxy group (indicating shortened elution times equivalent to 7 and 4 carbons, respectively). The retention times for the proposed species were 20.77 (1050), 19.82 (1072), 19.47 (1074), 16.75 (1076), and 22.80 min (1078). Retention of these compounds fits well with the predicted retention times using the theoretical carbon number (TCN). Recording of negative ion masses over the range of 1040 to 1200 *m/z* excluded any core ALD as well as any mixed ALD and KET function derivatives, the DNPH derivatives of which would have been of a lower mass.

It was estimated that the major species of core ALD identified

TABLE 2
TAG Core Mono-ALD (8:0, 9:0, 12:1, and 13:2) in Autoxidized Sunflowerseed Oil Identified by RP-HPLC/ESI/MS[a]

No.	Molecular structure	ACN : DB	Mass[b]	Rt	TCN	Retention times							
						0 d	2 d	4 d	6 d	8 d	10 d	12 d	18 d
1	18:3/9:0 ALD/18:3	45 : 6	946	9.91	30.71	9.79	10.04	9.91	10.05	9.75	9.88	9.97	9.91
2	18:3/13:2 ALD/18:3	49 : 8	998	9.52	30.93								9.52
3	18:3/18:3/13:2 ALD	49 : 8	998	9.75	31.38	9.75							
4	18:2/18:3/13:2 ALD	49 : 7	1000	10.26	32.49								10.26
5	18:3/18:3/9:0 ALD	45 : 6	946	11.03	31.16	11.14	11.24	10.66	10.89	11.07	10.96	11.09	11.19
6	18:2/9:0 ALD/18:3	45 : 5	948	11.18	31.88	11.25	11.24	10.94	11.06	11.41	11.17	11.15	11.19
7	18:2/8:0 ALD/18:2	44 : 4	936	11.33	32.27	11.33							
8	18:2/18:2/8:0 ALD	44 : 4	936	11.58	32.72		11.60				11.56	11.63	11.51
9	18:2/18:3/9:0 ALD	45 : 5	948	11.69	32.33	11.78	11.53	11.58	11.54	11.54	11.60	11.90	12.05
10	18:2/9:0 ALD/18:2	45 : 4	950	11.89	33.27	11.87	12.05	11.90	11.80	11.83	11.98	11.78	11.90
11	18:2/18:2/9:0 ALD	45 : 4	950	12.19	33.72	12.24	12.29	12.16	12.17	12.20	12.14	12.20	12.11
12	18:3/12:1 ALD/18:3	48 : 7	986	12.26	31.20		12.47			12.10	12.21	12.24	12.30
13	16:0/8:0 ALD/18:2	42 : 2	912	12.58	34.11						12.58		
14	18:3/18:3/12:1 ALD	48 : 7	986	12.67	31.65		12.91						12.42
15	18:1/8:0 ALD/18:2	44 : 3	938	12.88	33.60	13.09	12.66						
16	16:0/13:2 ALD/18:2	47 : 4	976	13.25	35.27	13.24			13.20			13.26	13.29
17	18:2/12:1 ALD/18:3	48 : 6	988	13.29	32.37			13.29					
18	18:1/9:0 ALD/18:2	45 : 3	952	13.42	34.60	13.34	13.35	13.36	13.48	13.58	13.44	13.33	13.45
19	16:0/9:0 ALD/18:2	43 : 2	926	13.46	35.11	13.39	13.37	13.59	13.37	13.67		13.64	13.20
20	16:0/18:2/13:2 ALD	47 : 4	976	13.59	35.72			13.61					13.56
21	18:2/12:1 ALD/18:2	48 : 5	990	13.67	33.76	13.88	13.51		13.77	13.50			
22	18:1/18:2/9:0 ALD	45 : 3	952	13.70	35.05	13.73	13.72	13.68	13.64	13.79	13.66	13.66	13.69
23	18:1/9:0 ALD/18:1	45 : 2	954	13.71	36.15	13.73	13.80	13.74	13.73	13.66		13.59	
24	16:0/18:2/9:0 ALD	43 : 2	926	13.75	35.56	13.80	13.77	13.73	13.77	13.80	13.73	13.84	13.56
25	18:1/13:2 ALD/18:3	49 : 6	1002	13.83	33.37	13.73				13.86			13.90
26	18:1/18:2/8:0 ALD	44 : 3	938	13.91	35.11				14.09			13.79	13.86
27	16:0/12:1 ALD/18:2	46 : 3	966	13.93	35.60	13.82		13.78	14.12	13.94			13.98
28	18:2/13:2 ALD/18:2	49 : 6	1002	14.03	33.81	14.02	14.10	14.02	14.01	14.02	14.05	14.01	
29	18:2/18:2/12:1 ALD	48 : 5	990	14.08	34.21	14.07	14.18	14.09	14.09	14.04			14.01
30	18:0/9:0 ALD/18:3	45 : 3	952	14.17	35.66	14.22	14.22	14.17	14.17	14.10	14.10		14.21
31	18:1/18:3/13:2 ALD	49 : 6	1002	14.29	33.82	14.54	14.24	14.21	14.17				
32	18:0/8:0 ALD/18:2	44 : 2	940	14.42	36.11	14.48		14.35					
33	18:0/18:3/9:0 ALD	45 : 3	952	14.58	36.11	14.58	14.55	14.61	14.54	14.56			
34	18:1/18:1/9:0 ALD	45 : 2	954	14.62	36.60	14.68	14.69	14.54	14.54			14.67	
35	18:2/18:2/13:2 ALD	49 : 6	1002	14.70	34.26	14.70							
36	16:0/13:2 ALD/18:2	47 : 4	978	14.84	35.27	14.84							14.73
37	16:0/9:0 ALD/18:1	43 : 0	928	14.94	36.38	14.93		15.07		14.90	14.84		
38	16:0/12:1 ALD/18:1	46 : 1	968	15.19	37.15	15.20		15.12	15.14		15.32	15.13	15.23
39	16:0/18:1/9:0 ALD	43 : 1	928	15.27	36.83	15.26	15.12	15.34		15.26		15.35	
40	18:0/9:0 ALD/18:2	45 : 2	954	15.29	37.11	15.32	15.34	15.31	15.27	15.22	15.30	15.29	15.27
41	16:0/18:1/12:1 ALD	46 : 2	968	15.34	37.60	15.34							
42	18:1/13:2 ALD/18:2	49 : 5	1004	15.42	34.76		15.48	15.35					
43	18:1/13:2 ALD/18:1	49 : 4	1006	15.42	36.37	15.42							
44	18:1/12:1 ALD/18:2	48 : 4	992	15.46	35.37	15.66		15.44		15.28			
45	16:0/16:0/9:0 ALD	41 : 0	902	15.48	37.34	15.48							
46	16:0/18:2/12:1 ALD	46 : 3	966	15.58	36.05	15.68	15.39		15.53		15.70		
47	16:0/18:2/13:2 ALD	47 : 4	978	15.62	35.72		15.75			15.48			
48	18:1/18:2/13:2 ALD	49 : 5	1004	15.63	35.21	15.63	15.77	15.56				15.58	15.62
49	18:1/18:2/12:1 ALD	48 : 4	992	15.70	35.82	16.03	15.78	15.44	15.53				
50	18:0/18:2/9:0 ALD	45 : 2	954	15.78	37.56	15.90	15.78	15.84	15.81	15.78	15.76	15.70	15.70
51	18:1/12:1 ALD/18:1	48 : 3	994	15.80	37.34	15.80							
52	16:0/13:2 ALD/16:0	45 : 2	954	15.90	37.11	15.90							
53	16:0/16:0/12:1 ALD	44 : 1	942	16.17	37.83						16.05		16.29
54	16:0/13:2 ALD/18:1	47 : 3	980	16.48	36.60		16.48						
55	18:0/9:0 ALD/18:1	45 : 1	956	16.95	38.38	16.98	16.94	16.89				16.98	
56	16:0/12:1 ALD/18:0	46 : 1	970	17.30	39.38		17.16		17.51		17.24		
57	18:0/12:1 ALD/18:1	48 : 2	996	17.31	39.15						17.23		17.38
58	18:1/18:1/12:1 ALD	48 : 3	994	17.39	37.79	17.19			17.76				17.23
59	18:0/18:1/9:0 ALD	45 : 1	956	17.44	38.83	17.56	17.53	17.39			17.40	17.34	
60	18:0/18:1/12:1 ALD	48 : 2	996	17.53	39.60						17.53		
61	18:0/12:1 ALD/18:2	48 : 3	994	17.61	37.60	17.61							
62	16:0/18:0/9:0 ALD	43 : 0	930	18.38	39.34			18.23	18.52				
63	18:0/18:0/8:0 ALD	44 : 0	944	19.13	40.34	19.23		19.10	19.07				
64	22:0/18:2/8:0 ALD	48 : 2	996	19.45	40.56	19.45							
65	18:0/18:0/9:0 ALD	45 : 0	958	19.83	41.34	19.89	19.97	19.92	19.81	19.70	19.78	19.76	19.79
66	18:0/13:2 ALD/18:0	49 : 2	1010	20.25	41.11		20.25						
67	22:0/18:2/9:0 ALD	49 : 2	1010	20.35	41.56		20.25	20.45					
68	22:0/12:1 ALD/18:2	52 : 3	1050	20.88	41.54	21.05	20.77	20.80	20.93	20.61	21.11		
69	22:0/18:2/12:1 ALD	52 : 3	1050	22.01	42.05								22.01

[a]Abbreviations: ACN, acyl carbon number; DB, double bond; Rt, retention time; TCN, theoretical carbon number; DPNH, 2,4-dinitrophenylhydrazone; RP, reversed-phase; for other abbreviations see Table 1.
[b]Nominal mass of [M + DNPH].

TABLE 3
TAG Epoxy Core ALD (12:1 and 13:2) in Autoxidized Sunflowerseed Oil Identified by RP-HPLC/ESI/MS[a]

No.	Molecular structure	ACN : DB	Mass[b]	Rt	TCN	Retention times							
						0 d	2 d	4 d	6 d	8 d	10 d	12 d	18 d
1	18:3/13:2 ALD-O-/18:3	49 : 8	1014	6.42	26.31						6.36		6.47
2	18:2/18:3/13:2 ALD-O-	49 : 7	1016	6.41	27.94								6.41
3	18:3/12:1 ALD-O-/18:3	48 : 7	1002	7.42	26.58	7.42	7.37	7.43				7.49	7.37
4	18:2/12:1 ALD-O-/18:3	48 : 6	1004	7.81	27.75	7.94	7.85	7.93		7.90			7.44
5	18:1/13:2 ALD-O-/18:3	49 : 6	1018	8.17	28.75			8.29					8.05
6	18:2/18:3/12:1 ALD-O-	48 : 6	1004	8.24	28.27	8.26	8.24	8.24		8.13			8.32
7	18:2/12:1 ALD-O-/18:2	48 : 5	1006	8.25	29.14	8.32	8.21	8.22					
8	18:2/13:2 ALD-O-/18:2	49 : 6	1018	8.50	29.19					8.50			
9	18:1/18:2/13:2 ALD-O-	49 : 5	1020	8.82	30.66		8.62						9.01
10	16:0/13:2 ALD-O-/18:2	47 : 4	992	9.32	30.65						9.25	9.31	9.39
11	18:2/18:2/12:1 ALD-O-	48 : 5	1006	9.71	29.66	9.74	9.77	9.74	9.70	9.69	9.65	9.67	9.71
12	18:1/18:3/13:2 ALD-O-	49 : 6	1018	9.73	29.27	9.60			9.86		9.74		
13	16:0/18:2/13:2 ALD-O-	47 : 4	992	9.93	31.17					9.83	9.94	9.98	9.97
14	16:0/13:2 ALD-O-/18:1	47 : 3	996	10.43	31.98			10.06				10.80	
15	18:1/13:2 ALD-O-/18:2	49 : 5	1020	10.62	30.14							10.62	
16	16:0/13:2 ALD-O-/18:2	47 : 4	994	10.66	30.65				10.54	10.83		10.88	10.39
17	18:1/12:1 ALD-O-/18:2	48 : 4	1008	10.67	30.75	10.66		10.67	10.55	10.76	10.85	10.54	10.66
18	16:0/12:1 ALD-O-/18:2	46 : 3	982	10.81	30.98	10.88	10.81	10.94	10.85		10.84	10.76	10.59
19	18:1/12:1 ALD-O-/18:1	48 : 3	1010	10.96	32.72	10.98					10.98	10.98	10.90
20	18:1/18:2/12:1 ALD-O-	48 : 4	1008	11.03	31.27	11.05	10.98	10.84	11.06	11.08	11.12	11.12	10.95
21	18:1/18:1/12:1 ALD-O-	48 : 3	1010	11.20	33.24		11.21						11.19
22	16:0/18:2/13:2 ALD-O-	47 : 4	994	11.20	31.17							11.20	11.19
23	16:0/12:1 ALD-O-/18:1	46 : 1	984	11.30	32.53	11.49			11.32	11.26	11.18	11.09	11.44
24	16:0/18:2/12:1 ALD-O-	46 : 3	982	11.38	31.50	11.42	11.26		11.32		11.36	11.60	11.33
25	16:0/13:2 ALD-O-/16:0	45 : 2	970	11.48	32.49								11.48
26	16:0/18:1/12:1 ALD-O-	46 : 2	984	12.59	33.05	12.84		12.47	12.54		12.69	12.57	12.45
27	18:0/12:1 ALD-O-/18:2	48 : 3	1010	12.03	32.98				12.00	12.06			
28	18:0/18:2/12:1 ALD-O-	48 : 3	1010	12.56	33.50	12.72		12.63	12.70	12.70		12.40	12.20
29	18:0/12:1 ALD-O-/18:1	48 : 2	1012	11.84	34.53		11.86				11.81		
30	18:0/18:1/12:1 ALD-O-	48 : 2	1012	12.33	35.05	12.17	12.50			12.29		12.36	
31	16:0/12:1 ALD-O-/18:0	46 : 1	986	12.26	34.76		12.47			12.10	12.21	12.24	12.30
32	18:1/13:2 ALD-O-/18:1	49 : 4	1022	12.52	31.75						12.52		
33	16:0/18:0/12:1 ALD-O-	16 : 1	986	12.67	35.28		12.91						12.42
34	18:0/12:1 ALD-O-/18:0	48 : 1	1014	13.32	36.76								13.32
35	22:0/12:1 ALD-O-/18:2	52 : 3	1066	13.93	36.98		14.18	13.63					13.99
36	18:0/13:2 ALD-O-/18:0	49 : 2	1026	13.93	36.49			13.93					
37	18:0/18:0/12:1 ALD-O-	48 : 1	1014	13.97	37.28								13.97
38	22:0/18:2/12:1 ALD-O-	52 : 3	1066	15.63	37.50					15.70			15.56

[a]Abbreviations: -O-, epoxy; for other abbreviations see Tables 1 and 2.
[b]Nominal mass of [M + DNPH].

as 9-oxononanoyl (70%)-, 12-oxo-9,10-dodecenoyl (10%)-, and 13-oxo-9,11-tridecenoyl (5%)-containing acylglycerols contributed a maximum of 70, 10, and 5%, respectively, of the total DNPH derivatives, with smaller amounts of simple and mixed-chain-length di-ALD, and keto and epoxy mono-ALD-containing acylglycerols contributing a total of 15% of the DNPH-reactive material.

Validation of quantification. Figure 9 shows that a nearly linear relationship ($y = 75455x + 5 \times 10^6$, $R^2 = 0.9457$) between the injected amount of standard core ALD (18:0/18:0/9:0 ALD DNPH) and the LC/ESI/MS total ion current response existed over a relatively limited range (0–1000 ng). At higher injection levels, the response leveled off or fell (1000–3000 ng). Since the amounts of the core ALD injected into the LC/ESI/MS system were below 1000 ng, the peak area proportions and ion intensities in Figures 3–5 provide a quantitative indication of the relative proportions of the oxidation products at 0, 4, and 12 d of autoxidation. The total amounts of the core aldehydes isolated at these times are given in Table 1.

Figure 10 shows that a linear relationship ($y = 2073.8x + 11{,}974$, $R^2 = 1$) was obtained between the amount of injected standard and the UV detector response (358 nm) over a much wider range of concentrations (0 to 5000 ng) than that obtained with LCI/ESI/MS.

DISCUSSION

Sunflowerseed oil contains linoleic and oleic acids as the major unsaturated species; these are interesterified with palmitic and stearic acids to yield mixed acid TAG (13) as the major source of TAG core ALD following oxidation with *tert*-butyl hydroperoxide (TBHP). The presence of TAG core ALD in autoxidized sunflowerseed oil was first noted during the TLC removal of triphenylphosphine following prepara-

TABLE 4
TAG Hydroxy Core ALD (9:0, 12:1, and 13:2) in Autoxidized Sunflowerseed Oil Identified by RP-HPLC/ESI/MS[a]

No.	Molecular structure	ACN : DB	Mass[b]	Rt	TCN	Retention times 0 d	2 d	4 d	6 d	8 d	10 d	12 d	18 d
1	18:1/13:2 ALD-OH/18:3	49 : 6	1018	6.16	25.01								6.16
2	18:3 OH/18:3/9:0 ALD	45 : 6	962	6.25	22.99								6.25
3	18:3/13:2 ALD-OH/18:3	49 : 8	1014	6.41	22.57						6.36		6.45
4	18:2/18:3/13:2 ALD-OH	49 : 7	1016	6.41	24.32								6.41
5	18:2/13:2 ALD-OH/18:2	49 : 6	1018	6.46	25.45								6.46
6	18:2/9:0 ALD/18:3 OH	45 : 5	964	6.49	23.71								6.49
7	18:2/18:3 OH/9:0 ALD	45 : 5	964	6.81	23.97								6.81
8	18:1/9:0 ALD/18:2 OH	45 : 3	968	7.12	26.43			7.19				7.10	7.07
9	18:2/9:0 ALD/18:2 OH	45 : 4	966	7.13	25.10								7.13
10	18:2 OH/18:3/9:0 ALD	45 : 5	964	7.19	24.16								7.19
11	18:1 OH/18:2/9:0 ALD	45 : 3	968	7.56	27.65								7.56
12	16:0/13:2 ALD-OH/16:0	45 : 2	970	7.84	28.75			8.05				7.55	7.91
13	16:0/13:2 ALD-OH/18:2	47 : 4	992	7.91	26.91							7.91	
14	18:1/13:2 ALD-OH/18:2	49 : 5	1020	7.91	26.40					8.23		7.48	8.01
15	16:0/13:2 ALD-OH/18:2	47 : 4	994	8.59	26.91								8.59
16	18:1/18:2/13:2 ALD-OH	49 : 5	1020	8.78	27.04		8.62					8.71	9.01
17	18:1/13:2 ALD-OH/18:1	49 : 4	1022	6.59	28.01	6.64	6.60	6.57				6.54	
18	18:1/18:2 OH/9:0 ALD	45 : 3	968	8.01	27.75		8.03	7.97			7.93		8.09
19	16:0/9:0 ALD/18:2 OH	43 : 2	942	8.02	26.94		8.05	7.99					
20	18:1/18:1 OH/9:0 ALD	45 : 2	970	8.05	27.89			8.05					
21	16:0/18:2 OH/9:0 ALD	43 : 2	942	8.27	27.20								8.27
22	16:0/18:2/13:2 ALD-OH	47 : 4	994	8.88	27.55								8.88
23	16:0/18:1 OH/9:0 ALD	43 : 1	944	8.54	28.12					8.51		8.56	
24	16:0/16:0/13:2 ALD-OH	45 : 2	970	8.60	29.39								8.60
25	16:0/13:2 ALD-OH/18:1	47 : 3	996	9.28	28.24						9.28		
26	18:0/18:2 OH/9:0 ALD	45 : 2	970	9.39	29.20		9.39						
27	16:0/12:1 ALD-OH/18:2	46 : 3	982	9.73	26.89		9.75			9.69	9.72	9.85	9.64
28	18:1/18:2/12:1 ALD-OH	48 : 4	1008	9.58	27.36	9.61				9.46			9.66
29	16:0/12:1 ALD-OH/18:1	46 : 2	984	9.89	28.44				9.81	9.80		9.98	9.97
30	16:0/18:2/12:1 ALD-OH	46 : 3	982	10.09	27.59	10.59			10.04	9.93	9.98		9.92
31	16:0/12:1 ALD-OH/18:0	46 : 1	986	10.57	30.67							10.95	10.19
32	18:0/12:1 ALD-OH/18:0	48 : 1	1014	10.76	32.67				10.76				
33	18:0/12:1 ALD-OH/18:1	48 : 2	1012	10.78	30.44						10.93		10.62
34	16:0/18:1/12:1 ALD-OH	46 : 2	984	10.84	29.14		10.77		10.84	10.85	10.85		10.90
35	16:0/16:0/12:1 ALD-OH	44 : 1	958	10.89	29.37						10.89		
36	16:0/18:0/12:1 ALD-OH	16 : 1	986	11.10	31.37							11.18	11.02
37	18:0/13:2 ALD-OH/18:0	49 : 2	1026	11.76	32.75						11.87	11.56	11.86
38	22:0/18:2 OH/9:0 ALD	49 : 2	1026	11.86	33.20								11.86
39	22:0/12:1 ALD-OH/18:2	52 : 3	1066	11.91	32.89					11.93			11.88
40	18:0/18:0/13:2 ALD-OH	49 : 2	1026	12.21	33.39						12.21		
41	18:0/18:0/12:1 ALD-OH	48 : 1	1014	12.22	33.37	12.22							
42	22:0/18:2/12:1 ALD-OH	52 : 3	1066	12.33	33.59					12.34		12.31	

[a]Abbreviations: OH, hydroxide; for other abbreviations see Tables 1 and 2.
[b]Nominal mass of [M + DNPH].

tion of DNPH derivatives. The DNPH derivatives appeared as dark bands resolved on the basis of their overall polarity as reported earlier for the TAG core ALD generated by oxidation with TBHP (13). The various core ALD making up the TLC bands from TBHP-treated oils had been identified previously by TLC/LC/ESI/MS, which was a laborious although a necessary task (13). In view of the detailed previous characterization of the core ALD resulting from chemical oxidation, core ALD resulting from autoxidation were identified by direct reversed-phase LC/ESI/MS examination of the autoxidized oil following preparation of the DNPH derivatives. By working in the negative ion mode, only those TAG molecules that had been converted to the DNPH derivatives because of the presence of a carbonyl function in the molecule were detected. Further simplification of peak identification was obtained by recording mass spectra over selected mass ranges.

The mechanism of autoxidation of the linoleic and oleic acid esters of glycerol leading to hydroperoxides was assumed to parallel that of autoxidation of the corresponding methyl esters, which has been reviewed in great detail by Porter *et al.* (2) and Frankel (22). Degradation of the hydroperoxides of TAG would be expected to lead to formation of the volatile ALD as described for the unsaturated FAME and to TAG core ALD (4). On the basis of previous discussion (13), it would be anticipated that linoleoyl TAG would yield 9-oxononanoyl and the 13-oxo-

TABLE 5
TAG Core Di-ALD (12:1 and 13:2) in Autoxidized Sunflowerseed Oil Identified by RP-HPLC/ESI/MS[a]

						Retention times							
No.	Molecular structure	ACN : DB	Mass[b]	Rt	TCN	0 d	2 d	4 d	6 d	8 d	10 d	12 d	18 d
1	18:3/9:0 ALD/9:0 ALD	36 : 3	1020	6.34	24.33		6.37						6.31
2	16:1/9:0 ALD/9:0 ALD	34 : 1	996	6.37	25.05								6.37
3	18:2/9:0 ALD/9:0 ALD	36 : 2	1022	6.54	25.78		6.50	6.57					6.54
4	18:3/13:2 ALD/13:2 ALD	44 : 7	1124	6.92	24.71								6.92
5	18:1/9:0 ALD/9:0 ALD	36 : 1	1024	7.35	27.05		7.35						
6	16:0/9:0 ALD/9:0 ALD	34 : 0	998	7.19	27.56		7.19						
7	18:2/13:2 ALD/13:2 ALD	44 : 6	1126	7.45	26.48		7.45						
8	18:2/12:1 ALD/12:1 ALD	42 : 4	1102	7.70	27.04					7.48			7.92
9	18:0/9:0 ALD/9:0 ALD	36 : 0	1026	7.85	29.56		7.85						
10	18:1/13:2 ALD/13:2 ALD	44 : 5	1128	8.85	27.43							8.85	
11	16:0/13:2 ALD/13:2 ALD	42 : 4	1102	8.75	27.94	8.56					8.93		
12	18:1/12:1 ALD/12:1 ALD	42 : 3	1104	9.67	29.01		9.67		9.68		9.67		
13	16:0/12:1 ALD/12:1 ALD	40 : 2	1078	9.80	28.82		10.40					9.04	9.95
14	20:0/9:0 ALD/9:0 ALD	38 : 0	1054	10.18	31.56						10.18		
15	18:0/12:1 ALD/12:1 ALD	42 : 2	1106	10.17	30.82		9.43		9.98	10.08	10.45	10.73	10.36
16	20:0/13:2 ALD/13:2 ALD	46 : 4	1158	10.34	31.94			10.34					
17	20:0/12:1 ALD/12:1 ALD	44 : 2	1134	10.74	32.82					10.22	11.25		10.76
18	22:0/12:1 ALD/12:1 ALD	46 : 2	1162	11.78	34.82		11.78						
19	22:0/9:0 ALD/9:0 ALD	40 : 0	1082	12.43	33.56								12.43
20	22:0/13:2 ALD/13:2 ALD	48 : 4	1186	12.43	33.94		12.30	12.34	12.64				

[a]For abbreviations see Tables 1 and 2.

9,11-tridecadienoyl TAG as major components, whereas the oleoyl TAG would yield 8-oxooctanoyl TAG as major components. Further autoxidation of TAG containing 13:2 ALD would be expected to yield TAG containing 12:1 ALD. The present study resulted in the isolation and quantification of all these core ALD in combination with both saturated and unsaturated FA in the acylglycerol molecule, as well as with their epoxy and hydroxy derivatives. The latter arose from the chemical reduction of the hydroperoxides with triphenylphosphine. Furthermore, the 13:2 ALD derivatives, which apparently were completely destroyed during the TBHP oxidation, were readily recovered under the milder autoxidation conditions, in accordance with the theoretical predictions (23).

However, the major reason for the successful isolation and identification of the core ALD from the autoxidation samples was the preparation of the DNPH derivatives. To avoid an overestimation of the core ALD owing to possible excessive destruction of their hydroperoxide precursors during isolation, the hydroperoxides were reduced to alcohols, which prevented generation of ALD during the sample workup. The resulting hydroxyl groups did not interfere with subsequent conversion of the ALD and KET into the DNPH derivatives. Furthermore, the TAG carrying the hydroxyl functions improved the chromatographic properties of the oxoacylglycerols over those of the hydroperoxyglycerols (better resolution, characteristic mass shift).

The major core ALD were shown to be the mono-ALD 18:2/18:2/9:0 ALD and 18:2/18:1/9:0 ALD, which reflected the high proportion of the 18:2/18:2/18:2 and 18:2/18:2/18:1 acylglycerols in the sunflowerseed oil. The 12:1 ALD had been

TABLE 6
TAG Diepoxy and Dihydroxy Core Di-ALD (12:1 and 13:2) in Autoxidized Sunflowerseed Oil Identified by RP-HPLC/ESI/MS[a]

						Retention times							
No.	Molecular structure	ACN DB	Mass[b]	Rt	TCN	0 d	2 d	4 d	6 d	8 d	10 d	12 d	18 d
1	18:1/13:2 ALD-OH/13:2 ALD-OH	44 : 5	1160	3.66	13.01							3.66	
2	18:0/13:2 ALD-OH/13:2 ALD-OH	44 : 4	1162	3.99	15.52				3.99				
3	18:3/13:2 ALD-O-/13:2 ALD-O-	44 : 7	1156	4.70	17.51				4.70				4.69
4	18:1/12:1 ALD-O-/12:1 ALD-O-	42 : 3	1136	5.34	20.28								5.34
5	18:0/12:1 ALD-O-/12:1 ALD-O-	42 : 2	1138	6.03	22.09					6.03			
6	18:2/13:2 ALD-O-/13:2 ALD-O-	44 : 6	1158	6.16	19.28						6.16		6.16
7	18:1/13:2 ALD-O-/13:2 ALD-O-	44 : 5	1160	5.82	20.23								5.82
8	16:0/13:2 ALD-O-/13:2 ALD-O-	42 : 4	1134	6.36	20.74								6.36
9	18:0/13:2 ALD-O-/13:2 ALD-O-	44 : 4	1162	6.89	22.74				6.89				
10	20:0/13:2 ALD-O-/13:2 ALD-O-	46 : 4	1190	6.46	24.74							6.46	
11	22:0/12:1 ALD-O-/12:1 ALD-O-	46 : 2	1194	8.20	26.09							8.20	
12	22:0/13:2 ALD-O-/13:2 ALD-O-	48 : 4	1218	8.96	26.74	8.94					8.98		

[a]For abbreviations see Tables 1–4.

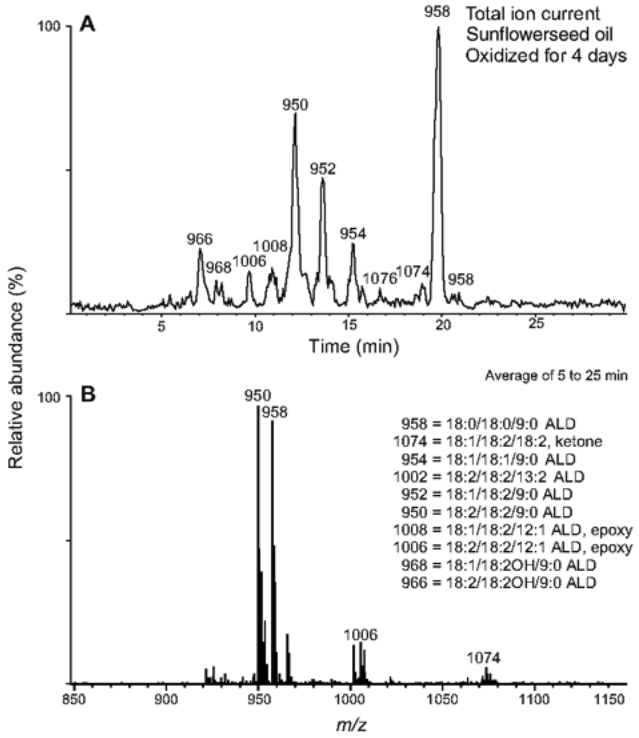

FIG. 4. The total negative ion current ESI/MS of DNPH-derivatized core ALD in 4-d oxidation sample of sunflowerseed oil (panel A), and the full mass spectrum (panel B) averaged over the core ALD elution range (5–25 min). The HPLC peaks are labeled as the major calculated neutral species and the identities of the masses are given in the figure. The tentative identities of all species are given as nominal mass of [M + DNPH] in Tables 2–6. LC/ESI/MS conditions are given in the Materials and Methods section. The solvent system is given in Figure 1. For abbreviations see Figures 1 and 2.

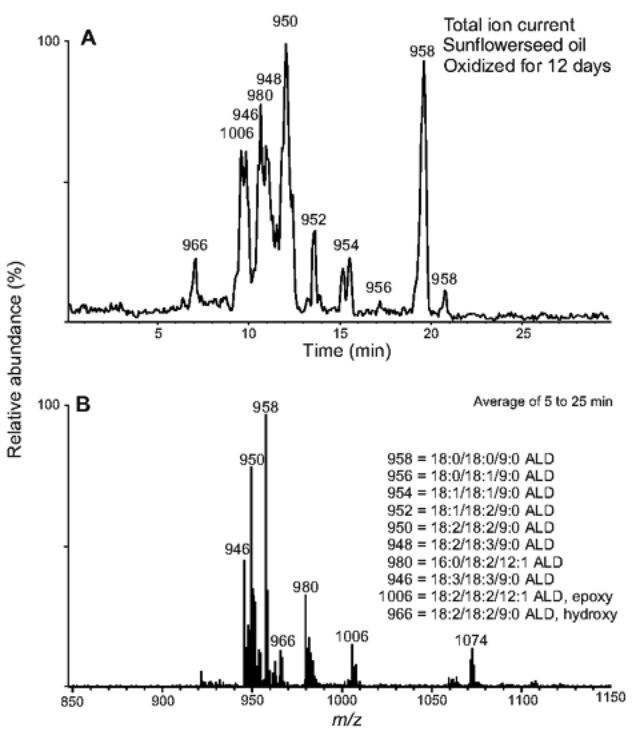

FIG. 5. The total negative ion current ESI/MS of DNPH-derivatized core ALD in 12-d oxidation sample of sunflowerseed oil (panel A), and the full mass spectrum (panel B) averaged over the core ALD elution range (5–25 min). The HPLC peaks are labeled as the major calculated neutral species, and the identities of the masses are given in the figure. The tentative identities of all species are given as nominal mass of [M + DNPH] in Tables 2–6. LC/ESI/MS conditions are given in the Materials and Methods section. The solvent system is given in Figure 1. For abbreviations see Figures 1 and 2.

recognized previously as the epoxy derivative, whereas the 13:2 ALD-containing acylglycerols had not been previously isolated (13). In addition, the present study identified TAG molecules that contained two ALD groups per acylglycerol molecule. The simple di-ALD (di-9:0 ALD) and mixed di-ALD (9:0 ALD/13:2 ALD, 9:0 ALD/12:1 ALD) had not been previously isolated, identified, or postulated.

Byrdwell and Neff (11,24) detected both neutral and polar chain-shortened products upon decomposition of the oxo-acylglycerols produced during autoxidation, which they tentatively identified as core ALD. Specific TAG core ALD, however, were not identified. Earlier, Byrdwell and Neff (25) had demonstrated dimerization of trioleoylglycerol along with chain-shortening of intact trioleoylglycerol. The present study provided no evidence for dimerization of TAG or for internal (within the molecule) aldol condensation of the di-ALD, but external (between two core ALD-containing molecules) aldol condensation could not be excluded, because the mass scans were cut off at 1200 *m/z*, which was below the molecular masses anticipated for dimers so produced. TAG core ALD could have undergone aldol condensation with the volatile ALD released from the methyl terminal end of the unsaturated FA. To be detected in our analysis system, the aldol condensation products would have had to possess a

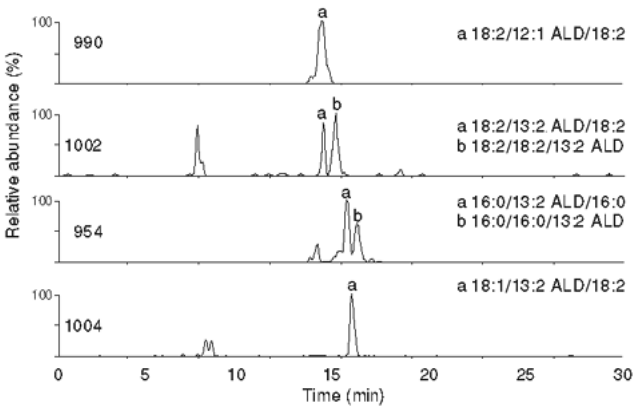

FIG. 6. Single-ion mass chromatograms of DNPH derivatives of selected mono-ALD representing *sn*-2- and *sn*-1(3)-isomers in sunflowerseed oil autoxidized for 18 d. The core ALD species are identified by their calculated neutral masses as given in the figure. The species are listed by the nominal mass of their [M + DNPH] derivatives in Tables 3 and 4. LC/ESI/MS conditions are described in the Materials and Methods section. The solvent system is given in Figure 1. For abbreviations see Figures 1 and 2.

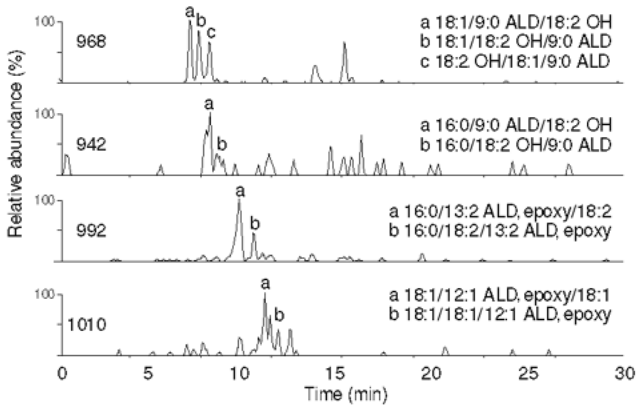

FIG. 7. The single-ion mass chromatograms of DNPH derivatives of major TAG core ALD carrying additional hydroxyl or epoxy groups in sunflowerseed oil autoxidized for 18 d. The core ALD species are identified by their calculated neutral masses as given in the figure. The species are listed by the nominal mass of their [M + DNPH] derivatives in Tables 3 and 4. LC/ESI/MS conditions are as described in the Materials and Methods section. The solvent system is given in Figure 1. For abbreviations see Figures 1 and 2.

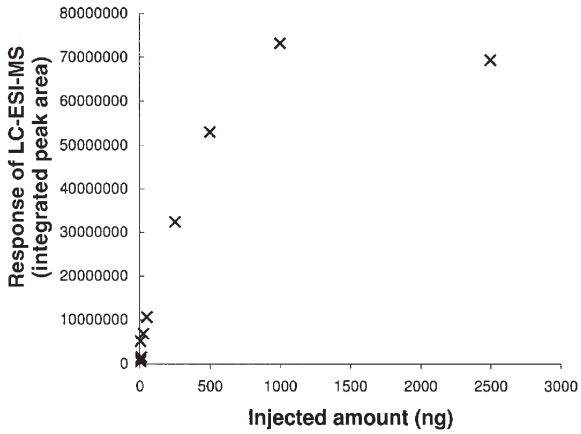

FIG. 9. Response of extracted ion current to injected amount of internal standard (18:0/18:0/9:0 ALD DNPH) as obtained by LC/ESI/MS. Detection was carried out in negative mode as $[M - H]^-$ ions. LC/ESI/MS conditions are described in the Materials and Methods section. The solvent system is given in Figure 1. For abbreviations see Figures 1 and 2.

DNPH group associated with one of the remaining fatty chains. The formation of the aldol condensation products was anticipated in view of the demonstration that the 5:0 ALD-containing glycerophosphocholines readily undergo external aldol condensation (26). DNPH derivatization could possibly reverse the aldol condensation just as it reverses the Schiff base formation (27). Core ALD also can convert easily to acids as reported recently (12).

The present study shows that preparation of the DNPH derivatives of the TAG core ALD was an effective way of isolating and identifying them following autoxidation of the sunflowerseed oil. In addition to excellent chromatographic properties, the DNPH derivatives had the advantage of UV absorption and gave excellent ESI in the negative mode. However, the DNPH derivatives eventually became hydrolyzed to free ALD on storage. It was observed that a purified standard 18:0/18:0/9:0 ALD DNPH derivative upon storage in dilute chloroform for 6 mon contained significant amounts of free ALD. The free ALD could be converted back into the DNPH derivative by reaction with fresh dinitrophenyldrazine.

The finding of TAG core mono-ALD in fresh sunflowerseed oil of commercial origin after several months of storage at 4°C in the dark was consistent with the slightly elevated PV and *p*-anisidine value, in agreement with results reported earlier (12,28).

Contrary to common belief, synthetic core ALD of high M.W. are chemically active and readily form Schiff bases with

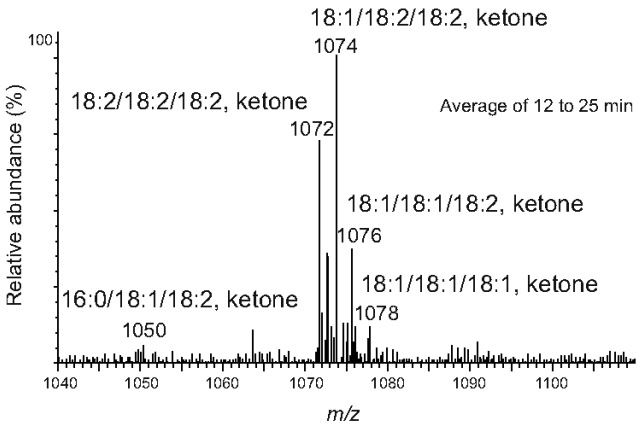

FIG. 8. The full mass spectrum (12–25 min) of the DNPH-derivatized TAG ketones in sunflowerseed oil autoxidized for 10 d at 60ºC. Peaks are identified in the figure (calculated masses of neutral species). LC/ESI/MS conditions are given in the Materials and Methods section. The solvent system is given in Figure 1. For abbreviations see Figures 1 and 2.

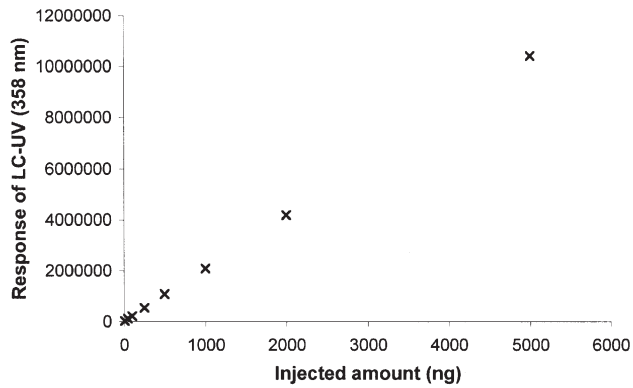

FIG. 10. UV absorption (358 nm) response to the injected amount of internal standard (18:0/18:0/9:0 ALD DNPH). HPLC conditions are described in the Materials and Methods section. The solvent system is given in Figure 1. For abbreviations see Figure 1.

aminophospholipids, nitrogenous bases, amino acids, and peptides (29,30). Hence, the core ALD may indeed attack cell membranes and plasma lipoproteins, potentially leading to degenerative disease, as suggested earlier (31). A determination of the structural motifs of oxidized PC responsible for antigenicity for EO6 (a monoclonal immunoglobulin M antibody that recognizes and binds to epitopes of oxidized LDL) showed that oxidized phospholipids containing *sn*-1 long-chain FA were not antigenic unless the *sn*-2-oxidized FA contained an ALD that first reacted with a peptide yielding a Schiff base or the *sn*-2-oxidized FA underwent an aldol-type self-condensation (26). In other studies, dietary trilinoleoylglycerol hydroperoxides (32) and dietary hydroperoxides of linoleic acid (33) were shown to decompose in the gastrointestinal tract to epoxy-KET and ALD, including 9-oxo-nonanoic acid as one of the products. The determination of the exact structure of the core ALD as well as a determination of their fate during fat absorption therefore constitutes a valid basis for further investigation.

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Effect of Ultrasonication and Grinding on the Determination of Lipid Class Content of Microalgae Harvested on Filters

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ABSTRACT: To ensure complete lipid extraction of algal samples collected on glass fiber filters, one must facilitate the access of extracting solvent to the lipids by using ultrasonication, grinding, or a combination of these two methods. This study examines the effect of these three treatments, in combination with storage time and sampling volumes, on the determination of lipid class composition of the diatom *Chaetoceros gracilis*. The TAG level was significantly influenced by treatment in relation to either storage time or sampling volume. FFA and other degradation products increased markedly with storage time at the expense of TAG and phospholipids (PL). Finally, sampling volume and storage time interacted in their effects on TAG and PL contents in an inverse manner.

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During the past decade, interest in the lipid content of marine microalgae has grown. In fact, lipids from marine microalgae have potential commercial applications in the human dietary supplement market and in fish and shellfish aquaculture. Additionally, they are pivotal to the understanding of energy and chemical fluxes in marine ecosystems. It is therefore important to determine lipid content and composition of microalgae accurately. However, sample handling for the determination of lipids in plankton is a major concern for several reasons.

Although no standard technique exists, ultrasonication appears to be one of the most common methods for disrupting cell membranes and ensuring free access of extracting solvents to the lipids. It also disrupts the link between cells and filter prior to lipid extraction (1,2). The extraction of organics from solid samples by using ultrasonication has been extensively investigated in the past (3–7) and can yield higher levels of pigments and FA than passive extraction in solvent (3). However, other published extraction methods, including grinding (8) and ultrasonication in combination with grinding (9), are also widely used. To the best of our knowledge, the efficiency of lipid class recovery from microalgae following ultrasonication and/or grinding has never been studied. In this context, "grinding" refers to homogenization of sample and filter with a tissue grinder.

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Abbreviations: AMPL, acetone-mobile polar lipids; PL, phospholipids; ST, cholesterol; TL, total lipids.

Extraction of lipids from large numbers of samples during the course of an experiment is usually impractical. Therefore, samples are stored for several days, weeks, or months at varying temperatures between −20 and −8°C (10,11), either in organic solvent, a nitrogen atmosphere, or both (1). The lipid classes and FA compositions of marine organisms are highly sensitive to hydrolysis and oxidation processes during storage (10,11); therefore, the decline of certain lipid classes and the formation of artifacts during storage is a constant issue in lipid studies.

Although the solvent-to-sample ratio necessary to yield maximal lipid recovery is well established (12,13), the interactive effects of sample volume, storage, and treatment on lipid class composition has never been investigated. In this study we address the effect of ultrasonication, grinding, and the combination of both treatments in addition to sample volume and storage time on the determination of lipid class composition of *Chaetoceros gracilis*.

MATERIALS AND METHODS

The diatom *C. gracilis* (clone CHGRA) was harvested during the exponential growth phase from batch cultures of 170 L (*ca.* 3×10^6 cells mL^{-1}). Samples of 5, 10, and 15 mL were filtered onto pre-ashed (450°C) GF/C filters (2.5 cm; VWR, Mont Royal, Canada) and stored in a precleaned glass vial in 1 mL of dichloromethane under a nitrogen atmosphere at −20°C until lipid extraction at 0, 1, or 6 mon. In this study, the commonly used chloroform was replaced by the less toxic dichloromethane (14). Analyses were performed on five replicate samples of each treatment combination for a total of 135 samples.

Prior to lipid extraction, one of three treatments was applied to algae samples from each sampling volume and storage time (Fig. 1). In treatment 1, samples were ultrasonicated three times for 10 min in 1.5 mL dichloromethane/methanol (2:1, vol/vol) in a sonic bath at *ca.* 5°C with an average output of 50 W (Model HT50; VWR, Nepean, Canada). Between each interval, the dichloromethane/methanol solution in the vial was poured into a 15-mL centrifuge tube for lipid extraction. The three solutions from the same sample were pooled for a total volume of 4.5 mL. In treatment 2, samples were homogenized with a glass pestle in a 7-mL Tenbroeck tissue grinder containing 4.5 mL dichloromethane/methanol (2:1, vol/vol). The dichloromethane/methanol solution in the tissue grinder was poured into a 15-mL centrifuge tube for lipid

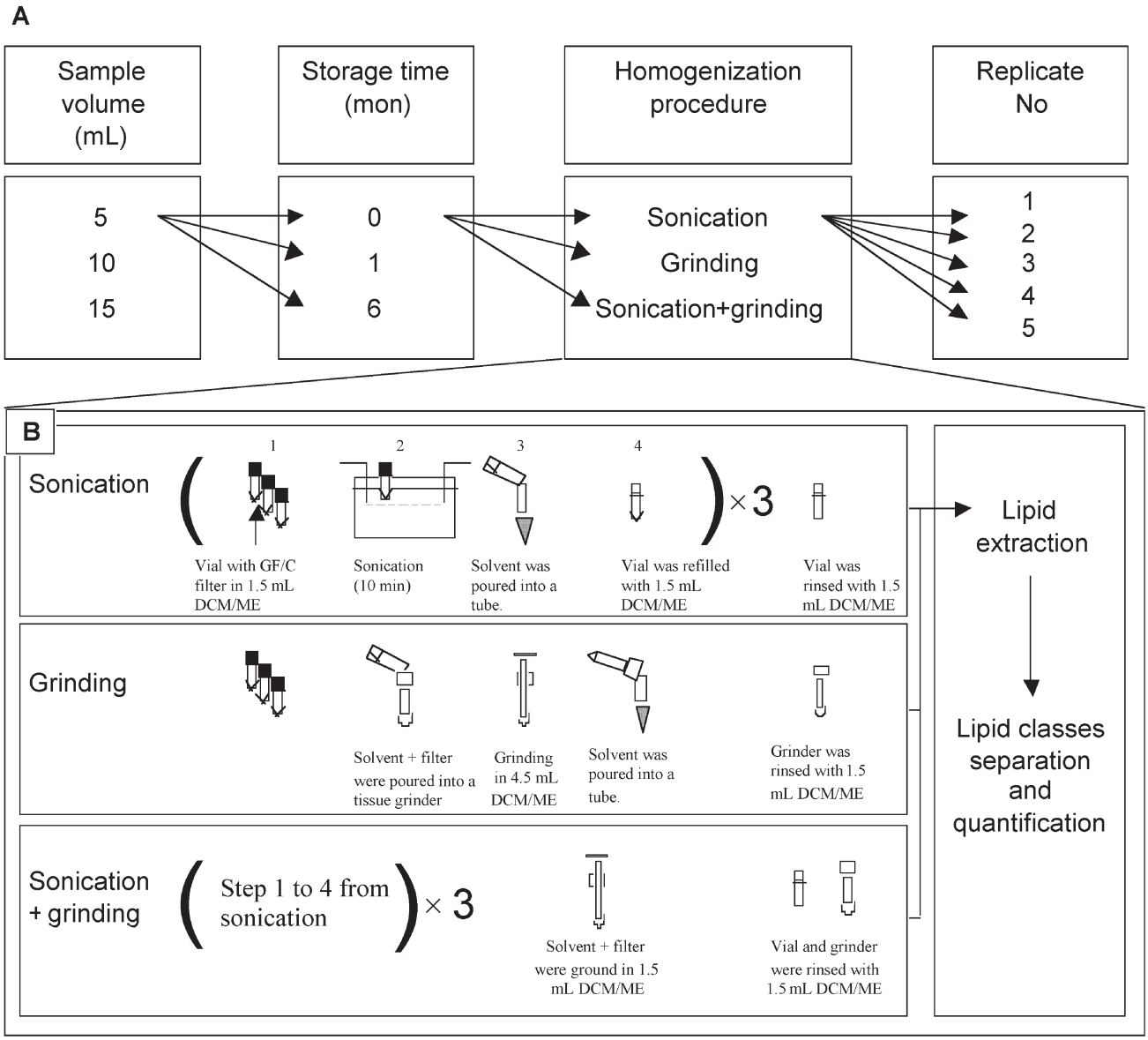

FIG. 1. Experimental design (A) and detail of the homogenization procedures (B). See the Materials and Methods section for details. DCM, dichloromethane; ME, methanol.

extraction. For treatment 3, samples were ultrasonicated three times as previously described. After the third sonication, the GF/C filter was homogenized in the 7-mL glass tissue grinder containing 1.5 mL dichloromethane/methanol (2:1; vol/vol). Glass vials and the tissue grinder were rinsed with 1.5 mL dichloromethane/methanol (2:1, vol/vol) to ensure complete lipid recovery.

KCl (0.88%) was added to the dichloromethane/methanol solutions to create a biphasic system of dichloromethane/methanol/KCl (2:1:0.75, by vol) (12). Homogenates were vortexed thoroughly for 30 s and centrifuged at 2000 rpm (800 × *g*) for 2 min. The lipid fraction was removed by double pipetting with Pasteur pipettes to penetrate the debris layer cleanly (8) and was then transferred into a clean tube. Two washes per sample were conducted. The solvent was evaporated under a flow of nitrogen, and the lipids were suspended in 0.1 mL CH_2Cl_2. All the manipulations were performed on ice and under nitrogen whenever possible. The lipid extract (4 µL) was spotted onto the S-III Chromarods (Iatron Laboratories Inc., Tokyo, Japan) using a Hamilton syringe. Four different solvents were used to separate aliphatic hydrocarbons, sterol esters and wax esters, ketones, TAG, FFA, cholesterol (ST), DAG, acetone-mobile polar lipids (AMPL), and phospholipids (PL) (15). Between each development, Chromarods were partially scanned by the FID of the Mark-V Iatroscan (Iatron Laboratories Inc.). Lipid classes were identified and quantified with the use of standard calibration curves obtained for each lipid class. Within each set of 10 rods, 9 were used for each combination of three treatments per 3 vol, and one was used for either lipid standards or total extraction blanks. Two sets of 10 rods were quantified per day, allowing a daily check of lipid standard and blank extraction.

The lipid class compositions were compared using a three-way multiple ANOVA with treatment (ultrasonication, grinding, or a combination of these two), storage (0, 1, and 6 mon), and sampling volume (5, 10, and 15 mL) as factors. Where overall differences were detected, the least-square mean multiple-comparison test was used to determine which means differed. Probability levels were divided by the number of DF of the tested factor (sequential Bonferroni correction). Homoscedasticity was tested by running Levene's test and was confirmed by graphical examination of the residuals (16). Data for FFA, ST, AMPL, and total lipids (TL) were log-transformed to achieve homoscedasticity.

RESULTS AND DISCUSSION

The lipid class composition of the diatom *C. gracilis*, according to treatment, storage duration, and sampling volume, is presented in Figure 2. Lipid extracted from *C. gracilis* consisted of relatively high concentrations of TAG, AMPL, and PL, with low concentrations of FFA and ST, as previously reported in the literature (1).

Effect of treatment on the determination of lipid class composition. There was a significant interaction between storage duration and extraction treatment for TAG ($P = 0.015$), FFA ($P = 0.046$), and TL levels ($P = 0.041$). After 3 d of storage, the combination of ultrasonication and grinding yielded a poorer TAG recovery (2.64 µg/10^6 cell) than either ultrasonication or grinding alone (2.90 µg/10^6 cell), although this difference represented only *ca.* 7% of the TAG level (Fig. 2). After either 1 or 6 mon of storage, no difference in TAG recovery was observed. The combination of ultrasonication and grinding led to higher FFA and TL levels than either ultrasonication or grinding alone only after 6 mon of storage, whereas after either 3 d or 1 mon of storage there was no difference.

A significant interaction between sample volume and treatment was detected for TAG ($P = 0.030$). The three treatments tested gave similar TAG contents for the 5- and 10-mL samples, whereas in the 15-mL samples the combination of ultrasonication and grinding yielded a significantly higher TAG content (3.51 µg/10^6 cell) than ultrasonication or grinding alone (3.42 and 3.21 µg/10^6 cell, respectively). Lyophilized samples of the oyster *Crassostrea gigas* exhibited lower TAG levels than nonlyophilized samples (17). It was suggested that extraction of hydrophobic lipids from lyophilized tissues is more difficult because proteins provide an ionic barrier to nonpolar solvents. This protein–TAG binding may explain why the combination of ultrasonication and grinding is more efficient in releasing TAG of large samples than ultrasonication or grinding alone. However, the discrepancy of treatment effect between small and large samples on TAG recovery remains obscure. Although very speculative, TAG might interact with the silica skeleton of the algae during the extraction procedure.

Effect of storage on the determination of lipid class composition. A significant effect of storage time on the lipid class composition of *C. gracilis* was also observed. FFA levels increased markedly after 6 mon of storage ($P < 0.001$) at the expense of both TAG ($P < 0.001$) and PL ($P < 0.001$; Fig. 2). FFA are usually detected in trace amounts in living organisms as these compounds are rapidly incorporated into TAG and PL. Consequently, a high level of FFA is an indication of lipolytic degradation of PL and TAG (8). For example, elevated amounts of FFA in the diatom *Skeletonema costatum* were related to the degradation of the more complex lipids (18), likely caused by lipase activity associated with the destruction of the intracellular compartment incurred during harvesting. Similarly, the polar lipid of the copepod *Calanus pacificus* decreased concomitantly with increased FFA during storage, thus suggesting enzymatic hydrolysis of PL (11).

PL showed a greater sensitivity to storage time than TAG as *ca.* 30% of the initial PL mass was degraded after 1 mon, whereas TAG level remained constant during this time (Fig. 2). Sasaki and Capuzzo (10) also noticed a higher degradation rate of PL than TAG in *Artemia* during storage. Furthermore, FFA production in algal samples can be inhibited by exposure to boiling water prior to lipid extraction, which apparently stabilizes PL while TAG remains constant (18), thus suggesting that FFA originates predominantly from PL.

Similar to FFA, ST levels increased after 6 mon of storage ($P < 0.001$), which has not been reported before. This may be an artifact of a poor separation between ST and DAG. Therefore, ST peak area augmentation likely reflects a misleading effect of the DAG increment, also considered as a degradation product. The augmentation of DAG during storage may result from hydrolysis of certain polar lipid classes.

The AMPL content, consisting primarily of glycolipids, pigments (e.g., chlorophyll) and MAG (15), also increased with storage duration ($P < 0.001$). This may be due to the conversion of PL and TAG into MAG, as it seems unlikely that pigment extraction would increase after storage in the solvent. For instance, the chlorophyll *a* content of plankton has been shown to decline with storage duration, and it is therefore recommended that plankton should be analyzed immediately after filtration (19).

In our study, the appearance of degradation products (FFA, ST, and AMPL) during storage was not completely counterbalanced by the decline of complex lipids (TAG and PL), as the TL concentration was highest after 6 mon ($P < 0.001$, Fig. 2). In contrast, the amount of PL loss during storage of the copepod *C. pacificus* was more than twice the increase of FFA (11). Our study suggests a higher overall lipid recovery after 6 mon of storage in a solvent than after either 3 d or 1 mon.

Effect of sample volume on the determination of lipid class composition. Surprisingly, the TAG content increased by factors of 1.5 and 1.8 with increases in sampling volume from 5 to 10 and 5 to 15 mL, respectively, although data were normalized ($P < 0.001$, Fig. 2). In contrast, FFA, ST (or DAG), and PL decreased with increasing sampling volume ($P < 0.001$, Fig. 2); therefore sampling volume did not affect TL content ($P = 0.386$). The hydrolysis of TAG during the freezing process may have been more pronounced for the small samples than for the larger ones, thus explaining the concomitant

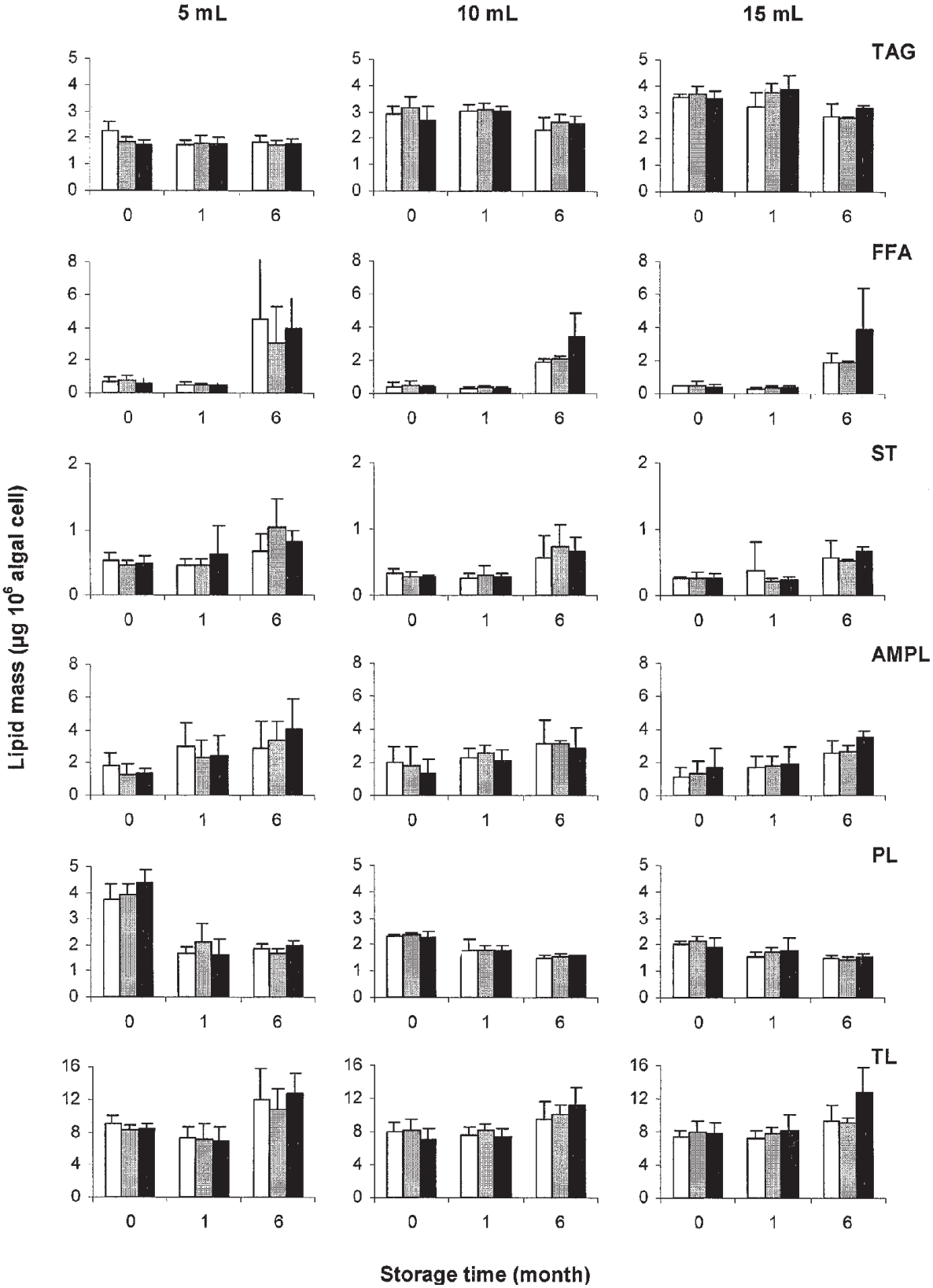

FIG. 2. Mass of each lipid class expressed in µg per 10^6 cells of the diatom *Chaetoceros gracilis* as a function of treatment (ultrasonication, white bars; grinding, gray bars; and a combination of these two, black bars), storage time (3 d, 1 and 6 mon), and sampling volume (5, 10, and 15 mL of algal culture) (±SD, $n = 5$). Lipid classes detected were TAG, FFA, cholesterol (ST), acetone-mobile polar lipids (AMPL), and phospholipids (PL). Total lipids (TL) were obtained by summation of each lipid class.

decline in degradation products. However, the pattern of PL associated with different sampling volumes remains obscure.

There was a significant interaction effect between volume and storage time on the TAG content of *C. gracilis* ($P < 0.001$). TAG levels were *ca.* 21% lower in both the 10- and 15-mL samples after 6 mon of storage than either of the shorter storage times. TAG levels of 5-mL samples were similar regardless of storage duration (Fig. 2). There was also a significant interaction effect of volume and storage on PL content ($P < 0.001$). In this case, the PL level in 5-mL samples was affected by the storage period to a greater extent than in the 10- and 15-mL samples (Fig. 2).

Our study shows that cell disruption treatment prior to lipid extraction influences the determination of lipid class composition of the diatom *C. gracilis*. Ultrasonication or grinding seems to allow a better TAG recovery than a combination of these two when extraction occurs within 1 mon of sampling. In contrast, a combination of ultrasonication and grinding seems to be more appropriate for higher TL recovery after 6 mon of storage. A combination of ultrasonication and grinding in larger samples yielded the highest TAG recovery. Finally, degradation of TAG during storage seems lower in small samples than in large ones, whereas degradation of PL is less pronounced in large samples. These conclusions refer to only a single diatom species. The result for other algal species with tough organic walls (e.g., some flagellates) may be different.

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Lipase-Catalyzed Methanolysis of Triricinolein in Organic Solvent to Produce 1,2(2,3)-Diricinolein

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ABSTRACT: The objective of this study was to find the optimal parameters for lipase-catalyzed methanolysis of triricinolein to produce 1,2(2,3)-diricinolein. Four different immobilized lipases were tested, *Candida antarctica* type B (CALB), *Rhizomucor miehei* (RML), *Pseudomonas cepacia* (PCL), and *Penicillium roquefortii* (PRL). *n*-Hexane and diisopropyl ether (DIPE) were examined as reaction media at three different water activities (a_w), 0.11, 0.53, and 0.97. The consumption of triricinolein and the formation of 1,2(2,3)-diricinolein, methyl ricinoleate, and ricinoleic acid were followed for up to 48 h. PRL gave the highest yield of 1,2(2,3)-diricinolein. Moreover, this lipase showed the highest specificity for the studied reaction, i.e., high selectivity for the reaction with triricinolein but low for 1,2(2,3)-diricinolein. Recoveries of 93 and 88% DAG were obtained using PRL in DIPE at a_w of 0.11 and 0.53, respectively. Further, NMR studies showed that a higher purity of the 1,2(2,3)-isomer vs. the 1,3-isomer was achieved at higher a_w (88% at a_w = 0.53), compared to lower a_w (71% at a_w = 0.11). The DAG obtained was acylated by the DAG acyltransferase from *Arabidopsis thaliana*. Therefore, this enzymatic product is a useful enzyme substrate for lipid biosynthesis. Accordingly, the use of PRL in DIPE at a_w 0.53 is considered optimal for the synthesis of 1,2(2,3)-diricinolein from triricinolein.

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The final step in the biosynthetic pathway of oil in castor seeds (*Ricinus communis* L.) is the acylation of 1,2-diricinolein to triricinolein (1). Triricinolein makes up the major part of the oil in castor seed, around 71% (2). The oil is highly viscous and is used industrially in products such as lubricants, coatings, polymers, and cosmetics (3). Unfortunately, castor seeds contain a potent toxin, ricin, which inhibits protein synthesis in mammalian cells by attacking the ribosome (4). In addition, they contain an allergen; therefore, work has been done in our laboratory to produce triricinolein in a plant lacking toxins. One important step has been to clone the cDNA for DAG acyltransferase (DGAT) in castor seeds, one of the enzymes involved in the biosynthesis of triricinolein from 1,2-diricinolein. To characterize the specific activity of the expressed DGAT enzyme, 1,2-diricinolein was needed as a substrate. Hence, the aim of this work was to synthesize a significant amount of 1,2-diricinolein.

There is also a more general interest in the synthesis of DAG, since they are useful as emulsifiers in food, pharmaceuticals, and cosmetics. DAG oil is a useful substitute for TAG oil in food (5). Furthermore, since 1,2-DAG can be used as starting material for the synthesis of various prodrugs (6), it is important to use an isomerically pure compound.

1,2-DAG are difficult to synthesize by using conventional organic methodologies. Instead, lipases can be used for higher selectivity of the reaction. Whichever method is used, enzymatic or chemical, the DAG formed are always a mixture of 1,2-, 2,3-, and 1,3-isomers, although the proportions vary. The optical isomers, 1,2- and 2,3-DAG, are usually referred to as 1,2(2,3)-DAG, since they are difficult to separate using common chromatography systems. For example, Fureby *et al.* (7) showed that lipases could be used for selective preparation of 1,2(2,3)-DAG from TAG by alcoholysis in organic media. In that work, 22 different lipases were tested for activity toward trilaurin, tricaprin, and tripalmitin, and a lipase from *Penicillium roquefortii* gave the highest yield of 1,2(2,3)-DAG. It is possible to produce 1,2-DAG from acylation (chemical or enzymatic) of enzymatically produced glycerol-3-phosphate followed by dephosphorylation (8). This approach has been used for chemical synthesis of labeled DAG. However, the presence of a hydroxyl group on ricinoleate makes chemical acylation difficult.

In this work, lipases were used to catalyze the hydrolysis and methanolysis of triricinolein to 1,2(2,3)-diricinolein. This approach is rather different from the one described above (7), since the presence of a hydroxyl group on the hydrocarbon chain probably alters the interaction of a lipase with triricinolein compared to saturated TAG. Further, there is a potential for polymerization of liberated ricinoleic acid, forming estolides, which also may affect the reaction.

Four different immobilized lipases were studied—Novozyme 435 *(Candida antarctica* type B, CALB), Lipozyme RM-IM *(Rhizomucor miehei,* RML), Lipase PS *(Pseudomonas cepacia*, PCL), and Lipase R_G *(P. roquefortii,* PRL). The reactions were performed in two different solvents, *n*-hexane and diisopropyl ether (DIPE)—at three water activities (a_w), 0.11, 0.53, and 0.97. 1,2(2,3)-Diricinolein was identified by MS, and its purity vs. 1,3-diricinolein was determined by ^{1}H and

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Abbreviations: AtDGAT, cDNA coding DGAT cloned from *Arabidopsis thaliana*, Columbia; a_w, water activity; CALB, *Candida antarctica* type B lipase; DGAT, DAG acyltransferase; DIPE, diisopropyl ether; ESI-MS, electrospray ionization MS; NP-HPLC, normal-phase HPLC; PCL, *Pseudomonas cepacia* lipase; PRL, *Penicillium roquefortii* lipase; RML, *Rhizomucor miehei* lipase; RRO, *sn*-1,2-diricinoleoyl-*sn*-3-oleoyl TAG; RT-PCR, reverse-transcriptase PCR.

^{13}C NMR. The conditions giving the highest yield of diricinolein were selected for synthesis.

EXPERIMENTAL PROCEDURES

HPLC system. A Waters Millenium HPLC system consisting of a computer, a gradient pump (model 600), an autosampler (model 717), and a UV-vis detector (model 2487) (Waters Associates, Milford, MA) was used. A Beckman Ultrasphere column, ODS 5 μm, 250 × 4.6 mm, was used for analysis (Beckman Instruments Inc., Fullerton, CA); a Phenomenex Luna column, C18(2) 100 Å, 5 μm, 250 × 15 mm (Phenomenex, Torrance, CA) was used for preparative chromatography; and a Phenomenex Luna column, CN 5 μm, 250 × 4.6 mm, was used for normal-phase HPLC (NP-HPLC).

Solvents. HPLC-grade 2-propanol, methanol, and hexane were obtained from Fisher Scientific (Fairlawn, NJ), DIPE was purchased from Fluka Chemie (Buchs, Switzerland), and methyl *tert*-butyl ether (MTBE) was obtained from Aldrich (Milwaukee, WI).

Chemicals. Accurel MP1000 was obtained from Membrana (Obernburg, Germany). Lithium chloride and methyl ricinoleate were purchased from Sigma (St. Louis, MO). Sodium sulfate and magnesium nitrate were obtained from J.T.Baker Inc. (Phillipsburg, NJ). Potassium sulfate, sodium phosphate, and disodium phosphate were purchased from Spectrum Chemical Mfg. Corp. (Gardena, CA).

Enzymes. Crude PCL and PRL were kind gifts from Amano Enzymes Inc. (Nagoya, Japan). Novozyme 435 (immobilized CALB, 10.000 propyl laurate units/g) and Lipozyme RM-IM [immobilized RML, 6.1 batch acidolysis units Novo (BAUN)/g] were generous gifts from Novozymes North America (Franklinton, NC). The activity for Novozyme 435 is based on ester synthesis of 1-propanol and lauric acid at 60°C for 15 min. Ester formation is calculated based on the acid values of the reaction mixture measured by titration before and after the reaction, and given as propyl laurate units/g. BAUN is based on acidolysis of high-oleic acid sunflower oil and decanoic acid at 70°C for 60 min. The rate of the reaction is determined by measuring the amount of decanoic acid incorporated into the 1- and 3-positions of the TAG.

Preparation of triricinolein. Triricinolein is not commercially available and was produced in our laboratory by preparative chromatography of castor oil, which is 71% triricinolein. Castor oil (0.5 mL at 0.5 g/mL in 2-propanol) was injected onto the preparative column. The mobile phase used was a gradient of methanol (A) and 2-propanol (B), and the flow rate was 7 mL/min, eluted with 100% A for 5 min, a linear gradient of A/B (80:20) over 10 min, and held for 5 min. The column was then reconditioned back to 100% A over 5 min and held for 5 min. Triricinolein was collected at a retention time of around 10 min. The solvent was removed under nitrogen with mild heating. The purity of the fractions was checked by analytical HPLC.

Immobilization of lipases. PCL and PRL were immobilized on a polypropylene powder (Accurel MP1000, formerly called EP100) by adsorption as described by Gitlesen *et al.* (9). Five grams of crude enzyme was mixed with 100 mL of phosphate buffer (pH 6.0, 20 mM) and then added to 5 g of Accurel MP1000 that had been prewetted with 15 mL of ethanol. The mixture was incubated overnight at room temperature while stirring. The following day the mixture was filtered, and the filtrate was washed with 5 mL of phosphate buffer (pH 7.0, 200 mM). The immobilized enzyme was dried overnight under vacuum.

Equilibration of the reaction system. The immobilized enzymes, reaction media (hexane and DIPE), and methanol were allowed to equilibrate for at least 48 h to a certain a_w over saturated salt solutions (10). This was achieved in airtight desiccators in which saturated water solutions of LiCl, $MgNO_3$, and K_2SO_4 gave a_w of 0.11, 0.53, and 0.97, respectively.

Methanolysis reaction. One hundred milligrams of triricinolein was dissolved in 5 mL of reaction media and 0.5 mL of methanol. One hundred microliters was taken out as a first fraction to determine the starting concentration of triricinolein (which was set to 100% yield, as shown on the axis of Fig. 5 and 6). Thereafter, 100 mg of immobilized enzyme was added, and the reaction took place in rotating glass tubes at room temperature. One hundred-microliter fractions were taken out at time intervals (after 0.5, 1, 2, 4, 6, 24, and 48 h), the solvent of each fraction collected was removed by nitrogen, and 0.5 mL of 2-propanol was added. The fractions were stored in a freezer at −20°C until HPLC analysis. The reactions were performed in duplicate.

HPLC analysis. Expected products of the lipase reaction, i.e., ricinoleic acid, methyl ricinoleate, diricinolein, and triricinolein, were analyzed by injection of 20 μL of sample on an RP-HPLC column, using a mobile phase of methanol (A) and methanol/water (90:10) (C). The gradient was as follows: from 100% C to 100% A in 20 min, then held for 10 min, and a gradient back to 100% C in 5 min, then held for 5 min. The total run time was 40 min. The flow rate was 1.0 mL/min, and the detection was performed at 205 nm. The peaks were quantified using external calibration with methyl ricinoleate at three different concentrations between 0.5 and 5.0 mg/mL.

Optimized methanolysis reaction. Enzymes and solvents were pre-equilibrated to a_w 0.53 for at least 48 h. One hundred milligrams of triricinolein was dissolved in 5 mL of DIPE and 0.5 mL of methanol. One hundred milligrams of PRL was added, and the mixture was allowed to react for 24 h during stirring at room temperature. The enzyme was removed by filtration at the end of the reaction time. The solvent was removed by nitrogen, and one mL of 2-propanol was added.

Fractionated collection of diricinolein. Portions of reaction product from above (0.5 mL) were injected onto the preparative column. A mobile phase consisting of methanol (A) and methanol/water (90:10) (C) was used at a flow rate of 7.0 mL/min. The gradient elution was from 100% C to 100% A in 15 min, then held for 15 min, back to 100% C in 5 min, and held for 5 min. The total run time was 40 min. The diricinolein peak was collected at a retention time of 16 min. Anhydrous sodium sulfate was added to the fractions collected, and the

tubes were shaken for 60 min. The sodium sulfate was removed by filtration, and the solvent was removed by nitrogen under mild heating. The recovered diricinolein oil was stored at –20°C.

MS. MS analysis was performed on an API QStar Pulsar Hybrid Quadrupole-TOF LC/MS/MS mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a Protana nanospray ion source (Proxeon Biosystems, Odense, Denmark). Prior to the electrospray ionization (ESI)-MS experiments, each sample was diluted with acetonitrile/water (1:1) containing 0.1% trifluoroacetic acid. The MS instrument was externally calibrated with Glu-Fib (Sigma). The samples were loaded into individual spray tips made of glass (PicoTip emitters; New Objective Inc., Woburn, MA) and inserted directly into the electrospray interface. The reactions were performed in duplicate and the mean values were reported.

NMR spectroscopy. NMR spectra were obtained at 27°C from samples in $CDCl_3$ with tetramethylsilane as an internal standard on a Bruker model ARX400 spectrometer (Bruker BioSpin Corp., Fremont, CA) at a frequency of 100.62 MHz for carbon and 400.13 MHz for protons. A 30° pulse at a 2.3-s repetition rate was used for carbon, and a 90° pulse at a 7- to 8-s repetition rate was used for protons. The number of attached protons for the ^{13}C signals was determined from distortionless enhancement by polarization transfer (DEPT) 90 and DEPT135 assays.

NP-HPLC. Peak purity of the 1,2(2,3)-isomer vs. the 1,3-isomer of diricinolein was also determined by NP-HPLC. Twenty-microliter samples in DIPE were injected onto a CN column, with a mobile phase consisting of *n*-hexane and MTBE (7:3), isocratic run. The total analysis time was 16 min. The flow rate was 1.0 mL/min with UV detection at 205 nm. The 1,3- and 1,2(2,3)-isomers eluted 2 min apart, at around 11 and 13 min retention time, respectively.

Cloning and expression of a cDNA encoding DGAT from A. thaliana*, Columbia (AtDGAT).* A full-length AtDGAT cDNA was amplified from an RNA sample extracted from *Arabidopsis* leaves by reverse transcriptase PCR (RT-PCR). Specific primers were designed based on the sequence information from GenBank (AF 051849): 5′-GAAATGGCGATTTTGGATTCT-GCT-3′ and 5′-TGACATCGATCCTTTTCGGTTCAT-3′. The cDNA was cloned into a pYES2.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA) and verified by complete sequencing in both directions using a PerkinElmer Big Dye Sequencing Kit (PerkinElmer, Seer Green, Buckinghamshire, United Kingdom). The recombinant protein was expressed in *Saccharomyces cerevisiae* strain INVSc-1 by using the pYES 2.1 TOPO TA Expression Kit according to the manufacturer's instructions (Invitrogen). Briefly, a single colony containing the pYES2.1/V5-His/*AtDGAT* construct was inoculated into medium containing 2% glucose and grown overnight at 30°C with shaking. Galactose (2%) was then added to the medium to induce expression of *AtDGAT* from the *GAL1* promoter. Cells were harvested after induction and the cell pellets were stored at –80°C until ready to use.

Assay for DGAT activity using lipase-catalyzed 1,2(2,3)-diricinolein as a substrate. Microsomes were isolated from harvested yeast cells as described by Urban *et al.* (11) and resuspended in 0.1 M Tris-HCl, pH 7.0, containing 20% glycerol and kept frozen at –80°C. Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). DGAT assays were performed as described in Cases *et al*. (12) with slight modifications. ^{14}C-Labeled oleoyl-CoA was synthesized according to McKeon *et al*. (13). The reaction mixture (100 μL) consisted of 0.1 M Tris-HCl, pH 7.0, containing 20% glycerol, microsomes (50 μg of protein), 1,2(2,3)-diricinolein (1.0 mM), and ^{14}C-oleoyl-CoA (20 μM, 200,000 cpm) and was incubated for 15 min at 30°C. The reactions were stopped and lipids were extracted using chloroform/methanol as described previously (14). Molecular species of acylglycerols were separated on a C18 column (25 × 0.46 cm, 5 μm, Ultrasphere C18; Beckman Instruments Inc.) using HPLC (15). Enzyme activity was determined based on the ^{14}C-label incorporated into the TAG products.

RESULTS AND DISCUSSION

A general reaction path for lipase-catalyzed hydrolysis of TAG is shown in Figure 1A. Lipases usually hydrolyze the outer positions first (*sn*-1 or *sn*-3), as these are more available than the inner *sn*-2 position. Once 2-MAG has been formed, most lipases easily break the last ester bond to form glycerol and FFA. Note that acyl migration occurs to form 1,3-DAG from 1,2(2,3)-DAG and 1(3)-MAG from 2-MAG. This type of migration is especially prominent in protic hydrophilic solvents such as ethanol and methanol and in aprotic hydrophobic solvents such as hexane, but it is lower in dipolar hydrophobic solvents such as ethers and ketones (16).

Figure 1B shows the details of the reaction studied in this work, i.e., the hydrolysis/methanolysis of triricinolein to 1,2(2,3)-diricinolein. The first step of Figure 1B describes the hydrolysis of the ester bond, which results in the release of 1,2(2,3)-diricinolein. The second step—hydration or methylation of the FA—is more rate limiting (17). The aim of this work was to find a lipase that selectively catalyzes this reaction (without further degradation of diricinolein to monoricinolein and glycerol, as described in Fig. 1A). Hence, the lipase should either show very low activity with 1,2(2,3)-diricinolein or give high yields of diricinolein before further degradation is initiated (i.e., much faster reaction kinetics with triricinolein than with diricinolein). Furthermore, acyl migration should be minimized, preferably by performing the reaction in a solvent such as ether.

HPLC analysis. The progress and yield of the reaction were monitored by determining the concentrations of triricinolein consumed and ricinoleic acid, methyl ricinoleate, and 1,2(2,3)-diricinolein formed. Monoricinolein was not found in the fractions, indicating that the last FA of the glycerol backbone was rapidly methanolyzed to give free glycerol and methyl ricinoleate. This effect also has been observed by others (7).

Figure 2 shows a chromatogram of a fraction from the PRL-catalyzed reaction of triricinolein in DIPE at a_w 0.11. The components considered were clearly well separated within 30 min using RP-HPLC. The peak eluting just before methyl ricinoleate, BHT, is a stabilizer in the DIPE solvent.

Diricinolein eluted as one peak in this RP-HPLC system, i.e., as a mixture of 1,2-, 2,3-, and 1,3-isomers. ESI-MS and MS-MS provided supporting evidence for identification of this peak as diricinolein. The ESI-mass spectrum of diricinolein is shown in Figure 3A. The protonated parent ion $(M + H)^+$ had a measured mass of 653.5 Da, which corresponds well to the calculated monoisotopic M.W. of protonated diricinolein, 653.5337 Da. This sample also gave rise to a sodium adduct $(M + Na)^+$ at *m/z* 674.5 and fragments at 652.5 $(M)^+$, 634.5 $(M - H_2O)^+$, 616.5 $(M - 2H_2O)^+$, and 598.5 $(M - 3H_2O)^+$. The parent ion was further fragmented by tandem MS-MS, giving the spectra in Figure 3B. The same ions were found as described above for the single MS mode, in addition to two further fragmented ions of *m/z* 337.3 and 355.3, respectively. The latter of these is the parent ion minus one of the FA chains (see Fig. 3B), and the former is the same but minus a water molecule. In conclusion, the MS experiments verified that it was diricinolein that was formed in the enzymatic reaction experiments.

1,2- and 2,3-Diricinolein are optical isomers (enantiomers), and they are difficult to separate using ordinary HPLC techniques. They may be separated as 3,5-dinitrophenyl urethanes using chiral HPLC (18), but the procedure is rather tedious. However, 1,3-diricinolein is a structural isomer that can be separated more easily from the 1,2(2,3)-isomers. Initially, some effort was made to separate 1,2(2,3)-diricinolein from 1,3-diricinolein using RP-HPLC and solvents such as methanol, 2-propanol, and water as the mobile phase. However, these structural isomers could be partly separated only by using the reversed-phase column. ^1H and ^{13}C NMR were used to determine the purity of 1,2(2,3)-diricinolein, but a less tedious

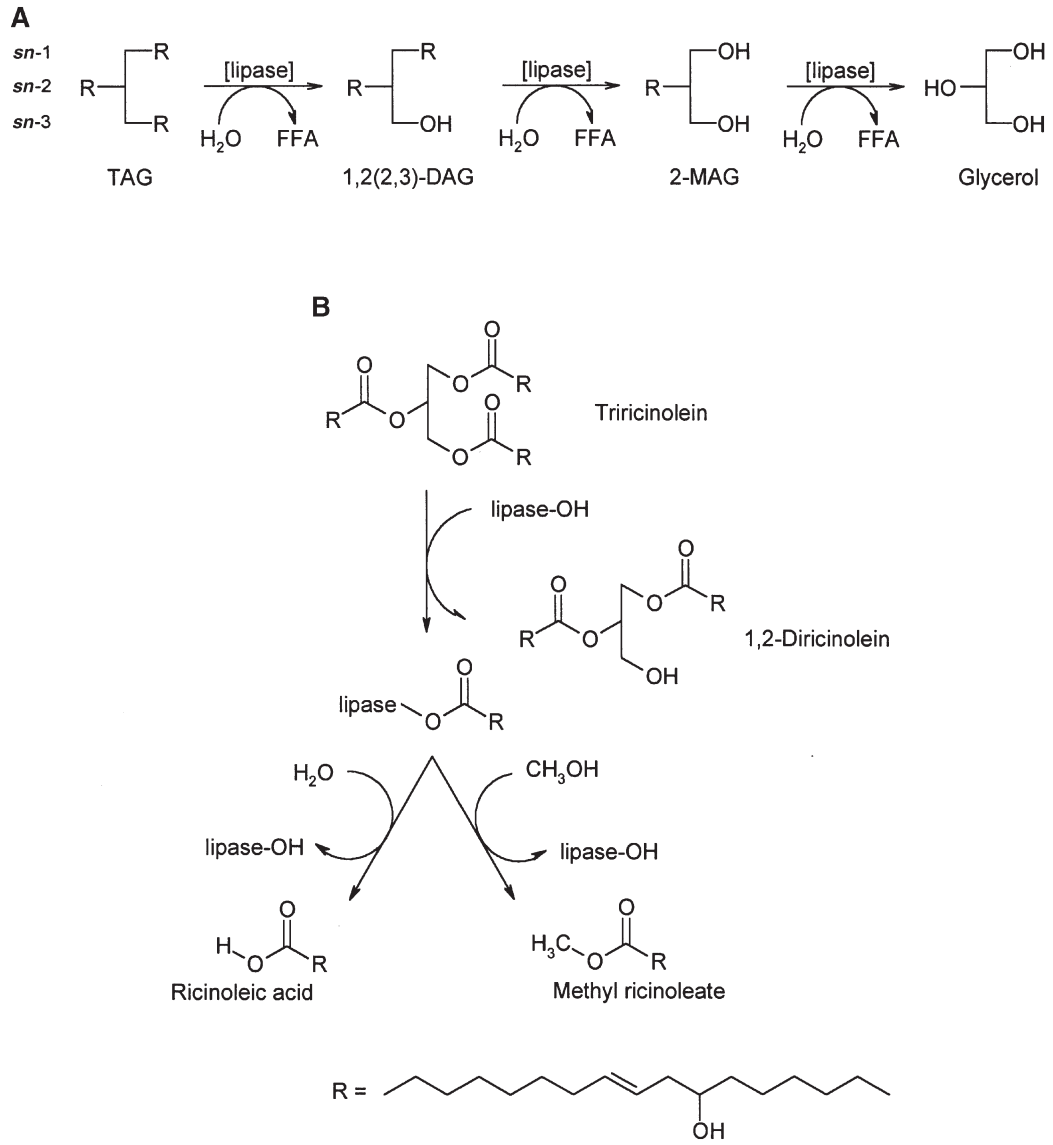

FIG. 1. (A) General reaction scheme for lipase-catalyzed hydrolysis of TAG to glycerol and FFA. (B) Reaction scheme for lipase-catalyzed hydrolysis/methanolysis of triricinolein to 1,2(2,3)-diricinolein and FFA/FAME.

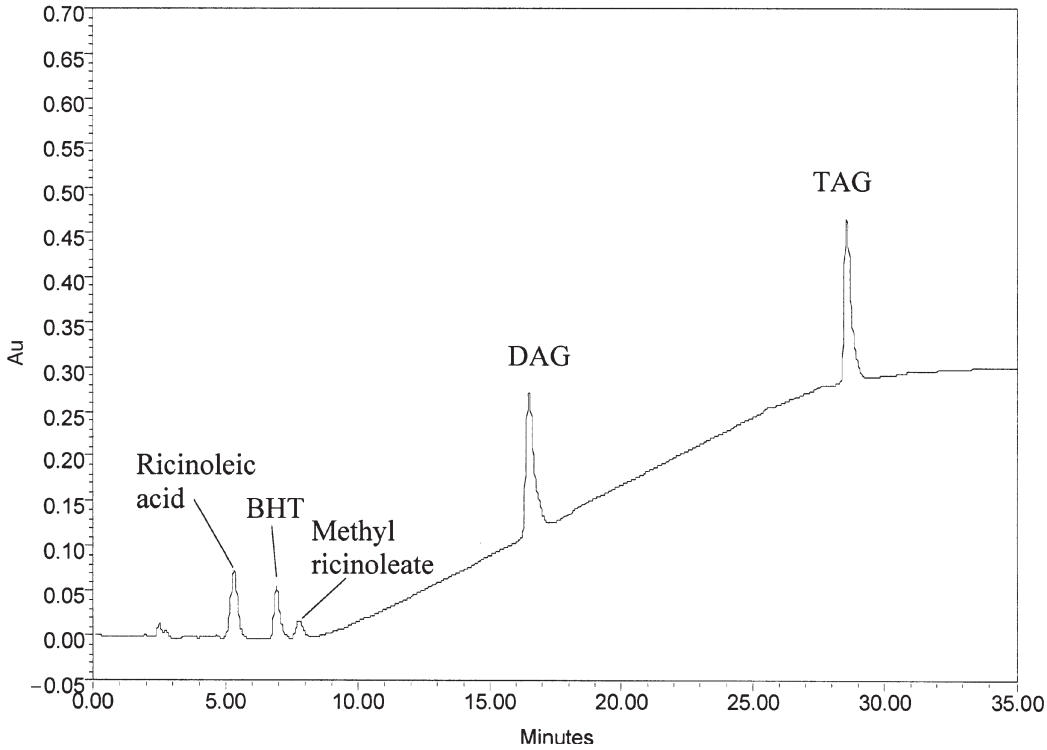

FIG. 2. RP-HPLC analysis of a fraction from the *Penicillium roquefortii* lipase (PRL)-catalyzed reaction in diisopropyl ether (DIPE) at water activity (a_w) of 0.11. An ODS column (Beckman Instruments Inc., Fullerton, CA) was used, and the mobile phase was based on methanol and water (see the Experimental Procedures section). The peaks were identified as ricinoleic acid, BHT, methyl ricinoleate, diricinolein (DAG), and triricinolein (TAG).

NP-HPLC procedure also was developed for the same purpose. Figure 4 shows the excellent separation of 1,3- and 1,2(2,3)-diricinolein isomers. This system was also used for fractionated collection of small amounts of pure 1,2(2,3)-diricinolein.

Choice of lipase. Four different lipases were tested in this study: CALB, RML, PCL, and PRL. In a previous study on ethanolysis of vitamin A esters in DIPE and hexane, CALB, RML, and PCL showed the best activity and stability of the six lipases studied (19). PRL was selected for the present study since it has demonstrated high specificity for conversion of TAG to 1,2(2,3)-DAG (7).

Twenty-four experiments were performed to evaluate the effects of solvents (hexane and DIPE) as well as a_w (0.11, 0.53, and 0.97) on the yield of 1,2(2,3)-diricinolein ($n = 2$). PRL was shown to have a higher specificity for the desired reaction than the other lipases investigated (Fig. 5).

Figure 5A shows the yield of diricinolein in DIPE at a_w 0.11 when CALB was used as the catalyst. The highest yield (40%) of diricinolein occurred after only 1 h of reaction, and it was then rapidly consumed for further production of methyl ricinoleate. RML and PCL showed similar trends. This behavior is common in lipases, as they usually drive the reaction toward degradation of TAG to either glycerol (nonspecific lipases) or 2-MAG (1,3-specific lipases) and FFA or FAME (see Fig. 1A).

Figure 5B shows the yield of diricinolein when PRL catalyzed the reaction in the same solvent and at the same a_w. In this case, the reaction was driven less toward methyl ricinoleate formation and more toward formation of diricinolein. The yield reached a maximum of 93% after around 24 h of reaction and remained stable for at least another 24 h. This result confirms that PRL selectively converts TAG to DAG but does not react further (7).

The maximum yields of diricinolein for all the reaction conditions tested are given in Table 1. PRL was clearly the preferred lipase for the reaction in question, giving diricinolein yields between 63 and 93% for a_w of 0.11 and 0.53 in hexane and DIPE.

Choice of solvent. Hexane and DIPE were investigated as reaction media for this study, since these solvents are commonly used for lipase-catalyzed reactions. In general, solvents of a higher log*P*-value (such as hexane: log*P* ~ 3.9) cause less inactivation of the enzyme than solvents of a low log*P*-value (such as ethanol and acetonitrile: log*P* < 0.2) (20). However, reaction rates are faster in solvents of a lower log*P*-value. Hence, a solvent of an intermediate log*P*-value, such as DIPE (log*P* ~ 1.7), may be a good compromise.

Of the two solvents studied, hexane and DIPE, the latter enabled slightly faster conversion of triricinolein to diricinolein using PRL (see Table 1). In hexane at any a_w, the maximum yield of diricinolein (83%) was not obtained until after 48 h of reaction. Therefore, DIPE was considered the better reaction medium for the reaction catalyzed by PRL. In addition, acyl migration was slower in DIPE than in hexane (16).

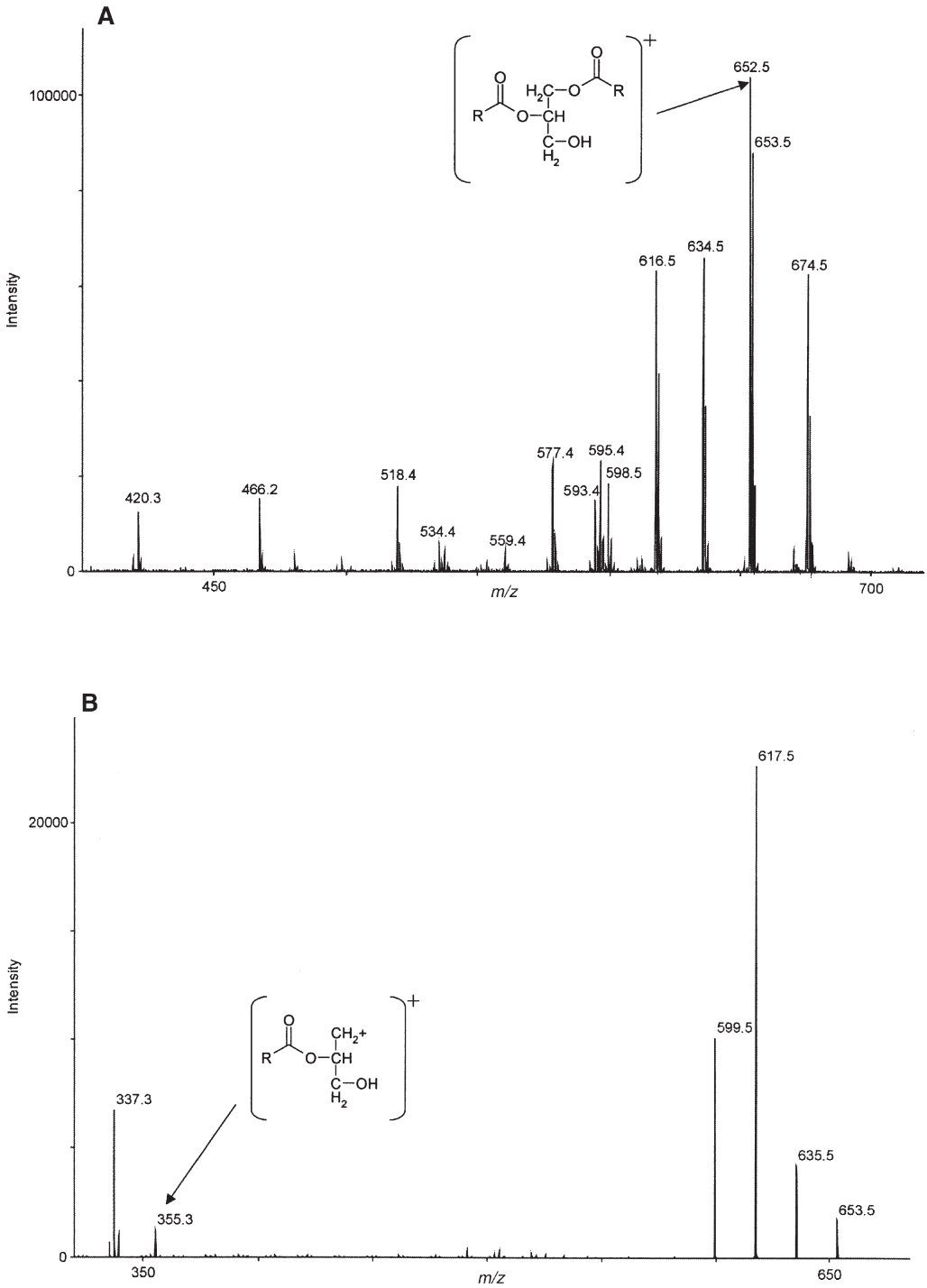

FIG. 3. (A) Mass spectrogram of diricinolein generated in the single MS mode, and the chemical structure of 1,2(2,3)-diricinolein. (B) Mass spectrogram of the parent ion of diricinolein generated in the tandem MS-MS mode, and the chemical structure of 1,2(2,3)-diricinolein minus one of its FA chains. R = ricinoleic acid, as illustrated in Figure 1.

Optimization of a_w. Three different a_w were studied, 0.11, 0.53, and 0.97. In general, a lower a_w gave a higher yield of diricinolein with any of the enzymes in any of the solvents. However, RML and PCL in DIPE produced diricinolein at higher yields with higher a_w (Table 1). This result is in agreement with work demonstrating that the initial reaction rate of PCL in DIPE was faster at higher a_w (21).

The PRL-catalyzed reaction in DIPE at a_w 0.53 and 0.97 is demonstrated in Figures 6A and 6B, respectively. The corresponding reaction at a_w 0.11 is shown in Figure 5B. These

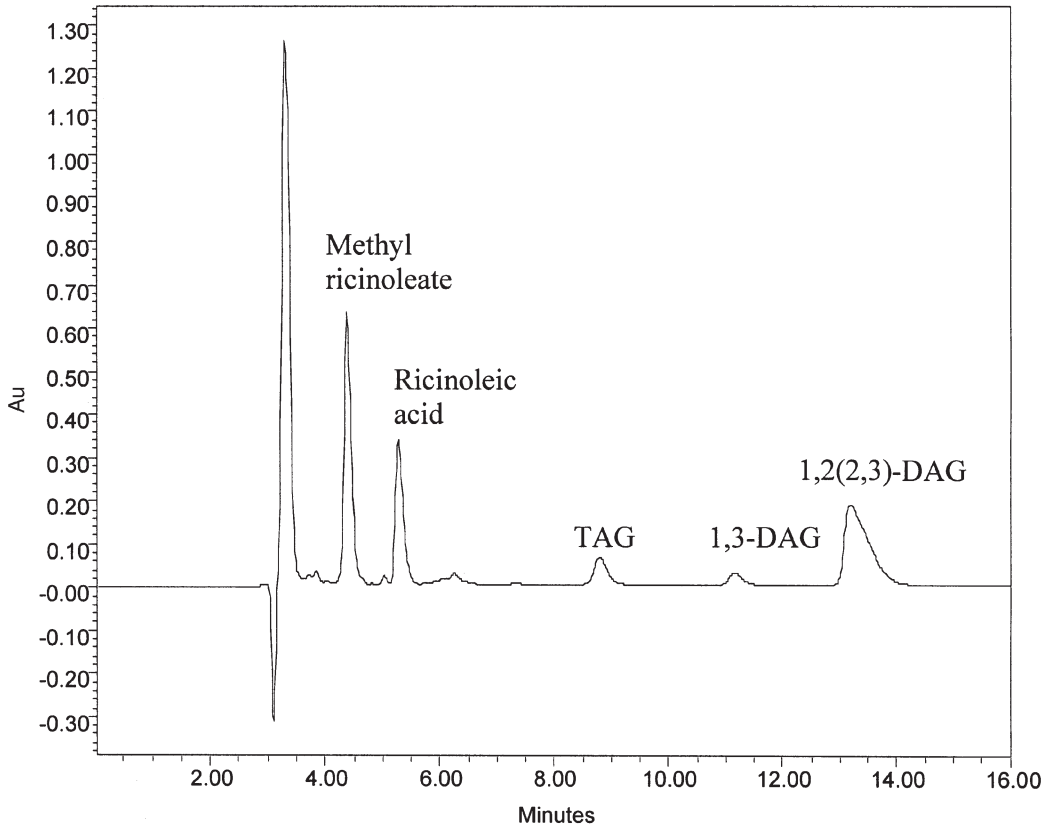

FIG. 4. Normal-phase HPLC analysis of a fraction from the PRL-catalyzed reaction in DIPE at an a_w of 0.53. A CN column (Phenomenex, Torrance, CA) and a mobile phase consisting of hexane and methyl *tert*-butyl ether (see the Experimental Procedures section) were used. The peaks were identified as methyl ricinoleate, ricinoleic acid, triricinolein (TAG), 1,3-diricinolein (1,3-DAG) and 1,2(2,3)-diricinolein [1,2(2,3)-DAG]. For other abbreviations see Figure 2.

figures demonstrate that, within the reaction time studied (48 h), the highest recoveries were obtained at a_w 0.11 and 0.53 (93 and 88%, respectively). At the highest a_w (0.97 in Fig. 6B), the reaction was very slow, and maximum yield was not reached even after 48 h of reaction. Since the reaction yields were similar at a_w 0.11 and 0.53, these two experiments were repeated for further examination.

The purity of 1,2(2,3)-diricinolein vs. 1,3-diricinolein was determined using ^{1}H and ^{13}C NMR. The isomeric structures of the mixture were identified by the chemical shift value of the proton attached to the *sn*-2 carbon of the glycerol backbone. For 1,2(2,3)-diricinolein, this proton was shown as a pentet at 5.080 ppm, whereas 1,3-diricinolein had a multiplet at 4.159 ppm. The observed data are compatible with those reported in the literature for triacetin and tristearin (absorption maxima at 242 and 368, respectively, in Ref. 22). The percentage of the isomeric components present in the mixture was calculated from the comparative integral value of each component in the spectral data of the mixture.

Although a_w 0.11 gave a higher yield of diricinolein, the proportion of 1,2(2,3)-isomer vs. 1,3-isomer was lower, only 73%, compared with that for a_w 0.53, where it was 88%. Therefore, an a_w of 0.53 was selected as optimal for the studied reaction. Additional experiments using a high a_w (~0.97) showed that the reaction took several days to give high yields, although the purity of 1,2(2,3)-diricinolein formed was 93%. Since the 1,2(2,3)-isomer was readily separated from the 1,3-isomer using NP-HPLC, giving a pure product (less than 1% of 1,3-diricinolein), for our purposes there was no point in carrying out the longer reaction to obtain a slight improvement in purity.

The DGAT catalyzes the final step in the Kennedy pathway for TAG biosynthesis and uses acyl-CoA to acylate 1,2-diricinolein. To test whether 1,2(2,3)-diricinolein could serve as an effective substrate for AtDGAT, an AtDGAT cDNA was expressed in yeast *S. cerevisiae* strain INVSc-1 by using a pYES2.1-TOPO TA Expression Kit. Microsomes containing AtDGAT proteins were extracted and used for the DGAT activity assay.

The DGAT activity was 510 pmol/min/mg protein when we supplied the microsomes with 1 mM 1,2(2,3)-diricinolein, whereas the activity was only 330 pmol/min/mg protein without adding diricinolein. The incorporation of ^{14}C-labeled oleoyl-CoA into ^{14}C-labeled *sn*-1,2-diricinoleoyl-*sn*-3-oleoyl

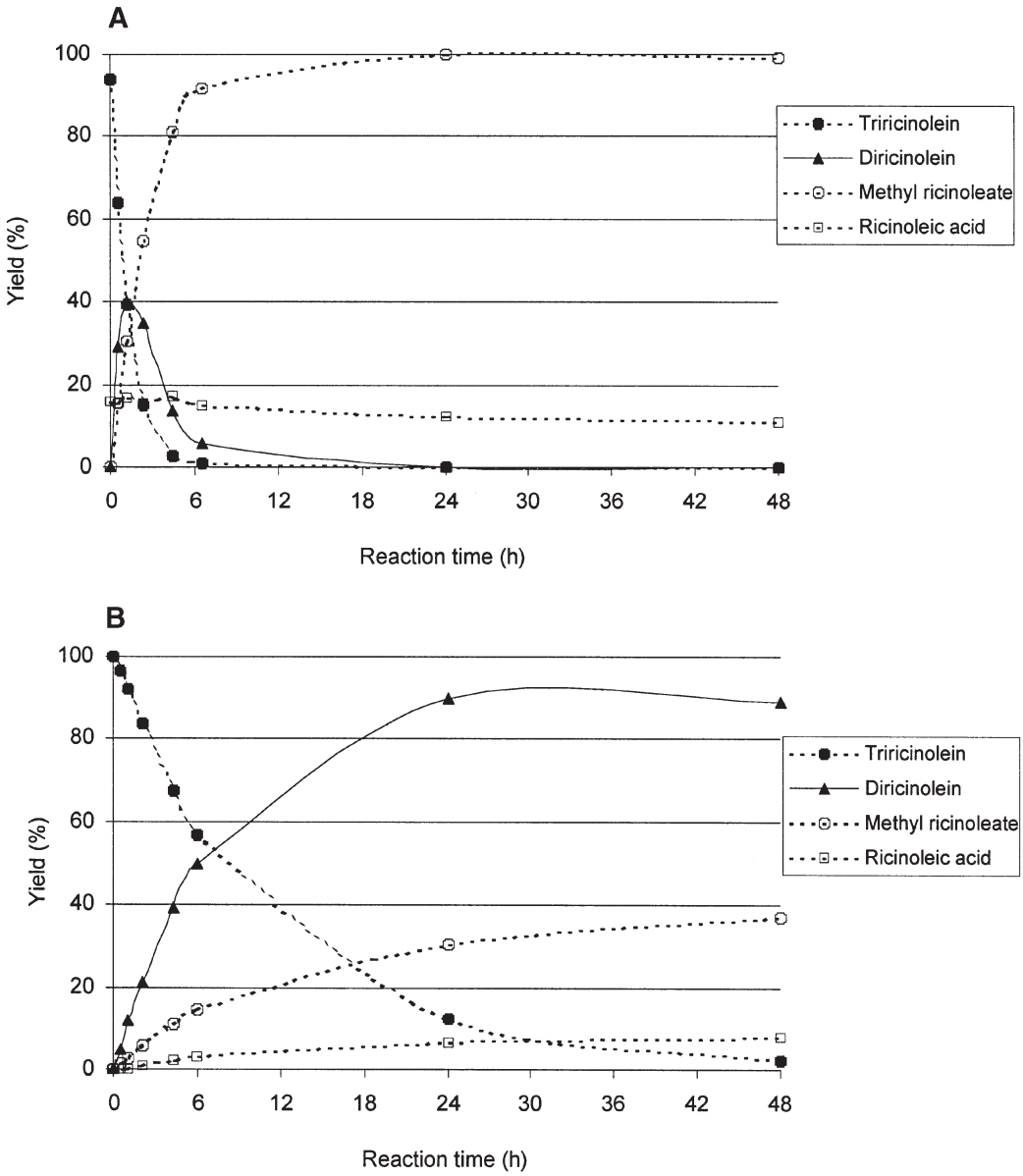

FIG. 5. Yield of diricinolein vs. reaction time for methanolysis of triricinolein in DIPE at an a_w of 0.11 catalyzed by (A) *Candida antarctica* type B lipase (CALB) and (B) PRL (n = 2). For abbreviations see Figure 2.

TABLE 1
Recoveries of Diricinolein (%) at Each Different Reaction Condition Tested (n = 2)[a]

Immobilized	a_w = 0.11		a_w = 0.53		a_w = 0.97	
lipase	Hexane	DIPE	Hexane	DIPE	Hexane	DIPE
CALB	40 (1 h)	40 (1 h)	16 (4 h)	26 (6 h)	20 (6 h)	17 (4 h)
RML	23 (1 h)	21 (1 h)	14 (1 h)	37 (1 h)	10 (48 h)	36 (1 h)
PCL	43 (7 h)	33 (4 h)	37 (1 h)	36 (4 h)	21 (1 h)	40 (4 h)
PRL	83 (48 h)	93 (24 h)	63 (48 h)	88 (48 h)	29 (48 h)	49 (48 h)

[a]The reaction times giving these maximum recoveries are shown within parentheses. a_w, water activity; CALB, *Candida antarctica* type B lipase; RML, *Rhizomucor miehei* lipase; PCL, *Pseudomonas cepacia* lipase; PRL, *Penicillium roquefortii* lipase; DIPE, diisopropyl ether.

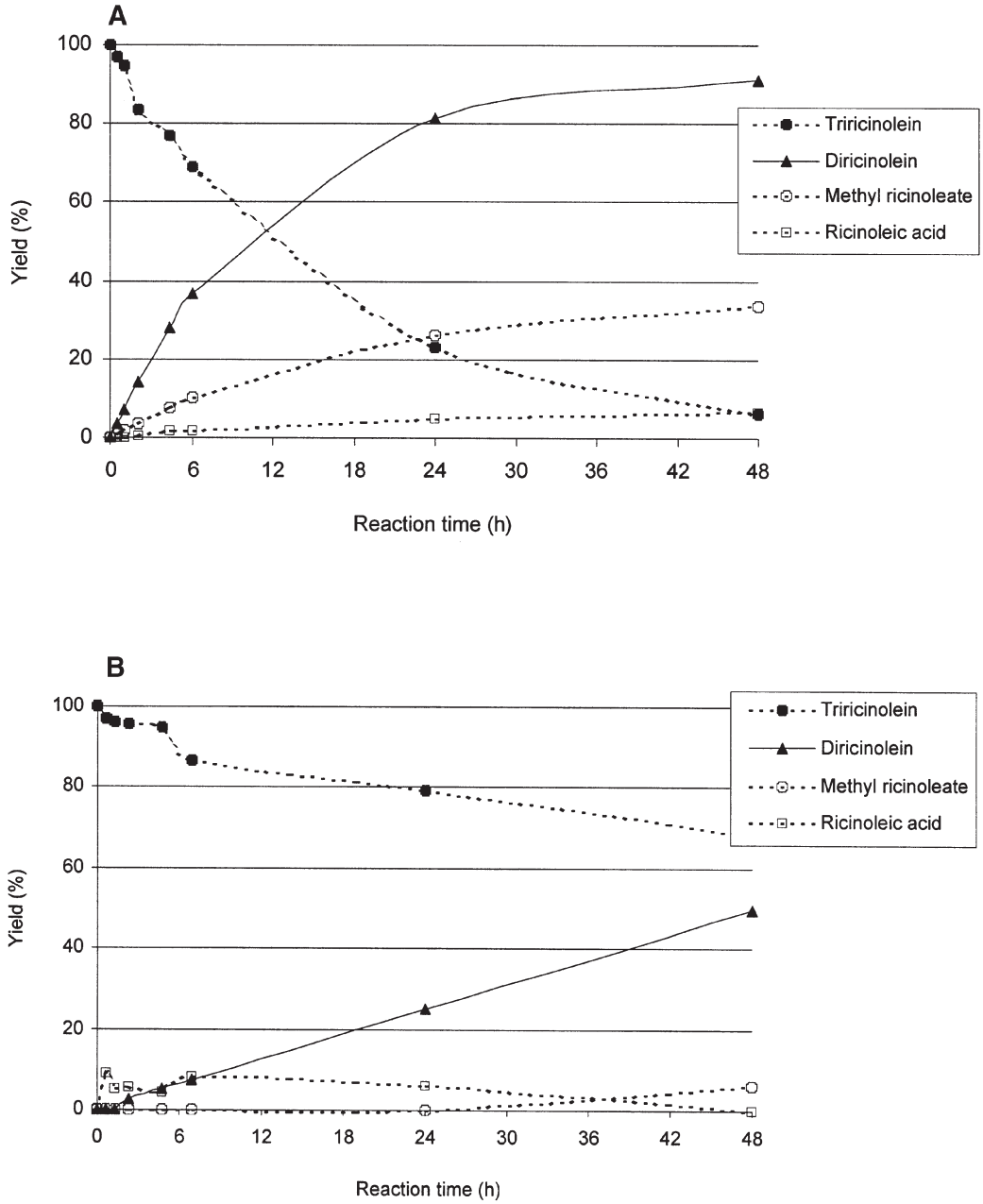

FIG. 6. Yield of diricinolein vs. reaction time for PRL-catalyzed methanolysis of triricinolein in DIPE at an a_w of (A) 0.53 and (B) 0.97 ($n = 2$). For abbreviations see Figure 2.

TAG (RRO) increased from 0 in the absence of diricinolein to 290 pmol/min/mg protein in the presence of 1 mM diricinolein. RRO was not produced in wild yeast cells when the same amount of 1,2(2,3)-diricinolein was supplied (data not shown). These results indicate that the diricinolein generated by lipase-catalyzed methanolysis is suitable as a substrate for the *Arabidopsis* DGAT, proving that diricinolein is suitable as a DGAT substrate.

In conclusion, PRL was chosen as the most suitable catalyst for the reaction, and DIPE was considered to be the better solvent, at an a_w of 0.53. The optimal reaction time was 24 h. These conditions gave a diricinolein yield of 88% and a purity of the 1,2(2,3)-isomer of 88%.

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COMMUNICATION

Occurrence of High Levels of Tetracosahexaenoic Acid in the Jellyfish *Aurelia* sp.

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ABSRACT: The FA composition of the pelagic jellyfish *Aurelia* sp. collected from off-shore Western Australia waters was determined by capillary GC and GC–MS, with confirmation of PUFA structure performed by analysis of 4,4-dimethyloxazoline derivatives. PUFA constituted 47.6% of the total FA, with the essential PUFA eicosapentaenoic acid (EPA), arachidonic acid, and DHA accounting for 34%. Of particular interest, the unusual very long chain PUFA 6,9,12,15,18,21-tetracosahexaenoic acid (THA, 24:6n-3) was present at 9.3%, and the rarely reported 6,9,12,15,18-tetracosapentaenoic acid (24:5n-6) also was detected at 0.8%. To our knowledge, this represents the first report of THA as a major PUFA in a pelagic marine organism.

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The signature lipid approach, in particular, FA and sterol profiles, has been used successfully to help understand marine trophodynamics (1,2). Recent studies using lipid biomarkers have demonstrated aspects of Antarctic ecology at the levels of both higher consumers and zooplankton communities that are not visible by conventional techniques (3–7). Similarly, the lipids of the phyllosoma (larval) and pueruli (postlarval) stages of the southern rock lobster, *Jasus edwardsii*, have been examined to improve our understanding of their nutrition, particularly in relation to the possible aquaculture of this commercially attractive species (8–10).

In a new Australian study, we are examining the lipid, FA, and sterol composition of larvae and potential prey of the western rock lobster, *Panularis cygnus*, to ascertain the possible food-chain links and nutritional condition of this species. As part of this research, we have collected and examined the lipids of 45 prey species. At the present time, little is known about the diet of the wild rock lobster phyllosoma, although it has been suggested that gelatinous zooplankton such as the jellyfish may be one important component of this lower food web (10). It was with this background that we examined the lipid profiles of potential prey, including the jellyfish, of western rock lobster phyllosoma. Here we report on the unusual occurrence of high levels of the very long chain PUFA (VLC-PUFA) tetracosahexaenoic acid (THA, 24:6n-3) in a pelagic marine animal, the jellyfish *Aurelia* sp.

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Abbreviations: AA, arachidonic acid; DMOX, 4,4-dimethyloxazoline; THA, tetracosahexaenoic acid; TPA, tetracosapentaenoic acid; VLC-PUFA, very long chain PUFA.

MATERIALS AND METHODS

Sample description. Four *Aurelia* sp. were collected from the oceanographic research vessel *RV Franklin* by square neuston-net surface trawls between November 21 and 25, 2000. Each trawl lasted 20 min. The trawls were taken between 24°11′ and 27°12′ S and 111°58′ and 112°27′ E (200–1300 m) off the Western Australia coast. Water temperature and salinity were 21.3–22.8°C and 34.98–35.36 ppt, respectively. All specimens of *Aurelia* sp. were sorted from the catch and frozen at −20°C. Frozen samples were shipped to Hobart for lipid analyses. Identification of the specimens as Semaeostone jellyfish of the genus *Aurelia* was based on the presence of the radial canals (11,12).

Lipid extraction. Samples were quantitatively extracted overnight using a modified Bligh and Dyer one-phase methanol/chloroform/water extraction (13). An aliquot of the total lipid was treated with methanol/HCl/chloroform to produce FAME (10).

Preparation of 4,4-dimethyloxazoline (DMOX) derivatives. The DMOX method was adapted from Christie (14). FAME were first saponified and, after extraction and removal of solvent, the resulting FFA were converted to their acid chlorides by using oxalyl chloride (1.0 mL) at ambient temperature overnight. After addition of Milli-Q water (Millipore, Milford, MA), the acid chloride products were extracted (2 × 2 mL; hexane/chloroform 4:1, vol/vol). Following removal of solvent under a stream of nitrogen, the products were reacted with 2-amino-2-methylpropanol in dichloromethane (1 mL of a 10 mg/mL solution, stored over beads of molecular sieves). After 1 h at ambient temperature, the solvent was removed under a stream of nitrogen, trifluoracetic acid anhydride (1 mL) was added, and the mixture was placed in an oven at 40°C for 1 h. The excess reagent was again removed under a stream of nitrogen and, after addition of Milli-Q water (2 mL), the DMOX products were extracted (2 × 2 mL) as before.

GC and GC–MS analysis. FAME and DMOX derivatives were analyzed by GC using a high-resolution, cross-linked methyl phenyl silicone (HP-5) quartz capillary column (50 m × 0.32 mm i.d., 0.17 μm film thickness; Agilent, Palo Alto, CA). The HP-5 column was chosen to minimize column bleed, particularly for the GC–MS analysis. GC operating conditions were as in Phleger *et al.* (10). FAME were quanti-

fied with Waters Millennium software (Waters, Milford, MA), and FAME and DMOX-derivative identification were performed with a Finnigan Thermoquest GCQ GC–MS system (Finnigan, San Jose, CA) (10,15).

RESULTS

The FA profile of *Aurelia* sp. was dominated by PUFA (47.6% of total FA) and saturated FA (38%), with MUFA at 8.3% (Table 1). In decreasing order of abundance, the main FA were 16:0, EPA, 18:0, arachidonic acid (AA), and DHA; these five components accounted for around 66% of the total FA. Of particular interest, one additional major FA was present, eluting immediately after the 23:0 internal injection standard (Fig. 1); it was the next most abundant FA (9.3%) present. EI-MS confirmed this FA to be a PUFA, with an M^+ of *m/z* 370 of low intensity; this M^+ corresponded to a structure of 24:6. Tetracosapentaenoic acid (TPA, 24:5n-6) was also present (0.8%), eluting immediately prior to THA.

Double bonds were located by EI GC–MS of DMOX derivatives of FAME by identifying in the MS the difference of 12 Da, which interrupts successive chain cleavages of methylene units (Ref. 15, and references therein). This rule states that if an interval of 12 Da, instead of the regular 14 Da, is observed between the peaks of clusters of fragments containing n and n − 1 carbon atoms in the acid moiety, a double bond occurs between carbons n and n + 1 in the molecule. The major VLC-PUFA was identified as 24:6n-3 (6,9,12,15, 18,21) based on the MS (Fig. 2). An M^+ of *m/z* 408 was present, and the fragmentations observed were consistent with this structure. The fragmentation pattern for 24:6n-3 [major ions at *m/z* 394, 380, 366, 354, 340, 326, 314, 300, 286, 274, 260, 246, 234, 220, 206, 194, 180, 166/167 (Δ-6 unsaturation), 152/153, 140, 126 (bp); Fig. 2] in *Aurelia* sp. was also similar to that observed in the identification of 28:8n-3 (4,7,10,13,16,19,22,25) in several dinoflagellates (15). As observed for the mass spectrum of the 28:8n-3, the last double bond (closest to the delta end) is an exception to the rule.

TABLE 1
FA Composition (%) of *Aurelia* sp. Isolated from Western Australia Waters (*n* = 4)

FA	Jellyfish (mean ± SD)	FA	Jellyfish (mean ± SD)
14:0	0.9 ± 0.3	20:2n-6	0.2 ± 0.0
15:0	0.7 ± 0.1	20:1n-9*c*	0.2 ± 0.1
16:1n-7*c*	1.1 ± 0.3	20:0	0.2 ± 0.0
16:1n-10*t*	0.5 ± 0.2	22:5n-6	0.7 ± 0.1
16:0	21.2 ± 0.8	22:6n-3, DHA	9.8 ± 1.6
7Me 16:1	2.2 ± 0.3	22:4n-6	0.6 ± 0.0
17:0	1.8 ± 0.1	22:5n-3	1.1 ± 0.2
18:2n-6/a18:0	1.0 ± 0.2	24:5n-6, TPA	0.8 ± 0.1
18:1n-9*c*[a]	3.5 ± 0.7	24:6n-3, THA	9.3 ± 1.8
18:1n-7*c*	1.8 ± 0.2	24:1	0.5 ± 0.2
18:0	10.9 ± 1.5	Other[b]	2.2 ± 0.2
18:0, FALD	3.1 ± 0.1	Sum SFA	37.9 ± 1.8
20:4n-6, AA	9.9 ± 2.3	Sum MUFA	8.3 ± 1.6
20:5n-3, EPA	14.1 ± 1.9	Sum PUFA	47.6 ± 1.8
20:4n-3	0.2 ± 0.0		

[a]Includes 18:3n-3 as a minor component. FALD denotes fatty aldehyde. AA, arachidonic acid; TPA, tetracosapentaenoic acid; THA, tetracosahexaenoic acid; SFA, saturated FA; MUFA, monounsaturated FA.
[a]Other refers to FA < 0.2%: 16:1n-5*c*, 16:1n-9*c*, 16:1, 18:1n-7*t*, 18:1n-5*c*, 20:0, 20:1n-7*c*, 18:4n-3.

DISCUSSION

The FA profile of *Aurelia* sp. was broadly similar to that of other marine jellyfish (e.g., 4,16,17), with 16:0, EPA, 18:0, AA, and DHA as the five main components accounting for around 66% of the total FA. The jellyfish *Cyanea capillata* from New Brunswick waters also contained elevated levels of 20:1 and 22:1, presumably from captured small Atlantic food herring eating copepods, or even directly from the copepods (16). Of interest, our analysis of *Aurelia* sp. showed higher proportions of EPA than DHA (Table 1) compared with both other potential prey items of the lobster phyllosoma and marine particulate matter collected from the same area (data not shown). In contrast, *A. aurita* collected from waters off Greece showed markedly lower levels of PUFA (18). Whether the lower levels of PUFA in the Grecian jellyfish were due to handling losses, dietary differences, or perhaps the presence of THA in place of other PUFA is not known.

VLC-PUFA ($>C_{22}$) generally occur only as minor components in marine organisms (e.g., 4,19–21). There have, however, also been selected reports on the occurrence of VLC-PUFA as more important constituents. For example, over 48% of the FA in a marine sponge have C_{23}–C_{28} chain lengths (22), although polyenes (>3 double bonds) are only minor components. The VLC-PUFA 28:8n-3 and 28:7n-6 were identified in seven species of marine dinoflagellates (15). The VLC-PUFA, THA, was reported in the lipids of crinoids, ophiuroids, echinoderms, and coelenterates (23,24) and in their predators such as the flatfish (25). Considerable variation (0–9.3%) occurred in the level of THA in flatfish species collected from different locations, from the same location at different times, and between lipid classes. Based on vastly different levels of THA in animals from various groups (starfish belonging to Asteroidea and brittlestar from Ophiuroidea within the Echinodermata) collected from the same area and with a similar detrital diet, it has been suggested that THA in Crinoidea and Ophiuroidea do not originate from the diet (23). Rather, the likely origin is either from chain elongation of DHA or inhibited chain-shortening of THA to DHA; this hypothesis is also consistent with our findings for *Aurelia* sp., which showed lower DHA than in other animals collected from the same environment.

To date, when THA and other VLC-PUFA have occurred at elevated levels, the organisms have been of a benthic nature. To our knowledge, few reports exist on the occurrence of elevated THA in pelagic marine organisms. The detection of around 10% THA in the pelagic jellyfish *Aurelia* sp. represents a novel finding and, to our knowledge, is the first report of this unusual FA as a major PUFA in a pelagic marine

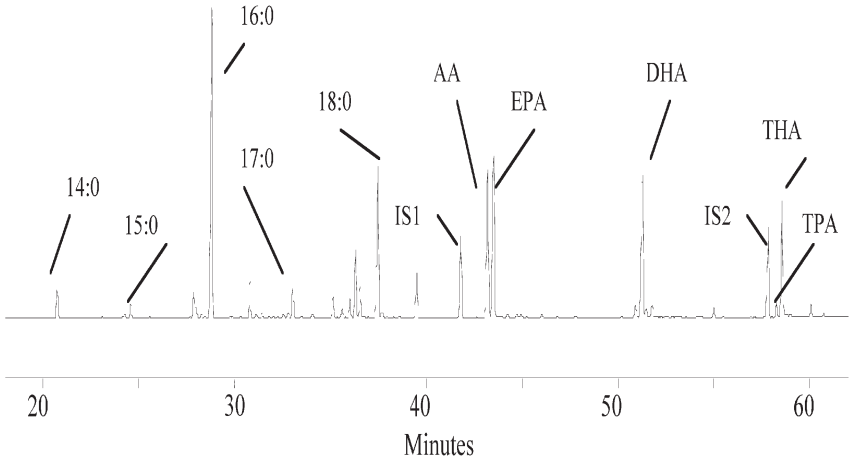

FIG. 1. Partial gas chromatogram of the total FA of the pelagic jellyfish *Aurelia* sp. HP-5 capillary column (50 m × 0.32 μm; Agilent, Palo Alto, CA). IS denotes FAME internal standards, IS1 = 19:0 and IS2 = 23:0. AA, arachidonic acid; THA, tetracosahexaenoic acid (24:6n-3); TPA, tetracosapentaenoic acid (24:5n-6).

organism; THA has not been reported previously as a major PUFA in any marine organism from Australian waters. Both THA and TPA are likely to be constituents of structural lipids, and some consideration of their potential role for the jellyfish in future research would be fruitful.

We aim to assess other potential prey items and the phyllosoma of the western rock lobster, *P. cygnus*, to ascertain whether this VLC-PUFA is in other members of the food web in this region. As mentioned earlier, the lack of a dietary origin for THA (23) suggests that this VLC-PUFA may not occur at high levels in other members of the lower food web in the Western Australia region. Jellyfish-derived FA (e.g.,

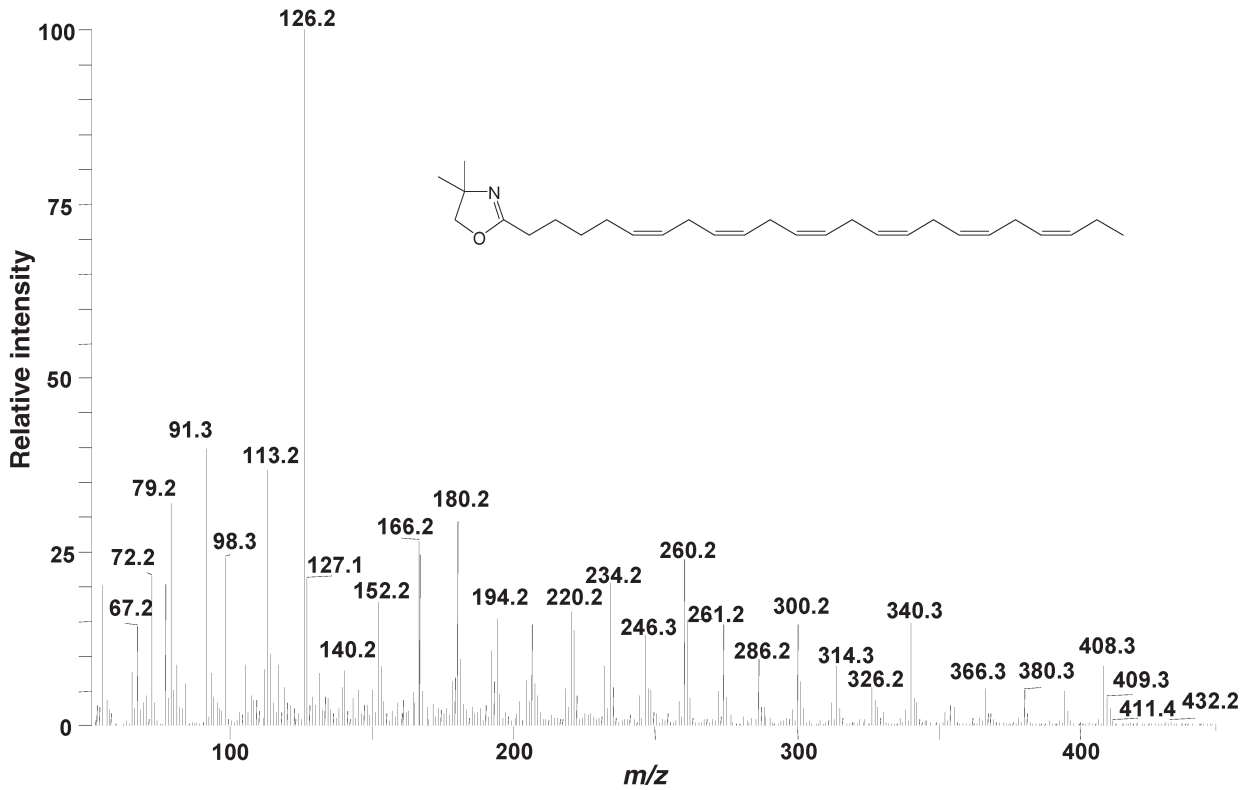

FIG. 2. EI mass spectrum of 4,4-dimethyloxazoline derivative of THA isolated from *Aurelia* sp. (relative intensity vs. *m/z*), showing $(M - 1)^+$ of *m/z* 408 and major ions at *m/z* 394, 380, 366, 354, 340, 326, 314, 300, 286, 274, 260, 246, 234, 220, 206, 194, 180, 166/167, 152/153, and 140.

trans-6-hexadecenoic acid and 7-methyl-7-hexadecenoic acid) also have been traced in the ocean sunfish *Mola mola* and marine turtles in tropical waters (26). It would also be fruitful to examine possible higher predators for *Aurelia* sp. from Western Australia waters for THA in a food web context.

With increasing recognition of the beneficial health attributes of marine n-3 FA (27), and given that the utilization of such oils in nutraceutical and other products will increase (28), the finding of the VLC-PUFA THA in lower members of the marine benthic and pelagic food web may have wider interest owing to its health benefits.

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Programming of Initial Steps in Bile Acid Synthesis by Breast-Feeding vs. Formula-Feeding in the Baboon

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ABSTRACT: We tested the hypothesis that breast- vs. formula-feeding differentially affects the enzymatic activity of three sterol hydroxylases critical in the initial steps of bile acid formation. Thirty baboons were either breast-fed or formula-fed for the first 14 wk of life before weaning to baboon chow. At 14 and 34 wk of age, liver biopsies were assayed for cholesterol 7α-hydroxylase (CYP7A1), 27-hydroxycholesterol-7α-hydroxylase (CYP7B1), and cholesterol 27-hydroxylase (CYP27A1). We also determined the kinetics of ^{3}H-27-hydroxycholesterol (27-OHC) turnover *in vivo* at both ages. At 14 wk of age, hepatic CYP7A1 activity was low but sevenfold higher among formula-fed vs. breast-fed baboons. By 34 wk, CYP7A1 activity had increased nearly 10-fold in both infant diet groups, and the sevenfold difference in CYP7A1 between previously breast- and formula-fed animals persisted. There were no differences in CYP7B1 activities between infant diet groups at either 14 or 34 wk of age although the activity increased in both groups by about 50% from 14 to 34 wk. CYP27A1 activity also increased between 14 and 34 wk of age, and, compared with CYP7A1, relatively small differences in CYP27A1 activity due to infant diet were observed at each age. Plasma 27-OHC turnover had a half-time of 2–4 min. We had previously reported that after weaning, the total bile acid synthesis rate was higher among baboons that were formula-fed than among breast-fed animals. The present results suggest that this difference is most likely due to significantly higher CYP7A1 activity among formula-fed vs. breast-fed animals.

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In previous experiments with baboons, we observed long-term differential effects (i.e., programming) of breast- vs. formula-feeding on cholesterol metabolism and plasma lipoprotein concentrations (1,2). Cholesterol-fed juvenile and adult baboons that as infants had been formula-fed had higher bile acid turnover rates than those that were breast-fed (1,3,4). Also, in the preweaning period several aspects of bile acid metabolism differed between formula-fed and breast-fed baboons (5). The current study was conducted to determine which of the key hydroxylating enzyme activities that initiate the two principal bile acid pathways (classic and alternative) are programmed by breast- and formula-feeding. We also measured these enzyme activities before and after weaning to assess their relative changes during early development. The classic pathway is initiated by the rate-limiting cholesterol 7α-hydroxylation of cholesterol by 7α-hydroxylase (CYP7A1) and occurs exclusively in the liver. This pathway is subject to hormonal regulation and feedback inhibition by bile acids (see review, 6) and produces cholic and chenodeoxycholic acids in approximately equal amounts (7). The response of CYP7A1 to dietary cholesterol varies among species (8,9), and in humans the effect of dietary cholesterol on bile acid synthesis appears to differ widely among individuals (10). The alternative pathway begins with the mitochondrial 27-hydroxylation of cholesterol by cholesterol 27-hydroxylase (CYP27A1) followed by hydroxylation by a second 7α-hydroxylase (27-hydroxycholesterol-7α-hydroxylase, CYP7B1) and in humans produces cholic and chenodeoxycholic acids in a ratio of about 1:2.5 (7). The regulation of this pathway is not as well understood, and although it is not responsive to the enterohepatic flux of bile acids in humans, it may be dependent on the availability of cholesterol for side-chain hydroxylation (11). The current study was designed to determine whether these three key enzymes, CYP7A1, CYP7B1, and CYP27A1, were affected by breast- or formula-feeding during the pre- and postweaning periods or whether their activities changed during early development in baboons. We also used these data to explain the differences in cholic and chenodeoxycholic acid biosynthesis and bile acid composition due to breast- or formula-feeding described previously (1,5).

MATERIALS AND METHODS

Experimental design and animal protocol. Thirty newborn baboons (*Papio* sp.) were derived from six breeding groups of 20 dams and one sire each and assigned to either breast-feeding or formula-feeding by a restricted random process designed to balance the diet groups by sire group and sex. The breast-fed infants remained with their mothers throughout the study and were weaned naturally to a solid baboon chow. The infants in the group fed Similac® formula (Ross Laboratories, Columbus,

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Abbreviations: 27-OHC, 27-hydroxycholesterol; CYP7A1, cholesterol 7α-hydroxylase; CYP7B1, 27-hydroxycholesterol-7α-hydroxylase; CYP27A1, cholesterol 27-hydroxylase; MTBE, methyl *t*-butyl ether; SFBR, Southwest Foundation for Biomedical Research; SPE, solid-phase extraction; StAR, steroidogenic acute regulatory; STE buffer, 0.25 M sucrose, 5 mM Tris, and 1 mM EGTA, pH 7.2; TMS, trimethylsilyl.

OH) in the nursery were weaned by adding the baboon chow to the formula in a bowl beginning at 15 wk of age. All animals were completely weaned by about 25 wk of age. The compositions of baboon breast milk and the Similac formula without iron are described elsewhere (12). The energy content of the baboon chow (Teklad 8715 Monkey Diet; Harlan-Teklad, Bartonville, IL) was 3.07 Cal/g: 75% carbohydrate, 18% protein, and 7% fat. Fatty acid distribution was 24% saturated, 25% monounsaturated, and 51% polyunsaturated. Cholesterol content was 0.02 mg/g.

At about 14 and 34 wk of age, several core liver biopsies (total 75–100 mg) were taken from each animal with a Menghini needle, 120 × 1.8 mm, under light sedation that included the following (per kg body weight): ketamine, 5.6 mg; rompin, 0.8 mg; acepromazine, 0.12 mg; and atropine, 0.012 mg. For the preweaning biopsies (14 wk), nursery-reared infants were fasted overnight (about 15 h) before biopsies were obtained. Breast-fed infants could not be fasted because they remained with their dams until the morning of the biopsy. The dams were sedated after an overnight fast, and infants were removed from them before 8:00 A.M. and sedated. All biopsies were taken between 8:55 and 10:20 A.M. to minimize the effects of a diurnal rhythm. A stomach tube was inserted, and the stomach was emptied to prevent aspiration during the procedure. All animals were fasted overnight before the postweaning (34-wk) biopsy was taken.

At 14 wk of age, the body mass of breast-fed baboons (1.63 ± 0.05 kg, mean ± SE) was not significantly different from those fed formula (1.53 kg ± 0.06 kg, $P > 0.1$). At 34 wk a similar difference in body mass was not statistically significant: 2.95 ± 0.11 kg for the previously breast-fed group and 2.83 ± 0.11 kg for those fed formula ($P > 0.1$). The males were about 140 g larger than females at 14 wk and 200 g larger at 34 wk.

The Institutional Animal Care Committee of the Southwest Foundation for Biomedical Research (SFBR) approved the animal protocols. SFBR and the University of Texas Health Science Center at San Antonio are accredited by the American Association of Accreditation of Laboratory Animal Care.

Enzyme assays. The liver biopsies were placed immediately in cold STE buffer containing 0.25 M sucrose, 5 mM Tris, and 1 mM EGTA, pH 7.2, and homogenized with a 2-mL glass/Teflon homogenizing apparatus (13). Nuclei and cellular debris were removed by centrifugation for 3 min at 1,100 × *g*. Mitochondria were isolated from the supernatant by centrifugation at 11,600 × *g* for 5 min. The mitochondrial pellet was washed with STE buffer and recentrifuged (14). The 11,600 × *g* supernatant was centrifuged at 16,000 × *g* for 5 min to remove mitochondrial fragments; the resulting supernatant was centrifuged at 100,000 × *g* for 15 min to isolate the microsomal fraction. The microsomes were resuspended in buffer and stored at –80°C before analysis. Protein concentrations of mitochondrial and microsomal suspensions were assayed using the Micro BCA™ Protein Assay Reagent from Pierce (Rockford, IL).

CYP27A1 activity was assayed within 1 h with freshly prepared hepatic mitochondria by modification of the method of Reiss *et al.* (15). To each of three test tubes (on ice) we added 0.25 μCi (7.7 μg) of purified 4-^{14}C-cholesterol dissolved in aqueous (45% wt/vol) 2-hydroxypropyl β-cyclodextrin to 100 μg of mitochondrial protein suspended in a total volume of 0.4 mL of 50 mM Tris-HCl buffer, pH 7.7, with added NADPH, isocitrate, and isocitrate dehydrogenase. Two of the tubes were incubated at 37°C for 30 min, and the third tube was kept on ice as a control. The assays were stopped by addition of 1 mL of ethanol containing 75 μg of BHT to minimize autoxidation, and the specimens were stored at –20°C before analysis. The sterols were isolated by adding 2 mL of isotonic saline to each tube and 5 mL of hexane/ethyl acetate (1:1, vol/vol), followed by vigorous shaking. The tubes were centrifuged for 5 min at low speed, and the upper phase was transferred to a 13 × 100 glass culture tube. The lower phase was again extracted with 2 mL of hexane/ethyl acetate (1:1). The combined extracts were evaporated to near dryness under N_2 gas, and the residue was stored at 4°C in isooctane/2-propanol (60:40, vol/vol) before HPLC separation. The sterols were fractionated with isooctane/2-propanol (97:3) on a Resolve™ silica radial compression cartridge, 10 μm particle size, 8 mm × 10 cm (Waters, Milford, MA). The cholesterol and 27-hydroxycholesterol[1] fractions were analyzed by liquid scintillation counting. The data were expressed both as the percentage of the labeled cholesterol substrate converted to 27-hydroxycholesterol and as pmol converted/min/mg mitochondrial protein.

CYP7A1 activity in microsomes was assayed by modifications of the method of Martin *et al.* (16). We added a solution of 5 μg of high-purity cholesterol dissolved in 5 μL of aqueous 2-hydroxypropyl β-cyclodextrin to 100 μg of microsomal protein suspended in 0.1 M potassium phosphate buffer, pH 7.4, with 1 mM EDTA in a total volume of 0.205 mL. The tubes were preincubated for 3 min at 37°C to suspend the substrate in the microsomal preparation, and NADPH was added to a final concentration of 1 mM in a total volume of 0.255 mL. After a 10-min incubation at 37°C, we terminated the reaction by adding 20 μL of 5 N NaOH. Identical tubes were kept on ice as controls. A deuterated internal standard of $^2H_5$-7α-hydroxycholesterol (77.5 pmol) in 25 μL of benzene was added to each tube. Assays and controls were performed in duplicate for 16 animals and stored at –80°C until the sterols were separated by HPLC as described below. The results from the control assays were subtracted from the incubated sample values to correct for endogenous 7α-hydroxycholesterol present in microsomes and that formed by autooxidation.

CYP7B1 activity was assayed in the same animals as assayed for CYP7A1 by a similar procedure except that the substrate was 5 μg of 27-hydroxycholesterol (Research Plus, Bayonne, NJ). After incubation, 102.7 pmol of $^2H_6$-7α,27-dihydroxycholesterol in chloroform/2-propanol (95:5) was added as an internal standard. The standardization was checked by GC–MS against

[1]The chemical name of this compound is (25*R*)-cholest-5-ene-3β,26-diol. The prevalent trivial name is 27-hydroxycholesterol, but the traditional name of 26-hydroxycholesterol may also be used, especially when the C-25 stereochemistry (or the stereochemical homogeneity) is not known. Although both isomers are produced in mammals, mitochondrial hydroxylation is believed to give only the 25*R* isomer.

purified $^2H_2$-7α,27-dihydroxycholesterol (16,16-dideutero) synthesized as described by Li *et al.* (17). The enzyme assays were performed for 16 animals in duplicate with a single unincubated control for each animal. The tubes were stored at −80°C until analyzed.

The assays for CYP7A1 and CYP7B1 were extracted with 2 × 2 mL of ethyl acetate. The combined extract was evaporated under argon gas and immediately resuspended in 0.2 mL of 2-propanol/isoctane (30:70, vol/vol). The samples were fractionated by HPLC using isooctane/2-propanol, 85:15, on a Radial-Pak silica column, 8 mm × 10 cm, and, depending on the assay, the 7α-hydroxycholesterol or 7α,27-dihydroxycholesterol fraction was collected. Immediately before GC–MS analysis, the solvent was evaporated to dryness, and the residue was silylated with 50 μL of Tri-Sil TBT (Pierce). The sterol derivatives were separated on a Trace 2000 gas chromatograph equipped with a DB-17MS capillary column with a 0.25 μm film thickness (0.25 mm × 15 m; J&W Scientific, Folsom, CA). Conditions were as follows: carrier gas, helium; flow rate, 1.2 mL/min; injector temperature, 240°C; injection mode, splitless; column temperature, 180°C held for 1 min followed by an increase to 280°C at 30°C/min. The mass ratios at 456:461 and 544:550 for CYP7A1 and CYP7B1, respectively, were measured by selected ion monitoring on a Finnigan SSQ700 instrument; ionization, electron impact with positive ion detection.

27-Hydroxycholesterol (OHC) turnover. The turnover of 27-OHC, the product of CYP27A1, was measured in three breast-fed and two formula-fed baboons. A side-chain ditritiated derivative of 27-OHC (predominantly 22,23-^{3}H) was synthesized as described by Ni *et al.* (18) and was purified before use by HPLC on a C_{18} Resolve Radial-Pak cartridge (Waters, Milford, MA) using a helium-sparged mobile phase of acetonitrile/2-propanol/methanol (20:3:7, by vol) at 0.8 mL/min. About 18 h before injection of the purified sterol, 40 μCi of ^{3}H-27-OHC stock solution was evaporated to dryness in a small glass vial and immediately suspended in 50 μL acetone. This solution was injected very slowly into a vial containing 2 mL of sterile baboon serum during gentle magnetic stirring. The acetone was partially evaporated with nitrogen for 1 min with stirring, and the vial was sealed and stored in the refrigerator overnight to equilibrate the ^{3}H-27-OHC with the serum lipoproteins. Immediately before injection into the baboon, the serum dose containing ^{3}H-27-OHC was filtered through a 0.22-μm syringe filter into a sterile vial. The radioactivity was precisely measured, and the dose of 35 to 45 μCi was injected intravenously into the sedated infant baboon. Blood samples (1 mL) were drawn from an indwelling catheter into tubes containing 1 mg EDTA and 50 μg BHT at 2, 4, 6, 8, 11, 15, 25, and 40 min and kept on ice while the animal remained sedated. The plasma was separated by centrifugation, and 20-μL portions were counted by liquid scintillation. The remainder of the plasma was stored at −80°C prior to analysis.

Plasma specific activity of ^{3}H-27-OHC. All solutions and solvents used in processing samples for analysis were sparged with argon for 5 min before use, and vessels were purged with argon during processing. Plasma samples (0.4–0.5 mL) were thawed and transferred to screw-capped tubes containing 200 ng of internal standard ($^2H_2$-27-hydroxycholesterol, 16,16-dideutero) (17). Saponification was accomplished by adding 1.5 mL of freshly prepared ethanolic KOH (15 g KOH in 100 mL 85% ethanol) and incubating in a 70°C water bath for 30 min. The samples were extracted three times with 5 mL methyl *t*-butyl ether (MTBE), and the combined extracts were dried over anhydrous Na_2SO_4. We used solid-phase extraction (SPE) with 500-mg silica cartridges (J&W Scientific) to isolate 27-OHC prior to GC–MS analysis. The SPE cartridge was conditioned with 0.7% ethanol in hexane that was freshly prepared from anhydrous, nondenatured ethanol (Sigma-Aldrich Corp., St. Louis, MO) and with hexane that had been sparged with argon before mixing. The MTBE extract was evaporated to dryness under argon, dissolved in 0.5 mL 0.7% ethanol in hexane, and applied to the silica cartridge. Cholesterol and other neutral lipids were eluted from the cartridge with 28 mL 0.7% ethanol in hexane. The fraction containing 27-OHC was eluted with 5 mL of 10% ethanol in hexane, evaporated to dryness under argon, and stored at −20°C prior to analysis. This material was converted to TMS ether derivatives and quantified by GC–MS analysis on a DB-5ms column using a ZAB-HF spectrometer in the selected ion monitoring mode as described previously (19). Total radioactivity recovery from the blood sample obtained 2 min after the dose injection was used as a correction for isotope recovery in the extracts of later specimens. Recovery of radioactivity from the 2-min samples averaged 70% from four turnover studies. In pilot experiments we determined that at 2 min, the accumulation of 27-OHC metabolites was insignificant. For samples at later times, we assumed that none of the acidic metabolites was extracted with MTBE from the saponified samples; therefore, the principal radioactive component extracted was 27-OHC. The specific radioactivity data for 27-OHC were fitted to a sum of two exponential functions by an exponential fit program (SAS PROC NLIN; SAS, Cary, NC), and parameters of a two-pool model were computed as described previously (19).

Studies on 27-OHC uptake and metabolism of 27-OHC ester. To test for a possible cause for the rapid turnover/uptake of injected 27-OHC, we measured the uptake of ^{3}H-27-OHC by blood cells. To 9.2 mL of fresh whole citrated baboon blood we added 5 μCi of ^{3}H-27-OHC in 800 μL of baboon serum and measured the hematocrit. The mixture was incubated at 37°C and nine 1-mL aliquots were removed at the same times as used for sampling in the turnover studies, i.e., 2–40 min. Each aliquot was centrifuged at 6,200 × *g* for 1 min and refrigerated immediately. A portion of the plasma was taken for radioactive counting by liquid scintillation spectroscopy.

In an experiment with an adult baboon, we injected intravenously 3-oleoyl-^{3}H-27-OHC (7.6 μCi), suspended in serum, to determine whether the 3-oleoyl ester of 27-OHC was metabolized and whether it had kinetics similar to those of the unesterified sterol. The labeled ester was custom-synthesized by Rann Research Corp. (San Antonio, TX). The animal was maintained under anesthesia and sampled 11 times over 4 h. The plasma was recovered and extracted twice with chloroform/methanol (2:1)

(20) without saponification. The lower phase was removed and washed with pure upper phase. The lower phase was dried, redissolved in 0.5 mL 1.5% 2-propanol in hexane, and applied to a 500-mg silica SPE column. The ester fraction was recovered by eluting with 3 × 5 mL portions of 1.5% 2-propanol in hexane, and the unesterified sterols were eluted with 5 mL of 12% 2-propanol in hexane. Radioactivity in each fraction was measured by liquid scintillation counting.

Statistical analyses. Data were analyzed using ANOVA for repeated measurements. The statistical model included the effects of infant diet, sex, age, and all interactions. Prior to analysis, CYP7A1 and CYP7B1 data were logarithmically transformed to better satisfy the assumption of the ANOVA.

RESULTS

Effect of infant diet on hydroxylase activities pre- and post-weaning. CYP7A1 activity was approximately sevenfold higher in the formula-fed group than the breast-fed group, both during the preweaning period at 14 wk ($P < 0.003$) and also at 34 wk of age, 3–4 mon after weaning ($P < 0.004$; Fig. 1, Table 1). In addition, the CYP7A1 activities in both infant diet groups increased about 10-fold from 14 to 34 wk ($P < 0.0001$). We did not observe a significant difference in CYP7B1 activity between breast- and formula-feeding at either 14 or 34 wk of age (Fig. 1, Table 1). The activity of CYP7B1 increased about 50% from 14 to 34 wk of age ($P < 0.0004$). There was a slightly higher CYP27A1 activity among formula-fed baboons at 14 wk and slightly lower at 34 wk compared with animals that were breast-fed (age by infant diet interaction, $P = 0.043$) (Fig. 2, Table 1). Between 14 and 34 wk of age, the hepatic CYP27A1 activity increased about 35% overall, approximately 56% among breast-fed baboons and 15% among those fed formula. The differences between infant diet groups when expressed as a percentage of cholesterol substrate hydroxylated (data not shown, age by infant diet interaction, $P = 0.041$) (3–5% converted) showed a trend similar to those expressed as mass.

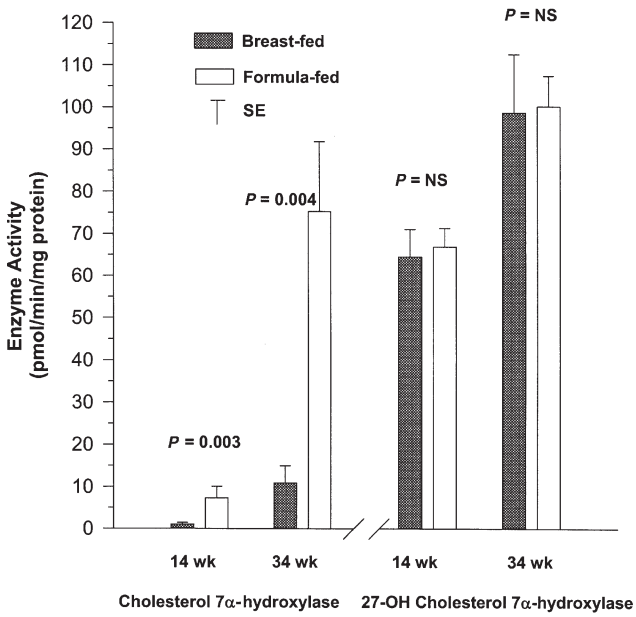

FIG. 1. Hepatic microsomal cholesterol 7α-hydroxylase (CYP7A1) and 27-hydroxycholesterol-7α-hydroxylase (CYP7B1) activities by infant diet group at 14 wk of age, prior to weaning, and at 34 wk, 3–4 mon after weaning. Data are presented as mean ± SE; NS, not significant ($P > 0.1$).

^{3}H-27-OHC turnover. The turnover of 27-OHC in infant baboons was very rapid, as was previously shown for adult baboons (19). The half-time of 27-OHC in the rapidly exchanging pool A, which includes the plasma compartment and probably the liver, was 2–4 min (data not shown). Because of the limited number of animals, the effects of infant diet or age were not tested statistically. However, at 14 wk of age the average

TABLE 1
Hepatic Steroid Hydroxylase Activities by Age and Infant Diet

Age	Infant diet	Cholesterol 7α-hydroxylase (CYP7A1)	27-OH Cholesterol 7α-hydroxylase (CYP7B1)	Cholesterol 27-hydroxylase (CYP27A1)
			(pmol/min/mg protein)	
14 wk	Breast-fed	1.03 ± 0.40[a]	64.5 ± 6.6	233.0 ± 22.0
	Formula-fed	7.33 ± 2.76	66.7 ±4.4	262.5 ± 22.6
	Ratio, formula/breast	7.12	1.03	1.12
	P value	0.003	NS[b]	NS
34 wk				
	Breast-fed	10.9 ± 4.1	98.6 ± 13.9	363.5 ± 22.9
	Formula-fed	75.2 ± 16.6	100.1 ± 7.3	300.8 ± 21.9
	Ratio, formula/breast	6.90	1.02	0.83
	P value	0.004	NS	0.058
Ratio, 34 wk/14 wk, breast-fed		10.6	1.53	1.56
Ratio, 34 wk/14 wk, formula-fed		10.7	1.50	1.15

[a]Values are mean ± SE.
[b]NS, not significant, $P > 0.05$.

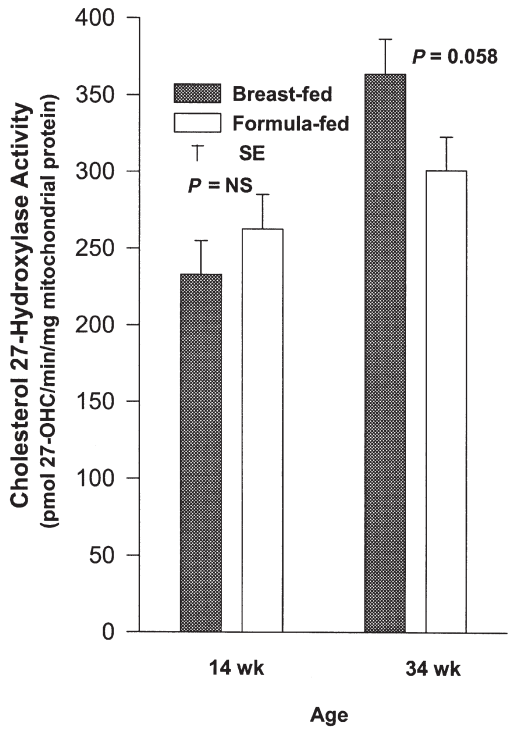

FIG. 2. Mitochondrial CYP27A1 activity at 14 and 34 wk of age by infant diet group. 27-OHC, 27-hydroxycholesterol; for other abbreviation see Figure 1.

mass of 27-OHC in pool A for the three breast-fed baboons was 44.5 nmol/kg body weight and for the two formula-fed infants was 28.5 nmol/kg body weight. At 34 wk, masses of pool A for the breast-fed and formula-fed groups were 50.4 and 24.4 nmol/kg body weight, respectively.

Sex differences. No differences between the sexes were observed for any of the variables measured at these young ages (data not shown).

Studies on 27-OHC uptake and metabolism of 27-OHC ester. In the *in vitro* experiment designed to measure the distribution of 27-OHC radioactivity between plasma and blood cells, we observed that the counts in the plasma were within 1.6% of the theoretical amount, if the distribution of counts was homogeneous between plasma and the cellular fraction. The total radioactivity in plasma was similar in all samples from 2 to 40 min. We interpret these observations to indicate that 27-OHC does not selectively bind to nor is preferentially taken up by blood cells *in vitro*, but is distributed uniformly between blood cells and plasma.

Although the 3-oleoyl ester of 27-OHC initially turned over as rapidly as the unesterified sterol (data not shown), after the 6-min sample the radioactivity increased by 8–9% for the next two time points and then began a very slow decrease up to 4 h. The mean recovery of the total counts in plasma samples that were extracted with chloroform/methanol was 91%, although only 74% of the total counts were recovered from the 4-h plasma sample. Of the extract applied to the SPE column, 91% of the counts were recovered in the ester fraction and less than 2% in the unesterified sterol fraction. The recovery of radioactivity primarily in the ester fraction was consistent across all of the time points up to 4 h. The results suggest that most of the 27-OHC ester was removed from circulation rapidly, whereas a smaller fraction was taken up slowly. The slower decay process may indicate incorporation into a lipoprotein fraction that is slowly taken up by cell receptors, or it may represent 27-OHC ester previously taken up by cells and slowly released intact back into the circulation.

DISCUSSION

These studies show that CYP7A1 activities of formula-fed baboons are sevenfold higher than among breast-fed baboons both during the infant diet period and 3–4 mon after weaning. In contrast, the hepatic activities of two of the initial enzymes of the alternative bile acid pathway are minimally affected by breast-feeding vs. formula-feeding. We conclude that breast- vs. formula-feeding differentially programs CYP7A1, the rate-limiting step in the classic bile acid pathway, for at least 4 mon beyond the infant feeding period. This effect of prior breast- vs. formula-feeding is consistent with our previous studies of the effects of infant diet on bile acid synthesis rates. Baboons that were formula-fed as infants and fed a high-cholesterol diet after weaning had higher total bile acid synthetic rates compared with those that had been breast-fed (3,4).

Preweaning. Although we observed large differences in CYP7A1 activity in the preweaning period between breast- and formula-feeding (Table 1, Fig. 1), total bile acid synthetic rates in a previous experiment (5) were not significantly different ($P > 0.1$) between these diet groups during the preweaning period. Because the CYP7A1 activity was quite low, it is likely that the classic pathway contributes relatively little to total bile acid synthesis during the preweaning period. Several lines of evidence support this inference. In contrast to CYP7A1, CYP27A1 and CYP7B1 activities were expressed at high levels during infancy and were not affected by breast- vs. formula-feeding, suggesting that the alternative bile acid pathway predominates during the preweaning period. Also, before weaning, the principal product of the alternative bile acid pathway, chenodeoxycholic acid, is the predominant bile acid in baboon bile and represents more than 65% of total bile acid synthesis (5).

Although not measured in this study, variation in cholesterol 12α-hydroxylase activity or the Δ4-3-keto 5β-reductase also could affect the cholic/chenodeoxycholic acid ratio. The extremely deleterious consequences of a defect in the CYP7B1 gene in a human infant (21), the high proportion of chenodeoxycholic acid in bile of the human fetus (22), and the gradual development of the classic pathway in the neonate suggest that the alternative pathway is also the major bile acid pathway during the perinatal period in humans. Unlike primates, mice do not develop full CYP7B1 activity until about 20–30 d of age (23), which causes greater dependence on the classic bile acid

pathway and CYP7A1 in young mice (24). The relative rates of development and regulation of the two major bile acid pathways also vary among other species (8,25).

Age-related changes: Pre- to postweaning. The nearly 10-fold increase in CYP7A1, and therefore probable enhancement of the classic bile acid pathway after weaning, is consistent with the nearly fivefold increase in total bile acid synthesis described previously (1,5). After weaning, cholic acid synthesis increased 10-fold and accounted for about 70% of the total bile acid production at 34 wk in our previous experiment (1,5). This effect increased the ratio of biliary cholic/chenodeoxycholic acids to greater than 3 at 34 wk of age (1), in contrast to the preweaning ratio of <0.5 (5). These age-related changes in bile acid synthesis rates and shift in types of biliary bile acid suggest that the classic pathway predominates after weaning. The alternative bile acid pathway likely contributes less to the age-related increase in bile acid synthesis, because after weaning, the hepatic activities of CYP7B1 and CYP27A1 increased by considerably less than the CYP7A1 activity (Table 1, Figs. 1,2).

Similar to baboons, CYP7A activities in pigs were low at birth and increased severalfold after weaning in one study (25) and by more than 10-fold in another (26), suggesting the increased importance of the classic pathway after weaning in the pig. In contrast to relatively small increases in the baboon, CYP7B1 activity in the pig increased more than threefold after weaning (25), but CYP27A1 activity in the pig did not change with age (25).

Postweaning effects of prior breast- vs. formula-feeding. In addition to the 10-fold increase in CYP7A1 activity in both infant diet groups after weaning, the sevenfold higher activity among the formula-fed vs. breast-fed baboons observed before weaning was maintained postweaning. At 34 wk, among previously formula-fed baboons compared with those that were breast-fed, both cholic acid and chenodeoxycholic acid synthesis rates were higher by about the same amount (1). That increase is consistent with the findings in humans that 7α-hydroxylation of cholesterol by CYP7A1, the rate-limiting step in the classic pathway, leads to similar amounts of cholic and chenodeoxycholic acids (7).

The limited data we have relating to the alternative pathway do not indicate that this pathway is programmed by breast- vs. formula-feeding. However, because CYP7A1 also 7α-hydroxylates 27-OHC (27), an increase in CYP7A1 activity could stimulate bile acid formation by the alternative pathway. We do not believe that this overlapping specificity of CYP7A1 could be a significant regulator of total flux through the alternative pathway because 27-OHC levels in blood and tissue are very low (19,28). Also, there is an absence of a coordinate regulation of expression of the CYP7 and CYP27 genes (29; see review, 30).

Additionally, the rate of 27-OHC synthesis may be limited not by level or activity of CYP27A1 but by transport of cholesterol by the StAR (steroidogenic acute regulatory) protein to the CYP27A1 enzyme located on the inner mitochondrial membrane (11). The StAR protein stimulates CYP27A1 activity in transfected COS-1 cells (31). Thus, further understanding of the regulation of flux through the alternative bile acid pathway should help clarify whether this pathway contributes to the programming effect of infant diet on bile acid synthesis.

Possible mechanisms. The mechanism of the early nutritional programming of bile acid synthesis is not known, although hormonal programming is a likely mediator. One possibility is altered thyroid hormone homeostasis. We observed differences in thyroid hormone levels in controlled studies with breast- and formula-fed baboons during both the pre- and postweaning periods (32,33). The promoter of the CYP7A1 gene has several binding sites for thyroid hormone receptors that mediate the significant effects of thyroid hormones on bile acid formation (34). However, the effects of thyroid hormones on CYP7A1 are dramatically different between humans and rodents, and this may explain some of the disparities in results of studies on bile acid metabolism in primates compared with other species. Studies of the role of hormonal differences among infant diet groups and understanding of the regulation of CYP7A1 gene expression will be necessary to relate hormonal changes with the programming effects of breast- vs. formula-feeding

A decrease in bile acid reabsorption or a more rapid enterohepatic circulation of bile acids by formula-fed compared with breast-fed baboons could accelerate bile acid turnover and increase CYP7A1 activities among formula-fed baboons. We are not aware of studies that confirm those possibilities in breast- or formula-fed animals.

27-OHC turnover. The rapid turnover of 27-OHC in these young baboons is similar to our previous report of half-times of several minutes for the rapidly exchanging 27-OHC compartment (pool A) in adult baboons (19). Similarly, in humans, labeled 27-OHC was metabolized rapidly and converted to cholic acid and chenodeoxycholic acid, in a ratio of about 1:2.5 (7,35). There was an apparent trend toward a larger, rapidly exchanging compartment of 27-OHC among baboons that had been breast-fed compared with those previously formula-fed, which is similar to the trend observed among several adult baboons that were breast- or formula-fed as infants (19). Further studies are necessary to determine whether breast- vs. formula-feeding affects these parameters.

Significant findings. Postweaning enhancement of CYP7A1 activity and therefore of the classic bile acid pathway is likely responsible for the higher bile acid synthetic rate reported among weaned baboons that were fed a high-cholesterol diet and formula-fed as infants compared with those that were breast-fed (1). The 10-fold increase in CYP7A1 activity after weaning is in contrast to modest increases in the activities of CYP27A1 and CYP7B1 of the alternative bile acid pathway. The two key enzymes in the alternative (acidic) bile acid pathway have high activities compared with CYP7A1 during the preweaning period, suggesting a predominance of the alternative pathway in infancy.

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Intramuscular Injection of Antigens and Adjuvant Preferentially Decreases 18:2n-6 and 18:3n-3 in Pig Neck Muscle

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ABSTRACT: Linoleic (18:2n-6) and α-linolenic acids (18:3n-3) have many important physiological functions including immunomodulation. We tested how immunization influences the metabolism of 18:2n-6 and 18:3n-3 in the neck muscle of pigs. At 35 d old, pigs received either an intramuscular neck injection containing hen egg white lysozyme (HEWL), killed *Mycobacterium tuberculosis,* and Freund's complete adjuvant (immunized) or PBS (control). At 49 d old, immunized pigs received a booster injection of HEWL and Freund's incomplete adjuvant, and the control pigs received PBS into the neck. At 56 d old, all pigs received an intradermal injection of *Mycobacterium bovis* into the hind leg to induce a delayed-type hypersensitivity (DTH) reaction. At 57 d old, immunized pigs had a twofold increase in serum haptoglobin, a 10-fold increase in antibodies to HEWL, and the skinfold at the DTH reaction site was 10 times thicker than the controls. Both 18:2n-6 and 18:3n-3 (% composition) were approximately 25% lower in muscle TG, 40% lower in FFA, 50% lower in phospholipids, but not different in cholesteryl esters of the neck muscle of immunized pigs. The antigens in this model induce an increased response in the innate (haptoglobin), humoral (antibodies), and cellular (DTH) immune systems as well as a preferential decrease of 18:2n-6 and 18:3n-3 in the inflamed neck muscle. It appears that 18:2n-6 and 18:3n-3 are preferentially metabolized (possibly β-oxidized) in response to antigens.

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The dietary need for fat and for at least two PUFA, linoleic (18:2n-6) and α-linolenic acids (18:3n-3), has been known since 1929 (1–4). Two other PUFA, arachidonic acid and EPA (20:4n-6 and 20:5n-3, respectively), have important roles in immunoregulation. The eicosanoid derivatives of 20:4n-6 and 20:5n-3 are signaling molecules that regulate immune function (5,6). PUFA also alter cytokine production and act as ligands for peroxisomal proliferator-activated receptors (7,8). Although many studies have evaluated the effects of PUFA on the function of the immune system (9,10), few have tested the effects of activation of the immune system on PUFA metabolism.

Humans in sepsis have a lower respiratory quotient, indicating lower carbohydrate oxidation and/or increased fat β-oxidation (11–13). Direct measurements indicate that fat oxidation is increased in sepsis (14). The extent to which the two parent PUFA (18:2n-6 and 18:3n-3) contribute to increased fat oxidation during immune response is not known. In a rat model of sepsis caused by puncture of the cecum, $^{14}CO_2$ recovery 6 h after an oral dose of ^{14}C-18:2n-6 was approximately two times higher than in controls (15). Rats infused with tumor necrosis factor-α have 10% less 18:2n-6 in carcass TG, suggesting that 18:2n-6 may be preferentially mobilized in this situation (16). Sepsis is a fairly extreme example of immune system activation, but even moderately increased antigen exposure in pigs can markedly increase whole-body β-oxidation of 18:2n-6 and 18:3n-3 and decrease their carcass total lipid content (17).

Therefore, various examples suggest that 18:2n-6 and 18:3n-3 are mobilized during immune system activation. Our objective was to study changes in PUFA metabolism during a controlled antibody and cell-mediated immune response in pigs. This was done by using an intramuscular (i.m.) injection of two antigens in adjuvant into the neck muscle and by inducing a delayed-type hypersensitivity (DTH) reaction (18). FA in skeletal muscle have been hypothesized to be an important fuel for immune cells (19), but to our knowledge this has not been tested. The FA profile of the inflamed muscle at the antigen injection site in the neck and at the cutaneous DTH site was measured to determine possible changes in 18:2n-6 and 18:3n-3 content. Based on our previous finding that nonspecific environmental antigen exposure decreased the carcass content of 18:2n-6 and 18:3n-3 (17), it was hypothesized that 18:2n-6 and 18:3n-3 would be preferentially decreased in these inflamed sites.

MATERIALS AND METHODS

Animals and treatment. The study was approved by the Shur-Gain Agresearch Animal Care Committee. At 14 d of age, 24 piglets were early-weaned into a clean nursery away from all non-littermates to minimize background antigen exposure (17). They were immediately started on a standard commercial pig feed, which was used throughout the study. The feed

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Abbreviations: 18:2n-6, linoleic acid; 18:3n-3, α-linolenic acid; 20:4n-6, arachidonic acid; 22:6n-3, DHA; CMI, cell-mediated immune/immunity; DTH, delayed-type hypersensitivity; FCA, Freund's complete adjuvant; HEWL, hen egg white lysozyme; HSL, hormone-sensitive lipase; i.d., intradermal; i.m., intramuscular; PPD, purified protein derivative.

(Shur-Gain Feed # 694, Guelph, Ontario, Canada) contained 477 g/kg of corn, 270 g/kg of soybean meal, 75 g/kg of wheat middlings, 74 g/kg of whey powder, 50 g/kg tallow, 36.2 g/kg of fishmeal, 10 g/kg of limestone, 7 g/kg of dicalcium phosphate, 3.3 g/kg of salt, and 17.6 g/kg of a vitamin/mineral supplement. The FA profile of the feed is shown in Table 1. At 35 d of age, 24 pigs were randomly allocated to one of two groups, the immunization group (n = 12) or the control group (n = 12).

Blood was collected from the retroorbital sinus of each animal prior to immunization (35 d of age) and at 38, 42, 45, 49, 52, 56, and 57 d of age. Feed was withdrawn 12 h prior to blood sampling to ensure fasting status. Blood was centrifuged at 1000 × g for 20 min and serum stored at –20°C for analysis of haptoglobin and antibodies to hen egg white lysozyme (HEWL). Pigs were anesthetized and killed with CO_2 at 57 d of age for FA analysis of neck muscle proximal to the site of antigen injection; liver; skin at the site of the DTH test site; the prefemoral lymph node; and adipose tissue immediately surrounding the lymph node. The muscle and DTH site were rinsed in saline, and all tissues were stored at –20°C until used for FA analysis.

Tissue FA analysis. Prior to lipid extraction, the epidermis at the DTH site was extracted and analyzed for FA. Total lipids of all tissues were extracted in chloroform/methanol (2:1, vol/vol). Cholesteryl heptadecanoate, 1,2-diheptadecanoyl-*sn*-glycerol-3-phosphocholine, triheptadecanoin, and heptadecanoic acid (Sigma Chemical Co., St. Louis, MO) were added as internal standards to an aliquot of the total FA extract to quantify total lipids. The total lipids were then recovered, and a portion of the extracted lipid was reconstituted in chloroform in preparation for TLC to separate lipid classes. Total lipid extracts and, in a separate lane, a standard containing cholesteryl heptadecanoate, 1,2-diheptadecanoyl-*sn*-glycerol-3-phosphocholine, triheptadecanoin, and heptadecanoic acid in chloroform/methanol were then streaked on Whatman K6F plates, precoated with 260 µm silica gel (Chromatographic Specialties, Brockville, Ontario, Canada), using a 25-µL Hamilton syringe. The plates were then developed for 35 min in a covered solvent tank containing petroleum ether, diethyl ether, and acetic acid (80:20:1, by vol). The plates were removed, dried in a fume hood, and sprayed with 0.02% 2′7′-dichlorofluorecein in methanol.

Phospholipid, FFA, cholesteryl ester, and TG bands were identified under UV light, scraped off the plate, and transferred to a screw-capped glass test tube. The phospholipid and cholesteryl ester bands, as well as an aliquot of the total lipid extract, were saponified in methanolic potassium hydroxide (60 g potassium hydroxide/L methanol) for 1 h at 100°C. All lipid fractions were then converted to FAME using 14% boron trifluoride/methanol at 100°C for 30 min (Sigma Chemical Co.) under nitrogen (20). FAME were analyzed by GLC using a capillary column (DB-23, 30 m × 0.25 mm; J&W Scientific, Folsom, CA) in a Hewlett-Packard 5890A gas–liquid chromatograph with automated sample delivery and injection and peak integration. Total lipid FA were quantified on the basis of the proportion in each chromatogram of the corresponding internal standard added to each sample.

TABLE 1
FA Profile of the Pig Feed[a]

FA	% of total FA	FA	% of total FA
∑ SFA	32.2 ± 0.2	18:3n-3	1.9 ± 0.1
∑ MUFA	28.9 ± 0.1	∑ n-3 LCPUFA	0.2 ± 0.1
18:2n-6	25.6 ± 0.3	n-6/n-3 PUFA	12.3 ± 0.2
∑ n-6 LCPUFA	0.2 ± 0.1	Total fat (mg/g)	68 ± 1.0

[a]Data presented as mean ± SD (n = 6). SFA, saturated FA; MUFA, monounsaturated FA; LCPUFA, long-chain PUFA.

Antibody and cell-mediated immune (CMI) response. Immune responses to antibody to HEWL (Sigma-Aldrich Canada Ltd., Oakville, ON) and CMI to *Mycobacterium* sp. were induced using standard protocols (18). Each immunized pig (35 d of age, Day 0 of the immunization schedule) received, by i.m. injection into two dorso-lateral neck muscles sites, 2 mL of a 50:50 vol/vol emulsion of Freund's complete adjuvant (FCA; Sigma-Aldrich) containing *M. tuberculosis* H37Ra, and a PBS solution of HEWL (Sigma-Aldrich), such that each animal received 10 µg of HEWL. Control pigs received 2 mL of PBS. At 49 d of age (Day 14 of the immunization schedule), immunized pigs received a second neck muscle injection of 10 µg HEWL in 2 mL of an emulsion made from equal parts of Freund's incomplete adjuvant (Sigma-Aldrich) and PBS. Control pigs received 2 mL of PBS. To test for CMI, a DTH response was induced. At 56 d of age (Day 21 of the immunization schedule), all pigs received 100 µL of purified protein derivative (PPD) of *M. bovis* tuberculin (Synbiotics, San Diego, CA) by intradermal (i.d.) injection from a 25-gauge needle and tuberculin syringe to the medial thigh. Adjacent to the PPD injection, pigs received 100 µL of PBS by i.d. injection from a 25-gauge needle and tuberculin syringe. The DTH reaction was quantified as percentage increase in double-fold skin thickness as measured using calipers (John Bull, Harpenden skinfold thickness calipers; Creative Health Products Inc., Ann Arbor, MI) at the site of injections prior to and 24 h after injection of PPD or PBS (18). At each site, three measurements were made prior to (0 h) and 24 h after injection and the mean value was determined.

With these values, the percent increase in skin thickness was calculated as follows:

$$\%\ \text{increase} = \frac{(\text{PPD 24 h} - \text{PPD 0 h})}{\text{PPD 0 h}} - \frac{(\text{PBS 24 h} - \text{PBS 0 h})}{\text{PBS 0 h}} \times 100 \quad [1]$$

Serum antibody to HEWL was measured by enzyme immunoassay as described previously (18). Briefly, 96-well plates (Immulon II HB; Fisher Scientific, Unionville, Ontario, Canada) were coated with HEWL at 1×10^{-4} mol/L in 0.05 M pH 9.6 carbonate–bicarbonate buffer by incubating at 4°C for 2 d. Plates were washed (ELx405 automatic washer; Bio-Tek Instruments Inc., Winooski, VT) with 0.05% Tween (Fisher Scientific) in PBS, blocked with 3% Tween 20 in PBS, and washed again; sera (100 µL of 1:5 and 1:125 dilu-

tions in 0.05% Tween) were then added such that four replicates of each dilution were included on each plate. Negative and positive anti-HEWL pig sera were similarly dispensed. Plates were incubated for 2 h at room temperature, then washed; next, rabbit anti-pig IgG-alkaline phosphatase (Sigma-Aldrich), 1:30,000 in 0.05% Tween–PBS, was added at 100 µL/well, incubated for 1 h at room temperature, and washed. The chromogenic substrate, *p*-nitrophenyl phosphate (Sigma-Aldrich), dissolved as recommended by the manufacturer in 10% diethanolamine, pH 9.8, was dispensed, 100 µL/well, and plates were incubated in the dark at room temperature for 30 min before the OD was measured for each well with an automatic 96-well plate reader (EL 808; Bio-Tek Instruments). For each pig serum, an index of anti-HEWL was determined using KCJr software (Bio-Tek Instruments) programmed to sum the average of the 1:5 and 1:125 dilutions. Test efficacy was ensured by comparison of values obtained for positive and negative standard sera to running means on a quality control chart. In the present experiment, OD values for each pig and day were used as such without declaration of "positive" or "negative" status with reference to negative control data.

Serum haptoglobin analysis. Serum haptoglobin (g/L) was determined by the Clinical Pathology service of the Animal Health Laboratory, The University of Guelph, using routine methods.

Statistical analysis. Data are expressed as the mean ± SD of 12 samples per group. Group means were tested for significance ($P < 0.05$) of difference using Student's *t*-test, and a rank correlation analysis was performed using Spearman's test. All statistical tests were performed with the Statistical Analysis System, version 8.02 (SAS Institute, Cary, NC).

RESULTS

Body weights and immune response. Body weights of the control and immunized pigs were not different at 35, 50, and 57 d of age (9.0 ± 0.8 vs. 8.9 ± 0.8 kg, 13.5 ± 2.0 vs. 12.8 ± 1.3 kg, and 16.7 ± 2.3 vs. 16.2 ± 1.5 kg, respectively). Serum haptoglobin did not differ between groups prior to treatment at 35 d of age, but was 83–365% higher in the immunized pigs between 38 and 57 d of age (Fig. 1A). Serum antibodies to HEWL were 2- to 10-fold higher in antigen-treated pigs between 42 and 57 d old (Fig. 1B). The DTH reaction induced a 94.0 ± 40.8% increase in double-fold skin thickness in immunized pigs compared to control pigs ($P < 0.01$).

Tissue FA profiles (% composition). Immunized pigs had lower total TG but higher FFA and cholesteryl esters in the neck muscle (Tables 2,3). Total TG and phospholipids were lower in the DTH reaction site (Table 4). Immunized pigs had lower 18:2n-6 and 18:3n-3 in the TG, phospholipids, and FFA of the neck muscle but not in the TG or phospholipids at the DTH site (Tables 2–4). Immunized pigs had higher 20:4n-6 and 22:6n-3 in the TG, phospholipids, and cholesteryl esters of the neck muscle (Tables 2,3). Immunized pigs had higher 20:4n-6 and 22:6n-3 in the phospholipids of the DTH site

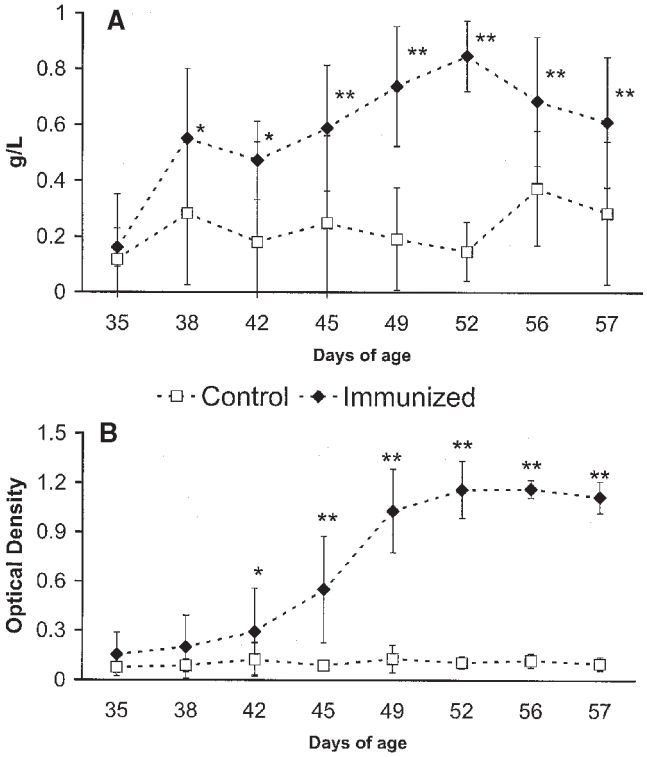

FIG. 1. Serum haptoglobin (A) and serum antibody response to hen egg white lysozyme (B) in control and immunized pigs over time. Immunization injections were given post-sampling at 35 and 49 d of age. *$P < 0.05$ vs. control; **$P < 0.01$ vs. control.

TABLE 2
TG and Phospholipid FA Profile at the Injection Site in Neck Muscle of Control and Immunized Pigs

FA	Control[a]	Immunized[a]
TG		
∑ SFA	36.0 ± 1.5	44.9 ± 8.8*
∑ MUFA	46.8 ± 1.5	41.3 ± 8.5
18:2n-6	14.5 ± 1.3	10.7 ± 2.5*
20:4n-6	0.4 ± 0.0	0.7 ± 0.7*
∑ n-6 PUFA	15.5 ± 1.3	12.5 ± 1.9
18:3n-3	0.8 ± 0.1	0.5 ± 0.2*
20:5n-3	0.1 ± 0.01	0.2 ± 0.2
22:6n-3	0.3 ± 0.0	0.6 ± 0.7*
∑ n-3 PUFA	1.5 ± 0.1	1.2 ± 0.8
Total FA (mg/g)[b]	55.9 ± 33.9	13.7 ± 12.3*
Phospholipid		
∑ SFA	36.1 ± 0.9	40.9 ± 3.7*
∑ MUFA	19.4 ± 1.4	23.1 ± 2.5*
18:2n-6	29.2 ± 1.4	14.1 ± 7.7*
20:4n-6	9.0 ± 0.9	11.5 ± 1.8*
∑ n-6 PUFA	40.6 ± 1.3	31.2 ± 5.3*
18:3n-3	0.5 ± 0.0	0.3 ± 0.2*
20:5n-3	0.8 ± 0.1	0.4 ± 0.1*
22:6n-3	1.2 ± 0.2	1.6 ± 0.4*
∑ n-3 PUFA	3.9 ± 0.4	3.8 ± 0.4
Total FA (mg/g)[b]	6.0 ± 0.7	6.5 ± 0.7

[a]% composition, $n = 12$ 57-d-old pigs per group; mean ± SD.
[b]mg/g of wet tissue. For abbreviations see Table 1. *$P < 0.05$ vs. control.

TABLE 3
FFA and Cholesteryl Ester FA Profile at the Injection Site in Neck Muscle of Control and Immunized Pigs

Tissue FA	Control[a]	Immunized[a]
FFA		
∑ SFA	21.6 ± 1.9	27.4 ± 3.2*
∑ MUFA	30.6 ± 3.3	32.7 ± 4.2
18:2n-6	27.3 ± 15.3	15.3 ± 6.0*
20:4n-6	10.3 ± 1.2	13.5 ± 2.2*
∑ n-6 PUFA	39.8 ± 2.6	34.3 ± 3.7*
18:3n-3	1.0 ± 0.1	0.3 ± 0.3*
20:5n-3	1.6 ± 0.3	0.5 ± 0.4*
22:6n-3	2.6 ± 0.5	2.3 ± 0.4
∑ n-3 PUFA	8.0 ± 1.1	5.6 ± 1.0*
Total FA (mg/g)[b]	2.7 ± 0.8	4.2 ± 1.0*
Cholesteryl ester		
∑ SFA	46.8 ± 7.7	25.8 ± 7.0*
∑ MUFA	37.8 ± 5.9	32.8 ± 5.5
18:2n-6	11.5 ± 3.6	13.5 ± 1.5*
20:4n-6	2.4 ± 1.2	16.2 ± 5.2*
∑ n-6 PUFA	14.2 ± 4.8	35.5 ± 9.7*
18:3n-3	0.7 ± 0.2	0.2 ± 0.1*
20:5n-3	0.6 ± 0.1	0.5 ± 0.1
22:6n-3	0.6 ± 0.1	2.9 ± 2.3*
∑ n-3 PUFA	2.0 ± 1.2	5.9 ± 2.6*
Total FA (mg/g)[b]	0.5 ± 0.5	1.5 ± 0.9*

[a]% composition, n = 12 57-d-old pigs per group; mean ± SD.
[b]mg of total FA per gram of tissue. For abbreviations see Table 1; for statistical parameters see Table 2.

TABLE 4
TG and Phospholipid FA Profile of the Delayed-Type Hypersensitivity Reaction Site of Control and Immunized Pigs

FA	Control[a]	Immunized[a]
TG		
∑ SFA	32.2 ± 1.5	32.8 ± 1.3
∑ MUFA	51.1 ± 1.3	50.1 ± 1.3
18:2n-6	13.9 ± 1.1	14.2 ± 1.0
20:4n-6	0.2 ± 0.0	0.2 ± 0.0
∑ n-6 PUFA	15.0 ± 1.2	15.2 ± 1.0
18:3n-3	0.7 ± 0.1	0.7 ± 0.1
20:5n-3	0.1 ± 0.02	0.1 ± 0.04
22:6n-3	0.2 ± 0.1	0.2 ± 0.0
∑ n-3 PUFA	1.4 ± 0.3	1.4 ± 0.3
Total FA (mg/g)[b]	270 ± 79	136 ± 53*
Phospholipid		
∑ SFA	39.4 ± 4.9	45.8 ± 5.2*
∑ MUFA	48.3 ± 6.2	34.8 ± 7.5*
18:2n-6	6.6 ± 1.3	7.2 ± 0.9
20:4n-6	1.9 ± 0.7	5.5 ± 2.5*
∑ n-6 PUFA	9.6 ± 2.2	15.2 ± 3.5*
18:3n-3	0.3 ± 0.1	0.4 ± 0.2
20:5n-3	0.3 ± 0.1	0.3 ± 0.1
22:6n-3	0.4 ± 0.2	1.0 ± 0.3*
∑ n-3 PUFA	2.2 ± 0.9	3.5 ± 0.8*
Total FA (mg/g)[b]	7.6 ± 3.6	3.8 ± 1.1*

[a]% composition, n = 12 57-d-old pigs per group; mean ± SD.
[b]mg/g of wet tissue. For abbreviations see Table 1; for statistical parameters see Table 2.

TABLE 5
Total Lipid FA Profile of the Liver and Prefemoral Lymph Node in Control and Immunized Pigs

Tissue FA	Control[a]	Treatment[a]
Liver total lipids		
∑ SFA	40.1 ± 1.81	41.0 ± 0.8
∑ MUFA	16.9 ± 1.3	16.3 ± 0.9
18:2n-6	14.8 ± 1.8	14.8 ± 0.4
20:4n-6	16.2 ± 0.7	15.4 ± 0.6*
∑ n-6 PUFA	32.9 ± 4.4	32.5 ± 0.8
18:3n-3	0.3 ± 0.1	0.3 ± 0.1
22:6n-3	5.8 ± 0.5	5.8 ± 0.5
∑ n-3 PUFA	9.8 ± 0.6	10.0 ± 0.3
Total FA (mg/g)[b]	41.6 ± 4.5	39.3 ± 7.1
Lymph total lipids		
∑ SFA	35.9 ± 2.3	35.7 ± 2.6
∑ MUFA	42.1 ± 2.5	41.9 ± 3.0
18:2n-6	13.6 ± 1.9	13.4 ± 1.8
20:4n-6	4.1 ± 1.7	4.3 ± 1.4
∑ n-6 PUFA	19.3 ± 0.8	19.5 ± 1.0
18:3n-3	0.7 ± 0.2	0.6 ± 0.1
22:6n-3	0.5 ± 0.1	0.5 ± 0.2
∑ n-3 PUFA	2.3 ± 0.2	2.4 ± 0.3
Total FA (mg/g)[b]	91.7 ± 7.4	84.7 ± 6.1

[a]% composition, n = 12 57-d-old pigs per group; mean ± SD.
[b]mg/g of wet tissue. For abbreviations see Table 1; for statistical parameters see Table 2.

(Table 4). The rise in 20:4n-6 and 22:6n-3 correlated with the percentage increase in double-fold skin thickness at the DTH reaction sites ($r^2 = 0.69$, $P < 0.01$, and $r^2 = 0.75$, $P < 0.01$, respectively). In liver total lipids, only 20:4n-6 was 5% lower in immunized pigs compared to controls. Changes in liver 20:4n-6 correlated negatively with serum haptoglobin ($r^2 = -0.58$, $P < 0.01$). There were no differences between treatments in any other liver total lipid FA profiles or concentrations. There were also no differences between groups in FA profiles or concentrations in the total lipid fraction of the prefemoral lymph node (Table 5) or the FA profile of the adipose surrounding the lymph node (data not shown).

DISCUSSION

The immunization protocol used in this study induced well-known responses from the innate (haptoglobin), humoral (antibodies to HEWL), and cell-mediated (DTH reaction) immune response systems (18,21). This study shows for the first time that a controlled immunization has a marked effect on the FA profile, especially the PUFA content, of the reactive tissues proximal to the immunization and at the DTH test sites. The neck muscle proximal to the site of injection of immunized pigs had preferentially decreased 18:2n-6 and 18:3n-3 in both the TG and phospholipid fractions. The decrease in muscle TG is consistent with the theory that these

FA are an important fuel during immune stimulation (19). More specifically, the β-oxidation of FA is increased in stimulated lymphocytes (22,23), and 18:2n-6 and 18:3n-3 from TG are most readily utilized (24). The decrease in muscle TG is also broadly consistent with our previous report showing decreased carcass content and increased β-oxidation of 18:2n-6 and 18:3n-3 in pigs chronically exposed to antigens (17). It is possible that some of the changes in FA metabolism seen are related to infiltration of immune cells. However, quantitatively this response is fairly small and mostly perivascular, and these immune cells do not contain significant amounts of TG (25–29). That there were no changes in the FA composition of the prefemoral lymph node or the phospholipid concentration of the muscle tissue also supports the notion that the changes observed were due to changes in tissue FA metabolism and not to the infiltration of immune cells.

The size of the dermal DTH reaction is an *in vivo* indicator of T cell function in pigs and humans (18,30). Although the 18:2n-6 and 18:3n-3 content of the DTH site did not change in immunized vs. control pigs, 20:4n-6 and 22:6n-3 increased in the DTH site phospholipids. The lower 18:2n-6 and 18:3n-3 in the muscle of immunized pigs but not in the DTH reaction site may reflect the different nature of these inflammatory reactions and the fact that antigens were injected into the neck muscle 14 d prior to sampling the site but only 1 d prior to sampling the cutaneous DTH reaction sites. The i.m. injection sites develop a severe, persistent, granulomatous reaction to the adjuvant and killed *M. tuberculosis,* which may become encapsulated abscesses some of which fistulate (31). The cutaneous DTH reaction is initiated by antigen-activating T-lymphocytes, the products of prior immunization. The resulting inflammatory cascade induces local infiltration, typically of mononuclear cells but also of neutrophils, basophils, and eosinophils in proportions that vary by time and by species (31,32). Unlike the FCA-induced granuloma, the DTH reaction is transient, typically peaking 24–48 h after antigen injection and regressing over the following days.

The liver of immunized pigs had a significantly decreased content of 20:4n-6. It is possible that at least some 20:4n-6 was mobilized from the liver to the inflamed neck muscle (where 20:4n-6 was increased). Despite increased 22:6n-3 in the neck muscle, there was no change in the 22:6n-3 content of the liver, so different mechanisms must account for the different effects of immunization on these two long-chain PUFA. The FA profile of prefemoral lymph nodes was also unchanged. This node, which drains the DTH test site field, might have been expected to be immunologically activated, resulting in possible changes in the FA profile of the node, but this was not observed. The adipose depot surrounding the prefemoral lymph node is rich in 18:2n-6 and 18:3n-3 and may actively influence activity of the immune system (33,34). However, under our experimental conditions, there was no effect of immunization on the node's total lipid FA profile or concentration (Table 5). In the future it may be instructive to examine the FA profile of lymph nodes draining the tissue field at i.m. sites of antigen injections.

Hormone-sensitive lipase (HSL) is the enzyme responsible for release of FA from TG; it is expressed in adipose tissue, skeletal muscle, and testes (35). A preferential increase in the release of 18:2n-6 and 18:3n-3 occurs during fasting and in *in vitro* models in which norepinephrine is used to stimulate HSL (36,37). Knockout mice not expressing HSL have increased 18:2n-6 in adipose tissue, further supporting the apparent selectivity of this enzyme (38). The preferential release of 18:2n-6 and 18:3n-3 is consistent with the decreased 18:2n-6 and 18:3n-3 observed in the inflamed muscle (Table 2). When released from TG, FA may bind with carnitine palmitoyltransferase, which also has a preferential affinity for 18:3n-3 and 18:2n-6 (39). Therefore, 18:2n-6 and 18:3n-3 released from TG during inflammation might be expected to undergo β-oxidation.

The immunization and DTH provocation methods used here induce responses from the innate, cell, and antibody-mediated immune systems of the pig and significantly alter the FA profile of the inflamed sites. In combination with stable isotope methodology and manipulation of dietary FA intake, this method could be used to study the relationship further between inflammation and PUFA metabolism. The immunization procedures used in this study preferentially decreased the 18:2n-6 and 18:3n-3 content of the neck muscle antigen and adjuvant injection sites, possibly using them as energy substrates.

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Ingestion of Plasmalogen Markedly Increased Plasmalogen Levels of Blood Plasma in Rats

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ABSTRACT: Plasmalogens, a subclass of phospholipids, are widely distributed in human and animals, and are taken into the body as food. However, no data exist on the intestinal absorption or fate of ingested plasmalogen. Here, we determined whether dietary plasmalogen is absorbed and whether blood and tissue concentrations increased in normal male Wistar rats by using four separate experiments. Phospholipids containing more than 20 wt% of plasmalogen extracted from the bovine brain were incorporated into test diets (10–15 wt%). In experiment 1, we estimated the absorption rate by measuring the plasmalogen vinyl ether bonds remaining in the alimentary tract of rats after the ingestion of 2 g of test diet containing 91 μmol plasmalogen. The absorption rate of plasmalogen was nearly 80 mol% after 4 h, comparable to the total phospholipid content in the test diet. In experiment 2, we observed no degradation of the plasmalogen vinyl ether bonds under *in vitro* conditions simulating those of the stomach and small intestinal lumen. In experiment 3 we confirmed a comparable absorption (36 mol%) by using a closed loop of the upper small intestine in anesthetized rats 90 min after injecting a 10 wt% brain phospholipid emulsion. Feeding a test diet containing 10 wt% brain phospholipids for 7 d increased plasmalogen concentration threefold in blood plasma and by 25% in the liver; however, no increases were seen in blood cells, skeletal muscle, brain, lungs, kidneys, or adipose tissue (experiment 4). We concluded that dietary plasmalogen is absorbed from the intestine and contributes to a large increase in plasmalogen levels in blood plasma.

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Plasmalogens are a subclass of phospholipids with vinyl ether double bonds in the *sn*-1 position (1-*O*-alk-1′-enyl). These lipids are widely distributed through human and animal tissues and in anaerobic bacteria; the average proportion of plasmalogens in the total phospholipids in the human body is about 18% (1). The brain, heart, lungs, muscle, and red blood cells contain relatively high levels of ethanolamine plasmalogens (2).

Although the role of plasmalogen is not fully understood, many reports suggest that plasmalogen is a structural component of the cell membrane that maintains cell membrane dynamics (3), and that it also has various functions within the cells. A protective role against oxidative stress has been proposed, especially in cerebral and cardiac tissues in which plasmalogen levels are higher than in other tissues (4). Plasmalogen also may be an endogenous lipidemic antioxidant, as the vinyl ether double bond is sensitive to oxidative agents (5–8). Further, the antioxidative effects of plasmalogen on lipoprotein (9,10) and its role in lipoprotein metabolism (11) have been reported. These earlier studies suggest that increases in plasmalogen levels in plasma lipoprotein protect against cholesterol oxidation and lower the incidence of coronary heart disease. Recently, Farooqui *et al.* (12) reported that plasmalogen deficiency is involved in nerve degeneration in Alzheimer's disease.

Several studies have shown that dietary alkylacylglycerol, another ether-linked lipid, is absorbed and incorporated as plasmalogen in tissues and red blood cell membranes (13), and alkylacylglycerol supplementation was found to restore tissue plasmalogen deficiency (14). However, no data have been reported on the absorption of dietary alkenyl phospholipids or plasmalogen, nor are data available on the fate of plasmalogen vinyl ether double bonds after the ingestion of foods that are common sources of plasmalogens (15).

The aims of the present study were (i) to examine the absorption of plasmalogen in bovine brain phospholipids (BPL) incorporated into test diets, and (ii) to determine whether the ingestion of plasmalogen increased plasmalogen levels in blood plasma, blood cells, and body tissues.

EXPERIMENTAL PROCEDURES

Animals and diets. Male Wistar rats (Japan SLC, Hamamatsu, Japan), weighing about 150 g, were given free access to deionized water and a semipurified stock diet (Table 1; 16–19) for a 7-d acclimation period. Four separate experiments were then performed with the acclimated rats. All rats used in the experiments were housed individually in stainless-steel cages with mesh bottoms. The cages were kept in a room in which the temperature (22–24°C), RH (40–60%), and lighting (lights on: 0800–2000 h) were controlled. This study was approved by the Hokkaido University Animal Committee, and animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

The test diets used in experiments 1 and 4 were casein–sucrose-based semipurified diets containing test lipids. Three test lipids were used in experiments 1, 2 (150 g lipid/kg diet), and 4 (100 g lipid/kg diet): soybean oil (SO; Wako Pure Chemical Industries, Tokyo, Japan), purified soybean phosphatidylcholine [SPL, more than 95 wt% phosphatidylcholine

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Abbreviations: BPL, brain phospholipids; SO, soybean oil; SPL, soybean phosphatidylcholine.

TABLE 1
Composition of Stock and Test Diets

		Test diets		
	Stock diet[a]	SO diet	SPL diet	BPL diet
	g/kg diet			
Casein[b]	250	250	250	250
Lipids[c]				
Corn oil	50			
SO		150 or 100		
SPL			150 or 100	
BPL				150 or 100
Mineral mixture[d]	40	40	40	40
Vitamin mixture[e]	10	10	10	10
Granulated vitamin E[f]	1.0	1.0	1.0	1.0
Choline bitartrate	4.0	4.0	4.0	4.0
Sucrose	to make 1 kg		to make 1 kg	

[a]The stock diet was given during the acclimation and recovery periods.
[b]Casein (ALACID; New Zealand Dairy Board, Wellington, New Zealand).
[c]In the stock diet, corn oil was used as a lipid. Three test lipids (SO, soybean oil; SPL, soybean PC; BPL, brain phospholipids) were added to the test diets as described in the Experimental Procedures section and in Table 2. Lipid levels were 150 g/kg diet in experiment 1 and 100 g/kg diet in experiment 4. Retinyl palmitate (7.66 μmol/kg diet) and ergocalciferol (0.0504 μmol/kg diet) were added to the lipids.
[d]The mineral mixture was prepared according to the method established at the AIN-76 Workshop held in 1989 (13). The mixture supplied the following minerals (mg/kg diet): Ca, 4491; P, 2997; K, 3746; Mg, 375; Fe, 100; I, 0.32; Mn, 10.0; Zn, 34.7; Cu, 6.00; Na, 4279; Cl, 6542; Se, 1.05; Mo, 1.00; Cr, 0.50; B, 0.50; V, 0.25; Sn, 2.00; As, 1.00; Si, 20.0; Ni, 1.00; F, 2.72; and Co, 0.20.
[e]The vitamin mixture was prepared in accordance with the AIN-76 mixture (14) except that menadione and L-ascorbic acid were added at 5.81 μmol/kg diet (15) and 284 mmol/kg diet (16), respectively.
[f]Vitamin E granules (Juvela; Eisai Co., Tokyo, Japan) supplied 423 μmol of all-*rac*-α-tocopheryl acetate per kilogram of diet.

(PC); EPIKURON200; Lucas Meyer GmbH, Hamburg, Germany], and BPL containing more than 20 wt% plasmalogen. Diet compositions are shown in Tables 1 and 2.

TABLE 2
Composition of Test Lipids

	Test lipids			
	BPL	SPL	SO	Corn oil
Phospholipid class (wt%)				
PC	50.8	98.0		
PE	44.1	Traces		
Others[a]	5.1	Traces		
Phospholipid subclass (mol%)				
Alkenylacyl	22.0	1.7		
Alkylacyl	1.0	1.4		
Diacyl	76.5	95.0		
Lysophospholipids	—	0.9		
Others	0.5	1.0		
FA (mol%)				
14:0	9.0	—	—	—
16:0	22.8	13.5	12.3	13.4
18:0	17.6	3.1	3.7	1.7
18:1	34.9	7.8	25.5	29.3
18:2n-6	1.3	68.2	52.3	54.9
18:3n-3	—	7.3	6.1	0.6
20:1	2.9	—	—	—
20:4n-6	3.9	—	—	—
22:5n-3	3.0	—	—	—
22:6n-3	4.5	—	—	—

[a]PI + PS + sphingomyelin. For abbreviations see Table 1. Dash, not detected.

BPL were extracted by the method of Folch (20) with some modifications. Briefly, homogenized bovine brain (approximately 400 g/brain) was completely washed with acetone and then with ethanol, and lipids were extracted from the washed brain homogenate using petroleum ether. After complete removal of the solvent, phospholipids were extracted by diethyl ether and again washed with acetone. The plasmalogen and total phospholipid contents in the extracted BPL fractions were 22.2 and 89.1 wt%, respectively, in experiment 1 and 27.9 and 93.2 wt%, respectively, in the other experiments.

Experiment 1 (dietary plasmalogen absorption in fasted rats). Acclimated rats were fasted for 1 d and then divided into three groups of six rats each. Each group of rats was fed one of the test diets described above (2 g diet/rat). After 2 or 4 h, aortic blood was collected from anesthetized rats (pentobarbital sodium, 50 mg/kg body weight; Abbott Laboratories, North Chicago, IL) into a heparinized syringe, and the rats were sacrificed. The stomach, small intestine, cecum, and colon and their contents were removed after ligation of both ends of each organ and stored at –40°C until analysis.

Experiment 2 (degradation of plasmalogen in the alimentary tract). To evaluate the survival of plasmalogen in the stomach after ingestion of a diet containing plasmalogen, 3 vol of 0.1 mol HCl/L was added to the test diet used in experiment 1 (BPL) and then incubated at 37°C for 1 h. We also examined the degradation rate of plasmalogen in a 10 wt% BPL emulsion under the same conditions as the test diet.

To evaluate the degradation of plasmalogen in the small and the large intestinal lumens, contents of the entire small and large intestines of four acclimated rats were collected between 1000 and 1100 h. The freshly prepared and pooled contents of the small intestine or cecum of four rats were added to a phosphate buffer (8 mmol phosphate/L, pH 7.4) containing BPL (final volume, 34 mL; 94 μmol plasmalogen and 360 μmol phospholipids). The cecal contents were gently suspended in the buffer and incubated under nitrogen gas at 37°C. Three small samples of the reaction mixture containing the contents of the small intestine or cecum were taken after 0, 0.5, 1, 2, or 4 h incubation.

Experiment 3 (absorption of plasmalogen from a closed loop of the small intestine). Six acclimated rats were anesthetized (pentobarbital sodium, 40 mg/kg body weight) and the upper small intestine was drawn out from a midline incision. A closed intestinal loop was made by ligating the pylorus and the mid small intestine (45 cm distal from the ligament of Treitz); 5 mL of BPL emulsion (100 g/L, 57.3 μmol plasmalogen/5 mL) emulsified with 10 g/L sodium taurocholate was then injected into the loop and the midline incision was closed. After 90 min, the rats were killed by withdrawing the aortic blood, and the closed loop with contents was removed. The contents were collected and the mucosa of the loop was also completely scraped away and collected. Both were stored at –40°C until subsequent analyses.

Experiment 4 (effects of feeding a diet containing plasmalogen for 7 d). Acclimated rats were divided into three groups of six rats and fed the test diets shown in Table 1 for

7 d. These test diets contained SO, SPL, or BPL (100 g/kg diet) as described above. Body weight and food consumption were measured every day. On the last day, aortic blood was collected from rats under pentobarbital anesthesia and was heparinized. The liver was removed after saline perfusion from the portal vein, and the brain, lungs, kidneys, heart, gastrocnemius muscle, and epididymal fat pad were removed and stored at −40°C. The stomach with its contents was also removed. The contents were collected and the pH was measured after weighing. All rats were killed between 1000 and 1130 h.

Analytical methods. The heparinized blood collected in experiments 1 and 4 was separated into plasma and blood cells by centrifugation, and the blood cells were then washed twice with saline. The frozen stomach, cecum, and colon were opened by cutting the wall, and the total contents were collected. Contents of the small intestine were collected together with the mucosa by applying pressure from outside the intestinal wall after thawing (21).

Total lipids in the blood plasma and cells, gastrointestinal contents, organs, and tissues were extracted with chloroform/methanol/saline (10:5:3, by vol) (22).

Plasmalogen in the BPL and in the total lipids extracted in the chloroform–methanol solution were measured by the iodine addition method (23–25). Briefly, an iodine solution (0.37 mmol/L) was added to the extracted lipid solution. After 10 min at room temperature, the reaction mixture was diluted with 95% ethanol. In this method, the plasmalogen vinyl ether double bonds reacted specifically with the iodine in the presence of methanol. As the iodine reacted with the vinyl ether moiety, the reduction of absorbance at 355 nm was measured photometrically. We observed that the BPL plasmalogen concentrations estimated by the iodine addition method were very closely correlated ($r = 0.984$) with those measured by the *p*-nitrophenylhydrazone method (26), in which plasmalogen was degraded to fatty aldehyde with 0.3 mol/L sulfuric acid. We also measured free fatty aldehyde in the liver by using the *p*-nitrophenylhydrazone method with 5 mmol/L sulfuric acid. Specificity of the iodine addition method for double bonds of the vinyl ether moiety in plasmalogen was very high; that is, iodine consumption by soybean lecithin and cholesterol was only 0.14 and 0.07 mol%, respectively, of that by plasmalogen. This method is convenient for tracing the vinyl ether double bonds in plasmalogen.

The phospholipid classes of the BPL and SPL were separated by TLC (chloroform/methanol/ammonia water, 65:35:8, by vol) and quantified after charring with sulfuric acid.

The phospholipid subclasses of the BPL and SPL were analyzed by HPLC. To do so, the BPL or SPL (approximately 5 mg) was suspended in 1.5 mL of Tris/HCl buffer (0.1 M, pH 7.0) containing 0.01 mmol $CaCl_2$. After the addition of 2 mL of diethyl ether and phospholipase C (*Bacillus cereus,* EC 3.1.4.3; Sigma, St. Louis, MO), the sample was vigorously mixed at room temperature for 3 h. After the diethyl ether layer was collected and evaporated, the sample was reacted with anhydride benzoic acid and 4-dimethylaminopyridine at room temperature for 3 h. Diradylglycerobenzoates were separated by HPLC (Waters 2695 separation module; Waters, Milford, MA) with TSK Silica gel 60 (4.6 × 250 mm; Tosoh, Tokyo, Japan) into three subclasses. The mobile phase was hexane/dichloromethane/acetonitrile (90:7.5:0.75, by vol) with a flow rate of 0.8 mL/min. Detection was performed at 230 nm using a Waters 2996 photodiode array detector (27).

Test lipids were hydrolyzed by KOH in ethanol, and their FA composition was measured by GLC (Shimadzu GC-14A; Shimadzu, Tokyo, Japan) with a CBP20-M25-025 capillary column (25 m i.d., 0.25 mm; Shimadzu), FID, and helium as carrier gas after methylation with 5% HCl–methanol. Initial temperature was 170°C, followed by an increase of 2°C/min to 250°C (28).

Phospholipid concentration was measured using a phosphate assay or choline oxidase procedure (Phospholipid-test Wako and Phospholipid B-test Wako; Wako Pure Chemical Industries, Osaka, Japan). Triglyceride (TG) and cholesterol concentrations in the plasma and liver were also assayed by enzymatic procedures (Triglyceride G-test Wako and Cholesterol C-test Wako; Wako Pure Chemical Industries).

Calculations and statistical analysis. We calculated the molar amounts of plasmalogen and phospholipids by taking the average M.W. of SPL, BPL, and plasmalogen in BPL as 777.3, 770.1, and 729.5, respectively.

In experiment 1, plasmalogen and phospholipid absorption rates were estimated from the disappearance rates of both lipids from the alimentary tract by using the following equation:

$$\text{absorption (\%)} = \frac{\text{[ingested plasmalogen or phospholipids (μmol)} - \text{remaining plasmalogen or phospholipids (μmol)]}}{\text{ingested plasmalogen or phospholipids (μmol)}} \times 100 \quad [1]$$

The remaining dietary lipids were estimated by subtracting the corresponding average values of the SO group from the values of the SPL or BPL group. The reliability of this method of estimation is discussed in the Discussion section below.

The results were analyzed by two-way (Table 3, Figs. 1 and 2) or one-way (Figs. 3–5) ANOVA. Duncan's multiple range test was used to determine whether mean values were significantly different ($P < 0.05$) (29). Correlation coefficients for the relationships between the results of the iodine addition method and the *p*-nitrophenylhydrazone method were assessed by the least squares method (30). These statistical analyses were performed by using the general linear model procedure of SAS (SAS version 6.07; SAS Institute Inc., Cary, NC).

RESULTS

Phospholipid classes and subclasses in the BPL. Test lipid compositions are shown in Table 2. The major phospholipid classes in the BPL were determined by TLC to be PC and phosphatidylethanolamine (PE), whereas the subclasses detected by HPLC were alkenylacyl (22.0 mol%), alkylacyl (1.0 mol%), and diacyl (76.5 mol%). No lysophospholipids were detected.

Experiment 1 (dietary plasmalogen absorption in fasted rats). In experiment 1, rats consumed the 2-g portions of the test diet within the first 10 min. However, considerable

TABLE 3
Amounts of Plasmalogen and Phopholipids in the Gastrointestinal Tracts and Their Contents[a] 2 and 4 h After Feeding 2 g of 15% Lipid Diets Containing SO, SPL, and BPL (45.5 mmol ethanolamine plasmalogen/kg diet)

	Plasmalogen (μmol)			Phospholipids (μmol)		
Position	2 h	4 h	*P* values	2 h	4 h	*P* values
Stomach						
SO	1.00 ± 0.16^{c}	0.82 ± 0.12^{c}	Lipid (L) <0.001	10.8 ± 2.7^{d}	11.5 ± 1.5^{d}	Lipid (L) <0.001
SPL	0.74 ± 0.17^{c}	0.56 ± 0.17^{c}	Time (T) <0.001	97.3 ± 12.4^{b}	18.1 ± 9.7^{d}	Time (T) <0.001
BPL	37.1 ± 1.62^{a}	7.01 ± 1.85^{b}	L × T <0.001	169 ± 9.4^{a}	64.8 ± 7.0^{c}	L × T <0.001
Small intestine						
SO	2.00 ± 0.49^{b}	1.45 ± 0.09^{b}	Lipid (L) <0.001	16.0 ± 1.0^{c}	17.1 ± 2.0^{c}	Lipid (L) <0.001
SPL	1.63 ± 0.10^{b}	1.44 ± 0.18^{b}	Time (T) 0.413	35.2 ± 2.9^{b}	17.9 ± 1.6^{c}	Time (T) 0.044
BPL	10.9 ± 1.49^{a}	9.43 ± 2.15^{a}	L × T 0.828	52.9 ± 7.0^{a}	46.7 ± 7.0^{a,b}	L × T 0.124
Cecum						
SO	4.01 ± 1.50^{b}	4.52 ± 1.07^{b}	Lipid (L) 0.007	6.55 ± 1.14^{b}	6.51 ± 0.94^{b}	Lipid (L) <0.001
SPL	5.40 ± 0.59^{b}	5.97 ± 1.56^{b}	Time (T) 0.072	5.04 ± 0.36^{b}	7.17 ± 1.40^{b}	Time (T) 0.003
BPL	6.11 ± 0.95^{b}	10.5 ± 1.24^{a}	L × T 0.196	6.11 ± 0.95^{b}	26.8 ± 5.08^{a}	L × T 0.020
Colon						
SO	0.415 ± 0.060	0.470 ± 0.092	Lipid (L) 0.381	0.667 ± 0.248	0.856 ± 0.242	Lipid (L) 0.807
SPL	0.307 ± 0.065	0.271 ± 0.098	Time (T) 0.646	0.697 ± 0.299	0.592 ± 0.227	Time (T) 0.432
BPL	0.337 ± 0.054	0.445 ± 0.211	L × T 0.808	0.592 ± 0.102	1.08 ± 0.486	L × T 0.606
Total						
SO	7.43 ± 1.60^{c}	7.26 ± 1.07^{c}	Lipid (L) <0.001	34.1 ± 3.1^{c}	35.9 ± 2.5^{c}	Lipid (L) <0.001
SPL	8.08 ± 0.79^{c}	8.23 ± 1.80^{c}	Time (T) <0.001	138 ± 11.3^{b}	43.7 ± 10.6^{c}	Time (T) <0.001
BPL	54.4 ± 2.19^{a}	27.4 ± 3.25^{b}	L × T <0.001	232 ± 10.6^{a}	139 ± 16.5^{b}	L × T <0.001

[a]Values are means ± SE (n = 6). These values contain mucosal plasmalogen and phospholipids. Values in the same position not sharing a common roman superscript letter differ significantly, $P < 0.05$, where "position" includes (e.g.) "Stomach" × "Plasmalogen" values. For other abbreviations see Table 1.

amounts of the test diet containing SPL were spilled. Intake of the test diets was 2.0 ± 0.0 g in the SO and BPL groups and 1.5 ± 0.1 g in the SPL group. Total phospholipids ingested were 289 (± 19) and 353 μmol in the SPL and BPL groups, respectively, and plasmalogen intake was 92 μmol in the BPL group.

Molar amounts of plasmalogen and phospholipids in the stomach, small intestine, cecum, and colon are shown in Table 3. The values in the SO group show the amounts of endogenous plasmalogen and phospholipids detected, including those from the mucosa, bile–pancreatic juice, and intestinal bacteria. The plasmalogen level in the cecal contents was the highest among all gastrointestinal segments in the SO group. No increases in plasmalogen and phospholipid levels in the colonic contents were observed in the BPL group. Plasmalogen and phospholipid levels in the cecal contents were higher in the BPL group than in the other groups 4 h after ingestion.

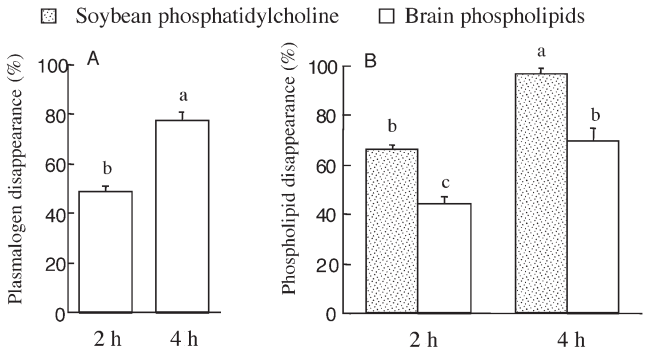

FIG. 1. Plasmalogen (A) and phospholipid (B) disappearance (absorption) from the entire alimentary tract in two groups of animals after ingestion of either 2 g of test diet containing brain phospholipids (BPL) or soybean phosphatidylcholine (SPL) in experiment 1. Details are provided in the Materials and Methods section. Each value shown is the mean ± SEM for six rats. *P* values estimated by one-way ANOVA for the plasmalogen disappearance rate were <0.001. *P* values estimated by two-way ANOVA for the phospholipid disappearance rate were <0.001 for lipid (P) and time (T), and 0.595 for P × T. Mean values not sharing a common letter are significantly different between groups ($P < 0.05$). (A) From animals fed the BPL diet (open bar); (B) from animals fed the BPL diet (open bar) and the SPL diet (stippled bar).

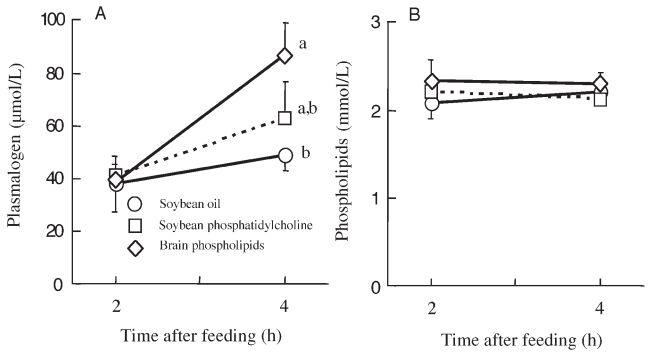

FIG. 2. Changes in plasmalogen (A) and phospholipid (B) concentrations in the blood plasma of fasted rats after feeding 2 g of test diet containing 150 g test lipid/kg diet in experiment 1. Test lipids were soybean oil (SO) (○), SPL (□), and BPL (◇). Each value shown is the mean ± SEM for six rats. *P* values estimated by two-way ANOVA for plasmalogen were 0.168 for lipid (P), 0.002 for time (T), and 0.171 for P × T. *P* values for phospholipids were 0.397 for lipid (P), 0.967 for time (T), and 0.757 for P × T. Mean values for times not sharing a common roman letter are significantly different between groups ($P < 0.05$). For abbreviations see Figure 1.

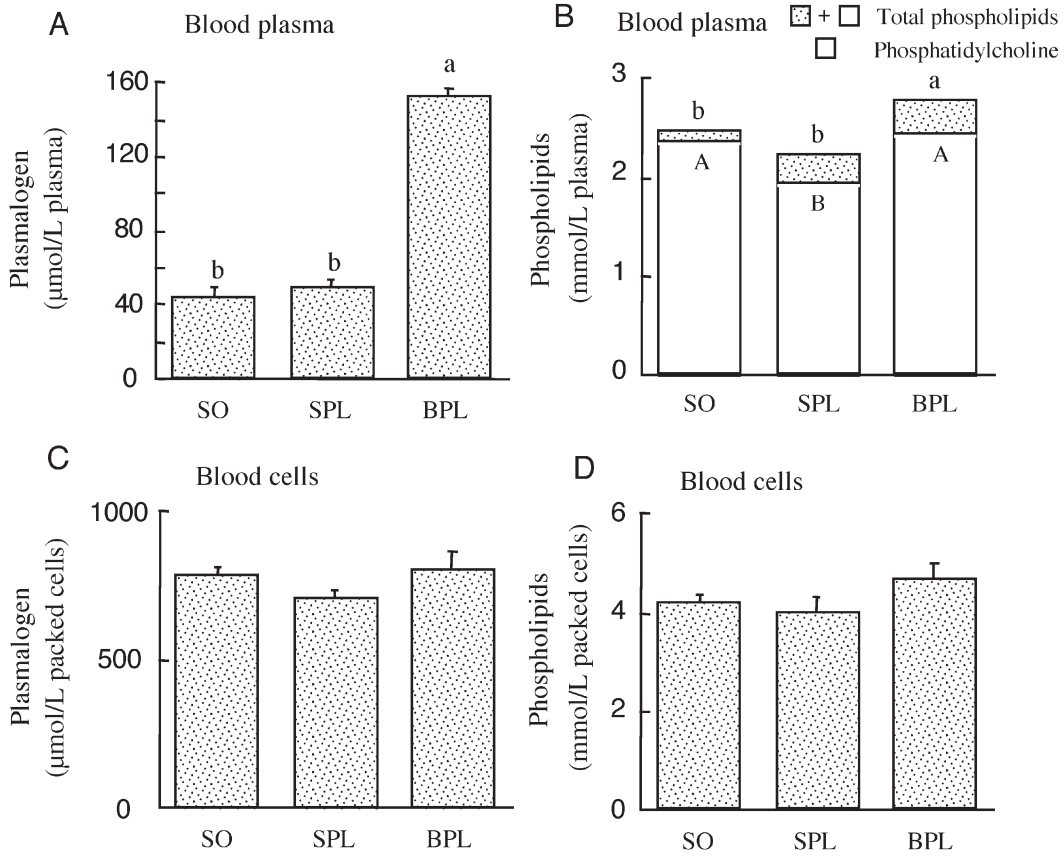

FIG. 3. Plasmalogen (panels A and C) and phospholipid (panels B and D) concentrations in the blood plasma (panels A and B) and blood cells (panels C and D) after feeding test diets containing 100 g test lipid/kg diet for 7 d in experiment 4. Test lipids were SO, SPL, and BPL. Details are provided in the Materials and Methods section. Each value shown is the mean ± SEM for six rats. *P* values were <0.001 (panel A), 0.016 and 0.022 for total and choline phospholipids (panel B), 0.435 (panel C), and 0.277 (panel D). Mean values not sharing a common roman letter are significantly different between groups ($P < 0.05$). For abbreviations see Figures 1 and 2.

The gastric content of phospholipids was higher in the BPL group than in the SPL group at 2 and 4 h. The proportions of phospholipids remaining in the gastrointestinal tract were 44.4 ± 2.62 and 15.2 ± 2.02 mol% of the total ingested lipids at 2 and 4 h, respectively, in the BPL group, and the solids contents in the stomach were 50.0 ± 1.90 and 15.9 ± 0.29 wt% at 2 and 4 h, respectively, in the BPL group, values that were comparable to those in the SO group.

Plasmalogen disappearance (absorption), as estimated from the lipids remaining in the entire alimentary tract, was approximately 50 mol% 2 h after feeding, and that amount was increased to nearly 80 mol% after 4 h (Fig. 1A). Phospholipid absorption in the BPL group was lower than that in the SPL group (Fig. 1B); however, the plasmalogen absorption rate was similar to that of phospholipids in the BPL group.

The plasmalogen concentration in blood plasma was unchanged 2 h after the ingestion of BPL. However, the concentration increased from 2 to 4 h and was two times higher than that after ingestion of SO (Fig. 2A). The total phospholipid concentration in plasma was not affected by time or diet (Fig. 2B).

Experiments 2 and 3 (examination of the fate of plasmalogen under in vitro *and* in situ *conditions).* No degradation of the plasmalogen vinyl ether bonds in the BPL diet was seen after the addition of 3 vol of 0.1 mol HCl/L solution to the diet and incubation at 37°C for 1 h (recovery rate was 100%). The pH of the medium was 3.6 after incubation. However, in the case of the emulsified BPL solution, 80 mol% plasmalogen was degraded under the same acid conditions. The pH of the medium containing 10 wt% BPL emulsion was 1.3 after incubation.

Plasmalogen in the emulsified BPL maintained its vinyl ether structure after 2 h incubation with the postprandial contents of the small intestine of rats, whereas the concentration of plasmalogen mixed and cultured with the cecal bacteria was reduced by 0.99, 7.73, and 16.0 mol% of the initial concentration at 1, 2, and 3 h after the start of the anaerobic culture at 37°C (each value was the mean of three observations). Recoveries of plasmalogen in the initial mixture (0 time) in the experiments using the small intestine and the cecum were 98 and 90 mol%, respectively.

The disappearance (absorption) of plasmalogen in the BPL from the closed loop of the upper small intestine of anesthetized rats was 36.0 ± 6.08 mol%, and that of phospholipids was 24.5 ± 8.05 mol% ($n = 6$) (experiment 3).

Experiment 4 (effects of feeding a diet containing plasmalogen for 7 d). Food intake and body weight gain in the SPL group were lower than those in the SO and BPL groups.

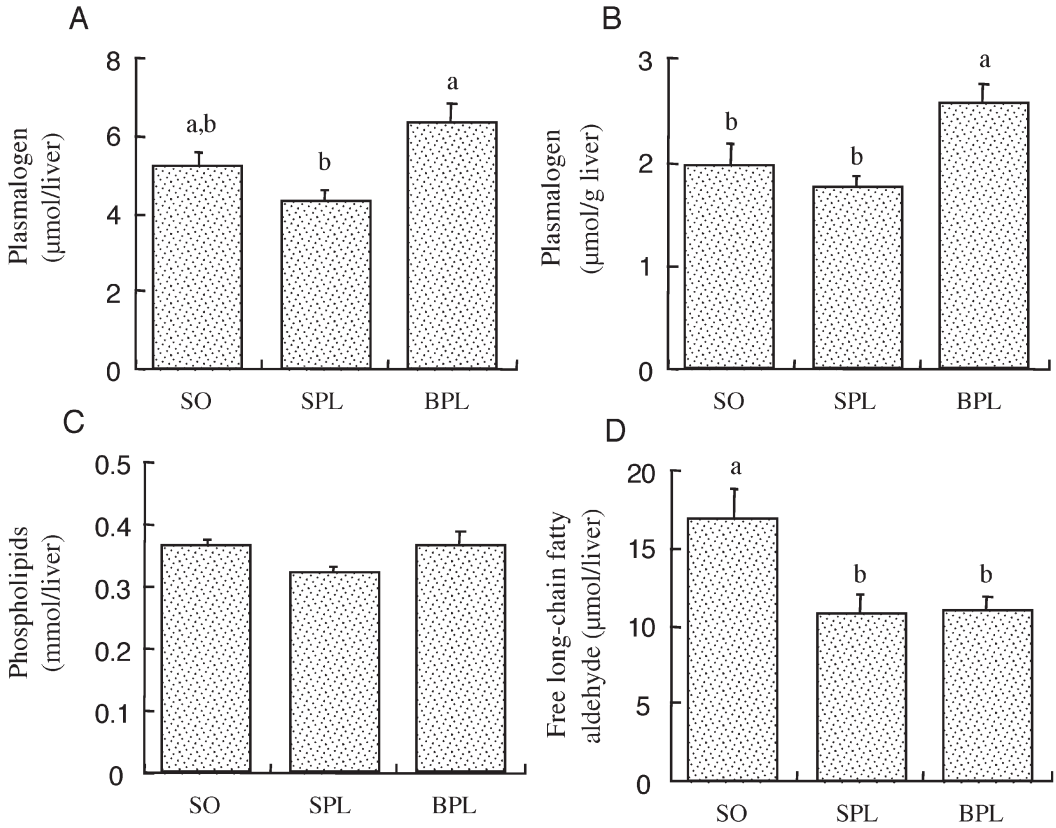

FIG. 4. Liver pools of plasmalogen (A), phospholipids (C), and free fatty aldehyde (D), and the liver concentration of plasmalogen (B) after feeding test diets containing 100 g test lipid/kg diet for 7 d in experiment 4. Test lipids were SO, SPL, and BPL. Each value shown is the mean ± SEM for six rats. *P* values were 0.007 (panel A), 0.007 (panel B), 0.094 (panel C), and 0.010 (panel D). Mean values not sharing a common roman letter are significantly different between groups ($P < 0.05$). For abbreviations see Figures 1 and 2.

Food intake in the SO, SPL, and BPL groups was 15.1, 12.0, and 14.7 g/d ($n = 6$, $P < 0.001$), respectively, and body weight gain was 7.87, 5.76, and 7.54 g/d ($n = 6$, $P < 0.001$), respectively.

Plasmalogen concentration in the blood plasma of rats fed BPL for 7 d was four times higher than that of rats fed SO or SPL (Fig. 3A). However, no differences were observed in the blood cell plasmalogen and phospholipid concentrations among the three groups (Figs. 3C and 3D). Total phospholipid concentration in the blood plasma was slightly, but significantly, higher in the BPL group than in the other groups, whereas the choline-containing phospholipid concentration was not affected by feeding the BPL diet (Fig. 3B).

In the liver, the plasmalogen concentration, but not the total phospholipid pool, was higher in the BPL group than in the SO or SPL group (Figs. 4B and 4C). The free fatty aldehyde pool was lower in rats fed SPL and BPL than in rats fed SO (Fig. 4D).

TG and cholesterol concentrations in the blood plasma were unchanged after feeding diets containing both phospholipids for 7 d (Figs. 5A and 5B). In contrast, the TG and cholesterol pools in the liver were largely decreased after feeding the SPL and BPL diets compared with those fed the SO diet (Figs. 5C and 5D).

There were no significant differences between the SO and BPL groups in the plasmalogen pools in tissues other than the liver. The average plasmalogen concentrations (sum of the SO and BPL groups, $n = 12$) were 60.5 μmol/g dry brain ($P = 0.731$, average dry weight: 308 mg), 21.8 μmol/g dry lung ($P = 0.902$, average dry weight: 182 mg), 7.32 μmol/g dry gastrocremius muscle ($P = 0.163$, average dry weight: 179 mg), 16.0 μmol/g dry kidney ($P = 0.729$, average dry weight: 347 mg), 1.51 μmol/g dry epididymal fat pad ($P = 0.409$), and 15.1 μmol/g dry heart ($P = 0.372$, average dry weight: 141 mg). The dry weight of the epididymal fat pad was lower in the BPL group than in the SO group (781 ± 63 and 981 ± 84 mg, $P = 0.039$).

The wet weight and pH of the gastric contents in the BPL group were very similar to those in the SO group. Means ± SE of both groups were 4.00 ± 0.461 g/rat ($n = 12$, $P = 0.828$) for the wet weight of the gastric contents, and 4.48 ± 0.100 ($n = 12$, $P = 0.526$) for the pH of the gastric contents.

DISCUSSION

To date, few data have been reported concerning the intestinal absorption of dietary plasmalogen or the fate of the ingested plasmalogen. In the present study, we demonstrated

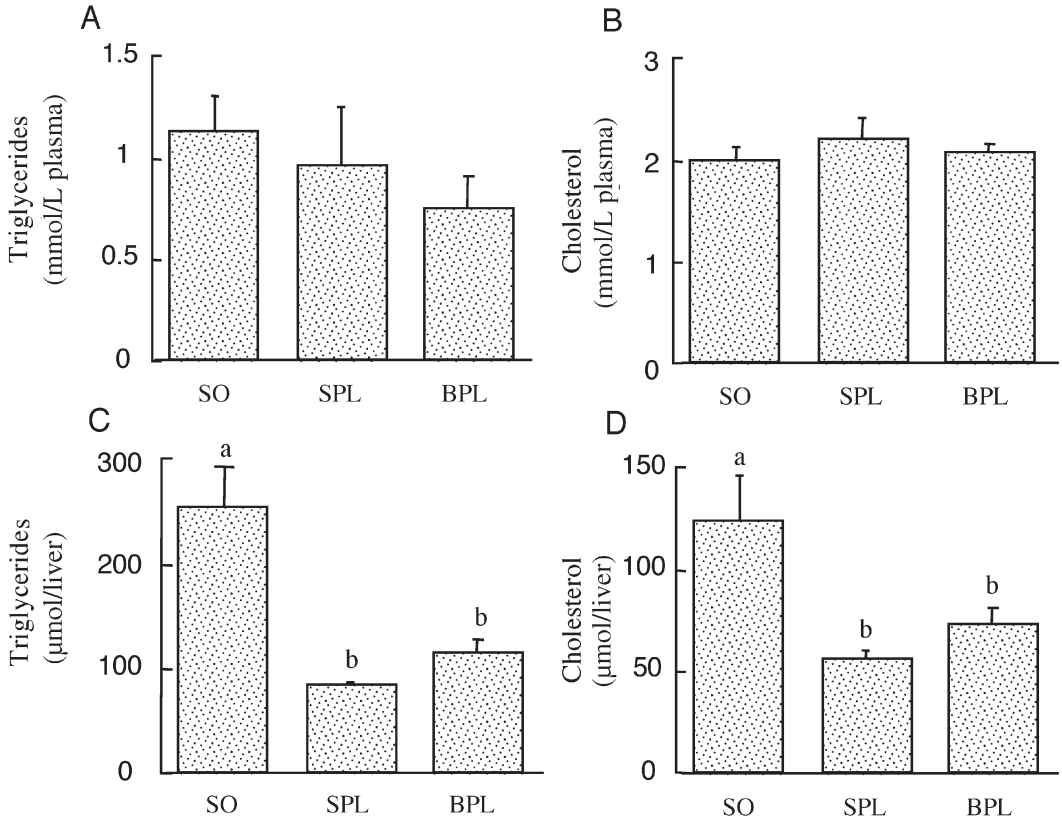

FIG. 5. Plasma concentrations of triglycerides (TG) (A) and cholesterol (B), and liver pools of TG (C) and cholesterol (D) after feeding test diets containing 100 g test lipid/kg diet for 7 d in experiment 4. Test lipids were SO, SPL, and BPL. Each value shown is the mean ± SEM for six rats. *P* values were 0.472 (panel A), 0.626 (panel), 0.001 (panel C), and 0.005 (panel D). Mean values not sharing a common roman letter are significantly different between groups ($P < 0.05$). For abbreviations see Figures 1 and 2.

that the major part of plasmalogen in BPL incorporated into a test diet disappeared from the alimentary tract 4 h after feeding; this result suggests that dietary plasmalogen is absorbed from the intestine. Vinyl ether double bonds in plasmalogen are known to be sensitive to acid. However, we showed that the acid-sensitive double bonds in the test diet were completely preserved during a 1-h exposure to excess amounts of HCl (gastric acid). This was due to the buffering action of some dietary components, such as dietary protein and some mineral salts, which prevented the pH from lowering. Actually, after incubation of a diet containing BPL with excess HCl in experiment 2, the pH was 3.6 and no degradation of the plasmalogen was observed. The pH of the stomach contents of rats was 4.5 after feeding the BPL diet in experiment 4, which was higher than the plasmalogen stability as tested by pH in experiment 2. We also found that the vinyl ether double bonds were not degraded by the contents of the small intestine. These results indicate that intestinal absorption, but not degradation, was responsible for the disappearance of ingested plasmalogen from the alimentary tract. A more important finding is the increase in plasmalogen concentration in blood plasma after feeding the test diet (Fig. 2). Also, a lower level of plasmalogen intake (approximately 2 wt% in diet) for 7 d caused a striking increase in the plasmalogen level in blood plasma, but not in blood cells (Fig. 3). We previously showed that enterally infused plasmalogen was released into the mesenteric lymph in rats cannulated into the mesenteric lymph duct (31). In the present study, we showed that the oral administration of dietary BPL in rats without lymph duct cannulation increased plasma BPL concentrations much more than in the previous study.

In the present study, plasmalogen absorption was estimated from the amount of this lipid remaining in the gastrointestinal tract together with its mucosa because the precise separation of luminal lipids from the mucosal cells was very difficult, especially in the small intestine. The amount of lipid remaining in the alimentary tract included luminal and mucosal plasmalogen derived from the diet, but endogenous lipids were excluded from our estimation of plasmalogen absorption by subtracting the values of the SO group. This means that the absorption rate in this study represents the release of vinyl ether moieties from the intestinal tract into systemic circulation.

We found that considerable amounts of plasmalogen reached the cecum, and we also observed that 16 mol% of plasmalogen was reduced by incubation with the cecal contents for 3 h. These results suggest that the cecal degradation of plasmalogen contributes in part to its disappearance from

the alimentary tract. However, the level of plasmalogen in the cecal contents was not increased 2 h after ingestion. Furthermore, the disappearance of plasmalogen from a closed loop of the upper small intestine was 36 mol% for 90 min in experiment 3. The absorption rate seems to agree with that shown in Figure 1 (50 mol% for 2 h). Thus, cecal degradation may not contribute significantly to the disappearance of plasmalogen. Also, plasmalogen was not excreted *via* the feces during the 4-h period because we observed no increase in the plasmalogen level in the colonic contents (Table 2). It has been reported that some kinds of intestinal bacteria metabolize plasmalogen (32), which agrees with our results. Also, we observed that endogenous plasmalogen, represented by the plasmalogen level in the gastrointestinal contents of the SO (control) group, was much higher in the cecum than in the other segments of the alimentary tract. This finding suggests that cecal bacteria contain plasmalogen. Anaerobic bacteria, including intestinal bacteria such as *Clostridium butyricum*, are known to be rich in plasmalogen (33).

Despite its abundance in various tissues and its importance to cell functions, few data are available on plasmalogen absorption, probably due to difficulties in the purification and quantification of intact plasmalogen. Partial hydrolysis and/or preparation of derivatives is necessary to quantify plasmalogen using chromatography (34). We adopted the iodine addition method to measure blood and tissue plasmalogen, which quantifies the vinyl ether double bonds in plasmalogen in the presence of methanol. The iodine addition method can trace the vinyl ether moiety in the lipid fraction and is suitable for the purposes of the present study. The results of this method represent the plasmalogen "activity" in the samples. Methods using isotopically labeled plasmalogen may be unable to trace intact vinyl ether double bonds, which are the most important structures of this phospholipid.

We found that the plasmalogen level in blood plasma was increased far beyond that in the control group (Fig. 3). Such increases in plasmalogen have not been reported previously. As described above, alkylacylphospholipid is converted to plasmalogen (35), which is possibly involved in the increase of plasmalogen in the blood. However, we did not find a significant amount of alkylacylphospholipid in the extracted BPL (1.0 mol%; Table 2). Thus, the increase in blood plasmalogen is derived from ingested BPL. This lipid is found in lipoprotein in the blood plasma (36). It was also reported that approximately half of the PE in lipoprotein is plasmalogen (37). As shown in Figure 3B, the total phospholipid concentration, but not the choline-containing phospholipid concentration, increased significantly after feeding a BPL diet. The increase in plasmalogen shown in Figure 3A contributed about 50% of the increase in phospholipids other than PC (Fig. 3B). The enrichment of plasmalogen in lipoprotein may reduce the susceptibility of the lipoprotein to oxidative stress and lower the incidence of coronary heart disease.

In contrast to the plasmalogen in blood plasma, feeding BPL did not increase the plasmalogen level in blood cells (Figs. 3C and 3D). The plasmalogen concentration in the blood cells was 20 times higher than that in the blood plasma (Figs. 3A and 3C), and it was reported that phospholipids in the plasma are rapidly incorporated into the red blood cell membrane (13). These results suggest that blood cell plasmalogen is maintained at a constant level.

Our results also show a small but significant increase in the liver plasmalogen concentration (Fig. 4B). Lipoproteins are synthesized in the liver, and a part of the plasmalogen in the lipoproteins may be incorporated into the liver. The increase in liver plasmalogen is possibly associated with the enrichment of plasmalogen in the blood plasma. Plasmalogen concentrations in the brain, heart, lungs, muscle, and fat tissue differed markedly, but were unchanged in spite of the large increase in blood plasma plasmalogen. Das and Hajra (35) also reported that ingested alkylglycerol is converted efficiently to plasmalogen and distributed to various tissues. However, they reported no changes in the level of plasmalogen. The level of plasmalogen in these organs or tissues may also be kept constant. The increase in plasmalogen in the blood plasma and liver described above seems to be much smaller than the amount absorbed from the intestine, as shown in Figure 1. This finding suggests the existence of high-clearance activities for the vinyl ether moiety in tissues. Recently, lysoplasmalogenase (EC 3.3.2.2, EC 3.3.2.5), by which lysoplasmalogen is degraded to fatty aldehyde, was found in the small intestine (38). The present study shows that the hepatic free fatty aldehyde pool did not increase, but rather decreased by feeding BPL. It seems that dietary plasmalogen is not degraded to free fatty aldehyde. However, we cannot exclude the possibility that the fatty aldehydes are immediately converted to fatty acids (FA) by aldehyde dehydrogenase. Thus, further work is needed to clarify the fate of absorbed plasmalogen.

Feeding a BPL diet lowered cholesterol and TG concentrations in the liver in a similar manner as feeding an SPL diet (Figs. 5C and 5D). These results show that the ethanolamine-rich phospholipid affects lipid metabolism as well as the SPL; however, the contribution of plasmalogen to the effects of BPL are not known.

We conclude that the major part of dietary plasmalogen disappeared from the intestine through intestinal absorption, not degradation into the intestinal lumen. The level of plasmalogen in blood plasma was markedly increased, which may provide increased protection against lipoprotein oxidation. In this study, we used a bovine BPL fraction as a ready source of plasmalogen; however, other sources may be available.

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Comparative Health Effects of Margarines Fortified with Plant Sterols and Stanols on a Rat Model for Hemorrhagic Stroke

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ABSTRACT: There is increased acceptance of fortifying habitual foods with plant sterols and their saturated derivatives, stanols, at levels that are considered safe. These sterols and stanols are recognized as potentially effective dietary components for lowering plasma total and LDL cholesterol. Our previous studies have shown that daily consumption of plant sterols promotes strokes and shortens the life span of stroke-prone spontaneously hypertensive (SHRSP) rats. These studies question the safety of plant sterol additives. The present study was performed to determine whether a large intake of plant stanols would cause nutritional effects similar to those seen with plant sterols in SHRSP rats. Young SHRSP rats (aged 26–29 d) were fed semipurified diets containing commercial margarines fortified with either plant stanols (1.1 g/100 g diet) or plant sterols (1.4 g/100 g diet). A reference group of SHRSP rats was fed a soybean oil diet (0.02 g plant sterols/100 g diet and no plant stanols). Compared to soybean oil, both plant stanol and plant sterol margarines significantly ($P < 0.05$) reduced the life span of SHRSP rats. At the initial stages of feeding, there was no difference in the survival rates between the two margarine groups, but after approximately 50 d of feeding, the plant stanol group had a slightly, but significantly ($P < 0.05$), lower survival rate. Blood and tissue (plasma, red blood cells, liver, and kidney) concentrations of plant sterols in the plant sterol margarine group were three to four times higher than the corresponding tissue concentrations of plant stanols in the plant stanol group. The deformability of red blood cells and the platelet count of SHRSP rats fed the plant sterol margarine were significantly ($P < 0.05$) lower than those of the plant stanol margarine and soybean oil groups at the end of the study. These parameters did not differ between the soybean oil and plant stanol margarine groups. These results suggest that, at the levels tested in the present study, plant stanols provoke hemorrhagic stroke in SHRSP rats to a slightly greater extent than plant sterols. The results also suggest that the mechanism by which plant stanols shorten the life span of SHRSP rats might differ from that of plant sterols.

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Currently, increased consumption of plant sterols (primarily campesterol, β-sitosterol, and stigmasterol) and their corresponding fully saturated derivatives, stanols (campestanol and sitostanol), are being promoted for the treatment of hypercholesterolemia, since they have potent cholesterol-lowering effects by reducing the intestinal absorption of both dietary and biliary cholesterol (1–8). Typical Western diets provide 160–360 mg plant sterols and 20–50 mg plant stanols per day, and at these levels of consumption, they have very little effect on cholesterol absorption (9). To achieve effective (10–15%) serum cholesterol reduction, the level of intake of plant sterols or stanols must be at least 2 g/d (10–13). At present, special margarines and a variety of other commercial food products fortified with 8–12% plant sterols or stanols are on the market in many countries. Consumption of these products may increase the intake of plant sterols by 10-fold and plant stanols by 100-fold compared to intakes from traditional dietary sources.

Consumption of plant sterols and stanols at recommended intakes of 2–3 g/d can be considered safe for the general population (13). The clinical studies that have examined the cholesterol-lowering efficacy of plant sterols and stanols so far have reported no adverse side effects with high consumption of these materials (1–12). This is most likely due to the lack of intestinal absorption of plant sterols and stanols by normal, healthy subjects (14). In contrast to the general population, some individuals absorb higher amounts of plant sterols than others, and consequently have elevated levels of plant sterols in the blood. A well-documented group of high absorbers of plant sterols are the patients with the rare autosomal recessive trait phytosterolemia (15). Infants receiving a daily infusion of total parental nutrition containing vegetable oil emulsions with plant sterols also have elevated levels of plant sterols in the blood (16). In these rare and special cases, elevated levels of plant sterols have been associated with atherosclerosis, xanthomata, thrombocytopenia, hemolytic anemia, poor clearance of cholesterol as bile acids, reduced bile acid synthesis, abnormal red cell morphology, decreased liver function, cholestatic liver disease, and painful arthritis of the knee and ankle joints. Several epidemiological studies also suggest that even a slight elevation in serum plant sterol levels could provoke premature coronary heart disease in a small number of hypercholesterolemic subjects, independent of serum cholesterol levels (17–20). Taken together, data from these studies suggest that in some individuals, there may be risks associated with consuming products enriched with high levels of plant sterols, particularly in those who absorb higher amounts of plant sterols. Compared to the general population, the number of

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Abbreviations: ABC, ATP transporter binding cassette; AIN, American Institute of Nutrition; DI, deformability index; LORCA, laser-assisted optical rotational cell analyzer; MUFA, *cis*-monounsaturated FA; PVP, polyvinylpyrrolidone; RBC, red blood cell; SHRSP rats, stroke-prone spontaneously hypertensive rats.

hyperabsorbers may be extremely small; however, it is difficult to predict the prevalence of "high absorbers" or to identify them in a given population.

In our previous studies, we observed adverse nutritional and biological effects of plant sterols in stroke-prone spontaneously hypertensive (SHRSP) rats (21,22), which are a model for human essential hypertension and hemorrhagic stroke (23). Consumption of plant sterols at levels of 0.1 to 0.2% of the total diet accelerated the onset of stroke and drastically reduced the life span of SHRSP rats. These effects were linked to excessive absorption and incorporation of plant sterols into various tissues with an accompanying reduction in the deformability of erythrocytes and in platelet number (21).

In normal humans and rats, the intestinal absorption of plant sterols appears to be about 4–5% of the dietary intake (24), with total plasma plant sterols in healthy humans following a normal diet ranging from 0.3 to 1.0 mg/dL (5,12). In contrast, plant stanols are virtually unabsorbable in the intestine (24,25); therefore, they are not found in the plasma of normal subjects (5). Because of the poor absorption, consumption of plant stanols might be considered safer than consumption of plant sterols. However, the safety of plant stanols has not been thoroughly studied. Therefore, in the present study we examined the effects of plant stanols on SHRSP rats. The objective of this work was to determine whether large intakes of stanols would affect the life span of SHRSP rats, as has been observed with dietary plant sterols. The study was performed using commercially available margarines enriched in plant stanols and sterols. Soybean oil, which contains a modest amount of plant sterols and virtually no plant stanols, was used as the reference dietary oil. In our previous studies, SHRSP rats fed soybean oil lived longer than SHRSP rats fed vegetable oils containing higher amounts of plant sterols (21,22).

MATERIALS AND METHODS

Test diets. Three casein-based semipurified diets were prepared using soybean oil (9 g oil/100 diet), a plant sterol-enriched margarine (14 g margarine/100 g diet), and a plant stanol margarine (14 g margarine/100 g diet) as test fats. The two margarines were purchased from a supermarket in Watertown, New York. The 14 g of margarine in the two margarine diets provided approximately 9 g fat per 100 g diet, which is equivalent to the fat content of the soybean oil diet. The other ingredients in the diets were (g/100 g diet) casein (20 g), cornstarch (50.95 g in the soybean oil diet and 45.95 g in the two margarine diets), granulated sugar (10 g), fiber (5 g), American Institute of Nutrition (AIN)-93G mineral mix (3.5 g), AIN-93 vitamin mixture (1 g), L-cystine (0.3 g), and choline bitartarate (0.25 g).

Animals and feeding phase. Young male SHRSP rats (aged 26–29 d, from the breeding colony in the Animal Resources Division of Health Canada, Ottawa; the breeding strain was originally obtained from SEAC Yoshimoti Ltd., Fukuoka-ken, Japan) were placed into three dietary groups of 28 rats per group in a completely randomized block design according to body weight. The rats were housed individually in metal cages in a climate-controlled room maintained at 22 ± 1°C and 60% RH with a 12-h day/12-h night cycle. Animals had free access to one of the three diets and drinking water. To induce earlier hypertension, the drinking water contained 10 g NaCl per liter. The diets were replaced every 4 d to minimize deterioration. Ten SHRSP rats from each group were killed after 31 d of dietary treatment (at 65–67 d of age) to collect tissue samples for biochemical analyses and pathological examination. The collection and storage of tissue samples were performed according to the procedure outlined previously (21). The remaining rats (18/group) were used to determine life span. Some rats died of acute stroke. Others were euthanized if they suffered (i) severe effects of stroke, such as being unable to move toward food and water; (ii) signs of severe pain; (iii) signs of paralysis; or (iv) inability to digest food. Canadian Council on Animal Care guidelines for the care and use of laboratory animals were followed, and the study protocol was approved by the Animal Care Committee of Health Canada.

Analytical methods. The FA compositions of dietary fats were determined by capillary GLC (22). Extraction of fat from the diet and tissues was performed according to procedures described previously (21). For determination of sterol composition, an accurately weighed sample (about 50 mg) of the lipids extracted from diets or tissue samples was mixed with 100 μg of 5α-cholestane (GC internal standard) and the mixture was saponified using ethanolic-KOH (0.5 g KOH, 0.4 mL water, 5 mL EtOH). The nonsaponifiable matter was extracted with hexane/methylene chloride (85:15, vol/vol; 2 × 9 mL) and purified with successive washes with water (2 × 9 mL) and water/EtOH (80:20, vol/vol; 1 × 9 mL). The extract was dried with anhydrous sodium sulfate, and solvents were evaporated in a hot water bath under a stream of nitrogen and then heated at 105°C for 15 min with a mixture of 150 μL of 1-methylimidazole/*N*-methyl-*N*-(trimethylsilyl)-heptafluorobutyramide (1:20, vol/vol) for converting free sterols to trimethylsilyl ether derivatives. The content and composition of the silylated sterols were determined in relation to the internal standard by GLC using a DB-1 (J&W Scientific, Folsom, CA) flexible fused-silica capillary column (30 m × 0.25 mm i.d.). Sterols were identified by comparing their GC retention times with those of authentic standards (Sigma Chemical, St. Louis, MO) and also by GC–MS analyses (Agilent 5973 mass selective detector coupled to an Agilent 6890 Series GC system; Agilent, Wilmington, DE).

Hematological parameters were measured using a Coulter Counter S-PLUS IV system (21). Red blood cell (RBC) deformability (i.e., the ability of RBC to deform in response to sheer stress) was measured using a laser-assisted optical rotational cell analyzer (LORCA; Mechatronics Instruments, Hoorn, The Netherlands). Under increasing sheer stress, RBC elongate and change the diffraction pattern of light from the laser beam. In this system, a thin layer of RBC suspension is sheared between two concentric cylinders with a gap of 0.3 mm. A laser light source traverses the blood suspension, and the amount of RBC deformation or elongation is measured as increasing shear stress is applied. For analysis, an aliquot of

EDTA-anticoagulated blood was diluted 200-fold (calculated from the sample hematocrit to ensure a constant 200× dilution of RBC) with polyvinylpyrrolidone (PVP, 300 g, Sigma PVP-360 in 6 L phosphate-buffered saline, pH 7.4, 300 mOsm/kg) solution at room temperature. A PVP–blood mixture (1.5 mL) was added to the cup of the LORCA and analysis was begun when the temperature of the cup reached 37°C. Increasing shear stress was applied to the sample by increasing the rotational speed of the cup, and the deformability index (DI) was determined at nine incremental shear stress points (from 0.5 to 49.79 Pa); DI vs. shear stress (Pa) was plotted on a logarithmic *x*-axis. For example, under the conditions used, normal RBC will deform with an DI index of 0.078 at 0.5 Pa to 0.594 at 49 Pa.

Statistical analyses of survival data were performed by using Wilcoxon's nonparametric test to compare survival curves for the effects of diet (26). The effects of diet on mean survival time and various biochemical parameters were examined by ANOVA using the STATISTICA system for personal computers (Release 5, 1997 edn.; Statsoft, Inc., Tulsa, OK). *Post hoc* comparisons of means were performed using Tukey's honest significant difference test. Differences were considered significant when $P < 0.05$. All data in tables are reported as means and SD.

RESULTS

Diets. Table 1 shows the sterol concentration of the three diets. The plant sterol-enriched margarine diet had the highest content of total plant sterols (1350 mg/100 g diet), whereas the plant stanol margarine diet had the highest content of plant stanols (1060 mg/100 g diet). The soybean oil diet had the lowest levels of plant sterols (22 mg/100 g diet) and contained no plant stanols. In all three diets, sitosterol and campesterol were the major plant sterols. Sitostanol and campestanol were the major plant stanols in the two margarines. The FA composition of the three diets is shown in Table 2. There were some differences in the FA profiles among the three diets. However, our previous study showed no relationship between the dietary FA composition and the life span of SHRSP rats (22). Thus, these differences in the FA profiles were not expected to have any influence on the development of stroke or on the life span of SHRSP rats. In all three diets, the total of the two EFA, linoleic and α-linolenic acids, accounted for more than 29% of the total FA. Since each diet contained 9 g fat/100 g diet, all the diets provided more than 2.6 g EFA/100 g diet. This level exceeded the EFA requirements for growing rats (27).

Growth. There were no significant differences in the average daily food intake among the three groups (average of the three groups, 11.8 ± 1.0 g/d). Rats in all three groups grew normally, and there were no significant differences in weekly body weights.

Length of survival. There was a significant effect of diet on survival rates (log-rank test, $P = 0.0009$, and Wilcoxon test, $P = 0.0041$) (Fig. 1). Both plant sterol margarine and plant stanol margarine significantly reduced the life span of SHRSP rats compared to soybean oil (plant sterol margarine vs. soybean oil: log-rank test, $P = 0.017$, Wilcoxon test, $P = 0.025$; plant stanol margarine vs. soybean oil: log-rank test, $P = 0.0002$; Wilcoxon test, $P = 0.0006$). At the initial stages of the feeding study, there was no difference in the survival rates between the two margarine groups (Wilcoxon test, $P = 0.27$), but after 50–55 d of feeding, the plant sterol margarine group had a slightly greater survival rate than the plant stanol margarine group (log-rank test, $P = 0.082$). The mean survival time in the soybean oil group was 87.6 (SD ± 9.3) d, significantly longer by 6 d than the plant sterol margarine group (81.7 ± 5.8 d; $P = 0.033$) or 9 d longer than the plant stanol margarine group (78.8 ± 4.3 d; $P = 0.001$). Relative to the soybean oil-fed SHRSP rats, the mean survival time of the SHRSP rats fed the plant stanol margarine was 10% lower, whereas the mean survival time of the SHRSP rats fed plant sterol margarine was only 6.7% lower than that of the soybean oil group. There was no significant difference in the mean survival times between the two margarine groups ($P = 0.417$).

General necropsy findings. Full necropsies followed by a complete histopathology were performed on all rats. Some animals died suddenly without having exhibited any symptoms;

TABLE 1
Sterol Concentration[a] (mg/100 g diet) of Diets Containing Soybean Oil, Plant Sterol Margarine, and Plant Stanol Margarine

Sterol	Soybean oil	Plant sterol margarine	Plant stanol margarine
Cholesterol	0.0 ± 0.0	5.6 ± 0.2	3.1 ± 0.0
Brassicasterol	0.0 ± 0.0	48.8 ± 1.3	4.2 ± 0.1
24-Methylene cholesterol	0.0 ± 0.0	10.4 ± 0.5	1.0 ± 0.0
Campesterol	4.4 ± 0.8	356.0 ± 9.9	20.8 ± 1.0
Stigmasterol	3.5 ± 0.9	245.6 ± 7.0	2.01 ± 1.0
β-Sitosterol	11.4 ± 1.4	652.0 ± 18.3	32.2 ± 2.1
Δ5-Avenasterol	0.9 ± 0.0	13.6 ± 0.4	0.0 ± 0.0
Campestanol	0.0 ± 0.0	8.8 ± 0.0	320.3 ± 21.0
Sitostanol	0.0 ± 0.0	16.8 ± 0.5	739.4 ± 55.1
Total plant sterols	22.0 ± 2.2	1350.4 ± 44.4	64.3 ± 4.2
Total plant stanols	0.0 ± 0.0	25.6 ± 0.4	1059.7 ± 76.1
Plant sterols + plant stanols	22.0 ± 2.2	1376.0 ± 44.9	1124.0 ± 80.3
Total sterols	22.0 ± 2.2	1381.6 ± 45.1	1127.4 ± 80.3

[a]Values are mean ± SD of three analyses.

TABLE 2
FA Composition (% total FA) of Soybean Oil, Plant Sterol Margarine, and Plant Stanol Margarine

FA	Soybean oil	Plant sterol margarine	Plant stanol margarine
14:0	0.1	0.1	0.1
16:0	9.8	6.1	7.1
18:0	3.7	6.2	4.9
ΣSFA[a]	13.9	13.3	13.0
18:1	24.6	40.5	46.1
Σ*cis*-MUFA[a]	25.3	42.1	47.2
18:2n-6	51.1	32.1	23.0
18:3n-3	8.0	3.9	6.0
Σ*cis*-PUFA[a]	59.1	36.1	29.0
t-18:1	0.0	7.3	9.2
t-18:2	0.6	0.8	0.6
t-18:3	1.1	0.5	1.0
Σ*trans* FA	1.7	8.6	10.8

[a]The sums for saturated FA (SFA), *cis*-monounsaturated FA (*cis*-MUFA), and *cis*-PUFA include minor FA not listed in the table.

occasionally animals died many days after showing minor transitional disturbances such as head tilt or slight tremors. Others were euthanized by anesthesia with isofluorene followed by collection of blood if they suffered (i) severe consequences of stroke such as hind-end paralysis, hemiplegia, or quadriplegia; (ii) signs of pain; (iii) any inability to feed themselves properly or to drink; (iv) extreme polyuria due to renal insufficiency; or (v) dehydration. After euthanasia the rats were necropsied immediately. The ones that died suddenly were placed in a refrigerator and a full necropsy was performed within the next 12 h. Acute intracranial and/or intracerebral hemorrhage was consistently observed in rats that showed typical symptoms or in ones that had died suddenly. Hemorrhage into the meninges tended to spread over the dorsal aspect of the extracerebral space on the surface of the hemispheres. Cerebral hemorrhage was characterized by the presence of blood in one or both ventricles, often leading to compression of adjacent structures. Rats that died in the later phase of the experiment had occasional focal areas of necrosis in the central nervous system. This was

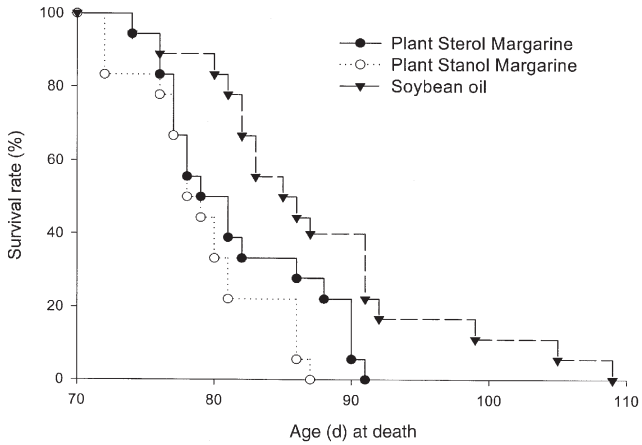

FIG. 1. Survival curves of stroke-prone spontaneously hypertensive (SHRSP) rats fed diets containing soybean oil, plant sterol-fortified margarine, and plant stanol-fortified margarine. Feeding was commenced at age 26–29 d. The mean survival times (mean ± SD, $n = 18$) of dietary groups were 87.6 ± 9.3, 81.7 ± 5.8, and 78.8 ± 4.3 d, respectively.

TABLE 3
Sterol Concentration of Serum[a] of Stroke-Prone Spontaneously Hypertensive (SHRSP) Rats Fed Soybean Oil, Plant Sterol Margarine, or Plant Stanol Margarine Diets for 31 d (mg sterol/100 g serum)

Sterol	Soybean oil	Plant sterol margarine	Plant stanol margarine
Cholesterol	89.7 ± 10.7[b]	74.2 ± 8.3[a]	90.5 ± 6.0[b]
Brassicasterol	0.1 ± 0.0[a]	0.1 ± 0.0[a]	0.1 ± 0.0[a]
24-Methylene cholesterol	0.6 ± 0.1[c]	0.3 ± 0.1[b]	0.1 ± 0.0[a]
Campesterol	6.9 ± 1.5[b]	16.4 ± 4.3[c]	1.9 ± 0.2[a]
Stigmasterol	0.3 ± 0.1[a]	0.6 ± 0.2[b]	0.4 ± 0.2[c]
β-Sitosterol	5.1 ± 1.1[b]	11.4 ± 3.2[c]	1.5 ± 0.1[a]
Δ5-Avenasterol	0.5 ± 0.1[c]	0.3 ± 0.1[b]	0.1 ± 0.1[a]
Campestanol	0.4 ± 0.3[a]	0.5 ± 0.1[a]	4.3 ± 0.9[b]
Sitostanol	0.1 ± 0.0[a]	0.2 ± 0.1[a]	4.2 ± 1.0[b]
Total plant sterols	13.6 ± 2.9[b]	29.8 ± 7.8[c]	4.3 ± 0.3[a]
Total plant stanols	0.9 ± 0.3[a]	0.7 ± 0.2[b]	8.5 ± 1.9[c]
Plant sterols + plant stanols	14.5 ± 3.2[b]	30.5 ± 8.0[c]	12.8 ± 2.2[a]
Total sterols	104.1 ± 13.9[a]	104.7 ± 15.7[a]	103.3 ± 6.9[a]

[a]Values are mean ± SD of 10 samples per group. Values in rows with different roman superscripts (a–c) are significantly different at the $P < 0.05$ level as determined by ANOVA analysis followed by Tukey's LSD test for equal *N*.

TABLE 4
Sterol Concentration of Red Blood Cells[a] of SHRSP Rats Fed Soybean Oil, Plant Sterol Margarine, or Plant Stanol Margarine Diets for 31 d (mg sterol/100 g red blood cells)

Sterol	Soybean oil	Plant sterol margarine	Plant stanol margarine
Cholesterol	112.7 ± 13.4[c]	61.2 ± 8.9[a]	90.2 ± 16.7[b]
Campesterol	4.6 ± 0.6[b]	17.9 ± 2.6[c]	2.2 ± 0.3[a]
β-Sitosterol	4.1 ± 0.6[b]	9.9 ± 3.7[a]	1.4 ± 0.3[c]
Stigmasterol	1.6 ± 0.4[c]	0.5 ± 0.3[a]	1.0 ± 0.2[b]
Δ5-Avenasterol	0.6 ± 0.1[c]	0.4 ± 0.1[b]	0.1 ± 0.1[a]
24-Methylene cholesterol	0.5 ± 0.1[b]	0.2 ± 0.1[a]	0.1 ± 0.0[a]
Campestanol	0.0 ± 0.0[a]	0.0 ± 0.0[a]	4.3 ± 0.6[b]
Sitostanol	0.0 ± 0.0[a]	0.1 ± 0.1[a]	2.5 ± 0.5[b]
Total plant sterols	13.0 ± 2.0[b]	30.2 ± 1.6[c]	5.2 ± 3.3[a]
Total plant stanols	0.0 ± 0.0[a]	0.1 ± 0.0[a]	6.8 ± 1.1[b]
Plant sterols + plant stanols	13.0 ± 2.0[a]	30.3 ± 1.7[c]	11.9 ± 4.4[a]
Total sterols	125.4 ± 14.9[c]	91.4 ± 12.8[a]	102.2 ± 17.9[a]

[a]Values are mean ± SD of 10 samples per group. Values in rows with different roman superscripts (a–c) are significantly different at the $P < 0.05$ level as determined by ANOVA analysis followed by Tukey's LSD test for equal *N*. For abbreviation see Table 3.

marked by inflammatory infiltrates that accompanied nests of macrophages filled with blood pigments. Such foci, less than 3 mm in diameter, tended to be disseminated throughout the periventricular white and gray matter. Although the rats generally appeared to be clinically healthy, with a few exceptions of dehydration and polyuria, there was a consistent observation of chronic interstitial nephritis with increased severity in older rats. In the hearts there were various focal myocardial discolorations, histologically identified as inflammatory changes.

The type and quality of abnormalities observed are consistent with those published previously (21,22). There were no differences in the type of changes observed among groups. Kidney lesions were noted throughout the groups. The severity of renal disease might be analyzed in a quantitative manner in additional investigations.

Sterol composition of tissues. The sterol composition of the diets affected the tissue sterol profiles (Tables 3–6). After 31 d of treatment, the total plant sterol content of serum, RBC, liver, and kidney was significantly higher in the plant sterol margarine-fed SHRSP rats than in the soybean oil and plant stanol margarine groups. This was primarily due to the higher concentrations of campesterol and β-sitosterol. On the other hand, the plant stanol margarine group had significantly higher tissue concentrations of campestanol and sitostanol compared to the soybean oil and plant sterol margarine groups. Although the plant stanol margarine contained a lower proportion of campestanol than sitostanol, the content of campestanol in all the tissues was similar to that of sitostanol. This might suggest a higher absorption of campestanol than sitostanol by SHRSP rats. Although the soybean oil diet contained no plant stanols, serum (Table 3), liver (Table 5), and kidney (Table 6) of the soybean oil-fed SHRSP rats showed some minor amounts of plant stanols, especially campestanol. This might suggest that the SHRSP rat has the ability to convert β-sitosterol and stigmasterol to their fully saturated

TABLE 5
Liver Sterol Concentration[a] of SHRSP Rats Fed Soybean Oil, Plant Sterol Margarine, or Plant Stanol Margarine Diets for 30 d (mg sterol/100 g liver)

Sterol	Soybean oil	Plant sterol margarine	Plant stanol margarine
Cholesterol	124.1 ± 18.5[b]	69.9 ± 15.5[a]	139.7 ± 18.9[b]
Brassicasterol	0.2 ± 0.1[a]	0.3 ± 0.1[a]	0.3 ± 0.1[a]
24-Methylene cholesterol	1.2 ± 0.7[a]	0.9 ± 0.2[a]	0.3 ± 0.0[a]
Campesterol	4.5 ± 1.5[a]	21.5 ± 4.7[b]	3.6 ± 0.3[a]
Stigmasterol	0.1 ± 0.1[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]
β-Sitosterol	4.8 ± 1.6[a]	17.1 ± 3.9[b]	2.9 ± 0.3[a]
Δ5-Avenasterol	0.5 ± 0.2[a]	0.5 ± 0.1[a]	0.2 ± 0.4[b]
Campestanol	0.2 ± 0.1[a]	0.2 ± 0.0[a]	6.9 ± 1.2[b]
Sitostanol	0.1 ± 0.0[a]	0.2 ± 0.0[a]	7.5 ± 1.5[b]
Total plant sterols	11.6 ± 3.0[b]	41.7 ± 8.6[c]	7.4 ± 0.6[a]
Total plant stanols	0.3 ± 0.1[a]	0.4 ± 0.0[a]	14.4 ± 2.7[b]
Plant sterols + plant stanols	11.9 ± 3.1[a]	42.1 ± 8.6[c]	21.8 ± 3.3[b]
Total sterols	134.6 ± 20.1[a]	112.0 ± 23.9[a]	161.5 ± 19.4[b]

[a]Values are mean ± SD of 10 samples per group. Values in rows with different roman superscripts (a–c) are significantly different at the $P < 0.05$ level as determined by ANOVA analysis followed by Tukey's LSD test for equal *N*. For abbreviation see Table 3.

TABLE 6
Kidney Sterol Concentration[a] of SHRSP Rats Fed Soybean Oil, Plant Sterol Margarine, or Plant Stanol Margarine Diets for 30 d (mg sterol/100 g kidney)

Sterol	Soybean oil	Plant sterol margarine	Plant stanol margarine
Cholesterol	299.9 ± 20.5[c]	216.2 ± 22.3[a]	266.2 ± 33.3[b]
Brassicasterol	0.3 ± 0.1[a]	0.8 ± 0.1[b]	0.3 ± 0.1[a]
24-Methylene cholesterol	0.8 ± 0.1[b]	0.9 ± 0.1[b]	0.4 ± 0.0[a]
Campesterol	6.2 ± 0.7[b]	28.8 ± 2.9[c]	3.4 ± 0.4[a]
Stigmasterol	0.8 ± 0.1[b]	1.4 ± 0.1[c]	0.2 ± 0.1[a]
β-Sitosterol	6.6 ± 0.8[b]	19.9 ± 1.8[c]	2.6 ± 0.3[a]
Δ5-Avenasterol	0.5 ± 0.1[a]	0.8 ± 0.1[c]	0.0 ± 0.0[a]
Campestanol	0.7 ± 0.1[a]	0.7 ± 0.1[a]	6.4 ± 0.7[b]
Sitostanol	0.0 ± 0.0[a]	0.0 ± 0.0[a]	6.6 ± 0.8[b]
Total plant sterols	17.2 ± 1.8[b]	54.7 ± 5.3[c]	7.8 ± 0.6[a]
Total plant stanols	0.7 ± 0.1[a]	0.7 ± 0.1[a]	13.0 ± 1.5[b]
Plant sterols + plant stanols	17.9 ± 1.9[a]	55.4 ± 5.4[c]	20.8 ± 2.1[b]
Total sterols	317.8 ± 21.7[b]	271.3 ± 27.2[a]	287.0 ± 34.1[a]

[a]Values are mean ± SD of 10 samples per group. Values in rows with different roman superscripts (a–c) are significantly different at the $P < 0.05$ level as determined by ANOVA analysis followed by Tukey's LSD test for equal *N*. For abbreviation see Table 3.

derivative, stanol, and campesterol to the corresponding campestanol. However, further work would be required to confirm these metabolic conversions. The RBC, however, contained no plant stanols (Table 4). The reason for this is not known, but it might reflect the general inability of RBC to perform any biochemical synthesis or metabolic conversions. Comparison of the tissue sterol levels of SHRSP rats fed the plant sterol margarine and those fed the plant stanol margarine shows that plant sterols were accumulated to a greater extent than the plant stanols. Additionally, the sum of plant sterol and stanol contents in the tissues was significantly higher in the plant sterol margarine group than in the plant stanol margarine group. These data indicate more efficient absorption and tissue accumulation of plant sterols than plant stanols by SHRSP rats.

Compared to the plant stanol margarine and soybean oil groups, SHRSP rats in the plant sterol margarine group had the lowest cholesterol content in serum and other tissues (Tables 3–6). Between the plant stanol margarine and soybean oil groups, cholesterol contents of RBC (Table 4) and kidney (Table 6) were significantly higher for the soybean oil group. However, there were no significant differences in the cholesterol contents of serum (Table 3) and liver (Table 5) between these two dietary groups.

Blood count. There was a significant effect of diet on platelet and white blood cell characteristics (Table 7). SHRSP rats fed plant sterol margarine had a lower platelet count, a higher platelet volume, and a higher white blood cell count than the SHRSP rats fed soybean oil or plant stanol margarine. However, these characteristics did not differ between the soybean oil group and the plant stanol margarine group. The other blood characteristics examined (RBC count, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean hemoglobin concentration,

TABLE 7
Blood Count[a] of SHRSP Rats Fed Soybean Oil, Plant Sterol Margarine, or Plant Stanol Margarine Diets for 31 d

Blood count	Soybean oil	Plant sterol margarine	Plant stanol margaine
WBC (10^9/L)	4.67 ± 0.81[a]	6.05 ± 1.23[b]	4.27 ± 0.47[a]
RBC (10^{12}/L)	7.98 ± 0.35[a]	8.44 ± 0.35[a]	7.82 ± 1.34[a]
HB (g/L)	146 ± 5[a]	151 ± 6[a]	148 ± 6[a]
MCV (fL)	54.3 ± 0.6[a]	53.1 ± 0.6[a]	54.3 ± 2.4[a]
MCH (pg)	18.4 ± 0.3[a]	18.0 ± 0.4[a]	18.3 ± 0.9[a]
MCHC (g/L)	338 ± 3[a]	338 ± 4[a]	337 ± 3[a]
RDW (%)	12.0 ± 0.8[a]	12.3 ± 0.5[a]	12.2 ± 0.6[a]
PLT (10^9/L)	631 ± 105[b]	348 ± 104[a]	593 ± 159[b]
MPV (fL)	6.5 ± 0.2[a]	9.7 ± 0.6[b]	6.6 ± 0.4[a]

[a]Values are mean ± SD of 10 samples per group. Values in rows with different roman superscripts are significantly different at the $P < 0.05$ level as determined by ANOVA analysis followed by Tukey's LSD test for equal *N*. WBC, white blood count; RBC, red blood count; HB, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean hemoglobin concentration; RDW, red cell distribution width; PLT, platelet count; MPV, mean platelet volume. For other abbreviation see Table 3.

TABLE 8
Red Blood Cell Deformability Index[a] with Increasing Shear Stress in SHRSP Rats Fed Soybean Oil, Plant Sterol Margarine, or Plant Stanol Margarine Diets

Shear stress (Pa)	Soybean oil	Plant sterol margarine	Plant stanol margarine	ANOVA (*P* value)
0.5	.117 ± .022	.112 ± .022	.126 ± .021	>0.1
0.89	.183 ± .023[a,b]	.176 ± .028[a]	.194 ± .022[b]	0.07
1.58	.271 ± .024[a,b]	.262 ± .028[a]	.281 ± .019[b]	0.05
2.81	.355 ± .024[a,b]	.347 ± .027[a]	.365 ± .017[b]	0.05
5.00	.425 ± .023[a,b]	.416 ± .026[a]	.431 ± .015[b]	0.07
8.88	.476 ± .022[a,b]	.467 ± .025[a]	.482 ± .015[b]	0.08
15.79	.515 ± .020	.505 ± .027	.519 ± .015	>0.1
28.14	.549 ± .020	.539 ± .025	.549 ± .014	>0.1
49.79	.584 ± .017	.578 ± .020	.586 ± .016	>0.1

[a]The deformability index was determined at necropsy just prior to end of natural life using a laser-assisted optical rotational cell analyzer (LORCA; Mechatronics Instruments, Hoorn, The Netherlands). Values are mean ± SD of 11–12 samples per group. Values in rows with different roman superscripts (a–c) are significantly different as determined by Tukey's LSD test. For abbreviation see Table 3.

and RBC distribution width) were not significantly affected by dietary plant sterols or stanols.

RBC deformability. At some shear stress points, the DI of RBC from rats fed plant sterols was lower than that of rats fed the stanol-containing margarine (Table 8). RBC from rats fed the stanol-containing margarine, however, demonstrated greater deformability than the other groups, although this increase was not statistically different from the soybean oil-fed group.

DISCUSSION

It is now well-recognized that diets rich in saturated and *trans* FA increase the risk of cardiovascular diseases, especially coronary heart disease (28–30). Replacement of saturated and *trans* FA by *cis*-monounsaturated FA (MUFA) and PUFA provides greater cardiovascular protection, although PUFA are somewhat more effective (30). The SHRSP rat model used in the present study is a widely used model for hemorrhagic stroke and essential hypertension (31–33). In this model, the FA composition of dietary fats had no major influence on the development of hemorrhagic stroke and life span of SHRSP rats, in contrast to models of cardiovascular diseases (21,22,34–36). In humans also, a recent epidemiological study showed that the risk of developing hemorrhagic stroke is not associated with dietary levels of MUFA and PUFA (37). However, a low intake of saturated fat is associated with increased risk in humans (37–42). In the present study, although there were differences in the levels of MUFA and PUFA among the three diets, the levels of saturated FA were the same (13–14%). Therefore, the differences observed in the life spans among the three SHRSP groups cannot be attributed to variations in the FA profiles of soybean oil, plant sterol margarine, and plant stanol margarine.

The two margarines in the present study contained modest levels of *trans* FA (9–11%), whereas soybean oil contained only 2%. There is only limited information on the effect of *trans* FA on the life span of SHRSP rats. A study by Miyazaki *et al.* (43) showed that partially hydrogenated canola oil and partially hydrogenated soybean oil containing 28 and 14% *trans* FA, respectively, shortened the life span of SHRSP rats by 40% as compared with unhydrogenated soybean oil containing no *trans* FA. However, when the two partially hydrogenated oils were compared with unhydrogenated canola oil, there were no differences in life span. This would suggest that *trans* FA are not the life-shortening factor in partially hydrogenated oils. Miyazaki *et al.* (43) hypothesized that a toxic factor may have been generated from minor components other than FA in the partially hydrogenated soybean oil and/or that a toxic contaminant derived from the materials used for commercial hydrogenation might be responsible for the life-shortening effect of soybean oil. The modest amounts of *trans* FA found in the two margarines of the present study suggest that these margarines were prepared from partially hydrogenated oils. Almost all refined unhydrogenated vegetable oils usually contain 1–2% *trans* FA (44); thus, the 2% *trans* FA found in the soybean oil used in the present study would indicate the absence of any hydrogenated material in the soybean oil diet. Therefore, there is some possibility that the toxic materials presumed by Miyazaki *et al.* (43) to be present in partially hydrogenated vegetable oils might have contributed to the shorter life span of SHRSP rats fed plant sterol and plant stanol margarines. However, this hypothesis is not consistent with the slightly higher survivability of SHRSP rats fed the plant sterol margarine compared to the plant stanol margarine, because both margarines contained similar levels of *trans* fat and hence most likely similar levels of partially hydrogenated vegetable oils.

In our previous studies (21,22), we showed that excessive tissue accumulation of plant sterols was associated with lower survivability of SHRSP rats fed vegetable oils high in plant sterols as well as fats fortified with plant sterols. Although structurally similar to plant sterols, several studies (1,24,25) have shown that plant stanols are negligibly absorbed by normal humans and animals. Because of this lack of absorption, we expected that plant stanols would be much safer than plant sterols for SHRSP rats. Contrary to this hypothesis, the results

of the present study clearly demonstrate that consumption of plant stanols also had detrimental effects on survival of SHRSP rats. Moreover, the survival curves and the mean life span data suggest that the plant stanols could exacerbate the development of hemorrhagic stroke and shorten the life span to a greater extent than plant sterols. The tissue sterol profile data of the present study demonstrated that the SHRSP rats absorbed and accumulated both plant sterols and stanols. Of the two dietary stanols, campestanol appeared to be absorbed more efficiently than sitostanol. However, the amount of total plant stanols in the various tissues of the SHRSP rats fed the plant stanol margarine was three to four times lower than the corresponding values for total plant sterols in the tissues of SHRSP rats fed the plant sterol margarine. These differences occurred despite the almost similar daily average intakes of plant stanols (0.13 g/d) in the plant stanol margarine group and plant sterols (0.16 g/d) in the plant sterol margarine group. Therefore, these data suggest that although plant stanols are absorbed by SHRSP rats, they are absorbed less efficiently than plant sterols, which is in agreement with studies in healthy humans (24) and rats (25). Despite this lower tissue content of plant stanols, death of SHRSP rats in the plant stanol margarine group occurred slightly earlier than in the plant sterol margarine group. This observation shows that plant stanols exacerbated hemorrhagic stroke to a slightly greater extent than plant sterols for SHRSP rats. Thus, it is apparent that plant stanols are more detrimental to survival for SHRSP rats than plant sterols.

Although hypercholesterolemic diets and higher circulating levels of cholesterol are risk factors for coronary heart disease (28–30), they are beneficial for the prevention of hemorrhagic stroke in SHRSP rats (31–33). A diet containing 1% cholesterol was shown to increase serum cholesterol, but this delayed the onset of hemorrhagic stroke and prolonged the life span of SHRSP rats (45). Diets with no added cholesterol greatly shortened poststroke survival. It has been suggested that higher circulating levels of cholesterol attenuate hypertension and prevent hemorrhagic stroke in SHRSP rats, probably by reducing vascular reactivity and decreasing the incidence of hypertensive vascular lesions (32). Thus, in addition to the high tissue content of plant sterols, the significantly lower content of cholesterol in serum and other tissues seen in SHRSP rats fed the plant sterol margarine diet in the present study might be another important factor contributing to the lower survivability of SHRSP rats in the plant sterol group. In contrast to the plant sterol margarine group, the serum and tissue cholesterol levels of the SHRSP rats fed the plant stanol margarine were not lower but significantly higher than those of the plant sterol margarine group and were similar to those of the soybean oil group. Thus, the lower survivability of SHRSP rats fed the plant stanol margarine cannot be attributed to serum or tissue cholesterol levels.

The findings of this study, that the plant stanol-fed SHRSP rats had serum cholesterol levels the same as those of controls and greater than those of the plant sterol group, are in contrast to the known hypocholesterolemic effects of stanols on healthy rats and humans. Studies on healthy Wistar rats have shown that when the diet contains no cholesterol, plant stanols and plant sterols reduce serum cholesterol levels to the same extent, and when cholesterol is included in the diet, plant stanols exhibit greater hypocholesterolemic activity (46–48). In healthy hypercholesterolemic subjects also, both stanols and sterols reduce serum cholesterol to the same extent (49–51), although some studies have claimed that stanols are more effective than sterols (52–56). The hypocholesterolemic action of sterols and stanols in normal rats and healthy humans is most likely related to their ability to inhibit intestinal absorption of both dietary and enterohepatically circulating cholesterol (46–48,54). In the present study, why plant stanols are ineffective in lowering serum cholesterol in SHRSP rats is not known, but might raise the possibility that plant stanols may not be interfering with intestinal absorption of cholesterol in the SHRSP rat model. On the other hand, it would appear that plant sterols lowered serum cholesterol in SHRSP rats probably by inhibiting intestinal absorption in a manner similar to that proposed for Wistar rats (46–48) and healthy humans (54).

Two other pieces of evidence suggest that the shortened survival seen in SHRSP rats fed the diet containing plant stanols may be due to a different mechanism than that seen in rats fed the sterol-containing margarine. Reduced platelet number and erythrocyte deformability were seen only in the rats fed the sterol-containing margarine; there was no evidence of a reduction in either of these parameters in the stanol-fed animals, even though both of these groups had significantly shorter life spans than the soybean oil-fed group.

It is now well established that dietary canola oil, compared to soybean oil, exacerbates hemorrhagic stroke and consequently shortens the life span of SHRSP rats (21,22,34,35). As pointed out above, increased fragility of the vascular membranes, which may be due to increased incorporation of plant sterols, can be a cause of early onset of hemorrhagic stroke in SHRSP rats given canola oil (21,22). After a series of studies with SHRSP rats, Naito and coworkers (57–60) proposed another possible explanation for the life-shortening effect of canola oil. They found that SHRSP rats given canola oil without salt loading for 4 wk became intolerant to low osmotic pressure (59) and that Na^+, K^+-ATPase activities in the aorta (57), brain, heart, and kidney (58) were enhanced compared to those in the group given soybean oil. The systolic blood pressure and plant sterol levels in plasma and erythrocyte membranes were also increased with canola oil feeding. They suggested that the enhanced Na^+, K^+-ATPase activity might be due to a direct effect of plant sterol incorporation into membranes. The enhanced activity of the enzyme facilitates Na^+ reabsorption in the renal tubules and causes the retention of body fluid and hypertension. Thus, Naito *et al.* (58) suggested that, when plant sterols are incorporated into membranes, they might play a role in the early onset of stroke in SHRSP rats by enhancing Na^+, K^+-ATPase. At present, no data exist on the effect of plant stanols on Na^+, K^+-ATPase activity. Therefore, in future research it would be of interest to learn whether the greater life-shortening effect of plant stanols compared to plant sterols on SHRSP rats might be due to further enhancement of Na^+, K^+-ATPase activities in various tissues.

Increased absorption of plant sterols and stanols is most likely due to a genetic defect of SHRSP rats. Recent studies have identified the molecular defect in SHRSP rats and have found that hyperabsorption of sterols is caused by a missense mutation in the ATP transporter binding cassette (ABC) G5 half transporter gene (61). This finding is consistent with previous reports on the identification of mutations in the human ABCG5 or ABCG8 genes in sitosterolemic patients (62–65). These mutations disrupt the mechanism that distinguishes between absorbed cholesterol and plant sterols and is characterized by hyperabsorption and impaired biliary elimination of dietary plant sterols.

The results of the present study and those of our previous studies (21,22) clearly demonstrate that increased intakes of both plant sterols and plant stanols provoke stroke in SHRSP rats. However, a few other vegetable oils, in particular olive oil, shorten the life span of SHRSP rats, despite lower amounts of phytosterols in these oils than in canola oil or corn oil (21,34,35). As discussed previously, the FA composition of vegetable oils has no major influence on the development of stroke in SHRSP rats (21,22,34,35). Therefore, in addition to phytosterols and stanols, there might be other components in common with vegetable oils that shorten the life span of SHRSP rats. As we pointed out in a previous report (21), the mechanism by which these unknown components shorten the life span might differ from that of plant sterols.

The dietary prevention of stroke demonstrated in SHRSP rats is expected to be valid for prevention of hypertension-related disorders in humans, since there is ample evidence for the effectiveness of such diets (23). Because increased consumption of plant sterols and stanols is considered beneficial for lowering serum cholesterol levels, it will be important for future studies to consider whether large daily intakes of these materials might exacerbate the development of hemorrhagic stroke in humans in a fashion similar to that seen in SHRSP rats.

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Comparative Effects of Flaxseed and Sesame Seed on Vitamin E and Cholesterol Levels in Rats

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ABSTRACT: Flaxseed and sesame seed both contain more than 40% fat, about 20% protein, and vitamin E, mostly γ-tocopherol. Furthermore, both contain considerable amounts of plant lignans. However, flaxseed contains 54% α-linolenic acid, but sesame seed only 0.6%, and the chemical structures of flaxseed and sesame lignans are different. In this study, we investigated the differential effects of flaxseed and sesame seed on plasma and tissue γ-tocopherol, TBARS, and cholesterol concentrations. Rats were fed experimental diets for 4 wk: vitamin E-free, (–VE), γ-tocopherol, flaxseed (FS), sesame seed (SS), flaxseed oil (FO), FO with sesamin (FOS), and defatted flaxseed (DFF). SS and FOS diets induced significantly higher γ-tocopherol concentrations in plasma and liver compared with FS, FO, and DFF diets. Groups fed FS, FO, and FOS showed lower plasma total cholesterol compared with the SS and DFF groups. Higher TBARS concentrations in plasma and liver were observed in the FS and FO groups but not in the FOS group. These results suggest that sesame seed and its lignans induced higher γ-tocopherol and lower TBARS concentrations, whereas flaxseed lignans had no such effects. Further, α-linolenic acid produced strong plasma cholesterol-lowering effects and higher TBARS concentrations.

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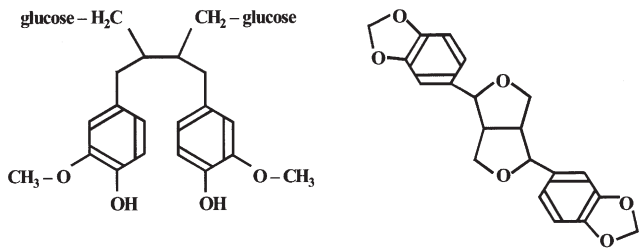
Flaxseed has received a great deal of attention as a healthful food that reduces the risk of chronic diseases such as cancer and coronary artery disease (1–3). Flaxseeds resemble sesame seeds in several ways. Flaxseeds are oily seeds containing more than 40% fat and about 20% protein; their vitamin E content is mostly in the form of γ-tocopherol. Furthermore, like sesame seeds, they contain abundant plant lignans. However, the chemical structure of flaxseed lignan is different from that of sesame lignan (Fig. 1).

In earlier experiments we found that sesame seed and its lignans produced elevated levels of α- and γ-tocopherol concentrations in plasma and tissues of rats (4,5). Recently, Parker and coworkers (6,7) and Ikeda *et al.* (8) reported that these higher tocopherol concentrations were due to the inhibiting effect of sesame lignans on the oxidation of the phytyl side chain in the metabolism of γ-tocopherol to γ-carboxyethylhydroxychroman (γ-CEHC).γ-Tocopherol has received relatively little attention in comparison to α-tocopherol since γ-tocopherol concentrations in plasma and tissues are about one-tenth that of α-tocopherol, in spite of the higher intake of γ-tocopherol in comparison to α-tocopherol (9). However, recent studies indicate that γ-tocopherol possesses unique features that distinguish it from α-tocopherol (10). For example, (i) the γ-tocopherol metabolite γ-CEHC possesses natriuretic activity that may be of physiological importance (11), (ii) γ-tocopherol can be converted to 5-NO_2-γ-tocopherol by the scavenging NO radical (12,13), and (iii) both γ-tocopherol and γ-CEHC, but not α-tocopherol, possess anti-inflammatory properties (14). Thus, we were interested in whether flaxseed lignans would produce higher γ-tocopherol concentrations in plasma and tissues in a manner similar to that of sesame lignans.

Further, Prasad (15) and Kitts *et al.* (16) showed that the flaxseed lignan secoisolariciresinol diglycoside (SDG) scavenged hydroxyl radicals *in vitro*. We also reported that sesame seed and its lignans produced strong antioxidative activities synergistically with γ-tocopherol (4). Further, Nakai *et al.* (17) reported that sesamin itself was converted to catechol substances that had strong antioxidative properties in rat liver. Therefore, we compared the antioxidative properties of flaxseeds and sesame seeds.

An additional point of interest in the two oily seeds is the difference in their FA composition. Flaxseed contains 54% α-linolenic acid (n-3 FA), 16% linoleic acid (n-6), and 20%



Secoisolariciresinol diglycoside in flaxseed **Sesamin in sesame seed**

FIG. 1. Primary lignans in flaxseed and sesame seed. The concentration of secoisolariciresinol diglycoside (SDG) is about 2 mg/g, and secoisolariciresinol is about 1 mg/g in flaxseed (15). The concentration of sesamin is about 3–5 mg/g in sesame seed.

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Abbreviations: AIN, American Institute of Nutrition; γ-CEHC, 2,7,8-trimethyl-2(2′-carboxyethyl)-6-hydroxychroman; DFF, diet containing defatted flaxseed; FO, diet containing flaxseed oil; FOS, diet containing flaxseed oil and sesamin; FS, diet containing flaxseed; MDA, malondialdehyde; SDG, secoisolariciresinol diglycoside; SS, diet containing sesame seed; T, diet containing γ-tocopherol; –VE, diet lacking vitamin E.

oleic acid, whereas sesame seed contains 0.6, 45, and 39% of these FA, respectively. A high concentration of α-linolenic acid in flaxseed may decrease blood lipid concentrations. However, reports concerning the effect on blood lipid concentrations are conflicting (18,19). Kelley *et al.* (20) demonstrated in humans and Prasad (21) demonstrated in rabbits that neither flaxseed nor flaxseed oil reduced blood cholesterol levels, yet Kim and Choi (22) showed that α-linolenic acid lowered serum cholesterol in rats. Furthermore, another component of seeds that may possibly cause lower plasma lipids is dietary fiber, of which there is 28% in flaxseed and 10.8% in sesame seed. Sugano and coworkers (23,24) reported that the sesame lignan sesamin reduced lipid levels in plasma and liver. Prasad (25) and Bhathena *et al.* (26) suggested that flaxseed lignan (SDG) reduced blood cholesterol and TG in animal models. Therefore, in the present study we investigated the effects of flaxseed and sesame seed on γ-tocopherol, TBARS, and plasma total cholesterol concentrations in rats fed diets containing only γ-tocopherol, without α-tocopherol, as well as 1% cholesterol and 0.25% sodium cholate.

MATERIALS AND METHODS

Materials. α-Tocopherol and γ-tocopherol were gifts of Eisai Co. (Tokyo, Japan). Sesamin was donated by Takemoto Oil & Fat Co. (Aichi, Japan). Roasted flaxseed, flaxseed oil, and defatted flaxseed flour were donated by Tuji Oil Mill Co. (Mie, Japan). White roasted sesame seed was donated by Shinsei Co. (Aichi, Japan). Vitamin E-stripped corn oil was purchased from Funahasi Nougyo Co. (Chiba, Japan). A vitamin E-free vitamin mixture and a mineral mixture were both made according to the American Institute of Nutrition (AIN)-93 formulation (27) by Nihon Nosan Kogyo (Yokohama, Japan). Solvents used for chromatography were HPLC grade from Katayama Chemicals Co., Ltd. (Osaka, Japan). Thiobarbituric acid was purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Animals and diets. Three-week-old male Wistar strain rats (Japan SLC Inc., Shizuoka, Japan) were housed individually in stainless-steel wire-mesh cages at 24.5°C and 55% RH, with a 12-h light/12-h dark cycle, and rats were maintained in accordance with the Guidelines for Animal Experimentation of Nagoya University. Rats were allowed free access to nonpurified diet for 3–5 d and then given an experimental diet for 4 wk. Diet compositions are shown in Table 1 and consisted of 20% protein, 12% fat, 0.06% γ-tocopherol, and 1% cholesterol with 0.25% sodium cholate. Flaxseed contained 21% crude protein, 43.8% crude fat, and 0.0151% γ-tocopherol, and sesame seed contained 18.5, 55.6, and 0.0302% of these components, respectively. All diets were adjusted to 20% protein, 12% fat, and (except for the vitamin E-free diet) 0.06% γ-tocopherol with casein, stripped corn oil, and γ-tocopherol, as shown in Table 1. Vitamins, excluding vitamin E, and minerals were in accordance with AIN-93 (27).

In Experiment 1, to compare the differential effects of flaxseed and sesame seed on γ-tocopherol and TBARS concentrations in plasma and liver as well as total cholesterol

TABLE 1
Composition of Diets (Experiments 1 and 2)

	Vitamin E-free (–VE)	γ-Toc (T)	Sesame seed (SS)	Flaxseed (FS)	Flaxseed oil (FO)	Flaxseed oil + sesamin (FOS)	Defatted flaxseed (DEF)
				g/kg diet			
Casein[a]	200	200	163	158	200	200	161
L-Cysteine	3	3	3	3	3	3	3
Cornstarch	467	467	415	397	467	465	395
Sucrose	100	100	100	100	100	100	100
Stripped corn oil	120	120	9	32	32	32	118
Cellulose powder	50	50	50	50	50	50	50
Mineral mixture (AIN-93)	35	35	35	35	35	35	35
Vitamin mixture [AIN-93 (–VE)]	10	10	10	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5
BHT	0.014	0.014	0.014	0.014	0.014	0.014	0.014
γ-Tocopherol	0	0.060	0	0.030	0.025	0.025	0.060
Cholesterol	10	10	10	10	10	10	10
Sodium cholate	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sesame seed[b]		—	200	—			
Flaxseed[c]	—	—	—	200			
Flaxseed oil[d]					88	88	
Defatted flaxseed[e]							112
Sesamin[f]						2	

[a]Vitamin-free casein (Wako Pure Chemical Industries, Osaka, Japan).
[b]Crushed roasted white sesame seed (Shinsei, Aichi, Japan) contained 18.5% protein, 55.5% oil, and 0.03% γ-tocopherol.
[c]Crushed roasted flaxseed (Tsuji Oil Mill Co. Ltd., Mie, Japan) contained 21% protein, 43% oil, and 0.015% γ-tocopherol.
[d]Flaxseed oil (Tsuji Oil Mill Co., Ltd.) contained 0.0395% γ-tocopherol.
[e]Defatted flaxseed (Tsuji Oil Mill Co., Ltd.) contained 35% protein, 1% oil, and no γ-tocopherol.
[f]Sesamin (Takemoto Oil & Fat Co., Ltd., Aichi, Japan) was a mixture of sesamin and episesamin.

levels in plasma, 24 rats were divided into four equal groups and fed for 4 wk a vitamin E-free diet (–VE), a γ-tocopherol-containing diet (T), a 20% sesame seed-containing diet (SS), or a 20% flaxseed-containing diet (FS).

In Experiment 2, we examined five diets containing either the fat fraction or the defatted fraction of flaxseed and sesame lignan, sesamin: a sesame seed-containing diet (SS); a flaxseed-containing diet (FS), a flaxseed oil-containing diet (FO), a flaxseed oil with sesamin diet (FOS), and a defatted flaxseed diet (DFF) as shown in Table 1. We found 0.0395% γ-tocopherol in flaxseed oil and no α-tocopherol in defatted flaxseed. The diets were adjusted for protein, fat, and γ-tocopherol contents in the same manner as in Experiment 1, and rats were fed each diet for 4 wk. In the present experiment, we did not confirm lignan concentrations in flaxseed and sesame seed.

After feeding experimental diets and a 24 h fast, rats were anesthetized with sodium pentobarbital, blood samples were drawn from the heart using heparinized needles and syringes, and plasma was prepared. The liver was excised after perfusing with physiological saline. The plasma and liver were kept at –80°C until analysis.

Analysis of samples. Analytical procedures for samples were essentially the same as described by Yamashita and coworkers (5,28). γ-Tocopherol concentrations in plasma and liver were analyzed by HPLC according to the method of Ueda and Igarashi (29). Instrumentation used for HPLC was a Shimadzu Model LC-9A with a Shimadzu RF-535 fluorescence detector (excitation, 298 nm; emission, 325 nm). The analytical column used was a Develosil 60-5 (4.6 mm i.d. × 250 mm; Nomura Chemical, Aichi, Japan). The mobile phase was hexane and isopropyl alcohol (99:1, vol/vol), and the flow rate was 1 mL/min. Lipid peroxides in plasma were determined by the thiobarbituric acid fluorometric method of Yagi (30), and in liver by the thiobarbituric acid colorimetric method of Ohkawa *et al.* (31). The concentrations of TBARS are presented in terms of nanomoles malondialdehyde (MDA). Total cholesterol in plasma was determined by enzymatic assay kits (Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis. Data were expressed as means ± SEM, $n = 6$. Results were analyzed by using one-way ANOVA with Fisher's *post hoc* test. Differences were considered statistically significant at $P < 0.05$.

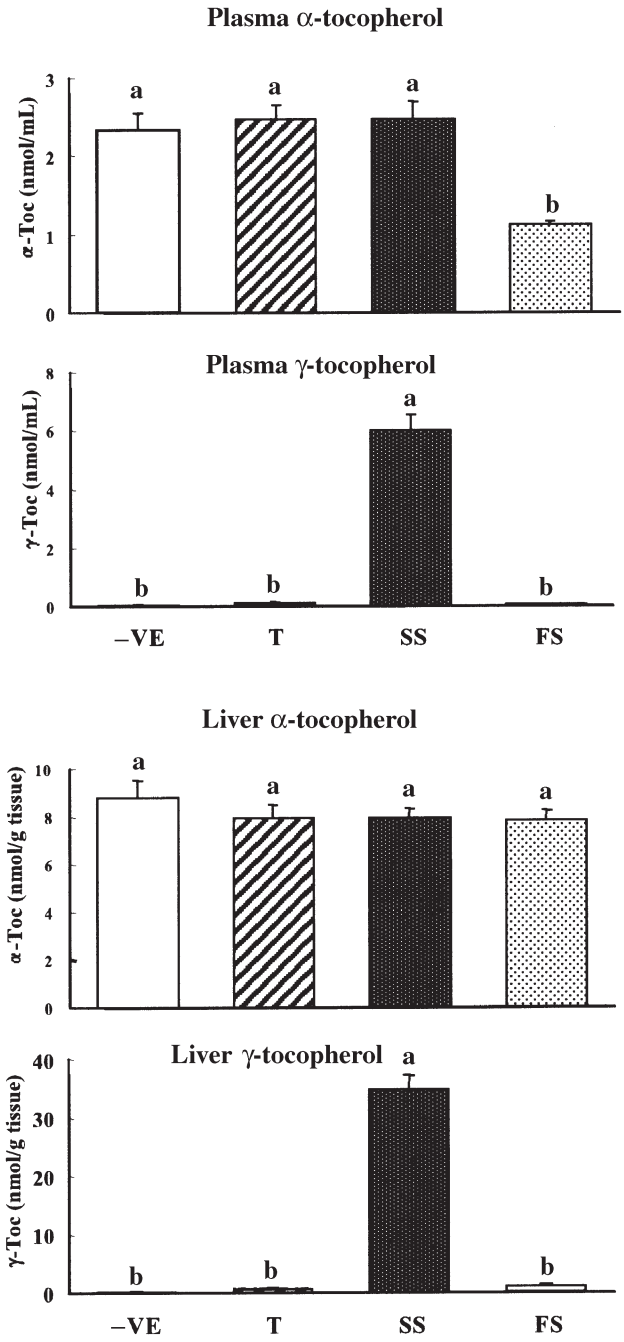

FIG. 2. α- and γ-Tocopherol concentrations in plasma (nmol/mL) and liver (nmol/g wet liver) of rats fed a vitamin E-free diet (–VE), 60 mg/kg γ-tocopherol-containing diet (T), 200 g/kg sesame seed-containing diet (SS), or 200 g/kg flaxseed-containing diet (FS) for 4 wk (Exp. 1). Values are mean ± SEM, $n = 6$. Different letters (a or b) indicate a statistically significant difference ($P < 0.05$).

RESULTS

In Experiment 1, we examined the concentrations of γ-tocopherol and TBARS in plasma and liver, and total cholesterol in the plasma of rats fed four experimental diets for 4 wk: –VE, T, FS, and SS. No significant effects of the diets on animal growth and food intake were observed (data not shown). In plasma and liver, γ-tocopherol concentrations in the SS group were higher ($P < 0.05$) than in the T or the FS group, even though γ-tocopherol intake in these diets was the same (Fig. 2). γ-Tocopherol concentrations in plasma and liver of the T and FS groups were almost the same as in the –VE group. Flaxseed did not induce higher γ-tocopherol concentrations in plasma or liver. Since the diets did not contain α-tocopherol, α-tocopherol concentrations in plasma of the T and SS groups were similar to those in the –VE group, whereas those of the FS group were lower than in the –VE group. Plasma TBARS concentrations of the T, SS, and FS groups were all lower than in the –VE group, but only slightly so in the FS group, where TBARS concentrations were considerably higher

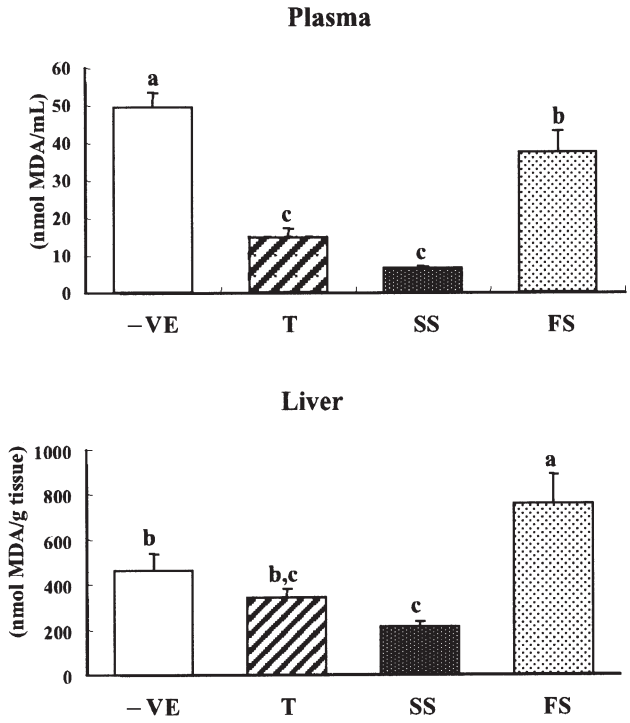

FIG. 3. TBARS concentrations in plasma and liver of rats fed –VE, T, SS, or FS diets for 4 wk (Exp. 1). The concentrations of TBARS are represented in terms of nanomoles of malondialdehyde (MDA). Values are mean ± SEM, $n = 6$. Different letters (a, b, or c) indicate a statistically significant difference ($P < 0.05$). For abbreviations see Figure 2.

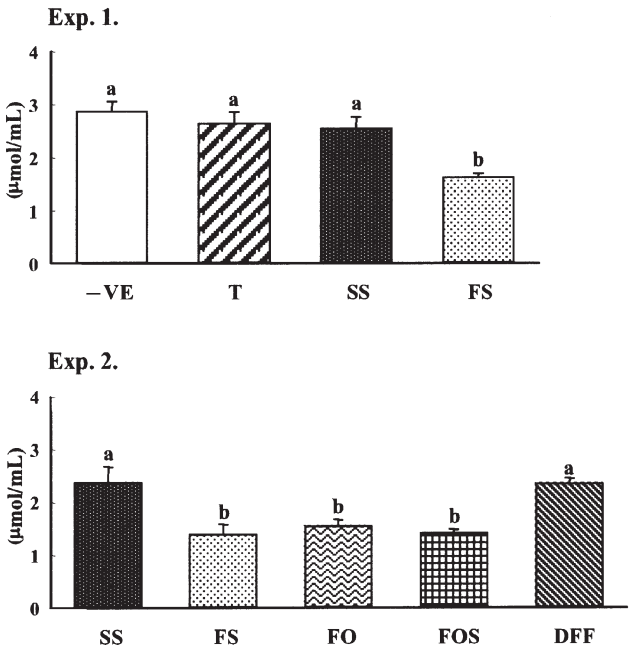

FIG. 4. Total cholesterol concentrations in plasma after 4 wk of feeding of rats with –VE, T, SS, or FS diets (Exp. 1) and of rats with SS, FS, flaxseed oil-containing (FO), FO with sesamin-containing (FOS), or defatted flaxseed-containing (DFF) diets (Exp. 2). Values are mean ± SEM, $n = 6$. Different letters (a or b) indicate a statistically significant difference ($P < 0.05$).

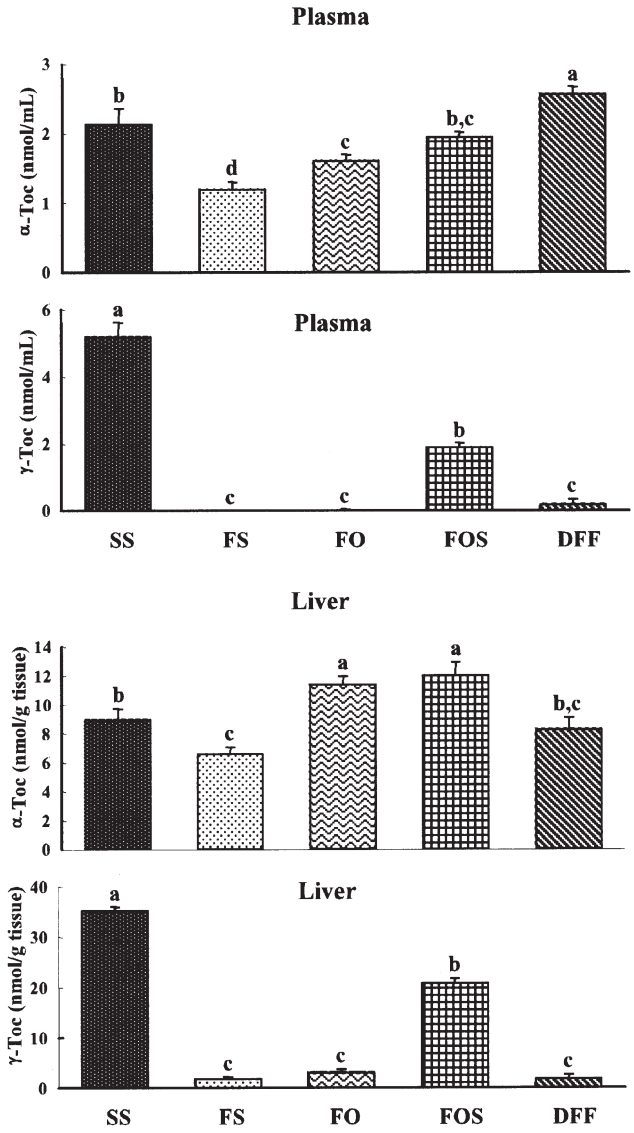

FIG. 5. α- and γ-Tocopherol concentrations in plasma and liver of rats fed SS, FS, FO, FOS, or DFF diets for 4 wk (Exp. 2). Values are mean ± SEM, $n = 6$. Different letters (a, b, c, or d) indicate a statistically significant difference ($P < 0.05$). TOC, tocopherol; for other abbreviations see Figures 2 and 4.

than in the T or SS groups. In liver, TBARS concentrations in the FS group were higher than in the –VE group (Fig. 3). The total cholesterol concentrations in the plasma of the FS group were significantly lower than in the other groups (Fig. 4).

In Experiment 1, the SS group showed higher γ-tocopherol concentrations and lower TBARS values in plasma and liver in comparison to the FS group, and the FS group had remarkably lower plasma cholesterol and higher TBARS values in plasma and liver in comparison to the SS group. We attributed these differences in response to the diets to flaxseed oil and sesame lignans. Therefore, in Experiment 2, we examined five diets: SS, FS, FO, FOS, and DFF, as shown in Table 1.

In plasma and liver, γ-tocopherol contents of the SS group and the FOS group were higher ($P < 0.05$) than in the FS, FO,

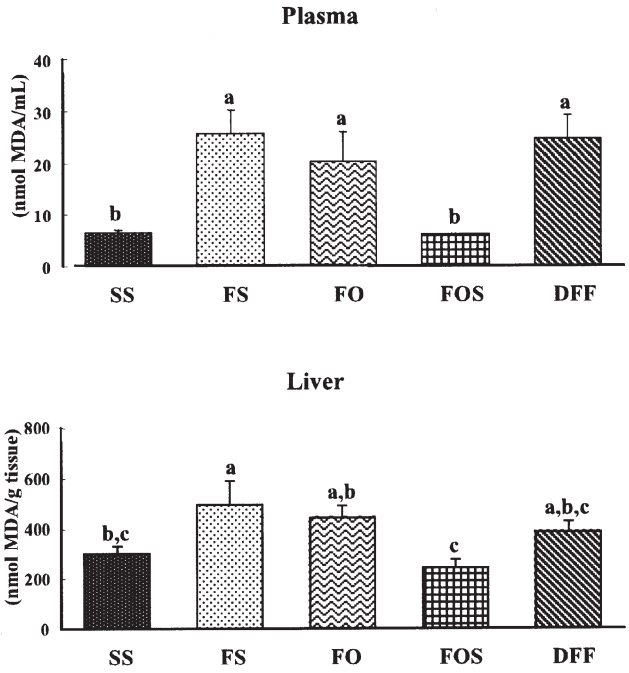

FIG. 6. Concentrations of TBARS in plasma and liver of rats fed the SS, FS, FO, FOS, or DFF diets for 4 wk (Exp. 2). The concentrations of TBARS are represented in terms of nanomoles of MDA. Values are mean ± SEM, $n = 6$. Different letters (a, b, or c) indicate a statistically significant difference ($P < 0.05$). For abbreviations see Figures 2–4.

and DFF groups, even though the γ-tocopherol concentration in the diets of these five groups was the same (Fig. 5). In plasma and liver, α-tocopherol concentrations in the FS group were significantly lower than in the other four groups. As shown in Figure 6, TBARS concentrations in plasma and liver of the SS and FOS groups were significantly lower than in the FS, FO, and DFF groups. Plasma total cholesterol concentrations in the FS, FO, and FOS groups were significantly lower than in the SS and DFF groups (Fig. 4).

DISCUSSION

Flaxseed closely resembles sesame seed on several points: Both oily seeds contain more than 40% fat, about 20% protein, and considerable amounts of plant lignans. Furthermore, their vitamin E is composed mostly of γ-tocopherol with no α-tocopherol. Previously, we reported that sesame lignans induced higher γ-tocopherol concentrations in plasma and tissue (4,5,8). Recently, γ-tocopherol has received increasing attention because it possesses unique features that distinguish it from α-tocopherol (10–14). The primary purpose in the present study was to investigate whether flaxseed lignans have a γ-tocopherol-elevating activity in plasma and tissue similar to sesame lignans.

In Experiment 1, we examined γ-tocopherol concentrations in plasma and liver in rats fed four experimental diets (Table 1). As shown in Figure 2, γ-tocopherol concentrations in plasma and liver of the T or FS group were very low compared with the SS group, and nearly the same as in the –VE group, although the T, FS, and SS groups were fed the same amount of γ-tocopherol. From the results one can see that sesame seed has the same γ-tocopherol-raising property reported previously (4,5). These diets did not contain α-tocopherol, but the α-tocopherol concentrations in plasma of the FS group were significantly lower than in the other groups including the –VE group. We speculate that the higher unsaturated FA content in flaxseed might induce decomposition of α-tocopherol and might therefore counteract the effect of flaxseed lignan on raising γ-tocopherol concentrations. As flaxseed lignans consist mainly of glucosides, their glucoside lignans would be in the defatted fraction. We considered that if flaxseed lignans showed a weak γ-tocopherol-elevating effect, the DFF group might show a slightly higher γ-tocopherol concentration than the FS group. Further, we already showed that sesame lignans, sesamin and sesaminol, induced higher γ-tocopherol concentrations (4). Therefore, we examined whether sesamin would induce higher concentrations of γ-tocopherol in rats fed a diet containing flaxseed oil (FOS group).

In Experiment 2, we examined the effects of flaxseed oil, defatted flaxseed, and flaxseed oil with sesamin on γ-tocopherol concentrations in rats fed the SS, FS, FO, FOS, and DFF diets shown in Table 1. Higher γ-tocopherol concentrations in plasma and tissue were observed only in the SS and FOS groups, and concentrations were very low in the FS, FO, and DFF groups (Fig. 5). These results (4,5,8) clearly showed that sesame lignans produced higher γ-tocopherol concentrations in plasma and tissue. Further, in human studies reports indicate that sesame seed (31) and sesame oil (32) produced higher γ-tocopherol concentrations in the blood. Parker and coworkers (6,7) as well as our own group (8) reported that higher γ-tocopherol concentrations were produced as a result of the inhibiting effect of sesame lignans on the metabolism of γ-tocopherol to γ-CEHC. However, the present results suggest that flaxseed lignans did not inhibit this metabolism since higher γ-tocopherol concentrations were not seen in the FS and DFF groups.

We also compared the antioxidative properties of flaxseed and sesame seed. Prasad (15) and Kitts *et al.* (16) showed that the flaxseed lignan, SDG, scavenged hydroxyl radicals in *in vitro* experiments. Previously, we reported that sesame seed and its lignans produce strong antioxidative activities synergistically with γ-tocopherol. Further, Nakai *et al.* (17) reported that sesamin itself was converted to catechol-like substances that had strong antioxidative properties in liver. Although there are many methods for measuring antioxidative properties, we limited ourselves to determining TBARS concentrations in plasma and liver as indices of antioxidative activity of flaxseed or sesame seed in Experiments 1 and 2. As shown in Figures 3 and 6, TBARS concentrations in plasma and liver were high in the FS and FO groups, and low in the SS and FOS groups. Its seems probable that the high α-linolenic acid content in the FS and FO diets induced high TBARS in plasma and liver. However, the addition of sesamin to the FO diet completely suppressed the induction of high TBARS levels by flaxseed oil. Sesamin exhibited

strong antioxidative properties because of its higher γ-tocopherol concentration and because of the antioxidants produced by sesamin itself, as also observed by Nakai *et al.* (17). The DFF group did not exhibit lower TBARS concentrations in plasma and liver. Thus, it was demonstrated that sesame seed and its lignan sesamin have a strong lowering effect on TBARS concentrations, whereas flaxseed and its lignan did not demonstrate the same antioxidative property.

Flaxseed has received a great deal of attention owing to its high concentration of α-linolenic acid (n-3 FA), which may produce beneficial effects on health by lowering plasma lipids. However, as already noted, reports concerning the effect of flaxseed on blood cholesterol concentrations are conflicting (18–22). As shown in Figure 4, a lower total cholesterol concentration in plasma was observed in the FS, FO, and FOS groups, all of which contained α-linolenic acid, but not in the SS or DFF groups. The present experiments clearly showed that higher levels of α-linolenic acid produced lower plasma cholesterol concentrations. Flaxseed also has received attention for its high dietary fiber content (28%) in comparison to sesame seed (11%) and therefore that dietary fiber reduces plasma cholesterol. Hence, we expected that the DFF group might show lower cholesterol levels since dietary fiber is present in the defatted fraction. However, no such effect on plasma cholesterol in the present study could be attributed to dietary flaxseed. Furthermore, we did not find that sesame seed exhibited a cholesterol-lowering effect, although sesame seed produced lower plasma cholesterol and TG levels in rats fed diets without added dietary cholesterol (Yamashita, K., and Obayashi, M., unpublished data). Sugano and coworkers (23,24) also have reported that sesamin produced lower plasma cholesterol. The FOS group showed lower plasma cholesterol, but in this study we had no way to determine whether sesamin in the FOS diet was related to lower plasma cholesterol.

In conclusion, flaxseed lignans did not induce higher γ-tocopherol concentrations, but flaxseed oil did demonstrate a strong cholesterol-lowering effect. Sesame seed and its lignans exhibited a strong γ-tocopherol-elevating effect as well as a TBARS-lowering effect, but no cholesterol-lowering effect. In the present study there were major differences in lignan contents among the diets. It would be advantageous to study lignans from plants whose contents are more nearly similar. Further investigation is needed to clarify the relationship between the structural properties of lignans and the inhibition of γ-tocopherol metabolism.

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Maternal Dietary FA Modulate the Long-Chain n-3 PUFA Status of Chick Cardiac Tissue

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ABSTRACT: The effect that egg yolk or maternal n-3 FA have on the cardiac tissue long-chain n-3 FA status of chicks during growth was investigated. Eggs with low, medium, and high levels of n-3 PUFA were obtained by feeding breeder hens a wheat/soybean meal-based diet containing 5% sunflower oil (Low n-3), 2.5% sunflower oil plus 2.5% fish oil (Medium n-3), or 5% fish oil (High n-3). The chicks hatched from Low, Medium, and High n-3 eggs were fed a diet containing 18:3n-3, but devoid of long-chain n-3 FA. The FA composition of cardiac tissue was determined on days 0, 14, 28, and 42. At day 0, the cardiac FA reflected maternal diet. With time, the level of all the long-chain n-3 FA decreased compared with day 0, and this was true especially by day 14. These data show that dietary 18:3n-3 fed to the chicks did not sustain high levels of EPA and DHA in cardiac tissue, despite the high content of long-chain n-3 FA in the maternal diet. At days 0 and 14, the chicks hatched from High and Medium n-3 eggs had higher 20:5n-3, 22:5n-3, and 22:6n-3 contents with a concomitant reduction in 20:4n-6 in the cardiac tissue compared with the Low n-3 egg group. Cardiac tissue of birds hatched from Medium n-3 eggs retained higher levels of 20:5n-3 up to day 42 of growth when compared with other treatments ($P < 0.05$). None of the treatments was effective in maintaining DHA levels after day 14 of growth.

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Dietary intervention studies support the concept that restricting saturated FA and increasing n-3 PUFA consumption decreases the incidence of coronary heart disease. n-3 PUFA may exhibit cardioprotective effects *via* incorporation into cell membrane phospholipids and through influences on the heart rate and function (1–3); by alterations of eicosanoid metabolism (4) and platelet function (5); by reduction of vascular resistance (6); and by modulation of ion channels to stabilize the cardiomyocytes (7).

Metabolic diseases such as ascites and sudden death syndrome (SDS) are the major cause of mortality in rapidly growing broiler chickens (8). Despite the high economic loss to the industry, very little research effort has been aimed at elucidating the etiology of cardiac diseases in broilers. Understanding the role of diet in the etiology of metabolic diseases may open new avenues for preventing sudden death in broiler birds. Broiler diets are predominantly based on corn and animal fat or vegetable oil and are high in *trans,* saturated, and linoleic FA (18:2n-6), the last of which is the precursor of 20:4n-6-derived eicosanoids. The n-3 eicosanoids derived from EPA reportedly inhibit platelet aggregation, constrict blood vessels, and decrease thrombus formation (4). An imbalanced state of eicosanoid synthesis may promote cardiac diseases and sudden death in fast-growing broiler birds. In the developing chick, the bulk of lipids in myocardial tissues, which are derived from the yolk lipids during the 21-d incubation period, are incorporated into the phospholipid fraction and serve as structural components. Changes in tissue phospholipid PUFA composition brought about by maternal diet may alter eicosanoid synthesis in the cardiac tissue. Previously, we reported that reduced activity of Δ-6 desaturase inhibited the formation of arachidonic acid (AA, 20:4n-6) in the hepatic tissue of chicks hatched from eggs high in n-3 PUFA (9). The changes in cardiac PUFA composition and eicosanoid formation during growth may result in a profound effect on cardiac health of broiler birds. It is hypothesized that increasing the yolk reserves of n-3 long-chain PUFA will increase the n-3 PUFA status of cardiac tissue of hatched chicks and broiler birds during periods of growth. The objectives of the present study were to investigate whether maternal or yolk n-3 PUFA reserves could modulate the retention of long-chain n-3 PUFA in cardiac tissue of broilers when faced with a diet containing 18:3n-3 but lacking long-chain n-3 FA during periods of growth.

MATERIALS AND METHODS

The experimental design was approved by the Faculty of Agriculture and Forestry Animal Policy and Welfare Committee, University of Alberta, and was conducted in accordance with the Canadian Council on Animal Care guidelines.

Maternal diet and egg alteration of n-6/n-3 PUFA ratio. Eggs with different ratios of n-6/n-3 PUFA were obtained by feeding breeder hens diets containing 5% (wt/wt) of sunflower oil rich in n-6 FA (Low n-3), 5% fish oil high in n-3 FA (High n-3), or 2.5% sunflower oil plus 2.5% fish oil (Medium n-3) (Table 1). The FA compositions of maternal or breeder hen diet and egg yolk are reported in Tables 1 and 2, respectively.

Chick diets. Five hundred of the newly hatched broiler chicks from Low, Medium, or High n-3 diets were randomly distributed into five deep-litter floor pens (100 chicks per pen), in an environmentally controlled house and at an appro-

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Abbreviations: AA, arachidonic acid, 20:4n-6; DHA, 22:6n-3; EPA, 20:5n-3; Low n-3, Medium n-3, and High n-3: diets low, medium, or high in n-3 FA; SDS, sudden death syndrome.

TABLE 1
FA Composition (%) of Maternal Diet[a]

FA	Low n-3	Medium n-3	High n-3
14:0	0.1	3.1	6.4
15:0	0.1	0.3	0.5
16:0	10.1	15.7	20.0
17:0	0.1	0.2	0.4
18:0	3.6	3.3	2.9
20:0	0.3	0.2	0.3
22:0	0.5	0.3	0.2
24:0	0.0	0.0	0.1
SAFA	14.8	23.1	30.8
14:1	0.0	0.1	0.2
15:1	0.0	0.0	0.1
16:1	0.2	4.2	8.9
17:1	0.0	0.10	0.2
18:1	16.9	15.6	14.4
20:1	0.2	0.6	1.3
MUFA	17.3	20.9	25.5
18:2n-6	65.0	41.5	17.8
18:3n-6	0.0	0.1	0.2
20:2n-6	0.3	0.1	0.1
20:3n-6	0.0	0.1	0.2
20:4n-6	0.0	0.3	0.6
Total n-6	65.0	42.0	18.9
18:3n-3	1.7	2.2	2.5
20:5n-3	0.0	3.9	8.6
22:5n-3	0.0	0.7	1.5
22:6n-3	0.0	3.1	6.5
Total n-3	1.7	10.0	19.3
n-6/n-3	37.1	4.2	1.0

[a]SAFA, saturated FA; MUFA, monounsaturated FA. Low, Medium, and High n-3 represent diets containing sunflower oil (5%), sunflower and fish oil (2.5% each), or fish oil (5%), respectively. Data are reported as the mean of two sample analyses.

TABLE 2
Major FA Content[a] of Egg Yolk from Broiler Breeder Hens Fed the Experimental Diets

FA	Low n-3	Medium n-3	High n-3
14:0	1.2 ± 0.0	1.8 ± 0.0	2.7 ± 0.1
16:0	80.4 ± 0.8	82.2 ± 0.9	87.3 ± 1.8
18:0	26.9 ± 1.7	25.5 ± 0.4	25.2 ± 0.2
SAFA	109.7 ± 2.6	110.9 ± 1.1	116.9 ± 2.0
16:1	9.4 ± 1.1	12.0 ± 0.6	15.7 ± 1.0
17:1	0.3 ± 0.0	0.5 ± 0.0	0.7 ± 0.1
18:1	107.0 ± 4.6	118.0 ± 8.4	115.5 ± 1.5
20:1	0.7 ± 0.0	0.9 ± 0.0	1.0 ± 0.0
MUFA	117.6 ± 5.8	131.7 ± 8.9	133.5 ± 0.4
18:2n-6	60.2 ± 5.6	45.2 ± 0.6	21.6 ± 1.8
18:3n-6	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:2n-6	0.7 ± 0.1	0.3 ± 0.0	0.1 ± 0.0
20:3n-6	0.6 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
20:4n-6	7.4 ± 0.2	3.8 ± 1.1	2.7 ± 1.2
Total n-6	69.3 ± 5.8	50.1 ± 0.5	25.0 ± 3.1
18:3n-3	1.0 ± 0.4	1.5 ± 0.4	1.7 ± 0.2
20:5n-3	0.0 ± 0.0	1.0 ± 0.1	2.9 ± 0.2
22:5n-3	0.3 ± 0.0	2.2 ± 0.5	4.3 ± 0.3
22:6n- 3	1.6 ± 0.1	11.0 ± 2.3	15.3 ± 1.7
Total n-3	2.9 ± 0.5	15.8 ± 2.5	24.3 ± 2.0
n-6/n-3	24.2 ± 5.8	3.2 ± 0.5	1.0 ± 0.2

[a]FA reported as mg/g of yolk.
[b]Data presented as mean ± SD for five sample analyses. For abbreviations see Table 1.

priate stocking density. All the broiler chicks were given free access to water and were fed a commercial broiler starter and finisher diets containing 18:3n-3 at 6.7 or 7.3% of total FA but devoid of long-chain (>20-carbon) n-6 and n-3 PUFA during the entire growth period (Table 3).

Sample collection. At hatch and at 2, 4, and 6 wk of age five birds (1 per pen, 5 per group) were randomly selected and killed by cervical dislocation; heart tissue was then harvested. The heart tissue was kept at –20°C until determination of FA composition.

FA analysis. The FA composition of the egg yolk, cardiac tissue, maternal and progeny diets were analyzed by the direct methylation method (10). Briefly, 50 mg of sample was weighed into a 50-mL Teflon-lined screw-capped tube containing tricosanoic acid (23:0, 2.0 mg/mL) as internal standard and was methylated using boron trifluoride/methanol. The mixture was tightly capped and refluxed in a water bath at 95°C for 1 h and allowed to cool at room temperature; next, the FAME were extracted with hexane. The FAME were then separated and quantified using an automated gas chromatograph equipped with an autosampler and FID (Model 3600; Varian Associates, Inc., Palo Alto, CA), using a 30 m × 0.25 mm i.d. fused-silica capillary column (Supelco Canada, Ltd., Oakville, Ontario). A Shimadzu EZChrom (Shimadzu Scientific Instruments, Inc., Columbia, MD) laboratory data integration system was used to integrate the peak areas. The initial GC column temperature was set at 70°C for 3 min, increased to 180°C at 30°C/min and held for 10 min, after which the column temperature was elevated to 230°C at 5°C/min and held at the final temperature for 3 min. The FID temperature was set at 240°C, and helium was used as a carrier gas at a flow rate of 3.0 mL/min. Liquid CO_2 was used to cool the injector. The identification of the FAME was done by comparison with the retention time of authentic FA standards.

Statistical analysis. All the data were analyzed by ANOVA, using the general linear model procedure of SAS (11). Treatment means were analyzed for significant differences using the least squares means test method at $P < 0.05$ (12).

RESULTS AND DISCUSSION

The n-6/n-3 ratios of the maternal diets were 37.1, 4.2, and 1.0 for the Low, Medium, and High n-3 diets, respectively (Table 1). Inclusion of sunflower oil rich in 18:2n-6 and menhaden oil rich in 20:5n-3 and 22:6n-3 resulted in the wide variation in n-6/n-3 ratio in the diet. The egg lipid FA composition reflected the dietary source, resulting in n-6/n-3 ratios of 24.2, 3.2, and 1.0 for Low n-3, Medium n-3, and High n-3 eggs, respectively ($P < 0.05$). DHA was the major n-3 FA in the yolk and ranged from 1.6 to 15.3 mg/g of yolk (Table 2). Manipu-

lation of the lipid content in hens' eggs with n-3 PUFA feeding is well documented through previous research (13,14). The predominant n-6 long-chain PUFA in egg yolk was AA (20:4n-6), constituting 7.4, 3.8, and 2.7 mg/g of yolk for Low n-3, Medium n-3, and High n-3 eggs, respectively (Table 2). Long-chain PUFA such as AA and DHA are involved in the development of the central nervous system in mammals and avians. Deficiencies of these PUFA are associated with learning disabilities and impairment of visual acuity in animal models and human infants (15,16). The incorporation of DHA and AA in the yolk of Low n-3 and High n-3 treatments indicates the ability of laying birds to desaturate and elongate 18-carbon precursors for incorporation to egg. The total n-6 and n-3 FA in the broiler starter and finisher diets were similar (Table 3). It is noteworthy that 18:3n-3 was the only n-3 FA in the commercial diets and that these diets did not contain AA, EPA, or DHA. This was expected because fat sources in most commercial diets in western Canada are of plant origin.

The FA compositions of chick cardiac tissue at different stages of growth are depicted in Tables 4–7. A significant effect of yolk long-chain PUFA reserves on the cardiac accretion of total n-3 FA was observed. As a percentage of total n-3 long-chain PUFA, DHA was the major FA in the cardiac tissue of day-old chicks in all the treatments, constituting 67, 67, and 58% in Low, Medium, and High n-3 chicks, respectively, suggesting the importance of DHA in cardiac cell membrane lipids (Table 4). EPA and DHA have been reported to reduce the *in vitro* beating rate of cardiac myocytes exposed to arrhythmogenic agents (17). Nair *et al.* (3) have reported the importance of readily available n-3 long-chain PUFA in the nonesterified FFA form in ventricular myocardium as protection against ischemia-induced arrhythmia. Although the birds were fed the same starter diet, at day 14, the cardiac tissue of High n-3 birds contained a 75% higher content of total n-3 FA compared with Low n-3 birds ($P < 0.05$) (Table 5). As growth progressed, chicks hatched from Medium and High n-3 eggs retained higher ($P < 0.05$) EPA, docosapentaenoic acid, and DHA levels in cardiac tissue up to

TABLE 3
FA Composition (%) of Broiler Starter and Finisher Diets[a]

FA	Broiler starter	Broiler finisher
14:0	0.3	0.3
16:0	11.7	9.3
18:0	3.5	2.3
20:0	0.4	0.5
22:0	0.2	0.3
SAFA	16.3	12.8
16:1	0.7	0.3
18:1	43.3	50.1
20:1	0.8	1.0
MUFA	47.1	51.9
18:2n-6	28.1	27.8
18:3n-6	0.1	0.1
20:2n-6	0.1	0.1
Total n-6	28.2	27.9
18:3n-3	6.7	7.3
n-6/n-3	4.2	3.8

[a]Data are reported as the mean of two sample analyses. For abbreviations see Table 1.

TABLE 4
FA Content[a] of Cardiac Tissue from Newly Hatched Broiler Chicks as Influenced by Maternal Diets[b]

FA	Low n-3	Medium n-3	High n-3
14:0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
16:0	4.1 ± 0.8	4.2 ± 1.1	4.9 ± 0.7
18:0	3.6 ± 0.2	3.4 ± 0.2	3.3 ± 0.2
SAFA	7.8 ± 1.0	7.7 ± 1.2	8.3 ± 0.8
16:1	0.1 ± 0.0^{b}	0.0 ± 0.0^{b}	0.2 ± 1.0^{a}
18:1	3.2 ± 0.9^{b}	3.4 ± 1.4^{b}	5.1 ± 1.2^{a}
MUFA	3.3 ± 1.0^{b}	3.5 ± 1.4^{b}	5.3 ± 1.3^{a}
18:2n-6	3.2 ± 0.7^{a}	3.3 ± 0.6^{a}	2.1 ± 0.3^{b}
20:2n-6	0.1 ± 0.0^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{c}
20:4n-6	4.0 ± 0.1^{a}	2.5 ± 0.3^{b}	1.3 ± 0.1^{c}
22:4n-6	0.2 ± 0.0^{a}	0.1 ± 0.0^{b}	0.0 ± 0.0^{b}
Total n-6	7.6 ± 0.7^{a}	6.0 ± 0.5^{b}	3.6 ± 0.3^{c}
20:5n-3	0.0 ± 0.0^{c}	0.3 ± 0.0^{b}	0.9 ± 0.2^{a}
22:5n-3	0.1 ± 0.0^{c}	0.2 ± 0.0^{b}	0.4 ± 0.0^{a}
22:6n-3	0.2 ± 0.0^{c}	1.0 ± 0.2^{b}	1.8 ± 0.1^{a}
Total n-3	0.3 ± 0.0^{c}	1.5 ± 0.2^{b}	3.1 ± 0.3^{a}
n-6/n-3	32.2 ± 8.7^{a}	4.0 ± 0.5^{b}	1.2 ± 0.1^{c}

[a]FA reported as mg/g of tissue.
[b]Data represented as mean ± SD for five samples. Means within rows with different roman letter superscripts are significantly different ($P < 0.05$). For abbreviations see Table 1.

TABLE 5
FA Content[a] of Cardiac Tissue of 14-d-old Broiler Chicks as Influenced by Maternal Diets[b]

FA	Low n-3	Medium n-3	High n-3
16:0	4.0 ± 0.7	3.7 ± 0.4	3.7 ± 0.3
18:0	3.8 ± 0.3	3.9 ± 0.5	3.8 ± 0.6
SAFA	7.8 ± 0.8	7.8 ± 0.6	7.7 ± 0.6
16:1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.2
18:1	5.9 ± 1.4	5.7 ± 0.3	6.6 ± 1.4
MUFA	6.4 ± 1.5	6.2 ± 0.3	7.2 ± 1.6
18:2n-6	6.0 ± 0.6	6.2 ± 0.8	6.2 ± 0.5
20:2n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:3n-6	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
20:4n-6	1.2 ± 0.1^{a}	1.0 ± 0.1^{b}	0.7 ± 0.1^{c}
22:2n-6	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
Total n-6	7.8 ± 0.8	7.7 ± 1.0	7.4 ± 0.6
18:3n-3	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.1
20:5n-3	0.1 ± 0.0^{b}	0.3 ± 0.0^{a}	0.3 ± 0.1^{a}
22:5n-3	0.0 ± 0.0^{b}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}
22:6n-3	0.0 ± 0.0^{b}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}
Total n-3	0.4 ± 0.1^{c}	0.5 ± 0.1^{b}	0.7 ± 0.1^{a}
n-6/n-3	21.0 ± 2.7^{a}	14.2 ± 1.6^{b}	10.7 ± 1.3^{c}

[a]FA reported as mg/g of tissue.
[b]Data represented as mean ± SD for five samples. Means within rows with different roman letter superscripts are significantly different ($P < 0.05$). For abbreviations see Table 1.

TABLE 6
FA Content[a] of Cardiac Tissue of 28-d-old Broiler Chicks as Influenced by Maternal Diets[b]

FA	Low n -3	Medium n-3	High n-3
16:0	4.2 ± 0.6	3.7 ± 0.6	3.8 ± 0.5
18:0	3.7 ± 0.2	3.8 ± 0.2	3.8 ± 0.4
SAFA	8.1 ± 0.5	7.8 ± 0.61	7.8 ± 0.9
16:1	0.4 ± 0.2	0.3 ± 0.2	0.3 ± 0.1
18:1	5.4 ± 0.9	4.6 ± 0.6	4.9 ± 1.0
MUFA	6.1 ± 1.0	5.2 ± 0.7	5.4 ± 1.1
18:2n-6	6.2 ± 0.5	6.1 ± 0.4	6.4 ± 0.5
20:2n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:3n-6	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
20:4n-6	1.4 ± 0.3	1.4 ± 0.1	1.4 ± 0.3
22:2n-6	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1
22:4n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Total n-6	8.2 ± 0.5	8.2 ± 0.3	8.4 ± 0.8
18:3n-3	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
20:5n-3	0.1 ± 0.0[b]	0.2 ± 0.0[a]	0.2 ± 0.0[a]
22:5n-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:6n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Total n-3	0.5 ± 0.0[b]	0.6 ± 0.0[a]	0.5 ± 0.1[a,b]
n-6/n-3	17.6 ± 2.6[a]	14.0 ± 1.2[b]	16.7 ± 1.7[a]

[a]FA reported as mg/g of tissue.
[b]Data represented as mean ± SD for five samples. Means within rows with different roman letter superscripts are significantly different ($P < 0.05$). For abbreviations see Table 1.

day 14 and higher EPA levels up to day 28 of growth compared with the Low n-3 egg group. The retention of AA was also lower ($P < 0.05$) in Medium and High n-3 chicks up to day 14 of growth, suggesting the role of maternal reserves in modulating the supply of long-chain n-6 PUFA to the chick cardiac tissue. As growth progressed, the total n-3 FA in the cardiac tissue decreased in all three treatments. However, chicks from Medium n-3 eggs retained higher ($P < 0.05$) total n-3 FA than Low and High n-3 up to day 28 of growth (Table 6). The increase in n-3 FA is further evidenced by the significant alteration in n-6/n-3 FA ratio in Medium n-3 chicks (Table 6). The retention of EPA was higher in Medium n-3 chicks up to day 42 of growth ($P < 0.05$) than other treatments (Table 7).

TABLE 7
FA Content[a] of Cardiac Tissue of 42-d-old Broiler Chicks as Influenced by Maternal Diets

FA	Low n-3	Medium n-3	High n-3
16:0	3.3 ± 0.3	3.1 ± 0.3	3.4 ± 0.5
18:0	3.7 ± 0.3	3.6 ± 0.2	3.4 ± 0.3
SAFA	7.3 ± 0.5	7.1 ± 0.0	7.0 ± 0.5
16:1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
18:1	4.2 ± 0.8	3.5 ± 0.4	3.8 ± 0.5
MUFA	4.7 ± 0.9	3.9 ± 0.4	4.1 ± 0.6
18:2n-6	5.5 ± 0.5	5.0 ± 0.4	4.9 ± 0.5
20:2n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-6	2.0 ± 0.4	2.2 ± 0.1	2.0 ± 0.4
22:2n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:4n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Total n-6	8.0 ± 0.4	7.8 ± 0.4	7.5 ± 0.5
18:3n-3	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:5n-3	0.1 ± 0.0[b]	0.2 ± 0.0[a]	0.1 ± 0.0[b]
22:5n-3	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.1
22:6n-3	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Total n-3	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.1
n-6/n-3	14.9 ± 1.0	13.8 ± 1.0	16.3 ± 2.6

[a]FA reported as mg/g of tissue.
[b]Data represented as mean ± SD for five samples. Means within rows with different roman letter superscripts are significantly different ($P < 0.05$). For abbreviations see Table 1.

The current study examined the lipid composition of chick whole-heart total lipids. These results may indicate that the pool of n-3 long-chain PUFA may be enhanced in the progeny through maternal diet manipulation. The metabolic disease SDS has been reported in flocks as early as 3 d of age with peak incidence between 21 and 42 d of growth (18). In view of the importance of long-chain PUFA in structural membrane and eicosanoid synthesis, the high retention of EPA and DHA and a reduced n-6/n-3 PUFA ratio during early growth may be valuable in providing cardiac protection when birds are faced with low dietary supply. Selected data on bird growth and mortality are shown in Table 8. At day 42, there were no significant differences between the final body weight of Low, Medium, or High n-3 birds. Excluding four culls, total mortality was 73 birds or 4.8% of the whole flock. Total mortality from SDS was 20 birds or 26% of the total mortality and approximately 1.3% of the whole flock. There seems to be no correlation between live-weight, feed intake, sex (19), and total causes of mortality. However, SDS as a contributory factor was random and not significant between treatments, with a wide range in the n-6/n-3 ratio. A plausible explanation might be the lack of mitigating factors, because other production parameters such as temperature and stocking density were normal, as the experiment was conducted in a university research farm. Imaeda (20) reported significantly higher mortality at higher stocking density in summer when compared with a lower stocking density in winter and autumn. In commercial broiler farms, high stocking density is common for economic reasons and such management systems could act as a mitigating factor or trigger in predisposing animals to SDS.

The overall data support the integral involvement of mater-

TABLE 8
Bird Growth and Mortality of Chicks Hatched from Low, Medium, or High n-3 Eggs[a,b]

Causes	Low n-3	Medium n-3	High n-3
Final body weight (g)	2258	2192	2188
Sudden death syndrome	6	6	8
Ascites	4	4	3
Total	10	10	11

[a]Low, Medium, and High n-3 represents eggs produced by feeding breeder hens diets containing wheat/soybean-meal basal diet with added sunflower oil (5%), fish oil (5%), or sunflower and fish oil (2.5% each).
[b]Mortality reported as number of chickens out of 500 birds per each Low, Medium, and High n-3 treatment.

nal reserves (yolk) in the retention of n-3 long-chain PUFA in the cardiac tissue of progeny. Normal broiler diets are high in saturated and n-6 FA and low in n-3 FA. This dietary imbalance and underlying requirement for n-3 FA for tissue incorporation during growth may ultimately contribute to the development of conditions that predispose broiler birds to metabolic diseases and SDS. Given that n-3 FA manifest a significant positive effect in reducing cardiovascular diseases in humans, the role of maternal diet in reducing metabolic diseases and SDS in broiler birds warrants further investigation.

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Effect of Starvation on Lipid Metabolism and Stability of DHA Content of Lipids in Horse Mackerel *(Trachurus japonicus)* Tissues

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ABSTRACT: For the purpose of characterizing the effect of starvation on 22:6n-3 (DHA) content in marine fish tissues, horse mackerel *(Trachurus japonicus)* were reared in a tank containing filtered, sterilized seawater under nonfeeding conditions for 107 d (survival rate of the fish was 96.5%). The crude total lipids (TL) of ordinary dorsal muscle, dorsal skin, and viscera of the starved individuals were separated into classes on silicic acid columns, and the constituents of the TL were quantified by gravimetric recovery from column chromatography. The TL, initially >85% TAG in dorsal muscle, and even more in skin lipids, decreased dramatically within the first 44 d of starvation, and then decreased more gradually during the remainder of the test period, whereas the visceral TL decreased more slowly. The percentages of both saturated and monoenoic FA in the muscle TL also decreased somewhat, but those of DHA increased significantly in muscle during the test periods. Decreases in PE and PC initially were much smaller than TAG, but DHA levels remained high in both PE and PC. These findings indicate that all of the FA in the depot lipids of horse mackerel tissues are easily metabolized for energy production during starvation, but DHA in muscle lipids of the starved fish was maintained at a consistently high level, indicating that starvation did not affect DHA stability in phospholipids. The findings suggest that preservation of DHA in cell membrane lipid PE and PC is necessary for self-protection functions in starving fish.

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Lipids play important roles in energy production processes in animal tissues and as a reserve for EFA. Our previous report (1) considered the FA compositions and seasonal variations of saturated and monoenoic FA levels in the tissues of the horse mackerel *(Trachurus japonicus)* from the East China Sea. We noted the occurrence of consistently high levels of PUFA, which were dominated throughout the year by DHA.

Starvation represents an extreme feeding regime that can provide a useful model for the energy budget in standard feeding experiments. Some reports (2–6) suggest that starvation may to some extent be beneficial for human nutrition because it produces a high DHA concentration in the edible tissues of fatty fish. Because a high intake of DHA supports the human body, information on variations in the levels of DHA in fish tissues consumed by people is of dietary significance for human health (7–9).

Considerable attention has been paid to the effects on metabolism, chemical composition, and physiological and microanatomical features of freshwater fish that result from starvation or periods of low energy intake (2–6,10–15). There is very little information, however, on the effect of starvation on the chemical components and physiology of marine fish (14,15), and in particular on the effect of starvation on lipid metabolism.

The present study was undertaken to elucidate the effect of starvation on lipid metabolism, with special attention on DHA stability, in the horse mackerel, an economically important marine fish. The basic purpose of the present study is to provide information on lipid metabolism in starved fish *in vivo* and to elucidate the stability and enrichment of DHA in this system. The potential utility of starved pelagic fatty fish in special formulations of value-added products relating to DHA is considered.

MATERIALS AND METHODS

Fish. Horse mackerel (body weight: 143.4 ± 6.7 g), *T. japonicus,* were caught by fishing with small- to medium-sized purse seines in offshore (10 km from the coast) surface waters near Nagasaki, Japan, and then transported to our laboratory in live condition.

Fish preservation. All fish were kept alive for starvation in an elliptical (11 × 7 × 1 m; major axis × minor axis × depth) indoor aquarium (50-ton capacity) at a stocking density of 4 fish/ton of filtered, sterilized seawater. The seawater was continuously refreshed at the rate of 50 ton/d. Water temperature variations (21.7–27.2°C) were measured daily. Salinity (29.00–35.40‰), dissolved oxygen (5.20–7.00 ppm), and pH (7.41–8.42) of the water were measured at 10-d intervals.

Preparation of sample for lipid analyses. The fork length (length between mouth and caudal furca) and body weight of the fish were measured. Ordinary dorsal muscles, dorsal skins (including the panniculus and a small amount of muscle), and viscera of the fish were then cut out using a clean scalpel for each tissue.

Lipid extraction and analyses of lipid classes. Each tissue sample was minced and homogenized with a mixture of chloroform/methanol (2:1, vol/vol). A portion of homogenized sam-

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Abbreviations: PL, phospholipid; SE, steryl esters; ST, sterols; TL, crude total lipids.

ple was extracted according to the procedure of Folch *et al*. (16). The crude total lipids (TL) were separated into classes on silicic acid chromatographic columns, and the constituent lipids were quantified by gravimetric recovery of column fractions. The first eluent (dichloromethane/*n*-hexane, 2:3, vol/vol) was used to collect the fraction containing steryl esters (SE). The second eluent (dichloromethane) removed the TAG fraction. This was followed with dichloromethane/ether (9:1, vol/vol), eluting sterols (ST); dichloromethane/methanol (10:1, vol/vol), eluting FFA; dichloromethane/methanol (1:1, vol/vol), eluting PE; and dichloromethane/methanol (1:20, vol/vol), eluting PC. Individual lipids within each lipid class were identified by comparison with standard samples by using TLC (thickness: 0.25 mm for analysis; Kieselgel 60, Merck, Darmstadt, Germany). All sample lipids were dried under nitrogen at room temperature and stored at –70°C in a small amount of dichloromethane.

Preparation of methyl esters and performance of GLC analysis. TL and individual lipid components were converted into FAME by direct transesterification with boiling methanol containing a catalytic amount of concentrated sulfuric acid under reflux for 1.5 h. The FAME obtained were separated from other by-products by column chromatography using silica gel (Silica gel 60, 0.063–0.200 mm; Merck) and elution with dichloromethane. Analyses of FAME were performed on a gas chromatograph (GC-17A; Shimadzu Seisakusho Co., Ltd., Kyoto, Japan) equipped with a capillary column (Omegawax-250, 30 m × 0.25 mm i.d., 0.25 μm film thickness; split ratio, 25:1; Supelco Japan Co., Ltd., Tokyo, Japan). The temperatures of the injector, column, and detector were maintained at 290, 205, and 290°C, respectively. Helium was used as the carrier gas at a constant inlet rate of 17 mL/min.

Statistical analysis. Regression analysis was conducted using Statview (Version 5.0, SAS Institute Inc., Cary, NC). Slopes of the regression lines (decreasing speed of chemical components) were compared by analysis of covariance (ANCOVA).

RESULTS AND DISCUSSION

Variations in TL of organs. Body size, condition factor, and TL contents of muscle, skin, and viscera, as determined at different intervals of starvation, are presented in Table 1. In general, the TL content in each tissue decreased during starvation. The levels of TL in both skin and muscle decreased more rapidly than those in the viscera in the initial 44 d. The average lipid contents fell from 6.8 to 2.3% in muscle ($Y = –0.09X + 6.18$, $r = –0.60$, $P < 0.05$, X: days, Y: lipid contents), and from 32.7 to 12.2% in skin ($Y = –0.43X + 29.72$, $r = –0.72$, $P < 0.001$). TL levels fell further in both the muscle and skin, respectively, to 2.2 ± 0.2% and 9.2 ± 0.8% at 107 d. On the other hand, the visceral TL hardly changed (56.0 to 51.4%) within the initial 44 d of starvation, and slowly decreased afterward, to 35.0 ± 5.0% at 107 d (Table 1).

It was reported that the TL level in eel liver decreased more rapidly than that in eel muscle during starvation (2). In carp the visceral and muscle TL both decreased at the same rate during starvation (6). The rapid decrease of TL in both dorsal muscle and skin of the horse mackerel under starvation differed from the results for these starved freshwater fish. The tissue phospholipids (PL) in the organs of all animals, including fish, are generally retained under starvation; therefore, the phenomenon of decreasing lipid levels suggests that the depot lipids in muscle and skin of seawater fish are mobilized for energy production before those in viscera during the initial stages of starvation.

Variations in FA composition and DHA level of TL during starvation. The variations in FA composition of TL during starvation are shown in Table 2. The DHA content in TL of dorsal muscle increased from 13.8 to 22.0% during the initial 44 d of starvation ($Y = 0.15X + 14.6$, $r = 0.53$, $P < 0.05$, X: days, Y: DHA content), and thereafter remained constant (20.0 ± 1.0%; average between 44 and 107 d). On the other hand, the content of saturated FA decreased significantly during the initial 44 d, from 34.2 to 30.8% ($Y = –0.10X + 34.67$, $r = –0.61$, $P < 0.005$, X: days, Y: saturated FA content). The contents of monoenoic FA also decreased, from 37.2 to 32.0%, during the same period although the decrease was not significant ($Y = –0.10X + 34.81$, $r = –0.32$, $P > 0.01$, X: days, Y: monoenoic FA content). The average contents of both saturated and monoenoic FA between 44 and 107 d reached their lowest levels (saturated FA, 32.0 ± 2.4%; monoenoic FA, 32.1 ± 2.5%). The content of PUFA rose from 19.0 to 25.2%, in

TABLE 1
Body Size, Condition Factor, and Crude Lipid Contents of Each Tissue from Starved Horse Mackerel[a]

Days starved (sample size)	Fork length (cm)	Body weight (g)	Condition factor[b]	Crude lipid in dorsal muscle (%)	Crude lipid in skin (%)	Crude lipid in viscera (%)
0 ($n = 4$)	21.8 ± 0.4	143.4 ± 6.7	13.9 ± 0.4	6.8 ± 1.3	32.7 ± 2.1	56.0 ± 5.4
2 ($n = 4$)	22.8 ± 0.3	158.7 ± 7.3	13.7 ± 0.2	6.0 ± 1.3	25.8 ± 3.4	60.9 ± 5.7
16 ($n = 5$)	23.0 ± 0.5	151.3 ± 10.1	12.3 ± 0.1	3.7 ± 0.9	23.3 ± 4.4	60.4 ± 4.6
30 ($n = 5$)	23.0 ± 0.4	157.4 ± 8.6	12.8 ± 0.3	3.6 ± 1.0	14.6 ± 3.3	54.7 ± 9.6
44 ($n = 4$)	21.9 ± 0.3	124.9 ± 5.3	12.0 ± 0.2	2.3 ± 0.5	12.2 ± 2.3	51.4 ± 3.2
59 ($n = 4$)	22.3 ± 0.1	131.0 ± 3.7	11.7 ± 0.3	3.0 ± 1.0	7.7 ± 1.3	39.8 ± 6.7
74 ($n = 5$)	23.1 ± 0.3	140.0 ± 6.5	11.4 ± 0.1	2.2 ± 0.5	10.4 ± 1.9	37.6 ± 5.7
89 ($n = 5$)	22.7 ± 0.4	134.8 ± 7.5	11.5 ± 0.2	1.7 ± 0.3	8.8 ± 2.3	30.6 ± 8.9
107 ($n = 5$)	22.6 ± 0.2	135.0 ± 4.2	11.7 ± 0.3	1.8 ± 0.4	6.9 ± 1.7	35.0 ± 5.0

[a]Data are mean ± SE.
[b]Condition factor = 1000 × body weight/(fork length)^3.

TABLE 2
FA Compositions (%) in Total Lipids in Muscle, Skin, and Viscera of Starved Horse Mackerel[a]

	Days starved (sample size)	Total saturated	Total monoenoic	Total polyenoic	22:6n-3
Muscle	0 (n = 4)	34.2 ± 1.1	37.2 ± 0.6	19.0 ± 1.2	13.8 ± 0.6
	2 (n = 4)	35.7 ± 1.4	31.8 ± 1.6	23.9 ± 2.0	15.7 ± 1.6
	16 (n = 5)	32.4 ± 0.9	34.8 ± 1.4	22.8 ± 1.5	16.5 ± 1.4
	30 (n = 5)	31.5 ± 0.5	29.1 ± 2.9	29.1 ± 3.0	20.5 ± 2.6
	44 (n = 4)	30.8 ± 0.3	32.0 ± 2.4	28.0 ± 2.1	22.0 ± 2.1
	59 (n = 4)	30.4 ± 0.4	32.5 ± 2.0	28.9 ± 2.4	20.2 ± 2.2
	74 (n = 5)	31.3 ± 0.7	33.4 ± 2.7	25.7 ± 2.7	18.0 ± 2.4
	89 (n = 5)	31.1 ± 0.5	31.7 ± 3.5	26.8 ± 2.9	20.8 ± 2.6
	107 (n = 5)	32.0 ± 2.4	32.1 ± 2.5	25.2 ± 1.7	19.5 ± 1.8
Skin	0 (n = 4)	31.4 ± 0.6	36.5 ± 0.5	21.6 ± 0.9	12.8 ± 0.3
	2 (n = 4)	31.1 ± 0.4	36.4 ± 1.6	21.2 ± 1.4	12.4 ± 0.4
	16 (n = 5)	31.0 ± 0.7	39.1 ± 0.9	20.1 ± 1.4	12.5 ± 0.8
	30 (n = 5)	29.2 ± 0.9	38.3 ± 0.7	23.8 ± 2.0	14.8 ± 1.3
	44 (n = 4)	30.6 ± 0.2	36.6 ± 1.5	21.9 ± 1.2	13.8 ± 1.0
	59 (n = 4)	28.4 ± 0.0	39.6 ± 0.0	23.4 ± 0.0	15.6 ± 0.8
	74 (n = 5)	30.3 ± 0.5	38.3 ± 0.9	20.1 ± 0.6	12.1 ± 0.5
	89 (n = 5)	31.3 ± 0.2	39.5 ± 1.1	18.7 ± 0.5	12.5 ± 0.4
	107 (n = 5)	31.0 ± 0.4	40.5 ± 0.6	17.9 ± 0.6	11.7 ± 0.3
Viscera	0 (n = 4)	34.6 ± 1.3	34.9 ± 1.0	21.3 ± 1.3	12.7 ± 0.9
	2 (n = 4)	31.4 ± 0.5	35.8 ± 0.7	21.7 ± 1.2	12.8 ± 0.2
	16 (n = 5)	31.8 ± 0.8	38.1 ± 1.2	19.3 ± 1.5	12.0 ± 0.5
	30 (n = 5)	28.7 ± 0.2	35.4 ± 0.9	27.0 ± 0.9	16.6 ± 0.2
	44 (n = 4)	31.6 ± 0.3	34.6 ± 1.5	23.4 ± 1.0	14.6 ± 0.3
	59 (n = 4)	30.4 ± 0.3	38.6 ± 0.5	21.0 ± 0.6	13.7 ± 0.9
	74 (n = 5)	31.3 ± 0.6	36.4 ± 1.0	22.2 ± 0.9	13.2 ± 0.8
	89 (n = 5)	32.4 ± 0.4	37.6 ± 1.1	18.9 ± 1.0	12.5 ± 0.7
	107 (n = 5)	31.8 ± 0.6	39.6 ± 1.1	18.3 ± 0.9	12.1 ± 0.6

[a]Data are mean ± SE.

parallel with that of DHA, during the starvation test. The contents of DHA in both skin and viscera did not change significantly during the starvation test (skin, 13.1 ± 0.4%; viscera, 13.4 ± 0.5%). From those results, it was clear that horse mackerel consistently preserved high levels of n-3 PUFA, such as DHA, in their dorsal muscle, whereas both saturated and monoenoic FA were quickly metabolized for energy production under conditions of starvation. Apparently, all the FA in both skin and viscera were metabolized at approximately the same rate. Thus, the findings revealed that all the TAG FA in depot lipids were easily metabolized for energy production regardless of the FA types, but the fish maintained DHA in muscle lipids while all the FA in its subcutaneous and visceral tissues were used indiscriminantly.

Variations in lipid class and FA compositions of TAG, PE, and PC. Variations in the amount of lipid classes of dorsal muscles of the starved fish are shown in Table 3. The contents of each lipid class were reduced during starvation periods. In particular, neutral lipids such as SE, TAG, ST, and FFA were markedly reduced. TAG, the principal constituent of TL, declined from 5807.4 to 1348.7 mg/100 g during the 107 d of starvation ($Y = -33.74X + 4557.2$, $r = -0.61$, $P < 0.001$, X: days, Y: TAG amount), but the levels of both PE and PC decreased slowly (PE, $Y = -0.66X + 124.6$, $r = -0.41$, $P < 0.005$; PC, $Y = -0.57X + 333.2$, $r = -0.24$, $P > 0.05$, X: days, Y: amount). The respective contents fell by over half (from 177.9 to 73.0 mg/100 g for PE), and by two-thirds of the initial value (from 420.0 to 290.0 mg/100 g for PC) at 107 d of starvation, although the decrease of PC content was not significant. The rates of decline of both PE (0.55%/d) and PC (0.29%/d) in muscle were markedly lower than that of TAG (0.72%/d). Such a rapid reduction of all the FA in TAG suggests that FA in TAG not only were used as an energy source but also were supplied to PE and PC if required by cell component turnover, and for the maintenance of membrane function. TAG occupied a large percentage in TL of both skin (98.1–90.9%) and viscera (97.1–91.1%), and the levels of PE (skin: trace–1.7%; viscera: 0.4–1.7%) and PC (skin: trace–5.0%; viscera, 0.8–1.2%) were very low throughout the starvation test.

The FA compositions of TAG, PE, and PC of the dorsal muscle are presented in Table 4. The levels of both PUFA and DHA in TAG of all organs gradually decreased during the starvation period (PUFA, from 22.6 to 14.6%; DHA, from 14.4 to 11.9%). The respective regression equations were $Y = -0.07X + 23.3$ ($r = -0.72$, $P < 0.001$, X: days, Y: ratio) and $Y = -0.03X + 15.4$ ($r = -0.55$, $P < 0.005$). The percentages of saturated FA in TAG were almost constant (30.8 ± 0.52%) during starvation, and that of muscle monoenoic FA increased from 36.9 to 45.8% ($Y = 0.08X + 34.6$, $r = 0.67$, $P < 0.001$, X: days, Y: monoenoic FA ratio in TAG). Moreover, the ratios of DHA in skin TAG (12.4–11.2%) and viscera (13.0–10.3% in TAG, 22.7–17.5% in PE, and 31.0–25.5% in PC) slightly

TABLE 3
Classes (mg/100 g) of the Lipids of Starved Horse Mackerel[a]

Days starved (sample size)	Steryl esters	TAG[b]	Sterol[b]	FFA	PE	PC
Muscle						
0 (n = 4)	93.8 (1.4)[c] ± 55.2	5807.4 (86.5) ± 1248.8	101.1 (1.5) ± 0.4	114.0 (1.7) ± 46.5	177.9 (2.6) ± 41.2	420.0 (6.3) ± 63.3
16 (n = 5)	25.2 (0.7) ± 13.8	2975.8 (87.4) ± 652.4	29.9 (0.9) ± 14.4	27.1 (0.8) ± 11.8	113.2 (3.3) ± 29.7	234.2 (6.9) ± 63.1
30 (n = 5)	20.3 (0.6) ± 5.1	3142.4 (88.2) ± 1046.5	16.4 (0.5) ± 3.0	26.6 (0.7) ± 6.5	42.0 (1.2) ± 6.8	316.7 (8.9) ± 20.0
59 (n = 4)	5.1 (0.2) ± 4.5	2573.4 (87.1) ± 867.6	9.7 (0.3) ± 5.5	15.2 (0.5) ± 6.0	78.7 (2.7) ± 15.7	271.8 (9.2) ± 19.2
89 (n = 5)	4.4 (0.3) ± 2.2	1333.4 (77.9) ± 360.1	1.3 (0.1) ± 1.3	16.3 (1.0) ± 3.5	72.8 (4.3) ± 10.5	284.1 (16.6) ± 10.1
107 (n = 5)	11.7 (0.7) ± 6.1	1348.7 (76.3) ± 860.0	15.1 (0.9) ± 7.5	29.9 (1.7) ± 4.1	73.0 (4.1) ± 9.3	290.0 (16.4) ± 17.6
Skin						
0 (n = 4)	89.8 (0.3) ± 48.3	31768.7 (98.1) ± 1741.2	212.2 (0.7) ± 76.8	308.9 (1.0) ± 35.2	Trace	Trace
107 (n = 5)	55.4 (0.8) ± 11.0	6263.9 (90.9) ± 1702.0	49.6 (0.7) ± 26.3	59.6 (0.9) ± 24.4	114.9 (1.7) ± 33.8	343.9 (5.0) ± 66.2
Viscera						
0 (n = 4)	103.7 (0.2) ± 32.8	54311.1 (97.1) ± 4413.9	284.9 (0.5) ± 95.6	495.7 (0.9) ± 380.0	250.0 (0.4) ± 166.6	464.7 (0.8) ± 467.5
107 (n = 5)	174.0 (0.5) ± 36.6	31761.0 (91.1) ± 7120.7	604.4 (1.7) ± 180.6	1286.3 (3.7) ± 570.3	605.7 (1.7) ± 246.0	414.1 (1.2) ± 282.6

[a]Data are mean ± SE.
[b]Includes trace DAG.
[c]Means % of total lipids.

TABLE 4
FA Compositions (%) in TAG, PE, and PC in Muscle, Skin, and Viscera of Horse Mackerel[a]

	Days starved (sample size)	Total saturated	Total monoenoic	Total polyenoic	C22:6n-3
Muscle					
TAG	0 (n = 4)	30.2 ± 0.3	36.9 ± 0.5	22.6 ± 0.5	14.4 ± 0.5
	16 (n = 5)	33.1 ± 0.5	37.7 ± 1.1	19.7 ± 0.8	13.5 ± 0.5
	30 (n = 5)	30.8 ± 0.7	34.1 ± 1.4	23.4 ± 1.3	17.2 ± 1.2
	59 (n = 4)	30.6 ± 0.4	38.4 ± 1.1	19.4 ± 1.2	13.4 ± 0.8
	89 (n = 5)	31.5 ± 0.3	40.8 ± 1.1	16.6 ± 0.8	11.6 ± 0.8
	107 (n = 5)	28.9 ± 0.3	45.8 ± 1.6	14.6 ± 1.7	11.9 ± 1.6
PE	0 (n = 4)	23.7 ± 0.7	13.6 ± 1.5	43.3 ± 2.0	38.2 ± 2.1
	16 (n = 5)	28.7 ± 2.5	12.0 ± 1.0	48.9 ± 2.8	44.7 ± 2.8
	30 (n = 5)	20.3 ± 0.8	10.6 ± 0.8	46.4 ± 1.5	42.5 ± 1.1
	59 (n = 4)	18.8 ± 1.3	13.8 ± 0.6	44.8 ± 0.9	41.3 ± 0.9
	89 (n = 5)	20.2 ± 0.3	14.0 ± 1.5	42.5 ± 1.3	39.1 ± 1.1
	107 (n = 5)	20.0 ± 1.4	16.2 ± 1.5	46.7 ± 2.7	43.7 ± 2.7
PC	0 (n = 4)	27.5 ± 2.0	11.0 ± 0.6	50.9 ± 1.6	40.6 ± 2.0
	16 (n = 5)	26.3 ± 0.5	10.9 ± 1.1	52.3 ± 2.2	45.6 ± 1.8
	30 (n = 5)	28.6 ± 0.3	10.9 ± 0.9	51.2 ± 0.7	41.3 ± 0.3
	59 (n = 4)	27.7 ± 0.3	13.9 ± 1.7	49.3 ± 1.6	41.1 ± 1.4
	89 (n = 5)	27.3 ± 0.5	12.8 ± 0.5	51.1 ± 0.4	43.6 ± 0.7
	107 (n = 5)	28.5 ± 0.6	15.6 ± 0.7	47.4 ± 3.1	37.6 ± 3.5
Skin					
TAG	0 (n = 4)	31.0 ± 0.6	37.1 ± 0.9	21.0 ± 0.8	12.4 ± 0.3
	107 (n = 5)	27.8 ± 0.3	48.4 ± 2.0	12.1 ± 0.5	11.2 ± 2.0
PE	0 (n = 4)	—[b]	—[b]	—[b]	—[b]
	107 (n = 5)	22.6 ± 0.5	27.1 ± 1.9	30.7 ± 1.8	26.6 ± 1.8
PC	0 (n = 4)	—[b]	—[b]	—[b]	—[b]
	107 (n = 5)	22.5 ± 3.0	20.5 ± 1.6	45.6 ± 3.2	36.4 ± 3.9
Viscera					
TAG	0 (n = 4)	31.4 ± 0.7	35.5 ± 1.3	22.1 ± 1.5	13.0 ± 0.4
	107 (n = 5)	31.5 ± 0.8	46.9 ± 2.5	14.4 ± 1.8	10.3 ± 0.8
PE	0 (n = 4)	32.1 ± 1.9	16.4 ± 3.0	27.9 ± 2.5	22.7 ± 2.2
	107 (n = 5)	32.7 ± 3.7	34.0 ± 4.4	20.2 ± 6.2	17.5 ± 6.1
PC	0 (n = 4)	27.7 ± 2.6	17.1 ± 1.3	38.0 ± 5.8	31.0 ± 4.6
	107 (n = 5)	26.9 ± 3.9	30.1 ± 3.8	29.9 ± 8.1	25.5 ± 8.1

[a]Data are mean ± SE.
[b]Means not examined because the amounts of both PE and PC in skin tissue were negligible.

decreased during 107 d of starvation. Although the levels of PUFA and DHA in TAG of each organ were decreased, those in PE and PC of dorsal muscle were maintained at a high level during the test period (PUFA: 43.3–46.7% in PE; 50.9–47.4% in PC. DHA: 38.2–43.7% in PE; 40.6–37.6% in PC).

In contrast, the levels of both PUFA and DHA in TAG generally increased for freshwater fish, such as rainbow trout (5) and *Tilapia nilotica* (3), during starvation. From the observation that PUFA and DHA increased in carp, Takeuchi and Watanabe (6) suggested that both saturated and monoenoic FA were used preferentially as an energy source by β-oxidation and that the remaining PUFA and DHA were less likely to be catabolized compared to saturated and monoenoic FA, which are preserved in the TAG of freshwater fish (11,17,18). In the present study, the levels of both PUFA and DHA in TAG decreased, unlike results obtained with freshwater fish. This contradiction might be caused by differences in the levels of PUFA and DHA in PL between marine and freshwater fish; the horse mackerel contained high levels of PUFA (in PE, 43.3%; in PC, 50.9% at the initial stage) and DHA (in PE, 38.2%; in PC, 40.6% at the initial stage). In contrast, the freshwater fish had low levels of PUFA. For example, the ratios of PUFA and DHA in the PL were, respectively, 31.6 and 17.2% for *T. nilotica* (6), and 28.1 and 17.0% for carp (11). The PUFA in TAG might be supplied to PL by turnover, for maintaining high levels in membrane lipids, and were not used only as an energy source by β-oxidation. In the horse mackerel, the percentages of PUFA and DHA in PL were higher than those of freshwater fish because of its marine habitat and prey items. In the wild it may be able to obtain small amounts of PUFA and DHA from its prey, similar to other seawater fish, even during starvation. This might be the reason for the reduction of its PUFA and DHA in TAG.

In light of this discussion, one may conclude that DHA in the tissues of horse mackerel was preserved and accumulated during starvation and that the occurrence of consistently high levels of DHA in tissues from starved fish depended on the DHA contained by PL because DHA in TAG was decreased during starvation. Although DHA is essential and important for marine fish as well as for freshwater fish, the horse mackerel has sufficient DHA to be able to afford to use some as an energy source.

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Two Distinct Pathways for the Formation of Hydroxy FA from Linoleic Acid by Lactic Acid Bacteria

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ABSTRACT: Twenty-three of 86 strains of lactic acid bacteria transformed linoleic acid into hydroxy FA. Two distinct conversion pathways were in operation. Two strains of *Lactobacillus acidophilus* and a strain of *Pediococcus pentosaceus* produced 13(*S*)-hydroxy-9-octadecenoic acid [13(*S*)-OH 18:1] and 10,13-dihydroxyoctadecanoic acid (10,13-OH 18:0) as main and minor products, respectively, whereas 13 strains, including *L. casei* subsp. *casei, L. paracasei* subsp. *paracasei, L. rhamnosus, L. lactis* subsp. *cremoris,* and *Streptococcus salivarius* subsp. *thermophilus* produced 10-hydroxy-12-octadecenoic acid (10-OH 18:1). Seven strains of *L. plantarum* converted linoleic acid to 10-hydroxyoctadecanoic acid (10-OH 18:0) through 10-OH 18:1. Linoleic acid at 2 g/L was converted by *L. acidophilus* IFO13951^T to 1.3 g of 13(*S*)-OH 18:1 and 0.09 g of 10,13-OH 18:0 in 7 d. *Lactobacillus paracasei* subsp. *paracasei* JCM 1111 produced 10-OH 18:1 in 91% yield, and *L. plantarum* JCM 8341, 10-OH 18:0 in 59% yield from linoleic acid (2 g/L) under optimal conditions. To our knowledge, this is the first report on the production of 13(*S*)-OH 18:1 by lactic acid bacteria other than ruminal bacteria, and of 10,13-OH 18:0 by any bacteria.

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There are many reports about the microbial conversion of oleic acid to hydroxy and keto FA. Little is known, however, about the microbial transformation of linoleic acid (1). Linoleic acid is converted to 10-OH 18:1 by *Lactobacillus acidophilus* AKU1137 (2), *L. plantarum* (3), *Streptococcus bovis* (4), *Nocardia cholesterolicum* NRRL 5767 (5), and *Flavobacterium* sp. DS5 (NRRL B-14859) (6). The hydroxy FA—13-OH 18:1, 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid, 9,10,13 (9,12,13)-trihydroxy-11*E*(10*E*)-octadecenoic acid, and 8(*R*)-hydroxy-9(*Z*),12(*Z*)-octadecadienoic acid—also were produced by ruminal strain *S. bovis* JB1 (4), *Clavibacter* sp. ALA2 (7), *Pseudomonas aeruginosa* PR3 (8), and the fungus *Leptomitus lacteus* (9), respectively.

Hudson *et al.* (4) has suggested that lactic acid bacteria are the major unsaturated FA-hydrating organisms in the rumen. However, there are no reports on the transformation of linoleic acid by lactic acid bacteria other than *L. acidophilus, L. plantarum*, and *S. bovis*. Further, the rate of conversion by these lactic acid bacteria is less than 30% (2–4).

The aim of this study was to screen for the ability among lactic acid bacteria to convert linoleic acid to hydroxy FA. Among 86 strains tested, we discovered two distinct routes of conversion from linoleic acid, one leading to 13-OH 18:1 and 10,13-OH 18:0, the other to 10-OH 18:1 and 10-OH 18:0. In this report, we describe the screening of lactic acid bacteria, product identification, and optimal conditions for product formation by selected strains.

MATERIALS AND METHODS

Chemicals. Linoleic acid (>99% purity) as substrate and linoleic acid methyl ester (>99% purity) as a standard sample for GC analysis were purchased from Nacalai Tesque Inc. (Kyoto, Japan) and Sigma-Aldrich Co. (St. Louis, MO), respectively. δ-Tridecalactone for internal standard and 13(*S*)-hydroxyoctadecanoic acid were obtained from Soda Aromatic Co., Ltd. (Tokyo, Japan) and Larodan Fine Chemicals (Malmö, Sweden), respectively. All other chemicals used were of analytical grade and are commercially available.

Microorganisms, media, and culture conditions. The 86 microbial cultures used in this study are listed in Table 1. All were incubated at 30°C using an Anaero Pouch (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan) except *L. acidophilus* (37°C).

Screening procedure. SMY and SMYG media were used to screen for the ability to transform linoleic acid into hydroxy FA. The SMY medium contained 100 g/L skim milk, 1 g/L yeast extract, and 2 g/L linoleic acid in distilled water adjusted to pH 6.8 with 0.1 M NaOH, and was autoclaved at 110°C for 15 min. The SMYG medium comprised 10 g/L glucose in SMY medium. The strains, precultivated on modified MRS plates for 2 d, were transferred to the screening media in screw-capped tubes (16 × 125 mm). After 7 d of growth with shaking once a day to mix the linoleic acid, a 1.5-fold volume of acetone was added and the culture preparations were stirred vigorously. After centrifugation, the supernatant was extracted with an equal volume of *n*-hexane containing δ-tridecalactone as an internal standard, and the solvent was evaporated to dryness. The products were converted to methyl esters with a 0.05 vol of hydrochloride in methanol

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Abbreviations: 10-OH 18:0, 10-hydroxyoctadecanoic acid; 10-OH 18:1, 10-hydroxy-12-octadecenoic acid; 10,13-OH 18:0, 10,13-dihydroxyoctadecanoic acid; 13(*S*)-OH 18:1, 13(*S*)-hydroxy-9-octadecenoic acid; RT, retention time.

TABLE 1
Strains[a] of Lactic Acid Bacteria Used in the Screening

Species	Strain
Lactobacillus acidophilus	IFO 13951, JCM 1021, JCM 1023, JCB 1028, JCM 1033, JCM 1034, JCM 1035, JCM 1038, JCM 1039, JCM 5342, JCM 10047, JCM 1229, CH-La5
L. brevis	IFO 3345
L. casei subsp. *casei*	IAM 1045, IFO 15883, JCM 8129, CH-L. casei 01
L. curvatus	IFO 15884
L. delbrueckii subsp. *bulgaricus*	IAM 1120, IFO 13953, CH-Lb 12
L. delbrueckii subsp. *delbrueckii*	IFO 3202
L. delbrueckii subsp. *lactis*	IFO 3073
L. helveticus	IFO 15019, CH-LhB02
L. paracasei subsp. *paracasei*	IAM 1043, IFO 3533, IFO 3953, IFO 14709, JCM 1109, JCM 1111, JCM 1133, JKCM 1161, JCM 1163, JCM 1172 JCM 1181, JCM 1556, JCM 2769, JCM 2770, JCM 8130, JCM 8131, JCM 8132, JCM 8133
L. paracasei subsp. *tolerans*	JCM 1171
L. plantarum	IAM 1041, IAM 1216, IAM 12477, JCM 5651, JCM 1055, JCM 1550, JCM 8341
L. rhamnosus	IFO 3532, IFO 3863, IFO 12521, IFO 14710, JKCM 1136, JCM 1165, JCM 1553, JCM 1561, JCM 1563, JCM 2771 JCM 2772, JCM 8134, JCM 8135, JCM 8136
L. sake subsp. *sake*	IFO 15893
L. lactis subsp. *cremoris*	IFO 3427
L. subsp. *lactis*	IFO 12007
Leuconostoc lactis	IFO 12455
Leuconostoc mesenteroides subsp. *mesenteroides*	IFO 3426
Pediococcus pentosaseus	JCM 5890, JCM 2023, JCM 2024, JCM 2026, JCM 2027, JCM 2028, JCM 2029, JCM 2030, JCM 2031
P. acidilactici	JCM 8797
Streptococcus salivarius subsp. *thermophilus*	IAM 1047, IAM 10064, CH-B01, CHK-St121, CH-TH3, CH-TH4

[a]Strain sources: IFO, Institute for Fermentation Osaka (IFO) Culture Collection; JCM, Japan Collection of Microorganisms; IAM, Institute of Molecular and Cellular Biosciences; CH-; commercial lactic acid bacteria purchased from Christian Hansen Holdings (Denmark).

(Kokusan Chemical Co., Ltd., Tokyo, Japan), and then analyzed by GC.

Bioconversion and isolation of hydroxy FA. The three strains selected (*L. acidophilus* IFO 13951, *L. paracasei* subsp. *paracasei* JCM 1111, and *L. plantarum* JCM 8341) were cultured in 2.5 L of SYM (*L. acidophilus* and *L. plantarum*) and SYMG (*L. paracasei*) media in anaerobic jars (3 L; Tomy Seiko Co., Ltd., Tokyo, Japan) for 7 d with shaking once a day. At the end of the cultivation, the products were extracted with acetone and *n*-hexane as described for the screening procedure. The resultant product methyl esters were separated on a silica gel column (25 cm × 40 mm i.d.) with a gradient from 0.17 to 0.83 vol of ethyl acetate in an *n*-hexane/ethyl acetate solvent system. The fractions containing hydroxy FA were further purified by TLC on a preparative silica gel plate using *n*-hexane/ethyl acetate (5:1, vol/vol). Spots were detected by spraying with 50 g/L phosphomolybdic acid in ethanol and using a hot plate.

Analysis of products. GC was routinely performed using a methylsilicone (DB-1) column (5 m × 0.25 mm i.d., 0.25 μm film; Agilent, Palo Alto, CA). N_2 was used as the carrier gas at 48 mL/min, and eluting compounds were detected by FID. The column, injector, and detector temperatures were maintained at 100 to 300 (20°C/min), 300, and 320°C, respectively. For chiral analysis, a Beta DEX^{TM} capillary column (30 m × 0.25 mm i.d., 0.25 μm film; Supelco, Bellefonte, PA), which was heated from a 100 to 240°C at 10°C/min, was used.

EI-MS readings were recorded on a JEOL JMS-GCmate mass spectrometer using a direct inlet system with an ionization energy of 70 eV. FABMS readings were measured with a JEOL JMS-SX 102 mass spectrometer. The ^{1}H and ^{13}C NMR spectra were measured with JEOL GSX-500 (500 MHz) instruments in $CDCl_3$ with tetramethylsilane as an internal standard.

Optimal conditions for the reaction. Bioconversions from linoleic acid were carried out in SNF medium consisting of 100

g/L skim milk and 2 g/L linoleic acid as the basal medium. Cultures were incubated for 7 d with vigorous shaking once a day and sampled at various intervals by removing 5 mL of entire culture and substrate controls. The products extracted with acetone and *n*-hexane from the samples were methylated and analyzed by GC as described above.

RESULTS AND DISCUSSION

Screening for hydroxy FA-producing strains. Of the 86 cultures tested, 23 strains were found to convert linoleic acid to more polar products. The methyl esters of these products gave peaks at retention times (RT) of 4.4, 4.5, and 4.6 min as compared with 3.7 min for the substrate linoleic acid and 2.1 min for the internal standard δ-tridecalactone on GC analysis. On the basis of retention time, the 23 active cultures can be separated into three groups (Fig. 1). Group I (3 strains, representing *L. acidophilus* and *P. pentosaceus*) produced the conversion product at a RT of 4.4 min, group II (13 strains including *L. acidophilus*, *L. casei* subsp. *casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus*, *L. lactis* subsp. *cremoris*, and *S. salivarius* subsp. *thermophilus*) produced a product with an RT of 4.5 min, and group III (*L. plantarum,* 7 strains) had a main product at 4.6 min. From groups I–III, *L. acidophilus* IFO 13951, *L. paracasei* subsp. *paracasei* JCM 1111, and *L. plantarum* JCM 8341, respectively, were selected as strains with high conversion rate that afforded products that were simple to purify.

Identification of products. Products A (660 mg) and B (74 mg) were obtained from the culture preparation (1,000 mL) of *L. acidophilus* 13951, and gave single spots at R_f 0.58 and 0.10 on TLC and single peaks at RT 4.4 and 5.3 min on GC analysis, respectively. FABMS data of product A gave a molecular ion at *m/z* 312 ($[M + H]^+$, 313) and a fragment ion at *m/z* 294 ($[M + H]^+ - H_2O$). The EI-MS spectrum of the tetramethylsilane derivative showed fragments at *m/z* 173 and 371, revealing cleavage of the molecule adjacent to C_{13}. The pattern of the tetramethylsilane derivative is similar to that reported by Hudson *et al*. (4). The ^1H and ^{13}C NMR spectral data are shown in Tables 2 and 3. Carbon NMR signals indicated the presence of the following carbon atoms: a double bond at 130.2 (C_9) and 130.0 ppm (C_{10}), and a –CHOH carbon at 72.0 ppm (C_{13}). Proton NMR absorption for the C_9 and C_{10} olefinic protons (–CH=CH–) was observed at 5.48 ppm, and there was one proton for –CH–O– at 3.25 ppm. Hydrogenation to product A using platinum black and H_2 was confirmed by the disappearance of the NMR peak derived from the double bond on ^{13}C and ^1H NMR analyses. The RT for the peak representing hydrogenated product A coincided in RT with that of authentic 13(*S*)-hydroxyoctadecanoic acid methyl ester on GC analysis using the chiral column. From these results, product A was deduced to be 13(*S*)-hydroxy-9-octadecenoic acid [13(*S*)-OH 18:1].

The production of 13-OH 18:1 from linoleic acid has already been identified by Hudson *et al*. (4) in the ruminant bacterium *S. bovis*, but no information on the stereospecificity of the product was given. In this study, *L. acidophilus* 13951 transformed linoleic acid into the (*S*)-form of 13-OH 18:1. Wanikawa *et al*. (10) reported the production of 10(*R*)-hydroxystearic acid from oleic acid by several strains of lactic acid bacteria. The difference in the stereospecificity of the products

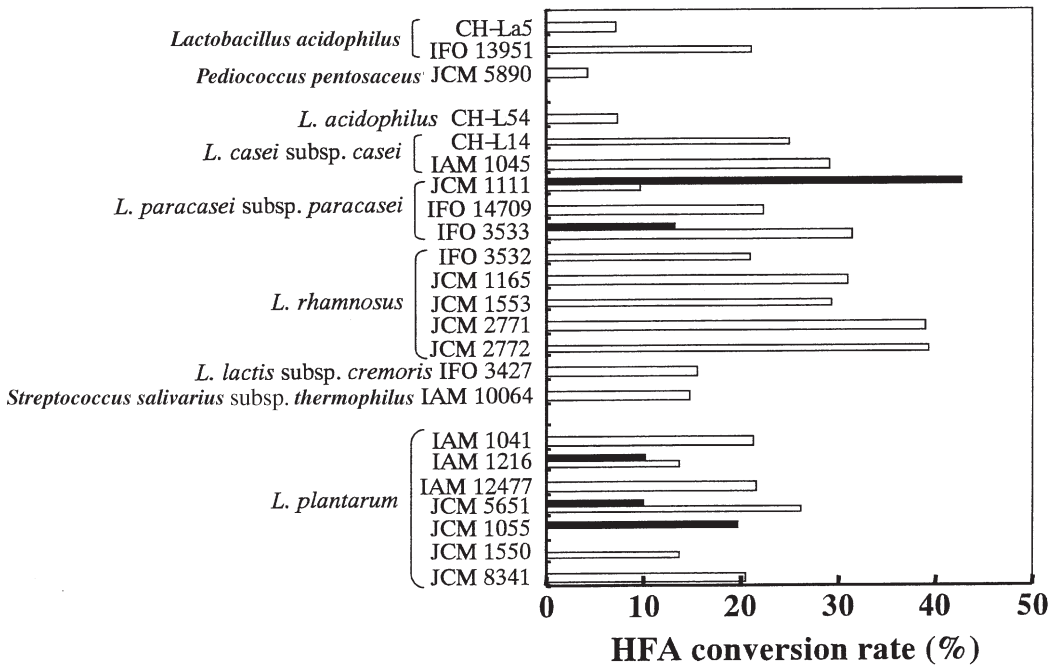


FIG. 1. Screening of strains for their ability to produce hydroxy FA (HFA) from linoleic acid (2 g/L) in SMY or SMYG culture broth. Incubation conditions are described in the Materials and Methods section. Open bar, HFA production in SMY medium; solid bar, HFA production in SMYG medium.

TABLE 2
^{1}H NMR Spectral Data[a] for FA Products (as methyl esters)

	Product A		Product B		Product C		Product D	
Carbon no.	δ	Mult.	δ	Mult.	δ	Mult.	δ	Mult.
2	2.32	*m*	2.32	*m*	2.32	*m*	2.32	*m*
3	1.63	*br, s*	1.63	*br, s*	1.63	*br, s*	1.63	*br, s*
4	1.29	*br, s*	1.29	*br, s*	1.29	*br, s*	1.29	*br, s*
5	1.29	*br, s*	1.29	*br, s*	1.29	*br, s*	1.29	*br, s*
6	1.29	*br, s*	1.29	*br, s*	1.29	*br, s*	1.29	*br, s*
7	1.35	*br, s*	1.29	*br, s*	1.29	*br, s*	1.29	*br, s*
8	1.99	*br, s*	1.29	*br, s*	1.29	*br, s*	1.29	*br, s*
9	5.48	*m*	1.43	*br, s*	1.43	*br, s*	1.43	*br, s*
10	5.48	*m*	3.25	*br*	3.25	*br*	3.56	*br*
11	1.98	*br, s*	1.43	*br, s*	2.10	*m*	1.43	*br, s*
12	1.49	*br, s*	1.44	*br, s*	5.48	*m*	1.29	*br, s*
13	3.25	*br*	3.25	*br*	5.48	*m*	1.29	*br, s*
14	1.45	*br, s*	1.43	*br, s*	1.94	*br, s*	1.29	*br, s*
15	1.29	*br, s*	1.29	*br, s*	1.31	*br, s*	1.29	*br, s*
16	1.29	*br, s*	1.29	*br, s*	1.28	*br, s*	1.29	*br, s*
17	1.32	*br, s*	1.38	*br, s*	1.32	*br, s*	1.38	*br, s*
18	0.99	*br, s*	0.99	*br, s*	0.99	*br, s*	0.99	*br, s*
OH	2.00	*br*	2.00	*br*	2.00	*br*	2.00	*br*
CH_3	3.67	*s*	3.67	*s*	3.67	*s*	3.67	*s*

[a]δ, chemical shift (ppm); mult., multiplicity (*br*, broad; *m*, multiplet; *s*, singlet).

might be attributed to the difference of stereospecificity of hydration enzyme(s) produced by lactic acid bacteria.

Similarly, product B showed a molecular ion at *m/z* 330 and fragment ions at *m/z* 312 ($[M + H]^+ - H_2O$) and 294 ($[M + H]^+ - 2H_2O$) on FAB-MS analysis. The fragments at *m/z* 291, 331, and 457 on EI-MS analysis of the TMS derivative showed cleavages adjacent to C_{10} and C_{13}. ^{13}C NMR signals indicated the presence of a –CHOH carbon at 72.3 (C_{10}) and at 72.2 ppm (C_{13}) (Table 3). ^{1}H NMR absorption revealed the absence of olefinic protons (–CH=CH–) and the existence of a proton for –CH–O– at 3.25 ppm (C_{10} and C_{13}) (Table 2). Based on these results, product B was deduced to be 10,13-dihydroxyoctadecanoic acid.

TABLE 3
^{13}C NMR Chemical Shifts (ppm) for FA Products (as methyl esters)

Carbon no.	Product A	Product B	Product C	Product D
1	174	174	174	174
2	34.1	34.1	34.1	34.1
3	25.6	25.6	25.6	25.6
4	29.2	29.2	29.2	29.2
5	29.3	29.2	29.2	29.2
6	29.6	29.4	29.4	29.4
7	29.5	29.6	29.6	29.6
8	34.2	25.6	25.7	25.6
9	130	37.5	37.6	37.5
10	130	72.3	72.0	72.0
11	26.8	31.3	40.5	37.4
12	38.2	31.7	130	25.0
13	72.0	72.2	130	29.7
14	38.0	37.9	33.3	30.3
15	23.2	23.2	29.3	29.3
16	32.2	32.2	31.8	31.9
17	22.7	22.7	22.8	22.7
18	14.1	14.1	14.1	14.1
OCH_3	51.4	51.4	51.4	51.4

Lactobacillus acidophilus might be able to hydrate both C_{10} and C_{13} in linoleic acid, because strain 13951 produced 10,13-OH 18:0 as well as 13-OH 18:1, and strain CH-La5 transformed linoleic acid into 10-OH 18:1 as a main product. Ogawa *et al*. (2) also reported that *L. acidophilus* AKU 1137 converted linoleic acid to 10-OH 18:1. The ability to hydrate linoleic acid at either C_{10} or C_{13} was speculated to depend on the capacity of the strain of *L. acidophilus*. Strain 13951 is considered to have a greater ability to hydrate at C_{13} than at C_{10}, and transforms linoleic acid into 13-OH 18:1 and hence into 10,13-OH 18:0, since this strain produced 10,13-OH 18:0 as a minor product and 13-OH 18:1 could be detected early on.

Purified product C (450 mg) was prepared from culture broth (1,000 mL) of *L. paracasei* 1111 and gave a single spot (R_f 0.58) and a single peak (RT 4.5 min). Product C gave a molecular ion at *m/z* 312 and a fragment ion at *m/z* 294 ($[M + H]^+ - H_2O$). The fragments at *m/z* 213 and 331 showed cleavage adjacent to C_{10} on EI-MS analysis of the TMS derivative (4). Olefinic protons (–CH=CH–) were observed at 5.48 ppm (C_{12} and C_{13}), and there was one proton (–CH–O–) at 3.25 ppm (C_{10}) on ^{1}H NMR analyses (Table 2). ^{13}C NMR showed the presence of a double bond at 130.4 (C_{12}) and 130.2 ppm (C_{13}), and a –CHOH– carbon at 72.0 ppm (C_{10}) (Table 3). These results indicate that product C is 10-hydroxy-12-octadecenoic acid (10-OH 18:1).

Purified product D (250 mg) from culture broth (1,000 mL) of *L. plantarum* 8341 was obtained as a white powder and gave a single spot at R_f 0.58 and a single peak at RT 4.6 min. FABMS of product D gave a molecular ion at *m/z* 314 and a fragment ion at *m/z* 296 ($[M + H]^+ - H_2O$). The fragments at *m/z* 215 and 331 showed cleavage adjacent to C_{10} on EI-MS analysis of the tetramethylsilane derivative. The ^{1}H and ^{13}C NMR spectra of product D revealed the absence of a double

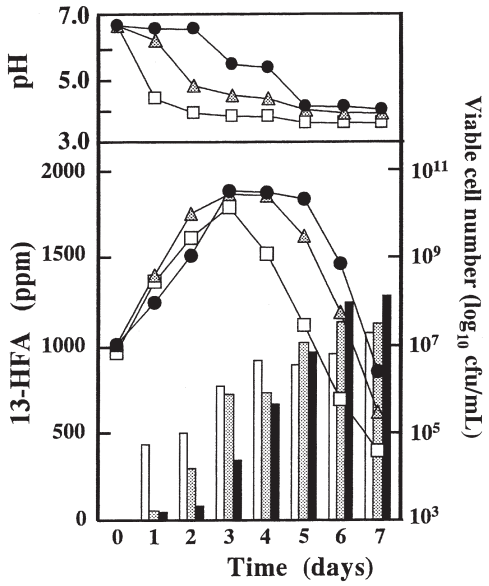


FIG. 2. Time course of 13-OH 18:1 production by *Lactobacillus acidophilus* 13951. Bacterium was cultured at 37°C in SNF medium containing 0.5 g/L proteose peptone as described in the Materials and Methods section. Products were extracted and assayed by GC. The viable cell number was counted by the pour plate method. Viable cell number and pH are indicated as symbols and 13-OH 18:1 as histogram bars. Open histogram bar and symbol, SNF medium containing 0.5 g/L proteose peptone; gray histogram bar and symbol, with 0.1 M phosphate buffer, pH 6.8; solid histogram bar and symbol, with 0.1 M citrate-phosphate buffer, pH 6.8. For abbreviation see Figure 1.

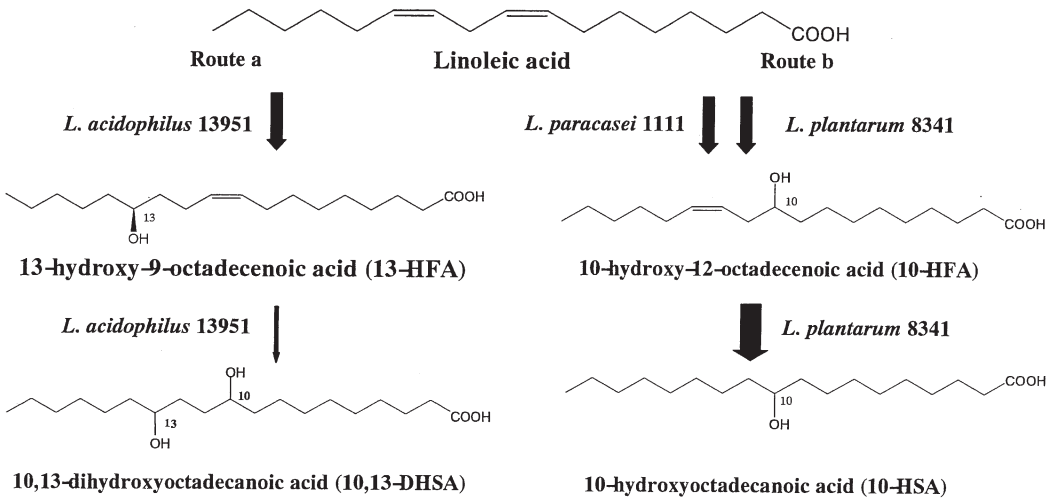
bond and the existence of a hydroxyl group at C_{10} (Tables 2 and 3). Based on these results, product D was identified as 10-hydroxyocta-decanoic acid (10-OH 18:0).

Optimized conditions for 13-OH 18:1 production by L. acidophilus *13951*. Elli *et al.* (11) reported that 1 g/L proteose peptone (Difco) promoted the growth of *L. acidophilus* in a medium containing 100 g/L skim milk. The production of 13-OH 18:1 from linoleic acid was also dependent on the concentration of proteose peptone in SNF medium, and productivity increased linearly up to 0.25 g/L of proteose peptone and reached a maximal level of 57% yield at 0.5 g/L. Further addition of yeast extract and polypeptone to the SNF medium did not increase the production of 13-OH 18:1. On the other hand, proteose peptone did not contribute to the increase in 10,13-OH 18:0 production (4% yields) from linoleic acid.

A typical growth curve of strain 13951 is shown in Figure 2. Growth reached a maximum of 1×10^{10} cfu/mL at 3 d and decreased rapidly thereafter to 4×10^4 cfu/mL at 7 d. The pH of the culture fell to 3.8 and 3.6 after 2 and 5 d, respectively. The addition of citrate-phosphate buffer (0.1 M, pH 6.5) to the medium resulted in a 64% increase in the 13-OH 18:1 yield after 7 d of growth, because the rapid decrease of intact cells was delayed and the final pH was maintainable at pH 4.

Optimized conditions of 10-OH 18:1 production by L. paracasei *1111*. The production of 10-OH 18:1 increased linearly up to 100 g/L of skim milk and reached a maximal level of 60% yield at 100 g/L. In the culture containing less than 25 g/L skim milk, linoleic acid remained on the surface of the culture as an oil drop. The addition of commercial casein protein instead of skim milk produced the same results as skim milk, suggesting that casein protein in skim milk serves to accelerate the conversion reaction, such as emulsification of linoleic acid. The optimal culture conditions for conversion to 10-OH 18:1 were as follows: strain 1111 was inoculated into a medium (pH 6.8) consisting of 100 g/L skim milk, 5 g/L polypeptone, 10 g/L glucose, and 2 g/L linoleic acid, and incubated at 30°C for 7 d. In this culture, linoleic acid at 2 g/L was converted to 10-OH 18:1 (1.8 g/L), and the viable cell number and pH of the end culture were 8 x 10^8 cfu/mL and 4.1, respectively.

Optimal conditions for 10-OH 18:0 production by L. plantarum *8341*. The bacterium transformed linoleic acid into 10-OH 18:0 with a 59% yield in the medium comprising 100 g/L skim milk, 7.5 g/L polypeptone, and 2 g/L linoleic acid at 30°C



SCHEME 1

for 7 d. The final viable cell number and pH were 7×10^8 cfu/mL and 4.4, respectively. At 24 h culture, the amount of 10-OH 18:1 was higher than that of 10-OH 18:0, whereas after 48 h culture 10-OH 18:0 production was strongly increased and 10-OH 18:1 production remained at the same level. Furthermore, the bacterium could transform 10-OH 18:1 produced by strain 1111 into 10-OH 18:0. These results suggest that 10-OH 18:1 may be an intermediate product in the conversion of linoleic acid to 10-OH 18:0.

From these results, we propose two alternative pathways for the hydroxylation of linoleic acid by lactic acid bacteria (Scheme 1). In the first pathway, the C_{13} atom across the double bond is hydrated by strains of *L. acidophilus* and *P. pentosaseus* other than the ruminal strain *S. bovis* JB1, which has already been reported by Hudson *et al*. (4). This route has been reported in only a limited number of strains up to now. However, more lactic acid bacteria hydrating the C_{13} atom might be isolated among intestinal flora, because *L. acidophilus* IFO 13951 and *S. bovis* JB1 were isolated from human (12) and bovine (4) feces, respectively. In the second pathway, the C_{10} atom across the double bond is hydrated by strains of *L. acidophilus, L. casei* subsp. *casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus*, *L. lactis* subsp. *cremoris*, *S. salivarius* subsp. *thermophilus,* and *L. plantarum*. Strains of *L. plantarum* further reduce 10-OH 18:1 to 10-OH 18:0. Most lactic acid bacteria of this group were detected in fermented foods.

The hydration at the C_{13} or the C_{10} position in the linoleic acid seemed to be catalyzed by the hydration enzyme in the lactic acid bacterial cells, because the products were detected from the cell extracts but not from the culture supernatants. Hou (13) reported that *Flavobacterium* sp. DS5 hydratase was a C_{10} positional-specific enzyme. Further investigation is required to determine the positional specificity of these hydration enzymes in the lactic acid bacteria.

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Linoleate Diol Synthase of the Rice Blast Fungus *Magnaporthe grisea*

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ABSTRACT: Mycelia of two strains of *Magnaporthe grisea,* Guy 11 and TH3, were incubated with linoleic acid, and the metabolites were isolated and identified by GC-MS and LC-MS. The two main metabolites were identified as 8-hydroxylinoleic and 7,8-dihydroxylinoleic acids, and the former was further oxidized by n-2 and by n-3 hydroxylation to 8,16- and 8,17-dihydroxylinoleic acids. Lipoxygenase metabolites of linoleic acid could not be detected. The sequence of the genome of *M. grisea* has been released from the Whitehead Institute; it contains a gene with close homology to the linoleate diol synthase gene of the take-all fungus *Gaeumannomyces graminis.* Both genes appear to have the same organization, with four exons and three short introns, and the intron–exon borders were determined by reverse-transcription PCR and sequencing. The linoleate diol synthase precursor of *G. graminis* consists of 978 amino acids, whereas the putative diol synthase precursor of *M. grisea* contains 987 amino acids. The diol synthases of *G. graminis* and *M. grisea* can be aligned with 65% identical and 78% positive amino acid residues, and catalytically important amino acid residues were conserved.

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Magnaporthe grisea is a filamentous ascomycete fungus that is best known as the causal agent of rice blast disease, a serious burden to rice-growing regions worldwide (1). Other pathogenic forms of *M. grisea* may infect millet, barley, and wheat. *Magnaporthe grisea* is closely related to the take-all fungus *Gaeumannomyces graminis*, which is a pathogen of wheat, oats, and other grasses (2,3), but the diseases differ. Rice blast disease is a leaf spot disease, whereas take-all is a disease of the roots.

In the field, *M. grisea* reproduces asexually by producing three-celled spores, conidia (1). The conidia attach to rice leaves and germinate. A penetration structure known as an appressorium then forms and punctures the leaf surface. Infection hyphae develop from this and ramify through the plant. After 72 h, about 10% of the rice plant biomass is fungal. This highly effective infectious process has been studied intensively, yet the complex series of genes and signal molecules that control the events from sporulation to infection and full-blown disease have only recently begun to emerge (1). Secondary metabolism is associated with fungal development, and hormone-like substances may affect the chain of events from sporulation to infection. Rapid degradation of lipids and glycogen reserves occurs during appressorium turgor generation (4).

In plants, important signal molecules are formed by oxygenation of linoleic and α-linolenic acids by lipoxygenases, whereas lipoxygenase and cyclooxygenase oxygenate arachidonic acid to leukotrienes, prostaglandins, and other biological mediators in mammals (5,6). Oxygenation of FA may also form signal molecules in fungi. This was discovered by Champe and coworkers (7,8), who identified sporulation factors (PsiA, -B, and -C) of *Aspergillus nidulans* as oxygenated unsaturated C_{18} FA, e.g., 8*R*-hydroxylinoleic (8-HODE; PsiBα) and 5*S*,8*R*-dihydroxylinoleic (5,8-DiHODE; PsiCα) acids. Oliw and coworkers (9–12) found that 8-HODE was formed by *G. graminis.* They elucidated the mechanisms of biosynthesis and purified the enzyme to homogeneity. Linoleic acid was oxidized by a hemeprotein, designated linoleate diol synthase (LDS), first to 8*R*-hydroperoxylinoleic acid (8-HPODE), which was then isomerized to 7*S*,8*S*-dihydroxylinoleic acid (7,8-DiHODE) by LDS or reduced to 8-HODE by enzymatic or nonenzymatic routes. Cloning of the LDS gene, *LDS*, and further studies on the oxygenation mechanism showed that *LDS* shared homology with cyclooxygenases (11,13). Both enzymes are hemeproteins, and both form a tyrosine radical that initiates the oxygenation reaction by hydrogen abstraction of the FA substrate.

Predicted *LDS* homologs have been identified among expressed sequence tags (EST) from *M. grisea, Neurospora crassa*, *Mycosphaerella graminicola,* and *Glomus intraradices* (see www.ncbi.nlm.nih.gov). The LDS product, 8-H(P)ODE, but not the corresponding LDS-like gene, has been reported in *Leptomitus lacteus* (14), *Laetisaria arvalis* (15), and *A. nidulans* as discussed above. One homologous gene (*ssp1*) has been conclusively identified by cloning and sequencing in *Ustilago maydis* (14,16). The corresponding sporulation-specific protein (ssp1) is highly expressed in mature teliospores of *U. maydis*. The similarity between ssp1,

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Abbreviations: EST, expressed sequence tags; 5,8-DiHODE, 5*S*,8*R*-dihydroxy-9*Z*,12*Z*-octadecadienoic acid; 7,8-DiHODE, 7*S*,8*S*-dihydroxy-9*Z*,12*Z*-octadecadienoic acid; 8,16-DiHODE, 8*S*,16*R*-dihydroxy-9*Z*,12*Z*-octadecadienoic acid; 8,17-DiHODE, 8*S*,17*R*-dihydroxy-9*Z*,12*Z*-octadecadienoic acid; 8-HODE, 8*R*-hydroxy-9*Z*,12*Z*-octadecadienoic acid; 17-HODE, 17*R*-hydroxy-9*Z*,12*Z*-octadecadienoic acid; 8-HPODE, 8*R*-hydroperoxy-9*Z*,12*Z*-octadecadienoic acid; LDS, linoleate diol synthase; ssp1, sporulation-specific protein 1.

LDS, and cyclooxygenase suggests that ssp1 might also be an oxygenase, but its catalytic activity is unknown. Targeted gene disruption of *ssp1* did not lead to an obvious phenotype, and its function in sporulation is unknown (16).

Champe and El-Zayat (7) hypothesized that the amounts of sexual spores (ascospores) increased and asexual spores (conidia) decreased by the action of 8-HODE and 5,8-DiHODE, and this hypothesis gained support in recent studies on *A. nidulans* by Keller and coworkers (17,18). Many fungi may thus express proteins (LDS, ssp1) that are structurally related to cyclooxygenases and might oxygenate PUFA, but unlike plants and mammals, only few reports on lipoxygenases in fungi exist (19).

Lipoxygenase activity has been described in *Saprolegnia parasitica* (20), *Pityrosporum orbiculare* (21,22), and *G. graminis* (23). The lipoxygenase of *G. graminis* has been cloned and sequenced; it differs from plant and animal lipoxygenases, as it contains a manganese catalytic center, has linoleate 11*S*- and 13*R*-lipoxygenase activities, and is secreted (19,24).

The first objective of the present study was to determine whether two strains of *M. grisea,* Guy 11 and TH3, could metabolize linoleic acid by the LDS or the lipoxygenase pathways. We report that both strains expressed LDS activity, whereas significant lipoxygenase activity could not be detected. The second objective was to search the genome of *M. grisea* for genes homologous to *LDS*, as the entire genome of *M. grisea* has been sequenced at the Whitehead Institute (www-genome.wi.mit.edu/annotation/fungi/magnaporthe/), and to confirm the expression of the corresponding mRNA.

MATERIALS AND METHODS

Experimental. Linoleic acid (18:2n-6), precoated TLC plates with silica gel (Kieselgel 60, 0.25 mm), most organic solvents, and salts were from Merck (Darmstadt, Germany). *Magnaporthe grisea* strains Guy11 (25) and TH3 (26) were grown in liquid media as described previously (10) for 7–8 d with shaking (150 rpm) at room temperature. The mycelia were collected by filtration (3MMM filter paper), washed with water, used directly for enzymatic analysis, and stored at –80°C for subsequent preparation of RNA. Mycelia (10 g wet weight) were incubated in 50 mL 0.1 M sodium borate buffer (pH 8.0) with 50 mg linoleic acid added to 0.15 mL ethanol) for 2–6 h at room temperature (9). Incubation was ended by filtration with modest mechanical squeezing of the mycelia. The metabolites of the filtrate were extracted twice with 1.5 vol of ethyl acetate, dried over Na_2SO_4, and evaporated to dryness under vacuum. The residue was dissolved in 2 mL ethanol. Part of this extract was either analyzed directly or after methylation with ethereal diazomethane by preparative TLC (ethyl acetate/heptane/acetic acid, 6:4:0.1 and 5:5:0.1, by vol, respectively; *cf.* Ref. 10). Methyl esters were hydrolyzed as described previously (9). After dilution from 1:50 to 10:50, as judged from TLC analysis, 1 µL of the crude extract was also analyzed by HPLC-electrospray ionization-MS.

LC-MS analysis. The pump for HPLC (P2000; ThermoFinnigan, San Jose, CA) was equipped with a degasser (Degasys DG-1310; Uniflow, Tokyo, Japan). The column contained octadecasilane silica (5 µm, 250 × 2 mm; Kromasil 5 C_{18} 100A; Phenomenex, Torrance, CA). The HPLC column was eluted with methanol/water/acetic acid, 80:20:0.01, by vol, at 0.4 mL/min, and connected to an ion trap mass spectrometer (LCQ; ThermoFinnigan). The capillary temperature was 230°C, the capillary voltage 4.5 kV, and the collision energy 40% for MS-MS and MS-MS-MS analysis. Negative ions were monitored. Prostaglandin $F_{1\alpha}$ (10 ng/µL; Upjohn, Kalamazoo, MI) was infused for tuning into the mass spectrometer with a syringe pump at a rate of 10 µL/min.

GC-MS analysis. A capillary gas chromatograph (3100; Varian, Palo Alto, CA) with a nonpolar column (30 m, DB-5; J&W Scientific, Folsom, CA; film, 0.25 µm; diameter, 0.25 mm; carrier gas He, 15 psi), and an ion trap mass spectrometer (ITS40; ThermoFinnigan) were used (9). After splitless injections of samples in heptane, the gas chromatograph was programmed from 120 to 200°C at 40°C/min, to 260°C at 28°C/min, and then to 285°C at 3°C per min. C-values were determined by the retention times of FAME (18:0, 20:0, 22:0, 24:0). Trimethylsilyl ether derivatives were prepared as described previously (9).

Sequence analysis. The genome of *M. grisea* was analyzed by the TBLASTN algorithm at the Whitehead Institute Center for Genome Research (www.genome.wi.mit.edu) with the protein sequence of LDS. The LDS-like gene of *M. grisea* was further analyzed by the Lasergene program (DNASTAR) and PBLAST (www.ncbi.nlm.nih.gov/).

Total RNA was prepared from mycelia of Guy 11 and transcribed. The following primers (Cybergene, Huddinge, Stockholm) were based on the genomic sequence of LDS of *M. grisea* (i.e., strain 70-15, back-crossed to Guy11) and located on both sides of the three putative introns: F323, 5′-CCAACGGCAAGAAGGTCACA; F342, 5′-AGACGGGCTCATCACGGGACTG; R934, 5′-CCCATGCGGCAGTGTCGTCTC; R971, 5′-AGCCGCCCGGTCTGGAACAAGTC. PCR (50 µL) contained 0.4 µM of each primer (F323 and R971), 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM dNTP, 1.5 U Taq DNA polymerase (Promega), and 1 µL cDNA template (~0.1 µg). The PCR protocol was: 94°C for 3 min (1 cycle), followed by 94°C for 1 min, 60°C for 45 s, 72°C for 1 min (30 cycles), and a final extension step (72°C, 10 min). This yielded an amplicon band of the expected size, 648 bp, on agarose gel electrophoresis. An aliquot of this PCR was used as a template for nested PCR (primers F342 and R934; 20 cycles, as explained above), and the expected amplicon of 592 bp was obtained. Both amplicons were sequenced (ABI PRISM; Applied Biosystems, Foster City, CA).

RESULTS AND DISCUSSION

Mycelia of *M. grisea* (strains TH3 and Guy11) transformed linoleic acid into two major metabolites in the course of 2 h,

as judged from TLC and LC-MS analyses. LC-MS analysis showed that 8-HODE and 7,8-DiHODE were formed, and the latter was the main metabolite (Fig. 1A). Their MS-MS spectra were as reported previously (27). Little linoleic acid could be recovered after 2 h, as judged by TLC analysis (ethyl acetate/heptane/acetic acid, 6:4:0.1 by vol), which mainly showed the bands of 8-HODE (R_f ~ 0.5) and 7,8-DiHODE (R_f ~ 0.4) after charring with phosphomolybdic acid.

Following incubation of mycelia for 6 h with linoleic acid, a series of additional oxygenated metabolites accumulated.

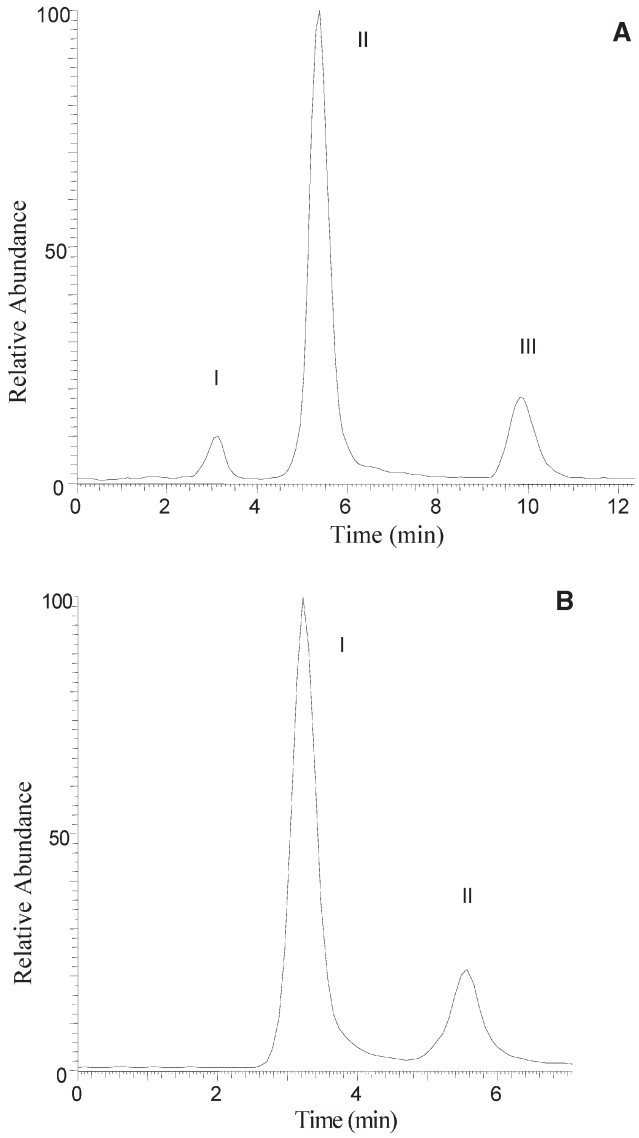

FIG. 1. LC-MS analysis of products formed from linoleic acid by mycelia of *Magnaporthe grisea* (Guy 11). (A) Products formed during 2 h incubation of mycelia with linoleic acid. 7*S*,8*S*-Dihydroxylinoleic acid (7,8-DiHODE) eluted after 5.4 min, 8*R*-hydroxylinoleic acid (8-HODE) after 9.8 min, and small amounts of 8*S*,16*R*-dihydroxylinoleic acid (8,16-DiHODE) and 8*S*,17*R*-dihydroxylinoleic acid (8,17-DiHODE) coeluted after 3.3 min. (B) Separation of 7,8-DiHODE and 8,17-DiHODE, which comigrated as methyl esters on TLC. Relative abundance denotes the total ion current of *m/z* 293 plus 311 (the carboxylate anions of mono- and dihydroxy linoleic acids) in A and *m/z* 311 in B; about 0.2 µg of metabolites were analyzed.

They were separated by TLC (ethyl acetate/heptane/acetic acid, 5:5:0.1, by vol) as methyl esters into four major broad bands with R_f values ranging from about 0.1 to 0.4 as described previously (10). The metabolites of these bands were first analyzed by GC-MS and then, after saponification, by LC-MS. In addition to methyl 8-HODE (R_f ~ 0.4) and methyl 7,8-DiHODE (R_f ~ 0.2), we also identified 17*R*-hydroxylinoleic acid (17-HODE) (R_f ~ 0.3); 8*S*,17*R*-dihydroxylinoleic acid (8,17-DiHODE) (R_f ~ 0.1); and 8*S*,17*R*-dihydroxylinoleic acid (8,16-DiHODE) (R_f ~ 0.2). The mass spectra of the trimethylsilyl ether methyl ester derivatives and the C-values of these five metabolites were as reported previously from incubations with *G. graminis* (10). The methyl esters of 8,16-DiHODE and 7,8-DiHODE thus comigrated on TLC, but they were separated by GC (C-values ~21.5 and ~21.7, respectively) and as FFA by HPLC (Fig. 1B). The MS-MS spectra of the carboxylate anion (A^-) of 8,16-DiHODE and 8,17-DiHODE differed slightly. MS-MS (*m/z* 311 → full-scan) of 8,16-DiHODE yielded strong signals at *m/z* 293 (A^- – 18), 275 (293 – 18), 267 (A^- – 44, loss of CO_2), 253 (A^- – 58, loss of O=CH–CH_2–CH_3), and 157 (possibly O=CH–$(CH_2)_6$–COO^-). The corresponding mass spectrum of 8,17-DiHODE lacked the signal at *m/z* 253 and showed a strong signal at *m/z* 267 (A^- – 44, loss of CO_2 and/or O=CH–CH_3). Finally, 8-HPODE could be identified by MS-MS-MS analysis in some samples as described previously (27).

The rice blast fungus is known to induce rice lipoxygenase as an early response of the host to the pathogen (19). We could not detect significant lipoxygenase activity in the mycelia of *M. grisea,* but the 16- and 17-hydroxy metabolites of 8-HODE were likely formed by cytochrome P450. Little is known about these fungal n-2 and n-3 hydroxylases, except that the alcohols have an *R* configuration, at least when formed by *G. graminis* (28).

Our results show that mycelia of *M. grisea* rapidly oxygenate linoleic acid to 8-HODE and 7,8-DiHODE. It seems very likely that *M. grisea* expresses an enzyme homologous to the LDS of *G. graminis.* This is in agreement with recently published EST of *M. grisea*, which cover about 330 nucleotides of the LDS gene, and by analysis of the genome of *M. grisea* at the Whitehead Institute/MIT Center for Genome Research (www-genome.wi.mit.edu). An LDS-like gene was found in contig 2.1121 and a smaller part in contig 2.2091. Contig 2.1121 contains over 65,000 nucleotides, and the coding region of the LDS-like gene spanned from nucleotide 45,253 ($A^{45,253}$ TG) to 48,321 followed by a stop codon ($T^{48,322}$ AG). We confirmed that the mRNA of LDS of *M. grisea* was expressed by the strain Guy 11 and confirmed the three intron borders, which followed the gt/ag rule, by reverse-transcription PCR and sequencing (Table 1). Reverse-transcription PCR and sequencing of the entire cDNA of LDS of *M. grisea* is now underway (GenBank accession number AY372822).

When the deduced LDS sequence of *M. grisea* was subjected to PBLAST analysis, alignment with LDS of *G. graminis* yielded 629:957 (65%) identities, 756:957 (78%) positives, and 25:957 (2%) gaps. Corresponding figures for

ssp1 were 32, 49, and 10%, respectively, whereas chicken and rainbow trout PGH synthase-2 could be aligned with 24% identities, 42–43% positives, and 21% gaps.

It seems appropriate, based on 65% sequence identity, to group LDS of *G. graminis* and the putative LDS of *M. grisea* to the same subfamily of FA dioxygenases. A BLAST alignment of LDS of *M. grisea* with LDS of *G. graminis* is shown in Figure 2. The putative proximal and distal heme-binding histidine residues (His-213 and His-387, respectively) and the tyrosine residue (Tyr-384), which likely forms a tyrosyl radical and abstracts a hydrogen atom during catalysis, are conserved in the LDS of *M. grisea*.

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LDS_Mg  MASSSSSGSSTRSSSPSDPPSSFFQKLGAFLGLFSKPQPPRPDYPHAPGN   50
LDS_Gga MTVSTHHDDSPGLSG----------RLRDLLHHVFGNQKSPTVYPNAPGN   40

LDS_Mg  SAREEQTDITEDIQKLGFKDVETLLLYLNSSVKGVNDDKQLLLERLIQLL  100
LDS_Gga SAKPVPTGLADDIDKLGFKDIDTLLIFLNSAVKGVNDDQQFLLEKMIQLL   90

LDS_Mg  SKLPPTSTNGKKVTDGLITGLWESLDHPPVSSLGEKYRFREADGSNNNIH  150
LDS_Gga AKLPPASREGKKLTDGLINDLWDSLDHPPVASLGKGFSFREPDGSNNNIH  140

LDS_Mg  NPTLGVAGSHYARSAKPMVYQNPNPPAPETIFDTLMARDPAKFRPHPNQI  200
LDS_Gga LPSLGAANTPYARSTKPLVFQNPNPPDPATIFDTLMVRDPAKFRPHPNKI  190
                    ○
LDS_Mg  SSVLFYFATIITHDIFQTSSRDPSINLTSSYLDLSPLYGRNLEEQLSVRA  250
LDS_Gga SSMLFYLATIITHDIFQTSPRDFNINLTSSYLDLSPLYGRNHDEQMAVRT  240

LDS_Mg  MKDGLLKPDTFCSKRVHGFPPGVGVLLIMFNRFHNYVVTSLAKINEGNRF  300
LDS_Gga GKDGLLKPDTFSSKRVIGFPPGVGAFLIMFNRFHNYVVTQLAKINEGGRF  290

LDS_Mg  KKPVG-DDTAAWEKYDNDLFQTGRLITCGLYVNIVLVDYVRTILNLNRVD  349
LDS_Gga KRPTTPDDTAGWETYDNSLFQTGRLITCGLYINIVLGDYVRTILNLNRAN  340
                                          ∇   ●
LDS_Mg  SSWILDPRTEEGKSLLSKPTPEAVGNQVSVEFNLIYRWHCGMSQRDDKWT  399
LDS_Gga TTWNLDPRTKEGKSLLSKPTPEAVGNQVSVEFNLIYRWHCTISERDDKWT  390

LDS_Mg  TDMLTEALGGKDPATATLPEFFGALGRFESSFPNEPEKRTLAGLKRQEDG  449
LDS_Gga TNAMREALGGQDPATAKMEDVMRALGMFEKNIPDEPEKRTLAGLTRQSDG  440

LDS_Mg  SFEDEGLIKIMQESIEEVAGAFGPNHVPACMRAIEILGMNQARSWNVATL  499
LDS_Gga AFDDTELVKILQESIEDVAGAFGPNHVPACMRAIEILGIKQSRTWNVATL  490

LDS_Mg  NEFREFIGLKRYDTFEDINPDPKVANLLAEFYGSPDAVELYPGINAEAPK  549
LDS_Gga NEFRQFIGLTPHDSFYHMNPDPKICKILAQMYDSPDAVELYPGIMAEAAK  540

LDS_Mg  PVIVPGSGLCPPSTTGRAILSDAVTLVRGDRFFTVDYTPRNLTNFGYQEA  599
LDS_Gga PPFSPGSGLCPPYTTSRAILSDAVSLVRGDRFYTVDYTPRNITNWGFNEA  590

LDS_Mg  ATDKSVDNGNVIYKLFFRAFPNHYAQNSIYAHFPFVIPSENKKIMESLGL  649
LDS_Gga STDKAVDWGHVIYKLFFRAFPNHFLPNSVYAHFPFVVPSENKLIFEGLGA  640

LDS_Mg  ADKYSWQPPQRKPATQMIRSHAAAVKILNNQKDFKVVWGESIGFLTKFPT  699
LDS_Gga ANKYSWDPPKARAPIQFIRSHKAVLEVLSNQKDYKVTWGPAIKMLS----  686

LDS_Mg  GENPGLGFALAGDAPANQQSRDQLMKCIFSPKAWEDEVRQFCEATTWDLL  749
LDS_Gga -GDPATSFALAGDEPANAASRHHVIAALTAPKQWRDEVRRFYEVTTRDLL  735

LDS_Mg  RRYSAKVQDKGPHLKVHTHEIDVIRDVISLANARFFAAVYSLPLKTENGD  799
LDS_Gga RRHGAPVHGVGAG--PRTHEVDVIRDVIGLAHARFMASLFSLPLKEEGKE  783

LDS_Mg  DGVYSDHEMYRSLMLIFSAIFWDNDVSKSFKLRRDARAATQKLGALVEKH  849
LDS_Gga EGAYGEHELYRSLVTIFAAIFWDSDVCNSLKLHQASKAAADKMSALIAEH  833

LDS_Mg  IVEMGS------LFHSFKH--SHSAVSDKTNGLANGGANGHANGNANGHT  891
LDS_Gga VREMEAGTGFLGALGKLKDLITGNDVHANGNGVYTNGNGVYTNGNGV-HT  882

LDS_Mg  NGNGIHQNGGAAP---SMLRSYGDLMLRRMIEAYGEGKSVKEAVYGQIMP  938
LDS_Gga NGNGVHTNGNGVPHAAPSLRSFGDQLLQRMLSQ--DGRSIEETVSGTILP  930

LDS_Mg  SIAAGTANQTQIMAQCLDYYMSDDGAEHLPEMKRLASLETPEAFNTLMK   987
LDS_Gga VVMAGTANQTQLLAQCLDYYLGV-GEKHLPEMKRLAMLNTSEADEKLLK   978
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FIG. 2. Alignment of the deduced protein sequences of the linoleate diol synthetase (LDS) precursor of *M. grisea* (LDS_Mg) with the LDS precursor of *Gaeumannomyces graminis* (LDS_Gga). The putative heme-binding histidine residues and the tyrosine residue of catalytic importance are marked ○, ●, and ▽, respectively.

TABLE 1
Intron–Exon Borders and Putative Lariat Sequences of the Deduced Linoleate Diol Synthase Gene of *Magnaporthe grisea*

Intron	(bp)	5′-Donor	Lariat sequence[a]	3′-Acceptor
1	(81)	TAC CGgtatgt Tyr Arg	atctgac	tagA TTC Phe-140
2	(74)	CAG ACTgtaggt Gln Thr	tgctgac	tag TCA Ser-220
3	(79)	GCC AAgtaagc Ala Lys	ttctaac	cagG ATC Ile-295

[a]The consensus lariat (branch signal) for splicing of mRNA of filamentous fungi is C/TNCTA/GAC/T. bp, base pair.

There is an interesting difference between LDS and the corresponding enzyme of *A. nidulans*: LDS transforms 8-HPODE into 7,8-DiHODE, whereas 8-HPODE (PsiBα) might be transformed to 5,8-DiHODE (PsiCα) in *A. nidulans*. The biological function of LDS is unknown, at least in *G. graminis* and *M. grisea*. In their extensive studies on ssp1, Huber *et al.* (16) found no phenotype associated with the targeted gene disruption of ssp1 in *U. maydis* (16). It will be of interest to determine the catalytic properties of the LDS-like oxygenase of *A. nidulans* and *U. myadis* and to determine whether disruption of the gene of LDS of *M. grisea* and the corresponding gene of *A. nidulans* will affect sporulation or induce other physiological changes.

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Regiospecific Analysis by Ethanolysis of Oil with Immobilized *Candida antarctica* Lipase

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ABSTRACT: A mixture of oil/ethanol (1:3, w/w) was shaken at 30°C with 4% immobilized *Candida antarctica* lipase by weight of the reaction mixture. The reaction regiospecifically converted FA at the 1- and 3-positions to FA ethyl esters, and the lipase acted on C_{14}–C_{24} FA to a similar degree. The content of 2-MAG reached a maximum after 4 h; the content was 28–29 mol% based on the total amount of FA in the reaction mixture at 59–69% ethanolysis. Only 2-MAG were present in the reaction mixture during the first 4 h, and 1(3)-MAG were detected after 7 h. After removal of ethanol from the 4-h reaction mixture by evaporation, 2-MAG were fractionated by silica gel column chromatography. The contents of FA in the 2-MAG obtained by ethanolysis of several oils coincided well with FA compositions at the 2-position, which was analyzed by Grignard degradation. It was shown that ethanolysis of oil with *C. antarctica* lipase can be applied to analysis of FA composition at the 2-position in TAG.

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The position of FA on the glycerol backbone is receiving a great deal of attention from the viewpoint of nutrition and TAG metabolism. Regiospecific analysis has been conducted so far by Grignard degradation (1,2) or by hydrolysis with a 1,3-position-specific lipase, such as lipases from pancreas, *Rhizopus oryzae*, and *Rhizomucor miehei* (3). After degradation of a desired oil, FA compositions at the 2- and 1,3-positions can be determined by TLC based on the contents of FA in 2-MAG or 1,3-DAG recovered from the reaction products.

The two methods, however, include some drawbacks. Grignard degradation requires close attention to technique because even a little moisture greatly impedes the reaction. Meanwhile, a lipase method cannot be expected to provide an exact analysis because the 1,3-position-specific lipases used so far do not act on all FA to a similar degree and because the 2-MAG are hydrolyzed easily after migration of FA at the 2-position to the 1(3)-position. In particular, regiospecific analysis of PUFA-containing oils cannot be achieved by this enzymatic method. Additionally, fractionation of the reaction products by TLC includes a risk of decomposition of unstable FA: Their oxidation starts rapidly as soon as the solvents evaporate.

It was recently reported that *Candida antarctica* lipase B acted on saturated and unsaturated C_{14}–C_{24} FA to a similar degree (4,5). In addition, when oils underwent ethanolysis with the immobilized lipase in the presence of large amounts of EtOH, FA at the 1- and 3-positions were converted regiospecifically to their ethyl esters (4,6). This paper shows that the ethanolysis system is effective for analysis of FA composition at the 2-position in oils containing PUFA.

MATERIALS AND METHODS

Materials. A single-cell oil produced by *Mortierella alpina* and containing 40 wt% arachidonic acid (TGA40 oil) was a gift from Suntory Ltd. (Osaka, Japan). Cocoa fat, borage oil, and tuna oil were donated by Showa Sangyo Co. Ltd. (Tokyo, Japan), Nippon Supplement, Inc. (Osaka, Japan), and Maruha Corp. (Tokyo, Japan), respectively. *Candida antarctica* lipase B (Novozym 435) immobilized on a macroporous resin was from Novozymes (Bagsvaerd, Denmark). Olive oil and ethanol were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and monoolein and oleic acid ethyl ester were from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). DAG-rich oil (Econa Cooking Oil; TAG/DAG/MAG = 6.0:89.9:4.1, by wt; Kao Corp., Tokyo, Japan) was purchased from a local supermarket. Other chemicals were of reagent grade.

Preparation of 1,3-dicapryloyl-2-eicosapentaenoyl glycerol (CEC). CEC was synthesized according to Kawashima *et al.* (5). In brief, trieicosapentaenoin was synthesized by esterification of eicosapentaenoic acid (purity, 95%; Maruha) with glycerol using immobilized *C. antarctica* lipase. The trieicosapentaenoin was purified by silica gel column chromatography and underwent acidolysis with caprylic acid using immobilized *R. oryzae* lipase. CEC was purified with an HPLC system (LC-9A; Shimazu Co., Kyoto, Japan) connected to an octadecyl silica column (20 × 500 mm; SH345-5; Yamamura Chemical Co. Ltd., Kyoto, Japan). The reaction mixture was eluted with a mixture of acetone/acetonitrile (1:1, vol/vol) at a flow rate of 7.0 mL/min. The purified preparation contained 95.4 wt% CEC and was contaminated with 3.9% 1(3),2-dicapryloyl-3(1)-eicosapentaenoyl glycerol (CCE).

Preparation of randomly interesterified oil. The arrange-

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Abbreviations: CCE, 1(3),2-dicapryloyl-3(1)-eicosapentaenoyl glycerol; CEC, 1,3-dicapryloyl-2- eicosapentaenoyl glycerol; FAEE, FA ethyl ester; TGA40 oil, single-cell oil containing 40% arachidonic acid.

ment of FA in TGA40 oil was randomized according to the conventional interesterification method (7). TGA40 oil (30 g) was stirred at 55°C for 30 min with 1.0% Na-methylate in a round-bottomed flask overlain with nitrogen gas. The interesterified products were extracted with 150 mL *n*-hexane after addition of 50 mL water, and the solvent was then removed by evaporation. The resulting oil contained *ca.* 5 wt% partial acylglycerols. To remove them, the oil was introduced onto a silica gel column (250 g; 4 × 45 cm; Merck, Darmstadt, Germany), and TAG were eluted with a 2-L mixture of *n*-hexane/ethyl acetate (98:2, vol/vol). The FA composition of randomly interesterified oil was the same as that of the original TGA40 oil. The yield was 24.5 g.

Reactions. Standard ethanolysis of oils was conducted as follows: A mixture of 30 g oil/EtOH (1:3, wt/wt; 1:57–59, mol/mol) and 1.2 g immobilized *C. antarctica* lipase was shaken at 30°C in a 50-mL screw-capped vessel overlain with nitrogen gas. Ethanolyses of cocoa fat, borage oil, and tuna oil were conducted at 30°C for 4 h in a 20-mL vessel covered with nitrogen gas, in which 16 g oil/EtOH (1:3, wt/wt; 1:57–59, mol/mol) and 0.64 g immobilized lipase were introduced. Ethanolysis of CEC was also conducted similarly: A mixture of 3.4 g CEC/EtOH (1:3, wt/wt; 1:41, mol/mol) and 0.14 g immobilized lipase was shaken at 30°C for 4 h.

Purification of MAG. The 2-MAG or a mixture of 2- and 1(3)-MAG was purified from the reaction mixture by silica gel column chromatography at room temperature (8). EtOH in the reaction mixture was first evaporated, and the resulting mixture (1–3 g) was introduced onto a silica gel column (17 g; 1.5 × 15 cm). After TAG and DAG were removed by stepwise elution with mixtures of 250 mL *n*-hexane/ethyl acetate (95:5, vol/vol) and 200 mL *n*-hexane/ethyl acetate (80:20, vol/vol), 2-MAG or a mixture of 2- and 1(3)-MAG was eluted with a 200-mL mixture of *n*-hexane/ethyl acetate (50:50, vol/vol). The yield of MAG was >90%.

Analyses. The contents of FA ethyl ester (FAEE), TAG, 1,3-DAG, 1(3),2-DAG, and MAG were measured by a TLC/FID analyzer (Iatroscan MK-5; Iatron Laboratories Inc., Tokyo, Japan) after double development (8). The first and second developments were performed using solvent mixtures of *n*-hexane/ethyl acetate/acetic acid (90:10:1, by vol) and *n*-hexane/ethyl acetate (65:5, vol/vol), respectively.

2-MAG and 1(3)-MAG were detected by TLC on boric acid-impregnated silica gel 60 plates (Merck) (9). The TLC plate was prepared as follows: The plate, on which 3% boric acid had been sprayed, was dried overnight at room temperature and was then baked at 120°C for 10 min before use. The sample spotted onto the plate was developed in a mixture of chloroform/acetone/acetic acid (96:4:1, by vol). Components of the reaction products were visualized by spraying 50% sulfuric acid in methanol, followed by heating at 150°C.

FA in acylglycerols were converted to their methyl esters in 3 mL methanol containing 1.0% Na-methylate by heating at 70°C for 15 min. The FAME were analyzed with a Hewlett-Packard 5890 gas chromatograph (Avondale, PA) connected to a DB-23 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA) (10). The column temperature was raised from 150 to 210°C at 2°C/min, and the temperatures of injector and detector (FID) were set at 250°C. The contents of FAEE in a reaction mixture comprising FAEE and acylglycerols were analyzed by direct injection of the reaction mixture into the gas chromatograph, followed by analysis under the same conditions as described above. The contents of FAME and FAEE were determined from their peak areas.

Regiospecific analysis by the Grignard method was performed by degradation of oil with allyl magnesium bromide (1), followed by isolation and analysis of the 1,3-DAG. The 1,3-DAG were isolated by TLC. FA located at the 2-position were calculated from the FA composition of the oil and 1,3-DAG.

RESULTS AND DISCUSSION

Ethanolysis of TGA40 oil with C. antarctica *lipase*. Ethanolysis of TAG with immobilized *C. antarctica* lipase was reported to produce an accumulation of 2-MAG in the early stage of the reaction (4,6). However, a change in the content of 2-MAG has not been reported when the reaction is extended. To determine FA composition at the 2-position from 2-MAG obtained by this ethanolysis, a full time-course of 2-MAG production needs to be studied.

TGA40 oil underwent ethanolysis with different amounts of EtOH by using 7% of immobilized *C. antarctica* lipase by weight of the reaction mixture (Fig. 1). An increase in the amount of EtOH accelerated the velocity of ethanolysis. 1(3),2-

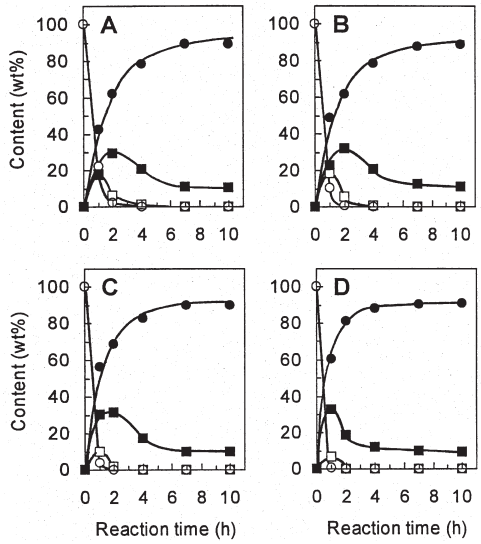


FIG. 1. Effect of ethanol (EtOH) amount on ethanolysis of single-cell oil containing 40% arachidonic acid (TGA40) with immobilized *Candida antarctica* lipase. A 30-g mixture of the oil and EtOH was shaken at 30°C with 2.1 g immobilized lipase. Part of the reaction mixture (*ca.* 1 g) was periodically withdrawn, and the contents of TAG, DAG, MAG, and FA ethyl ester (FAEE) were analyzed. (A) Ratio of TGA40 oil/EtOH = 1:2, w/w; (B) TGA40 oil/EtOH = 1:3, w/w; (C) TGA40 oil/EtOH = 1:4, w/w; (D) TGA40 oil/EtOH = 1:6, w/w. ○, TAG; □, 1(3),2-DAG; ●, FAEE; ■, MAG [2-MAG or 2- and 1(3)-MAG].

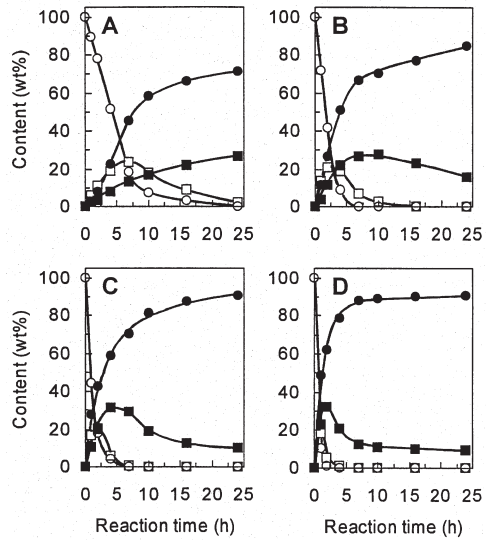


FIG. 2. Effect of the amount of immobilized *C. antarctica* lipase on ethanolysis of TGA40 oil. A 30-g mixture of TGA40 oil/EtOH (1:3, w/w) was shaken at 30°C with different amounts of immobilized lipase. Part of the reaction mixture (*ca.* 1 g) was periodically withdrawn, and the contents of TAG, DAG, MAG, and FAEE were analyzed. (A) Amount of immobilized lipase was 1% by weight of the reaction mixture; (B) immobilized lipase, 2 wt%; (C) immobilized lipase, 4 wt%; (D) immobilized lipase, 7 wt%. ○, TAG; □, 1(3),2-DAG; ●, FAEE; ■, MAG [2-MAG or 2- and 1(3)-MAG]. See Figure 1 for abbreviations.

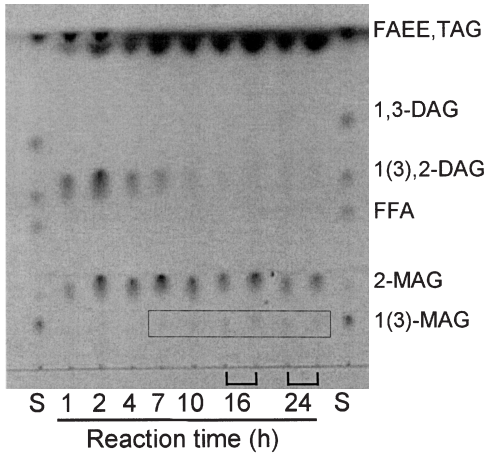


FIG. 3. TLC of reaction mixtures withdrawn from the reaction in Figure 2C. Three volumes of sample were spotted on the right lanes of 16- and 24-h reactions. Small amounts of 1(3)-MAG were detected in the box shown with the dotted line. A mixture of oleic acid ethyl ester, olive oil, DAG, oleic acid, and monoolein was spotted on lane S.

DAG were detected in the reaction mixture, but 1,3-DAG were not. Only 2-MAG were detected in the early stage of the reaction, and 1(3)-MAG were detected in the late stage of the reaction (see below). In addition, the content of MAG decreased gradually after reaching a maximal value (29–33 wt%; 26–30 mol% based on the total amount of FA in the reaction mixture).

Ethanolysis of TGA40 oil was next conducted with three proportions of EtOH and different amounts of immobilized lipase (Fig. 2). A decrease in the amount of immobilized lipase resulted in a several-hour delay in MAG reaching maximal value. 1(3)-MAG were detected after 7 h in reaction with 1–4 wt% of the lipase, and after 4 h in reaction with 7 wt% of the lipase (data not shown). When the reaction was conducted with 2 wt% of immobilized lipase, the content of MAG [2-MAG or a mixture of 2- and 1(3)-MAG] reached a maximal value after 10 h (27.3 wt%; 24.5 mol% based on total amount of FA in the reaction mixture). At this reaction time, the molar amount of total acylglycerols declined to 77.1% of the initial amount. On the other hand, the reactions with 4 and 7 wt% of lipase accumulated the highest content of 2-MAG (31–32 wt%; 28–29 mol% based on the total amount of FA in the reaction mixture) after 4 and 2 h, respectively. In addition, the molar amounts of total acylglycerols after the 4- and 2-h reactions were 95.9 and 98.2% of the initial amount, respectively.

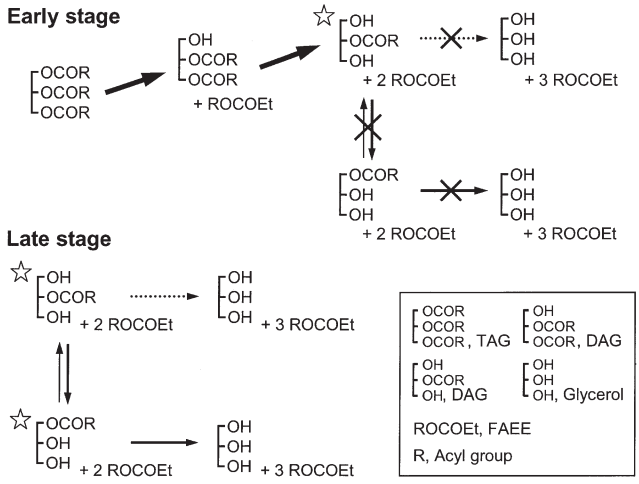
Considering these results, we decided that the optimal ratio of TGA40 oil/EtOH was 1:3 wt/wt and the optimal amount of immobilized lipase was 4% by weight of the reaction mixture. Ethanolysis was repeated for five cycles at 30°C by transferring immobilized enzyme to a fresh substrate mixture every 24 h. A decrease in the activity was not observed, indicating that the lipase was stable under these conditions.

Estimated pathway in ethanolysis of TGA40 oil. Reaction mixtures withdrawn from the reaction in Figure 2C were analyzed by TLC (Fig. 3). Only 2-MAG were detected during the first 4 h, and a small amount of 1(3)-MAG was detected after 7 h. 1,3-DAG were not detected at all during the reaction. TAG and 1(3),2-DAG were detected during the first 4 h, and their contents after 7 h declined to <1.0 wt% (Fig. 2C). Furthermore, the total content of acylglycerols did not decrease significantly during the first 4 h, and then fell gradually: 98.2% of the initial amount remained after 4 h; 79.2% after 7 h; 49.7% after 10 h; 33.5% after 16 h; and 25.4% after 24 h (calculated from Fig. 2C). These results suggested that the TGA40 oil underwent ethanolysis through the pathway shown in Scheme 1, when the reaction was conducted at 30°C using 4 wt% of immobilized lipase.

In the early stage of the reaction (during the first 4 h), FA at the 1- and 3-positions underwent very fast ethanolysis, and 2-MAG accumulated. Also, the total molar amount of TAG, DAG, and MAG was almost the same as the initial amount of substrate TAG. These results indicated not only that FA at the 2-position did not undergo ethanolysis but also that very little acyl migration of FA from the 2-position to the 1(3)-position occurred. Even though acyl migration occurred, FA migrated from the 2- to the 1(3)-position would easily undergo ethanolysis.

In the late stage of the reaction (after 7 h), the reaction mixture contained 2- and 1(3)-MAG and FAEE, but not DAG or TAG. Detection of 1(3)-MAG showed that acyl migration of 2-MAG to 1(3)-MAG occurred. The velocity of ethanolysis of 1(3)-MAG slowed because the reaction approached equilibrium. Consequently, the ratio of the content of 1(3)-MAG to that of 2-MAG increased gradually.

FA composition of 2-MAG. Ethanolysis of TGA40 oil was conducted under the same conditions as those in Figure 2C. After



SCHEME 1

EtOH was evaporated from the reaction mixture, the resulting mixture was subjected to silica gel column chromatography to recover MAG: at <4 h, 2-MAG was detected; from 7 to 24 h, a mixture of 2- and 1(3)-MAG was identified. The FA composition of the MAG is shown in Figure 4. FA composition of MAG was constant during the first 7 h, showing that the FA were located at the 2-position of TGA40 oil. The content of each FA in MAG, obtained from the 4-h reaction, is shown in Table 1.

Effect of FA specificity of the lipase on regiospecific analysis. As all TAG were not converted to 2-MAG during ethanolysis, the FA selectivity of *C. antarctica* lipase may affect regiospecific analysis somewhat. Hence, FA specificity of the lipase was studied.

Candida antarctica lipase showed strong activity on FA esters at the 1- and 3-positions of TAG in the ethanolysis. Since constituent FA in TGA40 oil do not distribute equally on the glycerol backbone, randomly interesterified TGA40 oil was used as a substrate for studying FA specificity of the lipase. Relative activity of the lipase on each FA in ethanolysis (K_{act})

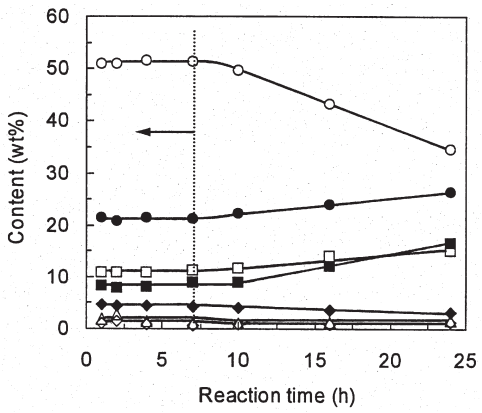


FIG. 4. Change in FA compositions of MAG obtained by ethanolysis of TGA40 oil. The reaction was conducted under the same conditions as those in Figure 2C. △, 16:0; ◇, 18:0; □, 18:1n-9; ●, 18:2n-6; ◆, 18:3n-6; ■, 20:3n-6; ○, 20:4n-6. For abbreviation see Figure 1.

TABLE 1
FA Composition at the 2-Position in TGA40 Oil

FA	$K_{act, 1h}$ [a]	FA composition (wt%) Observed [b]	$F_{MAG, 7h}$ [c]	F_{cor} [d]
16:0	1.04	1.6	1.1	1.1
18:0	1.00	0.8	0.6	0.6
18:1	0.97	10.9	11.3	11.2
18:2	0.95	20.5	21.0	20.7
18:3	1.02	4.4	4.2	4.2
20:3	0.96	8.1	8.9	8.8
20:4	1.00	51.7	50.9	50.9

[a]Relative activity of *Candida antarctica* lipase on each constituent FA in randomly interesterified single-cell oil containing 40% arachidonic acid (TGA40 oil). Activity was calculated from the FA composition after a 1-h reaction according to Equation 1 and was compiled from Figure 5B.
[b]The content of each FA in 2-MAG obtained after 4 h, which was compiled from Figure 4.
[c]The content of each FA in MAG [mixture of 2- and 1(3)-MAG] after 7 h, which was compiled from Figure 4.
[d]The content of each FA in 2-MAG, which was corrected according to Equation 2.

is expressed according to the equation

$$K_{act} = F_{ee}/F_{gly} \quad [1]$$

where F_{ee} and F_{gly} are the contents (mol%) of a particular FA in the FAEE fraction after the ethanolysis and in the substrate oil, respectively.

Ethanolysis of randomly interesterified TGA40 oil was conducted at 30°C. The time course of the contents of FAEE and acylglycerols was similar to that of the ethanolysis of TGA40 oil (Figs. 2C, 5A). The difference in K_{act} on the constituent FA was the largest when ethanolysis was started and fell gradually during the time span of the reaction (Fig. 5B). The maximal differences in K_{act} fell within the range of 0.93 to 1.04, thus confirming that *C. antarctica* lipase acted on constituent FA in TGA40 oil to a similar degree. The K_{act} of each FA at 1 h is shown in Table 1.

In the reaction with 4 wt% immobilized *C. antarctica* lipase, hardly any acyl migration occurred during the first 4 h, and also 2-MAG did not undergo ethanolysis. The total amount of acylglycerols decreased after 7 h, indicating that 1(3)-MAG, converted from 2-MAG, underwent ethanolysis. The FA composition of MAG [mixture of 2- and 1(3)-MAG] obtained by a 7-h reaction may therefore include an effect of FA specificity of the lipase.

To determine the average decrease in the amount of acylglycerols after 7 h, ethanolysis of TGA40 oil was conducted independently six times. Ethanolysis after 7 h was 73.2 ± 6.8% (mean ± SD). In this reaction mixture, the content of MAG FA in the total FA amount in the reaction mixture was 22.2 ± 4.0 mol%, and the amount of acylglycerols declined to 76.4 ± 16.2 mol% of the initial amount. These results showed that 23.6 mol% of 2-MAG underwent ethanolysis after migration of FA at the 2-position to the 1(3)-position. The contents of FA at the 2-position of TGA40 oil were corrected using the FA composition of MAG obtained after 7 h and the largest FA specificity (K_{act}) at 1 h. The

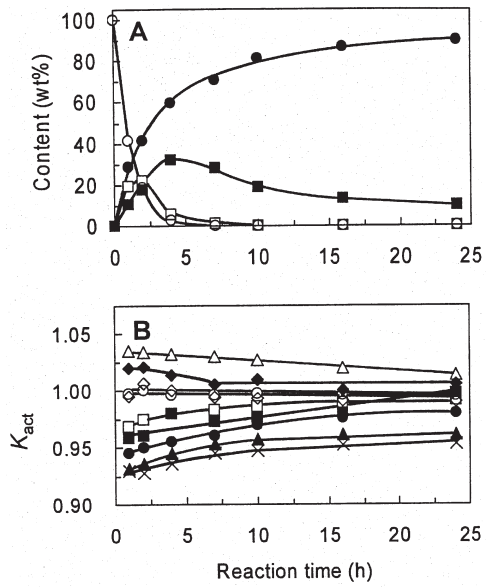


FIG. 5. Ethanolysis of randomly interesterified TGA40 oil with immobilized *C. antarctica* lipase. A 30-g mixture of oil/EtOH (1:3, w/w) was shaken at 30°C with 4 wt% immobilized lipase. (A) Time course of the contents of acylglycerols and FAEE. ○, TAG; □, 1,2-DAG; ●, FAEE; ■, MAG [>4 h, 2-MAG; 7–24 h, mixture of 2- and 1(3)-MAG]. (B) Relative activity (K_{act}) of *C. antarctica* lipase on each FA in the substrate oil. K_{act} was calculated according to Equation 1. △, 16:0; ◇, 18:0; □, 18:1n-9; ●, 18:2n-6; ◆, 18:3n-6; ■, 20:3n-6; ○, 20:4n-6; X, 22:0; ▲, 24:0. See Figure 1 for abbreviations.

corrected content (F_{cor}) can be expressed according to the equation

$$F_{cor} = F_{MAG,\ 7h} \times 0.764 + F_{MAG,\ 7h} \times (1 - 0.764) \times K_{act,\ 1h} \quad [2]$$

where $F_{MAG,\ 7h}$ is the content of a particular FA in MAG [mixture of 2- and 1(3)-MAG] obtained after 7 h in the reaction at 30°C (Fig. 4), and $K_{act,\ 1h}$ is the relative activity of the FA after 1 h (Fig. 5B). The contents of F_{cor} are shown in Table 1. Even though the correction was conducted, the contents of FA at the 2-position differed little from the observed contents after 4 h. This result indicated that a fairly accurate analysis of FA at the 2-position in TAG could be achieved owing to a combination of strong activity of *C. antarctica* lipase on FA at the 1,3-position in TAG and little selectivity toward the constituent FA.

Evaluation of the enzymatic method. It is believed that FA composition at the 2-position in TAG can be analyzed most exactly by the Grignard method. Hence, FA compositions at the 2-position in cocoa fat, borage oil, and tuna oil were analyzed by Grignard degradation and enzymatic ethanolysis, as developed here. Ethanolyses of cocoa fat, borage oil, and tuna oil were conducted at 30°C for 4 h with immobilized *C. antarctica* lipase. The degrees of ethanolysis of cocoa fat, borage oil, and tuna oil reached 68.6, 67.3, and 59.4%, respectively, and the contents of 2-MAG were 32.2 wt% (29.1 mol%), 32.1 wt% (29.0 mol%), and 32.1 wt% (28.9 mol%), respectively. After the MAG had been recovered by silica gel column chromatography, the FA composition was analyzed (Table 2). The FA compositions determined by the two methods coincided well, showing that this enzymatic method is effective for analysis of FA composition at the 2-position in TAG.

In addition, the FA composition at the 2-position in high-purity CEC, which contained 3.9 wt% CCE, was analyzed by enzymatic ethanolysis. When ethanolysis of CEC was conducted at 30°C for 4 h, the content of 2-MAG was 44.3 wt% (28.4 mol%) at 69.4% ethanolysis. The content of caprylic acid

TABLE 2
FA Compositions at the 2-Position in Several Oils, Which Were Determined by Grignard Degradation and Enzymatic Ethanolysis

		FA composition (wt%)									
Oil	Method	8:0	16:0	18:0	18:1 n-9	18:2 n-6	18:3 n-6	20:3 n-6	20:4 n-6	20:5 n-3	20:6 n-3
TGA40	—[a]	ND[b]	9.8	7.1	6.7	9.5	2.7	3.9	42.1	4.3	10.4
	Grignard[c]	ND	1.9	1.0	11.1	21.0	4.9	8.2	50.4	ND	ND
	Enzymatic[d]	ND	1.6	0.8	10.9	20.5	4.4	8.1	51.7	ND	ND
Cocoa	—[a]	ND	25.3	36.4	34.3	2.9	ND	ND	ND	ND	ND
	Grignard[c]	ND	0.9	1.5	90.5	6.0	ND	ND	ND	ND	ND
	Enzymatic[e]	ND	1.0	1.3	90.7	5.9	ND	ND	ND	ND	ND
Borage	—[a]	ND	10.7	4.1	17.2	35.9	21.3	ND	ND	ND	ND
	Grignard[c]	ND	0.6	ND	15.3	36.8	45.9	ND	ND	ND	ND
	Enzymatic[e]	ND	0.7	ND	15.6	37.7	44.1	ND	ND	ND	ND
Tuna	—[a]	ND	16.4	4.2	23.0	1.0	ND	ND	2.0	6.5	23.7
	Grignard[c]	ND	19.1	1.3	11.1	1.3	ND	ND	2.3	6.4	39.9
	Enzymatic[e]	ND	18.5	1.3	12.1	0.8	ND	ND	2.0	5.9	40.6
CEC	—[a]	49.1	ND	ND	ND	ND	ND	ND	0.5	48.1	2.1
	Enzymatic[e]	2.1	ND	ND	ND	ND	ND	ND	0.8	93.0	4.1

[a]FA composition of each oil.
[b]Not detected.
[c]The content of each FA at the 2-position was calculated from the FA compositions of TAG and 1,3-DAG.
[d]Compiled from Table 1.
[e]Ethanolysis was conducted at 30°C in a mixture of oil/ethanol (1:3, w/w) using 4 wt% immobilized *Candida antarctica* lipase. The content of each FA at the 2-position was determined by analysis of MAG obtained after 4 h.

was 2.2 wt% (4.3 mol%), and the total content of EPA, arachidonic acid, and DHA of 2-MAG was 97.8 wt% (95.7 mol%) (Table 2). Thus, this enzymatic method can be used for analysis of FA composition at the 2-position in structured TAG containing medium-chain FA and PUFA.

In this paper, we analyzed FA composition by GC of FAME prepared from MAG; at least 1 g of mixture was necessary for a reliable recovery of MAG by silica gel column chromatography. The regiospecific analysis may become possible on a milligram scale through a combination of NMR spectroscopy or HPLC by direct injection of a reaction mixture.

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Simple Chemical Syntheses of TAG Monohydroperoxides

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ABSTRACT: For the purpose of synthesizing standards to be used in the quantification of TAG hydroperoxides, three TAG (1,2-dioleoyl-3-palmitoylglycerol, 1-oleoyl-2-linoleoyl-3-palmitoylglycerol, and triolein) monohydroperoxides were chemically synthesized as authentic specimens. TAG were prepared by using a simple condensation in pyridine of glycerol and the corresponding acid chlorides. These TAG were then converted into monohydroperoxides by a photosensitized peroxidation. The synthesized monohydroperoxides were analyzed by normal-phase and RP-HPLC. The results of normal-phase HPLC analysis showed that monohydroperoxides from a corresponding TAG were a mixture of regioisomers. In RP-HPLC, however, the regioisomers of monohydroperoxides were not separated and gave a single peak, which may improve the sensitivity for the detection of TAG monohydroperoxides. In this study TAG monohydroperoxide standards were synthesized; these will be useful for the study of yet unknown biological and pathological roles of TAG hydroperoxides.

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A causal relation between oxidized LDL and the development of atherosclerosis is well established. Attention has been drawn to cholesteryl ester hydroperoxide in plasma as a product of lipid peroxidation and of experimentally oxidized LDL (1–4). The role of TAG hydroperoxides in the development of atherosclerosis is obscure, although some evidence indicates that atherosclerosis can develop as a result of changes in the metabolism of TAG-rich lipoproteins (5,6).

Here we report a convenient chemical synthesis of hydroperoxides of three TAG, 1,2-dioleoyl-3-palmitoylglycerol (OOP), 1-oleoyl-2-linoleoyl-3-palmitoylglycerol (OLP), and triolein (OOO), which together account for over 60% of total TAG in both VLDL and adipose tissue (7,8), for use as standards in the measurement of TAG hydroperoxides in plasma and adipose tissue.

MATERIALS AND METHODS

Linoleic acid, oleic acid, and palmitic acid were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and used after chromatographic purification. Dimethylethylsilylimidazole was also purchased from Tokyo Chemical Industry Co., Ltd. Oxalyl chloride and cytochrome C (from horse heart) were from Wako Pure Chemical Industry, Ltd. (Osaka, Japan). Glycerol and luminol were from Kanto Chemical Co., Inc. (Tokyo, Japan).

For column chromatography, Silica gel 60N (100–120 μm; Kanto Chemical Co., Inc., Tokyo, Japan) was used. All chemicals and solvents used were reagent grade. The chemical structures of synthesized compounds were confirmed by their ^{1}H NMR spectra. ^{1}H NMR and ^{13}C NMR spectra were recorded with JEOL JNM EX-400 and ECA 500 at 400 and 500 MHz, respectively, with tetramethylsilane as an internal standard; the abbreviations used were *s*, singlet; *d*, doublet; *dd*, doublet of doublets; *t*, triplet; *sext*, sextet; and *m*, multiplet. Melting points were measured by a micro hot plate m.p. apparatus (Mitamura Riken Co., Tokyo, Japan) and were uncorrected.

*Synthesis of monohydroperoxides. (i) 1-Palmitoylglycerol (**2**).* A solution of palmitic acid (10 mmol) and oxalyl chloride (15 mmol) in dichloromethane (10 mL) was refluxed for 2 h and then evaporated to remove solvent and excess reagent. The oily residue (palmitoyl chloride) was redissolved in dichloromethane (5 mL), and the solution was then added dropwise to a stirred solution of glycerol (**1**, 50 mmol) in pyridine (20 mL) at 0°C. The reaction continued with stirring for 1 h at 0°C and then the solution was poured into ice water. The mixture was extracted twice with chloroform. The combined organic layers were washed with ice-cold HCl (2 mol/L) and water and then dried over Na_2SO_4. After evaporation of the solvent, the residue was subjected to silica gel (SiO_2) column chromatography, with chloroform/methanol (15:1, vol/vol) as eluant, to give pure 1-palmitoylglycerol (**2**) as white platelets recrystallized from 1:1 ethyl acetate/*n*-hexane (2.48 g, 75%), m.p. 81–83°C. ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t*, *J* = 6.4 Hz, 3H, –C*H*$_3$), 1.20–1.65 (*m*, 26H, –C*H*$_2$–), 2.35 (*t*, *J* = 6.3 Hz, 2H, –OCO–C*H*$_2$–), 3.61 and 3.69 (*m*, each 1H, 3-*H*), 3.93 (*m*, 1H, 2-*H*), 4.15 (1H, *dd*, *J* = 11.7 and 5.8 Hz, 1-*H*), and 4.20 ppm (1H, *dd*, *J* = 11.7 and 4.7 Hz, 1-*H*).

(ii) 1-Oleoyl-3-palmitoylglycerol (**3**). Oleoyl chloride prepared from oleic acid (3 mmol) and oxalyl chloride (4.5 mmol), as described above, was dissolved in dichloromethane (5 mL). This solution was added dropwise to a solution of 1-palmitoylglycerol (**2**, 2 mmol) in pyridine (5 mL) at 0°C, and the solution was stirred at 0°C for 2 h. The reaction was monitored by TLC until 1-oleoyl-3-palmitoylglycerol was recognized as a major product. The reaction mixture was processed as described above.

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Abbreviations: OLP, 1-oleoyl-2-linoleoyl-3-palmitoylglycerol; OOO, triolein; OOP, 1,2-dioleoyl-3-palmitoylglycerol; POP, 2-oleoyl-1,3-dipalmitoylglycerol; PPO, 1-oleoyl-2,3-dipalmitoylglycerol.

The crude product was purified by SiO_2 column chromatography with *n*-hexane/ethyl acetate (10:1, vol/vol) as eluent to yield pure 1-oleoyl-3-palmitoylglycerol (**3**) as a colorless white powder recrystallized from 1:1 ethyl acetate/*n*-hexane (0.83 g, 70%), m.p. 44–46°C. ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t, J* = 6.4 Hz, 6H, –C*H*$_3$), 1.18–1.65 (*m*, 48H, –C*H*$_2$–), 2.00 (*m*, 4H, –C=C–C*H*$_2$–), 2.35 (*t, J* = 6.3 Hz, 4H, –OCO–C*H*$_2$–), 4.08 (*m*, 1H, 2-*H*), 4.15 (*m*, 4H, 1- and 3-*H*), and 5.35 ppm (*m*, 2H, –C*H*=C*H*–).

*(iii) OOO (**4**).* A dichloromethane (5 mL) solution of oleoyl chloride obtained from oleic acid (5 mmol) and oxalyl chloride (6 mmol), as described above, was added dropwise to a solution of glycerol (1 mmol) in pyridine (5 mL) at room temperature; the reaction mixture was stirred for 1 h and then was processed in the usual manner. The crude product was subjected to SiO_2 column chromatography, with *n*-hexane/ethyl acetate (20:1, vol/vol), to yield pure OOO as a colorless oil (0.71 g, 80%). ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t, J* = 6.4 Hz, 9H, –C*H*$_3$), 1.26–1.60 (*m*, 66H, –C*H*$_2$–), 2.00 (*m*, 12H, –C=C–C*H*$_2$–), 2.30 (*t, J* = 6.0 Hz, 6H, –OCO–C*H*$_2$–), 4.15 (*dd, J* = 11.7 and 5.5 Hz, 2H, 1- and 3-*H*), 4.28 (*dd, J* = 11.7 and 4.0 Hz, 2H, 1- and 3-*H*), 5.28 (*m*, 1H, 2-*H*), and 5.33 ppm (*m*, 6H, –C*H*=C*H*–). ^{13}C NMR ($CDCl_3$, δ): 62.18 (glyceryl C1 and C3), 68.95 (glyceryl C2), 129.79, 130.09 (–C=C–), 172.92, and 173.33 ppm (–COO–).

*(iv) OOP (**5**).* A solution of oleoyl chloride prepared from oleic acid (5 mmol) and oxalyl chloride (7.5 mmol) in dichloromethane (5 mL), as described above, was added dropwise to a stirred solution of **2** (1.5 mmol) in pyridine (20 mL) at room temperature. Stirring continued for 1 h. The reaction mixture was then processed as usual, and the crude product was purified by SiO_2 column chromatography by elution with *n*-hexane/ethyl acetate (20:1, vol/vol) to give pure OOP (**5**) as a colorless oil (1.24 g, 96%). ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t, J* = 6.5 Hz, 9H, –C*H*$_3$), 1.25–1.65 (*m*, 70H, –C*H*$_2$–), 2.00 (*m*, 8H, –C=C–C*H*$_2$–), 2.31 (*t, J* = 6.3 Hz, 6H, –OCO–C*H*$_2$–), 4.14 (*dd, J* = 12.2 and 6.0 Hz, 2H, 1- and 3-*H*), 4.29 (*dd, J* = 12.2 and 4.4 Hz, 2H, 1- and 3-*H*), 5.26 (*t, J* = 6.3 Hz, 1H, 2-*H*), and 5.34 ppm (*m*, 4H, –C*H*=C*H*–). ^{13}C NMR ($CDCl_3$, δ): 62.17 (glyceryl C1 and C3), 68.98 (glyceryl C2), 129.78, 130.09 (–C=C–), 172.92, and 173.37 ppm (–COO–).

*(v) OLP (**6**).* A solution of linoleoyl chloride prepared from linoleic acid (2 mmol) and oxalyl chloride (3 mmol) in dichloromethane (5 mL), as described above, was added dropwise to a stirred solution of **3** (1 mmol) in pyridine (5 mL) at room temperature. The reaction mixture was stirred for 1 h at room temperature and then processed as already described. The crude product was subjected to SiO_2 column chromatography by elution with *n*-hexane/ethyl acetate (20:1, vol/vol) to yield OLP (**6)** as a colorless oil (0.70 g, 82%). ^{1}H NMR ($CDCl_3$, δ): 0.86–0.90 (*m* by three overlapping triplets, 9H, –C*H*$_3$), 1.25–1.62 (*m*, 64H, –C*H*$_2$–), 2.01–2.07 (*m*, 8H, –C=C–C*H*$_2$–), 2.31 (*t, J* = 6.4 Hz, 6H, –OCO–C*H*$_2$–), 2.77 (*t, J* = 5.5 Hz, 2H, –C=C–C*H*$_2$–C=C–), 4.14 (*dd, J* = 12.2 and 6.3 Hz, 2H, 1- and 3-*H*), 4.28 (*dd, J* = 12.2 and 4.4 Hz, 1- and 3-H), 5.27 (*m*, 1H, 2-*H*), and 5.34 ppm (*m*, 6H, –C*H*=C*H*–). ^{13}C NMR ($CDCl_3$, δ): 62.07 (glyceryl C1 and C3), 68.86 (glyceryl C2), 87.11 (–*C*H–OOH–), 127.86, 128.05, 129.69, 129.97, 129.99, 130.21 (–C=C–), 172.82, and 173.25 ppm (–COO–).

(vi) Hydroperoxidation of TAG. Hydroperoxidation was carried out by a previously described method (11). Briefly, a solution of TAG (1 mmol) and hematoporphyrin (3 mg) in pyridine (10 mL) was irradiated by a 200-W tungsten lamp for 15–30 min at 15°C while bubbling O_2 gas into the solution through a glass ball filter. The reaction mixture was then poured into ice water and acidified with HCl (2 mol/L). The aqueous layer was extracted twice with peroxide-free diethyl ether. The combined organic layer was washed with water and saturated sodium chloride solution, then dried over Na_2SO_4. After evaporating the solvent, the residue was subjected to SiO_2 column chromatography by elution with *n*-hexane/ethyl acetate (10:1, vol/vol) to yield monohydroperoxides of TAG. These monohydroperoxides were obtained as a mixture of regioisomers, and the following ^{1}H NMR data were recorded on the isomeric mixture.

*(vii) OOO monohydroperoxides (**7**).* OOO monohydroperoxides were obtained from **4** as a colorless, viscous liquid with 30% yield (0.27 g). ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t, J* = 6.4 Hz, 9H, –C*H*$_3$), 1.26–1.62 (*m*, 64H, –C*H*$_2$–), 1.98–2.15 (*m*, 10H, –C=C–C*H*$_2$–), 2.29–2.33 (*t, J* = 6.2 Hz, 6H, –OCO–CH$_2$–), 4.14 (*dd, J* = 4.4 and 11.7 Hz, 2H, 1- or 3-*H*), 4.28 (*m*, 2H, 1- or 3-*H*), 4.31 (*m*, 1H, –C*H*OOH), 5.26 (*m*, 1H, 2-*H*), 5.32 (*m*, 4H, *cis* –C*H*=C*H*–), 5.36 (*m*, 1H, *trans* –C*H*=CH–C–OOH), and 5.73 ppm (*m*, 1H, *trans* –CH=C*H*–C–OOH). ^{13}C NMR ($CDCl_3$, δ): 62.19, 62.48 (glyceryl C1 and C3), 68.96 (glyceryl C2), 87.03 (–*C*H–OOH–), 128.57, 129.00, 129.79, 130.10, 136.60, 137.14 (–C=C–), 172.97, and 173.38 ppm (–COO–).

*(viii) OOP monohydroperoxides (**8**).* OOP monohydroperoxides were obtained from **5** as a colorless, viscous liquid in 32% yield (0.29 g). ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t, J* = 6.6 Hz, 9H, –C*H*$_3$), 1.25–1.58 (*m*, 68H, –C*H*$_2$–), 2.05 and 2.10 (*m*, 4H and 2H, –C=C–C*H*$_2$–), 2.31 (*t, J* = 6.5 Hz, 6H, –OCO–C*H*$_2$–), 4.14 (*dd, J* = 12.2 and 6.2 Hz, 2H, 1- and 3-*H*), 4.28 (*m*, 2H, 1- and 3-*H*), 4.30 (*m*, 1H, -C*H*OOH), 5.26 (*m*, 1H, 2-*H*), 5.33 (*m*, 2H, *cis*-C*H*=C*H*–), 5.35 (*m*, 1H, *trans* –C*H*=CH–C–OOH), and 5.73 ppm (*m*, 1H, *trans* –C*H*=C*H*–C–OOH). ^{13}C NMR ($CDCl_3$, δ): 62.19 (glyceryl C1 and C3), 68.96 (glyceryl C2), 87.02 (–*C*H–OOH–), 128.59, 129.01, 129.77, 130.10, 136.57, 137.10 (–C=C–), 172.90, and 173.41 ppm (–COO–).

*(ix) OLP monohydroperoxides (**9**).* OLP momohydroperoxides were obtained from **6** as a colorless, viscous liquid in 24% yield (0.22 g). ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t, J* = 6.6 Hz, 9H, –C*H*$_3$), 1.24–1.66 (*m*, 62H, –C*H*$_2$), 1.98–2.12 (*m*, 6H, –C=C–C*H*$_2$–), 2.31 (*t, J* = 6.2 Hz, 6H, –OCO–C*H*$_2$–), 2.77 (*m*, 0.6H, –C=C–C*H*$_2$–C=C–), 4.12 (*dd, J* = 4.3 and 12.3 Hz, 2H, 1- or 3-*H*), 4.28 (*dd, J* = 4.4 and 12.3 Hz, 2H, 1- or 3-*H*), 4.35 (*m*, 1H, –C*H*OOH), 5.25 (*m*, 1H, 2-*H*), 5.34 (*m, ca.* 4H, *cis* –C*H*=C*H*–), 5.37 (*m, ca.* 0.3H, *trans* –C*H*=CH–C–OOH–), 5.74 (*m, ca.* 1H, –CH=C*H*–C–OOH– and –CH=CH–CH=C*H*–C–OOH), 5.99 (*m, ca.* 0.4H, –CH=C*H*–CH=CH–C–OOH–), and 6.55 ppm (*m, ca.* 0.3H, –CH=CH–C*H*=CH–C–OOH–). ^{13}C NMR ($CDCl_3$, δ): 62.17 (glyceryl C1 and C3), 68.97 (glyceryl C2), 86.76, 86.83, 87.01 (–*C*H–OOH–), 127.96, 128.02, 128.17, 129.78, 130.06, 130.09 (–C=C–),

172.96, and 173.42 ppm (–COO–). H–H COSY showed correlation of the signals between 4.35 and 5.74 ppm, and C–H COSY also indicated correlation of the signals between about 87 and 4.35 ppm.

(X) Hydroperoxidation of 2-oleoyl-1,3-dipalmitoylglycerol (POP) and 1-oleoyl-2,3-dipalmitoylglycerol (PPO) for the study of selectivity at the 1- or 2-position of the glyceride. POP (0.5 mmol) and PPO (0.5 mmol) prepared as above were subjected to hydroperoxidation under the same conditions as above. POP monohydroperoxides and PPO monohydroperoxides were obtained as colorless, viscous liquids in yields of 28 (120 mg) and 26% (113 mg), respectively. POP monohydroperoxide: ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t*, *J* = 6.6 Hz, 9H, –C*H*$_3$), 1.21–1.32 and 1.52–1.61 (*m*, 74H, –C*H*$_2$), 1.98–2.12 (*m*, 2H, –C=C–C*H*$_2$–), 2.31 (*t*, *J* = 6.2 Hz, 6H, –OCO–C*H*$_2$–), 4.14 (*dd*, *J* = 4.4 and 12.5 Hz, 2H, 1- or 3-*H*), 4.25 (*m*, 1H, –C*H*OOH–), 4.30 (*dd*, *J* = 4.8 and 12.5 Hz, 2H, 1- or 3-*H*), 5.26 (*m*, 1H, 2-*H*), 5.36 and 5.77 ppm (*m*, each 1H, –C*H*=C*H*–). ^{13}C NMR ($CDCl_3$, δ): 62.40 (glyceryl C1 and C3), 68.97 (glyceryl C2), 87.11 (–*C*H–OOH–), 128.61, 129.10, 136.70, 137.22 (–C=C–), 173.07, 173.42 (–COO–). PPO monohydroperoxide: ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t*, *J* = 6.6 Hz, 9H, –C*H*$_3$), 1.21–1.32 and 1.52–1.61 (*m*, 74H, –C*H*$_2$), 2.04–2.12 (*m*, 2H, –C=C–C*H*$_2$–), 2.28 and 2.30 (*t*, *J* = 6.2 Hz, each 2H, –OCO–C*H*$_2$–), 4.14 (*dd*, *J* = 4.3 and 12.8 Hz, 2H, 1- or 3-*H*), 4.24 (*m*, 1H, –C*H*OOH–), 4.30 (*dd*, *J* = 4.5 and 12.8 Hz, 2H, 1- or 3-*H*), 5.26 (*m*, 1H, 2-*H*), 5.36 and 5.76 ppm (*m*, each 1H, –C*H*=C*H*–). ^{13}C NMR ($CDCl_3$, δ): 62.20 (glyceryl C1 and C3), 68.95 (glyceryl C2), 87.03 (–*C*H–OOH–), 128.59, 128.99, 136.62, 137.13 (–C=C–), and 173.00, 173.34 ppm (–COO–).

HPLC analysis. HPLC using a normal-phase column was performed with an SCL-10A system (Shimadzu Co., Kyoto, Japan) including the CTO-10A injector and the SPD-10 A UV-vis detector. The analytical column used was Mightysil Si 60 (4.5 × 250 mm, 5 μm; Kanto Chemical Co., Inc.). The mobile phase was a mixture of *n*-hexane/2-propanol (150:1, vol/vol). The flow rate was 1 mL/min, and the temperature was ambient. The detection wavelength was 210 nm. RP-HPLC with postcolumn chemiluminescence detection (9,10) also was performed with an SCL-6A system (Shimadzu Co.) including the CTO-10A injector, and the CLD-10A chemiluminescence detector. The column used was Inertsil ODS-2 (4.6 × 250 mm; GL Sciences Inc., Tokyo, Japan). The mobile phase was a mixture of ethanol/water (100:2, vol/vol), and the flow rate was 0.5 mL/min. Upon emerging from the column, the eluate was mixed with the luminescent reagent (0.5 mL/min) in a postcolumn mixing joint at 40°C. The luminescent reagent consisted of cytochrome C (10 μg/mL) and luminol (2 μg/mL) in 50 mM borate buffer (H_3BO_3/KCl-Na_2CO_3, pH 10).

GC–MS analysis. GC–MS was performed using a JEOL Automass (JEOL Co., Tokyo, Japan). The column used was a capillary fused-silica column, DB-1 (30 m × 0.25 mm; J&W Scientific Co., Folsom, CA), and the column temperature was programmed at 80 (initial) to 280°C (final) at a rate of 20°C/min. Helium was used as carrier gas at a flow rate of 60 mL/min. Both ion source and separator temperature were set at 250°C. The ionization energy was set at 70 eV.

Conversion of hydroperoxides into hydroxy FA and their derivatization for MS. Monohydroperoxides (0.2 mmol) were dissolved in 20 mL of ethyl acetate/benzene (1:1 vol/vol), and the solution was stirred under a H_2 atmosphere in the presence of catalytic amounts of platinum oxide at room temperature for 12 h. After removing the catalyst by suction, the filtrate was evaporated and the residue was dissolved in ethanol (50 mL). To this solution 2 mol/L methanolic KOH (10 mL) was added, and the solution was refluxed for 6 h. The reaction solution was evaporated to remove most of the solvent and then diluted with water. The aqueous solution was acidified with HCl (2 mol/L) and extracted twice with ether. The combined organic layer was washed with water and dried over Na_2SO_4. After evaporation, the residual oil was treated with diazomethane-etherate to give methyl esters. The crude methyl esters were then subjected to SiO_2 column chromatography by elution with *n*-hexane/ethyl acetate (9:1, vol/vol). A part of each fraction was treated with dimethylethylsilylimidazole as previously reported (11) and analyzed by GC–MS. The following results were obtained: Methyl 9-hydroxystearate, *m/z* (%): 371 (8), 339 (27), 273 (44), 243 (53), 155 (42). Methyl 10-hydroxystearate, *m/z* (%): 371 (9), 339 (38), 287 (48), 229 (72), 169 (25). Methyl 12-hydroxystearate, *m/z* (%): 371 (10), 339 (44), 315 (46), 210 (100), 173 (15). Methyl 13-hydroxystearate, *m/z* (%): 371 (4), 339 (31), 329 (23), 187 (88), 159 (15).

DISCUSSION

In previous reports on the measurement of plasma TAG hydroperoxides by HPLC, LLL hydroperoxide obtained by autoxidation of LLL was used as a standard (2,12). In those studies, the authors observed an HPLC peak similar to that for LLL hydroperoxide in stored human plasma. However, LLL is reportedly absent in human plasma (7,13,14), and the hydroperoxide they observed cannot be that of LLL. Further, the previous studies did not answer whether TAG hydroperoxide is present or absent in various pathological plasma containing accumulated TAG-rich lipoproteins or in the adipose tissue, where TAG are stored abundantly. To clarify this point, effective analytical methods using naturally occurring TAG hydroperoxides as standards are necessary.

The major TAG in human VLDL, or the major plasma lipoprotein species containing TAG as the preponderant lipid, are reported to be OOP (32.8%), OLP (22.3%), and OOO (12.2%) in healthy young Spanish men (7). A similar TAG composition has been reported for human adipose tissue (8). Hence, monohydroperoxides of the three major TAG are expected to be suitable standards for measuring TAG hydroperoxides in human plasma and adipose tissue; therefore, these hydroperoxides were initially synthesized in this study.

Numerous reports on the synthesis of TAG have appeared, and recent studies have focused on asymmetric synthesis, such as enzymatic syntheses of TAG (15–17). However, the

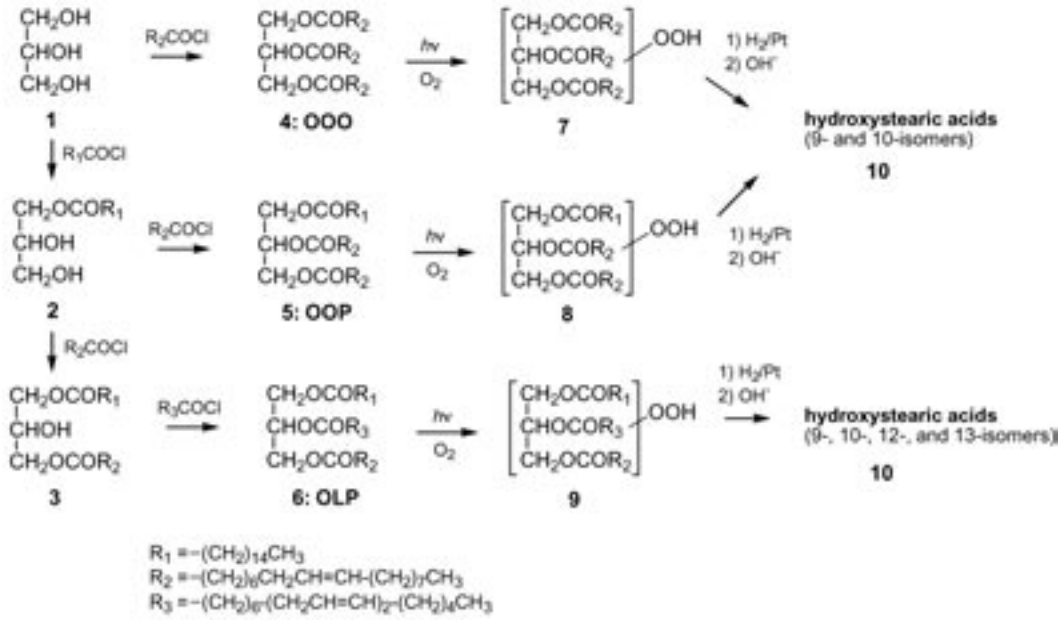

FIG. 1. Chemical syntheses of TAG and their monohydroperoxides. OOO, triolein; OOP, 1,2-dioleoyl-3-palmitoyl-glycerol; OLP, 1-oleoyl-2-linoleoyl-3-palmitoylglycerol.

selectivities in some lipase-catalyzed reactions were not as good as anticipated due to acyl migration, resulting in poor yields and impurities (18,19). Additionally, enzymatic synthesis is difficult because of the problems in choosing solvents, in selecting carriers for lipase immobilization, and in picking appropriate water activities. Furthermore, optimization is necessary for each new substrate (20).

In Figure 1 is shown the synthetic method for TAG and their hydroperoxides. This method is a stepwise combination of well-known acylations using freshly prepared acyl chloride. Polyacylation, an unfavorable side reaction in the conversion of glycerol (**1**) to monoacylglycerol **2**, is avoided by the use of a large excess of glycerol at 0°C, and the acylation at the 2-position was not observed. This acylation was also effective for the synthesis not only of **2** but also of other 1-acylglycerols. The second acylation of the monoacylglycerol **2** also was smoothly carried out by using an equimolar amount of oleoyl chloride to give **3** in good yield (70%). The chemical structure of the DAG **3** was confirmed by its H NMR, showing the signals of 1- and 3-methylene protons around 4.15 ppm as a pair of AB-type quartets. The use of excess oleoyl chloride at ambient temperature also gave OOP (**5**) in high yield (96%). OLP (**6**), with three different FA, was conveniently synthesized from the DAG **3** by the action of linoleoyl chloride. OOO (**4**) also was synthesized by the acylation of glycerol with excess oleoyl chloride.

The TAG (**4**–**6**) then were converted into hydroperoxides by photosensitized hydroperoxidation, as previously reported (11). It had been supposed that the hydroperoxidation of TAG would produce a complicated mixture, such as mono-, di-, and trihydroperoxides plus other oxidized compounds. In fact, hydroperoxidation under the above-reported reaction conditions resulted in "polyhydroperoxidation," leading to a complex product. However, during the course of a brief exposure to oxygen under the irradiation of a 200-W tungsten lamp, monohydroperoxidation preferentially took place, yielding monohydroperoxides (**7**–**9**) accompanied by small amounts of other oxidized products. The reaction of **9**, which contains a linoleoyl moiety, did not yield a detectable amount of intramolecular cyclization products, as determined by TLC. These monohydroperoxides were easily isolated by column chromatography as a regioisomeric mixture and were found to be stable for at least 2 mon at –80°C. The net yields of these monohydroperoxides were over 80%, calculated from the recovery of starting TAG. Thus, the other minor oxidized products were not further isolated and determined.

The chemical structures of the monohydroperoxide isomers were elucidated by ^{1}H and ^{13}C NMR. In their ^{1}H NMR spectra, the signals that appeared as a sextet around 4.3 ppm indicated the presence of a proton attached to a carbon with a hydroperoxyl group, and the ^{13}C NMR spectrum also showed the signal for a carbon with a hydroperoxyl group at around 87 ppm. The correlation of the signals at 4.3 and at 87 ppm was observed by the C–H COSY analysis. H–H COSY also indicated that the signal at 4.3 ppm correlated with the olefinic signal around 5.3 ppm.

Further investigation of the chromatographic characteristics of these synthesized hydroperoxides was carried out to determine whether these compounds could be used as standards in analyzing an individual hydroperoxide. The chromatograms from normal-phase HPLC for the monohydroperoxides of OOO, OOP, and OLP (**7**–**9**, respectively) are shown in Figures

2A, 2B, and 2C, respectively. The monohydroperoxides of OOO and OOP (**7** and **8**) were separated into four peaks, corresponding to the regioisomers. From our previous data (11), it was considered that the 9- or 10-positions on the two oleoyl side chains could undergo hydroperoxidation as a result of photosensitive oxidation under the present reaction conditions to give four possible isomers as a result of the conversion of *cis* into *trans* geometry of the double bond. In fact, H NMR showed the signals of *trans* olefinic protons between 5.7 and 6.7 ppm. Moreover, six peaks in the chromatogram of OLP monohydroperoxide (**9**) might correspond to the isomers with a hydroperoxyl group at 9, 10, 12, or 13 on the linoleoyl side chain and at 9 or 10 on the oleoyl side chain. In Figure 2C, at least six peaks could be distinguished; however, other possible isomers could be presented because of the transformation of *cis–trans* geometry of the double bond by hydroperoxidation. The positions of the hydroperoxyl group on the FA side chains were confirmed by the procedure used in an earlier report (11) as follows. These hydroperoxides (**7**–**9**) were initially subjected to a catalytic hydrogenation and then alkaline hydrolysis to give a mixture of isomeric hydroxystearic acids (**10**) as shown in Figure 1. After treatment with diazomethane, methyl esters from OOO and OOP monohydroperoxides (**7** and **8**) gave only two isomers of hydroxystearate, which were separated by SiO_2 column chromatography. 10-Hydroxystearate and 9-hydroxystearate were identified in the first and the second eluted fractions, respectively. The GC–MS analysis of these compounds clearly showed the characteristic fragment ions by α-cleavage at the C–C bond with a dimethylethylsilyloxy group at *m/z* 229 and 287 for the 10-isomer and at *m/z* 243 and 273 for the 9-isomer. The regioisomers of the mixture of OLP monohydroxides (**9**) were also determined to be 9-, 10-, 12-, and 13-isomers by the same procedure. The mass spectrum of the 12-isomer showed the fragment ion peaks at *m/z* 210 and 315 due to α-cleavage, and those of the 13-isomer were at *m/z* 187 and 329. These results indicate that the oleoyl side chains in OOP and OOO were subjected to hydroperoxidation at the 9- or 10-position.

It can be assumed that the positioning of the unsaturated FA moiety on the glycerol may not have an effect on photo-oxidation in the present study. The chromatograms of OOP and OOO monohydroperoxides showed almost the same ratio of isomers. To confirm this observation, OPP and POP were subjected to photo-oxidaion. The results showed that the production of monohydroperoxides from OPP and POP was almost the same, thus indicating that the positions on glycerol had no effect. However, it is also assumed that the active reaction species under the present conditions were "supersaturated," so the difference in the original reactivities between OPP and POP might not be distinct.

The linoleoyl side chain in OLP was also subjected to hydroperoxidation at the 9-, 10-, 12-, or 13-position as in the case of photosensitive oxidation of FFA and FA cholesteryl esters (11,21,22). However, the identification of each isomeric monohydroperoxide peak in the chromatograms shown in Figure 2 was not established. Linoleate is well known to be more easily oxidized than oleate, so there was a possibility of producing 9-, 10-, 12-, and 13-hydroxy derivatives only from the linoleoyl

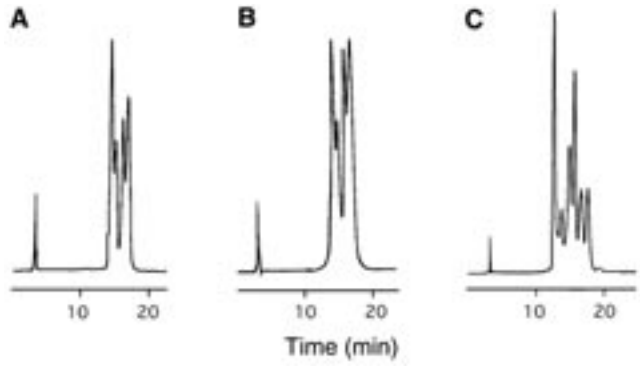

FIG. 2. Chromatograms of the TAG hydroperoxides by normal-phase HPLC with UV detection. (A) Monohydroperoxides of OOP; (B) monohydroperoxides of OOO; (C) monohydroperoxides of OLP. For abbreviations see Figure 1.

residue in the case of OLP. However, 9-, 10-, 12-, and 13-hydroxystearic acid derivatives from OLP were attributable to the hydroperoxidation of not only linoleoyl but also oleoyl chains. The ^{1}H NMR of OLP monohydroperoxides showed a signal for 11-H protons on the linoleoyl chain at 2.77 ppm with an integration intensity of 0.6H. This value indicated that 30% (0.6H/2H) of the linoleoyl chain remained without hydroperoxidation, that is, OLP monohydroperoxides contained 70% of hydroperoxidized linoleoyl and 30% of hydroperoxidized oleoyl side chains.

The chromatograms for the monohydroperoxides of OOO, OOP, and OLP in the RP-HPLC with chemiluminescent detection are shown in Figure 3. Peaks 1, 2, and 3 corresponded to monohydroperoxides of OOO (**7**), monohydroperoxides of OLP (**9**), and monohydroperoxides of OOP (**8**), respectively. Under these conditions, the separation of regioisomers could not be achieved; however, from the point of detecting TAG hydroperoxides in biological fluids, the high sensitivity would be obtained by the accumulation of each isomer. The detection limits for these monohydroperoxides were 0.5 pmol at a signal-to-noise ratio of 6 by chemiluminescence detection.

Our simple chemical syntheses of these TAG monohydroperoxides can be applied to various TAG hydroperoxides. The present method also enables one to synthesize TAG directly from glycerol and acid chlorides without any need for protection procedures, in contrast to such a synthesis using 2,3-isopropylidene glycerols as substrates for acylation (23). The disadvantage of the method is that optically active derivatives cannot be synthesized, which would not be a serious weakness in clinical use.

In this study, we synthesized monohydroperoxides of biologically important TAG. Regioisomers of these hydroperoxides were determined by a combination of chemical reactions and MS. These monohydroperoxides may be useful as standards for the determination of TAG monohydroperoxides. The elution order of regioisomers in normal-phase HPLC was not clearly established; however, each regioisomer could be determined by GC–MS. These methods and the hydroperoxides obtained might be useful in research on cardiovascular diseases, obesity, diabetes, cancer, inflammation, aging, and foods.

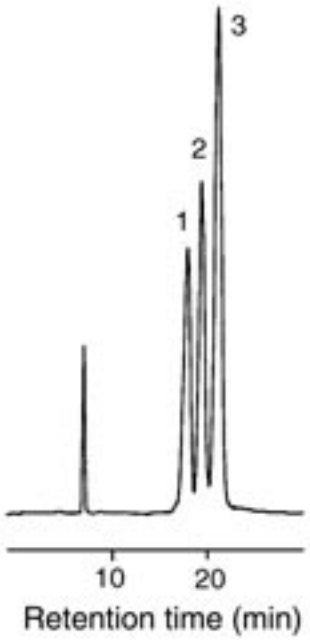

FIG. 3. Chromatogram of the mixed monohydroperoxides of three TAG (OOO, OOP, and OLP) in RP-HPLC with chemiluminescence detection. Peaks 1, 2, and 3 correspond to the monohydroperoxides of OOO, monohydroperoxides of OLP, and monohydroperoxides of OOP, respectively. For abbreviations see Figure 1.

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Synthesis of Novel Tri- and Tetrasubstituted C_{18} Furan Fatty Esters

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ABSTRACT: A methylene-interrupted C_{18} keto-acetylenic fatty ester (methyl 12-oxo-9-octadecynoate) was obtained from methyl ricinoleate by bromination-dehydrobromination followed by oxidation. Reaction of methyl 12-oxo-9-octadecynoate with bis(benzonitrile) palladium(II) chloride, allyl bromide, or methyl-allyl bromide furnished methyl 8-[5-hexyl-3-allyl-furan-2-yl]-octanoate (**1**, 56%) or methyl 8-[5-hexyl-3-(2-methyl-allyl)-furan-2-yl]-octanoate (**2**, 55%). Reaction of methyl 12-oxo-11-chloro- or 11-fluoro-9-octadecynoate (prepared from methyl santalbate—methyl 11-*E*-9-octadecynoate, found in sandalwood, *Santalum album,* seed oil) with bis(benzonitrile) palladium(II) chloride gave methyl 8-(4-chloro-5-hexyl-furan-2-yl)-octanoate (**3**, 59%) or methyl 8-(4-fluoro-5-hexyl-furan-2-yl)-octanoate (**4**, 50%), respectively. And when methyl 12-oxo-11-chloro- or 11-fluoro-9-octadecynoate was treated with a mixture of bis(benzonitrile) palladium(II) chloride, allyl bromide, or methyl-allyl bromide, the reaction yielded tetrasubstituted C_{18} furan derivatives, *viz.,* methyl 8-(3-allyl-4-chloro-5-hexyl-furan-2-yl)-octanoate (**5**, 54%), methyl 8-[4-chloro-5-hexyl-3-(2-methyl-allyl)-furan-2-yl]-octanoate (**6**, 54%), methyl 8-(3-allyl-4-fluoro-5-hexyl-furan-2-yl)-octanoate (**7**, 10%), and methyl 8-[4-fluoro-5-hexyl-3-(2-methyl-allyl)-furan-2-yl]-octanoate (**8**, 10%). The presence of a fluorine atom in the furan derivatives **4**, **7**, and **8** was readily characterized by the appearance of doublets for carbon nuclei, which were coupled to the fluorine atom in the ^{13}C NMR spectra. All furan fatty derivatives from this work were characterized by NMR spectroscopic and mass spectrometric analyses. The yields of compounds **7** and **8** were very low (10%) despite attempts to improve the procedure by increasing the amounts of the reactants and catalyst.

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Natural compounds containing a furan ring system are quite common (1–3). Many such heteroaromatic compounds are well known for their use as fragrances (4,5). FA containing a furan nucleus have been reported in plants (6–8), fish (9,10), invertebrates (11), marine sponges (12,13), algae (14), and bacteria (15,16).

The occurrence of furan FA in human blood cells also has been reported (17). Spiteller and coworkers (18,19) have studied some of the physiological properties of furan FA in humans. Of interest is the hypothesis that furan FA could be one of the metabolites from CLA involved in the anticarcinogenic properties of CLA (20).

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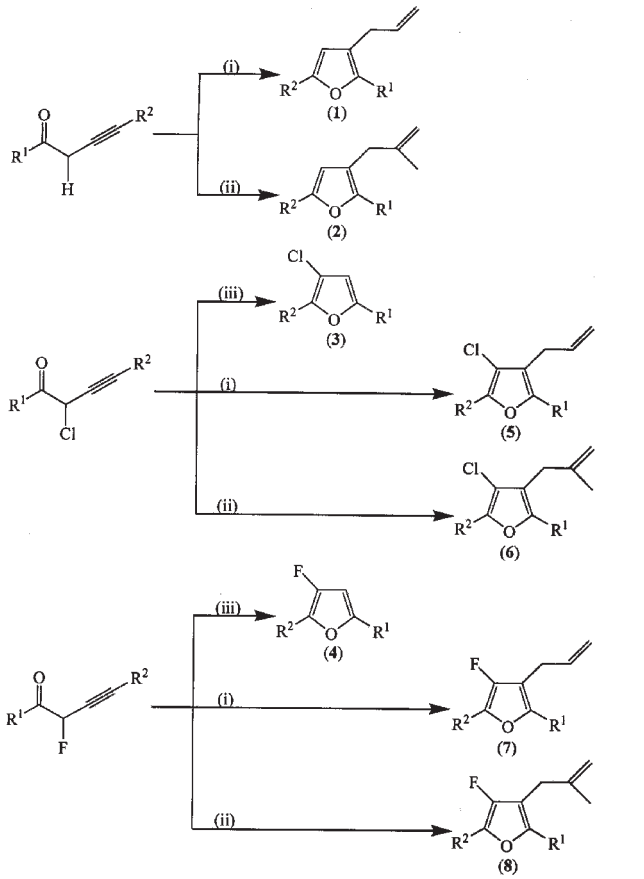
Abbreviations: HMBC, heteronuclear multiple bond correlation; r.t., room temperature; TMS, tetramethylsilane.

The functionalization of the furan ring, especially at the less-reactive sites, namely, the 3- and 4-positions of the heteroaromatic ring, has been one of the greatest challenges in furan syntheses (21–23). In the field of lipids, some natural tetrasubstituted furan FA are potent peroxidase inhibitors (24).

We have recently reported the preparation of an interesting oxo-allenic FA derivative from an oxo-acetylenic fatty ester derivative (methyl 12-oxo-9-octadecynoate), which was derived from methyl ricinoleate. This oxo-allenic FA derivative (methyl 12-oxo-9,10-octadecadienoate) was used in the synthesis of N,N-heterocyclic FA esters (25). We also have prepared the corresponding 11-chloro- and 11-fluoro-oxo-acetylene fatty ester derivatives with the halogen at the methylene group located between the oxo function and the acetylenic system. The halogen-containing fatty esters were chemically transformed to the corresponding chloro- and fluoro-oxo-allenic FA esters (26). This paper reports the successful bis(benzonitrile) palladium(II) chloride-catalyzed cyclization of (i) methyl 12-oxo-9-octadecynoate with allyl bromide and methyl-allyl bromide to yield C_{18} furan fatty ester derivatives containing an allyl and methyl-allyl group substituted at the 3-position of the furan ring, respectively, and (ii) the cyclization of methyl 12-oxo-11-chloro- and 12-oxo-11-fluoro-9-octadecynoate to give halo-substituted C_{18} furan fatty esters with the chloro- or fluoro- function at the 4-position of the furan ring (Scheme 1). Tetrasubstituted C_{18} furan fatty ester derivatives were subsequently prepared by reaction of methyl 12-oxo-11-halo-9-octadecynoate with allyl or methyl-allyl bromide under similar palladium-catalyzed reaction conditions.

MATERIALS AND METHODS

Instrumentation. Column chromatographic separation was performed on silica gel (Art. 7730, type 60, 70–230 mesh; Merck, Darmstadt, Germany) as the adsorbent using gradient elution with a mixture of *n*-hexane/diethyl ether as the mobile phase. The separation of the various components was monitored by TLC analysis of collected fractions using *n*-hexane/diethyl ether (4:1, vol/vol) as developer. IR spectra were recorded on a Bio-Rad FTS-165 FTIR spectrometer. Samples were run as neat films on NaCl plates. NMR spectra were recorded on a Bruker Avance DPX_{300} (300 MHz) Fourier-transformed NMR spectrometer from solutions in deuteriochloroform ($CDCl_3$) (0.2–0.3 mM) with tetramethylsilane (TMS) as the internal reference standard. Chemical shifts are given in δ-values in ppm downfield from TMS



$R^1 = CH_3(CH_2)_5$; $R^2 = (CH_2)_7COOCH_3$

Reaction conditions: (i) propylene oxide, allyl bromide, $Pd(PhCN)_2Cl_2$, r.t., 3 h
(ii) propylene oxide, methyl-allyl bromide, $Pd(PhCN)_2Cl_2$, r.t., 3 h
(iii) propylene oxide, $Pd(PhCN)_2Cl_2$, r.t., 3 h

SCHEME 1

(δ_{TMS} = 0 ppm). Mass spectral analyses were carried out on a Finnigan MAT-95 mass spectrometer. Methyl 12-oxo-9-octadecynoate, methyl 11-fluoro-, and 11-chloro-12-oxo-9-octadecynoate were prepared as described elsewhere (25,26).

*General procedure for the synthesis of tri- and tetrasubstituted C_{18} furan fatty ester derivatives as exemplified by the reaction of methyl 12-oxo-9-octadecynoate with allyl bromide in the presence of bis(benzonitrile) palladium(II) chloride. (i) Methyl 8-(3-allyl-5-hexyl-furan-2-yl)-octanoate (**1**).* A mixture of methyl 12-oxo-9-octadecynoate (0.5 g, 1.6 mmol), propylene oxide (4.7 g, 5.7 mL, 81.1 mmol), allyl bromide (2.36 g, 1.7 mL, 19.5 mmol), and bis(benzonitrile) palladium(II) chloride (0.31 g, 0.81 mmol) was stirred at room temperature (r.t.) for 3 h. The reaction mixture was poured into water (20 mL) and extracted with ethyl acetate (3 × 10 mL). The organic extracts were combined and washed successively with brine (20 mL) and water (20 mL). The organic solvent was evaporated under reduced pressure, and the residue was chromatographed on a silica (30 g) column by gradient elution using a mixture of *n*-hexane/diethyl ether to give pure methyl 8-(3-allyl-5-hexyl-furan-2-yl)-octanoate (**1**, 0.32 g, 56%). TLC on silica: R_f = 0.7 (*n*-hexane/diethyl ether, 4:1 vol/vol, as developer); IR (neat): 2930, 2857, 1743, 1436, 1197, 1172 cm^{-1}; ^1H NMR ($CDCl_3$, δ_H): 0.88 (*t*, *J* = 6.5 Hz, 3H, 18-*H*), 1.2–1.7 (*m*, 18H, CH_2), 2.29 (*t*, *J* = 7.5 Hz, 2H, 2-*H*), 2.46–2.56 (*m*, 4H, 8-*H* and 13-*H*), 3.03 (*dt*, *J* = 6.3 and 1.4 Hz, 2H, $-CH_2-CH=CH_2$), 3.66 (*s*, 3H, $COOCH_3$), 4.95–5.07 (*m*, 2H, $-CH_2-CH=CH_2$), 5.75 (*s*, 1H, 11-*H*), 5.79–5.94 ppm (*m*, 1H, $-CH_2-CH=CH_2$); ^{13}C NMR ($CDCl_3$, δ_C): 14.04 (C-18), 22.56 (C-17), 24.91 (C-3), 25.89 (C-8), 28.06 (C-13), 28.71, 28.90, 29.06, 29.44 ($-CH_2-CH=CH_2$), 31.59 (C-16), 34.06 (C-2), 51.38 ($COOCH_3$), 106.54 (C-11), 114.71 ($-CH_2-CH=CH_2$), 116.64 (C-10), 137.40 ($-CH_2-CH=CH_2$), 149.44 (C-9), 153.81 (C-12), 174.22 ppm ($COOCH_3$); *m/z* 348 (M^+, 75), 317 (M − 31, 5), 307 (13), 277 (4), 233 (4), 205 (100); high-resolution mass spectral analysis found 348.2666, $C_{22}H_{36}O_3$ requires 348.2664.

*(ii) Methyl 8-[5-hexyl-3-(2-methyl-allyl)-furan-2-yl]-octanoate (**2**, 55% yield).* TLC on silica: R_f = 0.7 (*n*-hexane/diethyl ether, 4:1 vol/vol, as developer); IR (neat): 2931, 2850, 1746, 1462, 1430, 1370, 1167 cm^{-1}; ^1H NMR ($CDCl_3$, δ_H): 0.88 (*t*, *J* = 6.8 Hz, 3H, 18-*H*), 1.2–1.66 (*m*, 18H, CH_2), 1.68 [*s*, 3H, $-CH_2C(CH_3)=CH_2$], 2.29 (*t*, *J* = 7.5 Hz, 2H, 2-*H*), 2.45–2.60 (*m*, 4H, 8-*H* and 13-*H*), 2.97 [*s*, 2H, $-CH_2C(CH_3)=CH_2$], 3.65 (*s*, 3H, $COOCH_3$), 4.69 (*s*, 1H, $-CH_2C(CH_3)=CH_2$], 4.72 [*s*, 1H, $-CH_2C(CH_3)=CH_2$], 5.72 ppm (*s*, 1H, 11-*H*); ^{13}C NMR ($CDCl_3$, δ_C): 14.01 (C-18), 22.16 [$-CH_2C(CH_3)=CH_2$], 22.55 (C-17), 24.89 (C-3), 25.83 (C-13), 28.00 (C-8), 28.04, 28.66, 28.86, 28.97, 29.00, 29.05, 31.57 (C-16), 33.66 ($-CH_2C(CH_3)=CH_2$], 34.03 (C-2), 51.34 ($COOCH_3$), 106.82 (C-11), 110.63 [$-CH_2C(CH_3)=CH_2$], 116.55 (C-10), 144.93 [$-CH_2C(CH_3)=CH_2$], 149.82 (C-9), 153.61 (C-9), 174.18 ppm ($COOCH_3$); *m/z* 362 (M^+, 100), 307 (14), 291 (7), 219 (90); high-resolution mass spectral analysis found 362.2821, $C_{23}H_{38}O_3$ requires 362.2821.

*(iii) Methyl 8-(4-chloro-5-hexyl-furan-2-yl)-octanoate (**3**, 59% yield).* TLC on silica: R_f = 0.70 (*n*-hexane/diethyl ether, 4:1 vol/vol, as developer); IR (neat): 2931, 2858, 1742, 1436, 1195, 1171 cm^{-1}; ^1H NMR ($CDCl_3$, δ_H): 0.88 (*t*, *J* = 6.7 Hz, 3H, 18-*H*), 1.2–1.7 (*m*, 18H, CH_2), 2.30 (*t*, *J* = 7.5 Hz, 2H, 2-*H*), 2.51 (*t*, *J* = 8.0 Hz, 2H, 8-*H*), 2.57 (*t*, *J* = 7.6 Hz, 2H, 13-*H*), 3.66 (*s*, 3H, $COOCH_3$), 5.88 ppm (*s*, 1H, 10-*H*); ^{13}C NMR ($CDCl_3$, δ_C): 14.03 (C-18), 22.55 (C-17), 24.89 (C-3), 25.34 (C-13), 27.72/27.75 (C-7, C-14), 28.07 (C-8), 28.67, 28.84, 28.93, 29.00, 31.47 (C-16), 34.04 (C-2), 51.42 ($COOCH_3$), 106.58 (C-10), 110.38 (C-11), 149.31 (C-12), 153.97 (C-9), 174.21 ppm ($COOCH_3$); *m/z* 342 (M^+, 45), 311 (M − 31, 6), 307 (100), 275 (30), 271 (8), 257 (7), 199 (11), 185 (11); high-resolution mass spectral analysis found 342.1956, $C_{19}H_{31}ClO_3$ requires 342.1962.

*(iv) Methyl 8-(4-fluoro-5-hexyl-furan-2-yl)-octanoate (**4**, 50% yield).* TLC on silica: R_f = 0.7 (*n*-hexane/diethyl ether, 4:1 vol/vol, as developer); IR (neat): 2931, 2861, 1742, 1657, 1576, 1457, 1435, 1416, 1354, 1235, 1194, 1167, 1127, 1065, 1019, 959, 776, 723 cm^{-1}; ^1H NMR ($CDCl_3$, δ_H): 0.88 (*t*, *J* = 6.7 Hz, 3H, 18-*H*), 1.2–1.75 (*m*, 18H, CH_2), 2.30 (*t*, *J* = 7.5 Hz, 2H, 2-*H*), 2.48 (*t*, *J* = 7.5 Hz, 2H, 8-*H*), 2.55 (*dt*, *J* = 7.4 and 1.5 Hz, 2H, 13-*H*), 3.66 (*s*, 3H, $COOCH_3$), 5.84 ppm (*s*,

1H, 10-*H*); ^{13}C NMR ($CDCl_3$, δ_C): 13.95 (C-18), 22.51 (C-17), 24.59 (*d*, *J* = 3.1 Hz, C-13), 24.83 (C-3), 27.63 (*d*, *J* = 1.6 Hz, C-14), 27.66 (C-7), 28.39 (C-8), 28.69, 28.79, 28.88, 28.96, 31.43 (C-16), 33.97 (C-2), 51.31 ($COOCH_3$), 98.04 (*d*, *J* = 20.4 Hz, C-10), 136.12 (*d*, *J* = 26.1 Hz, C-12), 148.44 (*d*, *J* = 243.2 Hz, C-11), 151.99 (*d*, *J* = 8.6 Hz, C-9), 174.11 ppm ($COOCH_3$); *m/z* 327 (M + 1, 19), 326 (M^+, 86), 306 (M – HF, 5), 295 (M – 31, 22), 277 (10), 263 (8), 255 (35), 239 (4), 223 (9), 198 (13), 197 (100), 184 (77), 183 ppm (27); high-resolution mass spectral analysis found 326.2257, $C_{19}H_{31}FO_3$ requires 326.2260.

*(v) Methyl 8-(3-allyl-4-chloro-5-hexyl-furan-2-yl)-octanoate (**5**, 56% yield).* TLC on silica: R_f = 0.7 (*n*-hexane/diethyl ether, 4:1 vol/vol, as developer); IR (neat): 2930, 2858, 1742, 1435, 1196, 1171 cm^{-1}; ^{1}H NMR ($CDCl_3$, δ_H): 0.88 (*t*, *J* = 6.7 Hz, 3H, 18-*H*), 1.2–1.7 (*m*, 18H, CH_2), 2.29 (*t*, *J* = 7.5 Hz, 2H, 2-*H*), 2.48 (*t*, *J* = 7.5 Hz, 2H, 8-*H*), 2.57 (*t*, *J* = 7.4 Hz, 2H, 13-*H*), 3.07 (*dt*, *J* = 6.0 and 1.5 Hz, 2H, $-CH_2-CH=CH_2$), 3.66 (*s*, 3H, $COOCH_3$), 4.95–5.03 (*m*, 2H, $-CH_2-CH=CH_2$), 5.75–5.95 ppm (*m*, 1H, $-CH_2-CH=CH_2$); ^{13}C NMR ($CDCl_3$, δ_C): 14.02 (C-18), 22.55 (C-17), 24.89 (C-3), 25.59 (C-13), 26.39 (C-8), 27.39 ($-CH_2-CH=CH_2$), 27.70, 28.32, 28.69, 28.91, 28.94, 29.02, 31.47 (C-16), 34.04 (C-2), 51.40 ($COOCH_3$), 111.81 (C-11), 115.03 ($-CH_2-CH=CH_2$), 115.38 (C-10), 135.84 ($-CH_2-CH=CH_2$), 148.54 (C-12), 149.76 (C-9), 174.21 ($COOCH_3$); *m/z* 382 (M^+, 75), 347 (100), 341 (9), 311 (10), 307 (45), 239 (49); high-resolution mass spectral analysis found 382.2271, $C_{22}H_{35}ClO_3$ requires 382.2275.

*(vi) Methyl 8-[4-chloro-5-hexyl-3-(2-methyl-allyl)-furan-2-yl]-octanoate (**6**, 54% yield).* TLC on silica: R_f = 0.7 (*n*-hexane/diethyl ether, 4:1 vol/vol, as developer); IR (neat): 2923, 2863, 1738, 1462, 1430, 1248, 1167 cm^{-1}; ^{1}H NMR ($CDCl_3$, δ_H): 0.88 (*t*, *J* = 6.6 Hz, 3H, 18-*H*), 1.2–1.7 (*m*, 18H, CH_2), 1.72 [*s*, 3H, $-CH_2C(CH_3)=CH_2$], 2.30 (*t*, *J* = 7.5 Hz, 2H, 2-*H*), 2.47 (*t*, *J* = 7.5 Hz, 2H, 8-*H*), 2.57 (*t*, *J* = 7.4 Hz, 2H, 13-*H*), 3.00 [*s*, 2H, $-CH_2C(CH_3)=CH_2$], 3.66 (*s*, 3H, $COOCH_3$), 4.62 [*s*, 1H, $-CH_2C(CH_3)=CH_2$], 4.74 ppm [*s*, 1H, $-CH_2C(CH_3)=CH_2$]; ^{13}C NMR ($CDCl_3$, δ_C): 14.03 (C-18), 22.37 [$-CH_2C(CH_3)=CH_2$], 22.57 (C-17), 24.91 (C-3), 25.62 (C-13), 26.43 (C-8), 27.72, 28.16, 28.67, 28.97, 29.05, 31.29 [$-CH_2C(CH_3)=CH_2$], 31.48 (C-16), 34.08 (C-2), 51.43 ($COOCH_3$), 110.81 [$-CH_2C(CH_3)=CH_2$], 112.21 (C-11), 115.36 (C-10), 143.48 [$-CH_2C(CH_3)=CH_2$], 148.46 (C-12), 150.15 (C-9), 174.22 ppm ($COOCH_3$); *m/z* 396 (M^+, 90), 365 (M – 31, 7), 361 (100), 341 (11), 325 (11), 307 (28), 253 (6); high-resolution mass spectral analysis found 396.2442, $C_{22}H_{35}ClO_3$ requires 396.2431.

*(vii) Methyl 8-(3-allyl-4-fluoro-5-hexyl-furan-2-yl)-octanoate (**7**, 10% yield).* TLC on silica: R_f = 0.7 (*n*-hexane/diethyl ether, 4:1 vol/vol, as developer); IR (neat): 2932, 2861, 1743, 1670, 1643, 1440, 1360, 1252, 1200, 1171 cm^{-1}; ^{1}H NMR ($CDCl_3$, δ_H): 0.88 (*t*, *J* = 6.7 Hz, 3H, 18-*H*), 1.2–1.7 (*m*, 18H, CH_2), 2.30 (*t*, *J* = 7.5 Hz, 2H, 2-*H*), 2.43 (*t*, *J* = 7.4 Hz, 2H, 8-*H*), 2.54 (*dt*, *J* = 7.5 and 1.6 Hz, 2H, 13-*H*), 3.06 (*dt*, *J* = 6.0 and 1.6 Hz, 2H, $-CH_2-CH=CH_2$), 3.66 (*s*, 3H, $COOCH_3$), 5.0–5.1 (*m*, 2H, $-CH_2-CH=CH_2$), 5.8–5.95 ppm (*m*, 1H, $-CH_2-CH=CH_2$); ^{13}C NMR ($CDCl_3$, δ_C): 14.03 (C-18), 22.56 (C-17), 24.75 (*d*, *J* = 3.1 Hz, C-13), 24.90 (C-3), 26.29 (*d*, *J* = 2.0 Hz, $-CH_2-CH=CH_2$), 26.51 (C-8), 27.60 (*d*, *J* = 1.5 Hz, C-14), 28.26, 28.77, 28.91, 28.94, 29.04, 31.49 (C-16), 34.06 (C-2), 51.41 ($COOCH_3$), 108.48 (*d*, *J* = 18.0 Hz, C-10), 114.98 ($-CH_2-CH=CH_2$), 135.41 (*d*, *J* = 25.4 Hz, C-12), 135.90 ($-CH_2-CH=CH_2$), 147.85 (*d*, *J* = 7.3 Hz, C-9), 149.30 (*d*, *J* = 244.8 Hz, C-11), 174.21 ppm ($COOCH_3$); *m/z* 367 (M + 1, 100), 347 (M – F, 4), 335 (M – 31, 14), 325 (20), 295 (22), 237 (16), 223 (84); high-resolution mass spectral analysis found 366.2552, $C_{22}H_{35}FO_3$ requires 366.2570.

*(viii) Methyl 8-[4-fluoro-5-hexyl-3-(2-methyl-allyl)-furan-2-yl]-octanoate (**8**, 10% yield).* TLC on silica: R_f = 0.7 (*n*-hexane/diethyl ether, 4:1 vol/vol, as developer); IR (neat): 2929, 2861, 1738, 1611, 1443, 1376 cm^{-1}; ^{1}H NMR ($CDCl_3$, δ_H): 0.88 (*t*, *J* = 6.7 Hz, 3H, 18-*H*), 1.2–1.7 (*m*, 18H, CH_2), 2.30 (*t*, *J* = 7.5 Hz, 2H, 2-*H*), 2.43 (*t*, *J* = 7.5 Hz, 2H, 8-*H*), 2.55 (*dt*, *J* = 7.4 and 1.5 Hz, 2H, 13-*H*), 2.99 [*s*, 2H, $-CH_2C(CH_3)=CH_2$], 3.66 (*s*, 3H, $COOCH_3$), 4.68 [*s*, 1H, $-CH_2C(CH_3)=CH_2$], 4.74 ppm [*s*, 1H, $-CH_2C(CH_3)=CH_2$]; ^{13}C NMR ($CDCl_3$, δ_C): 14.03 (C-18), 22.22 [$-CH_2C(CH_3)=CH_2$], 22.57 (C-17), 24.76 (*d*, *J* = 3.1 Hz, C-13), 24.90 (C-3), 26.47 (C-8), 27.60 (*d*, *J* = 1.5 Hz, C-14), 28.12, 28.74, 28.95, 29.05, 30.31 [*d*, *J* = 1.9 Hz, $-CH_2C(CH_3)=CH_2$], 31.47 (C-16), 34.06 (C-2), 51.41 ($COOCH_3$), 108.51 (*d*, *J* = 18.1 Hz, C-10), 110.68 [$-CH_2C(CH_3)=CH_2$], 135.46 (*d*, *J* = 24.48 Hz, C-12), 143.59 [$-CH_2C(CH_3)=CH_2$], 148.21 (*d*, *J* = 7.1 Hz, C-9), 148.43 (*d*, *J* = 244.8 Hz, C-11), 174.23 ppm ($COOCH_3$); *m/z* 361 (M^+ –F, 7), 320 (11), 251 (83); high-resolution mass spectral analysis found 380.2718, $C_{23}H_{37}FO_3$ requires 380.2727.

RESULTS AND DISCUSSION

Fukuda *et al.* (27) reported an elegant method for the synthesis of trisubstituted furans that involves the cyclization of β-oxo-acetylenic substrates (i.e., a methylene-interrupted oxo-acetylenic system) in the presence of bis(benzonitrile) palladium(II) chloride as the catalyst.

A β-oxo-acetylenic fatty ester substrate was readily obtained from methyl ricinoleate (methyl 12-hydroxy-9-*Z*-octadecenoate) by bromination-dehydrobromination of the olefinic bond to yield a hydroxy-acetylenic intermediate; the latter was then oxidized to give methyl 12-oxo-9-octadecynoate as described elsewhere (25). When methyl 12-oxo-9-octadecynoate was treated with either allyl or 2-methyl-allyl bromide in the presence of propylene oxide and bis(benzonitrile) palladium(II) chloride, the reaction gave the requisite 3-allyl (**1**) and 3-(2-methyl-allyl) (**2**) trisubstituted furan fatty esters, respectively (Scheme 1).

In an earlier paper we reported the ring-opening reactions of the epoxy derivative of methyl santalbate (methyl 11-*E*-octadec-9-ynoate from sandalwood seed oil, *Santalum album*) (26). Treatment of the epoxy derivative of methyl santalbate with chloride or fluoride anions gave exclusively the corre-

sponding 11-fluoro or 11-chloro halohydrin derivative. Oxidation of the latter derivatives gave methyl 11-chloro- or 11-fluoro-12-oxo-9-octadecynoate, which are therefore halogenated β-oxo-acetylenic fatty esters. Hence, the reaction of these halogenated β-oxo-acetylenic fatty esters with bis(benzonitrile) palladium(II) chloride in the presence of propylene oxide gave the requisite 4-chloro (**3**) and 4-fluoro (**4**) trisubstituted furan fatty esters, respectively (Scheme 1).

Methyl 11-chloro- and 11-fluoro-12-oxo-9-octadecynoate were subsequently used for the reactions with allyl bromide and 2-methyl-allyl bromide in the presence of bis(benzonitrile) palladium(II) chloride. This extension of the cyclization reactions led to the production of 3-allyl-4-chloro- (**5**), 3-allyl-4-fluoro- (**6**), 4-chloro-3-(2-methyl-allyl)-, (**7**), and 4-fluoro-3-(2-methyl-allyl)- (**8**) tetrasubstituted C_{18} furan fatty esters (Scheme 1).

The trisubstituted C_{18} furan fatty esters **1** (containing an allyl group at the 3-position of the furan ring) and **2** (containing a 2-methyl-allyl group at the 3-position of the furan ring) were readily characterized by the appearance of a singlet at δ_H 5.7, which was coupled to the carbon nucleus at δ_C 106 of the furan ring (^1H–^{13}C COSY). From the results of the HMBC (heteronuclear multiple bond correlation) analysis, the structures of compounds **1** and **2** were unambiguously determined. The couplings of the HMBC spectrum of compound **1** are shown in Figure 1. When methyl 11-chloro- and 11-fluoro-12-oxo-9-octadecynoate were each treated with bis(benzonitrile) palladium(II) chloride in propylene oxide, the corresponding 4-chloro- (**3**) and 4-fluoro- (**4**) trisubstituted C_{18} furan fatty esters were obtained, respectively. The presence of a fluorine atom in compound **4** was readily distinguished from the chloro-analog (**3**) by the appearance of split signals (doublets) in the ^{13}C NMR spectrum, which are due to the coupling of the fluorine atom with adjacent carbon nuclei. The four furan ring carbon atoms and carbons C-13 and C-14 (in the hexyl chain attached to the 5-position of the ring) were all affected by the fluorine atom. The $J_{C\text{-}F}$ constants reflected the distance of the fluorine atom from that carbon nucleus. Thus, the carbon atom (at the 4-position of the furan ring) to which the fluorine is attached had a very large $J_{C\text{-}F}$ = 243.2 Hz. The coupling constants for the 3- and 5-position ring carbons resulted in $J_{C\text{-}F}$ constants of 20–26 Hz, and the most distant carbon in the furan ring from the fluorine atom (2-position of the ring) gave a $J_{C\text{-}F}$ = 8.6 Hz. Small but distinct nuclear effects from the fluorine atom were also felt on C-13 and C-14 (methylene groups of the hexyl group at the 5-position of the furan) where the signals appeared as doubles with $J_{C\text{-}F}$ = 1.6 and 3.1 Hz, respectively. These $J_{C\text{-}F}$ values for compound **4** were in general agreement with NMR data reported for some closely similar furan molecules (28).

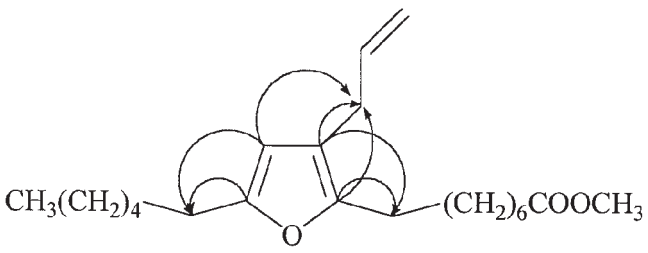


FIG. 1. Heteronuclear multiple bond correlation of methyl 8-(3-allyl-5-hexyl-furan-2-yl)-octanoate (**1**).

The NMR spectra of the tetrasubstituted furan fatty esters (**5–8**) showed that all ring carbon nuclei were of the quaternary type by DEPT (distortionless enhancement through polarization transfer) experiment. The carbon shift signals of the furan ring carbons of the fluorinated tetrasubstituted furan fatty esters (**7, 8**) appeared as doublets owing to the coupling between the fluorine and carbon atoms. This feature distinguished the fluoro-fatty esters readily from the chlorinated furan fatty esters (**5, 6**). The NMR results were in good agreement with carbon chemical shifts recorded by Marson and Harper (29) for some substituted furan FA derivatives.

Attempts were made to improve the low yields (10%) of compounds **7** and **8** by increasing the amounts of the palladium catalyst, reactants (allyl or methyl-allyl bromide), and the reaction time. The organic products isolated from these reactions showed that the substrate (methyl 11-fluoro-12-oxo-9-octadecynoate) had completely reacted. TLC analysis of the crude products showed the requisite derivatives (**7** and **8**) as a minor component (10% after isolation). The rest of the crude product appeared on the TLC plate as a streak. Further separation of the complex mixture of products was unsuccessful. The NMR spectral results of compounds **7** and **8** are of interest. It seems that the very electronegative fluorine atom in the substrate caused the palladium catalyst to produce a range of intermediates, which resulted in the formation of the many derivatives. Further work is planned to study the mechanism of the reactions.

It can be concluded from this work that interesting halogenated fatty acid esters can be produced from readily available natural FA. The presence of a chloro-, fluoro-, allyl, or methyl-allyl group offers a new direction for such exotic lipid derivatives to be developed in order to study their physiological and pharmaceutical properties.

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New Glyceroglycolipid and Ceramide from *Premna microphylla*

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ABSTRACT: Two new compounds (**1**,**2**) were isolated from the ethanolic extract of the leaves of *Premna microphylla*, together with five known compounds. The structures of compounds **1** and **2** were elucidated as (2*S*,3*S*,4*R*,11*E*)-2-[(2*R*)-2-hydroxytetracosanoylamino]-11-octadecene-1,3,4-triol (**1**) and 1-*O*-(9*Z*,12*Z*,15*Z*-octadecatrienoyl)-3-*O*-[β-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl] glycerol (**2**) by means of spectroscopic and chemical methods.

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Recent studies on the biological and chemical constituents of members of the genus *Premna* have resulted in the isolation of iridoids (1–3), sesquiterpenes (4,5), diterpenes (6–9), and phenylethnoids (10,11), some of which have exhibited significant antibacterial (4,6,8) and cytotoxic activities (9). *Premna microphylla* Turez., distributed mainly in the southern part of China, is a shrub whose leaves are applied widely to treat skin cuts and infections, malaria, dysentery, headaches, and viper bites in Chinese traditional medicine (12). The application of *P. microphylla* in folk medicine and the identification of biologically active isolates from members of this plant genus have led us to analyze the chemical composition of this plant. Except for the isolation of pectin (13), a more detailed chemical study of this plant has not been reported previously. In the current study, two new compounds, (2*S*,3*S*,4*R*,11*E*)-2-[(2*R*)-2-hydroxytetracosanoylamino]-11-octadecene-1,3,4-triol (**1**) and 1-*O*-(9*Z*,12*Z*,15*Z*-octadecatrienoyl)-3-*O*-[β-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl] glycerol (**2**), as well as five known ones, α-linolenic acid (**3**), 1-monolinolenin (**4**), 1-*O*-(9*Z*,12*Z*,15*Z*-octadecatrienoyl)-3-*O*-β-D-galactopyranosylglycerol (**5**), gingerglycolipid A (**6**), and 1-*O*-β-D-glucopyranosyl-(2*S*,3*S*,4*R*,8*Z*)-2-[(2*R*)-2-hydroxydocosanoylamino]-8-octadecene-1,3,4-triol (**7**), were isolated and structurally identified. Herein, we report for the first time the isolation and structural elucidation of two new compounds (**1**,**2**), together with five known compounds (**3**–**7**) from the leaves of *P. microphylla*.

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Abbreviations: CC, column chromatography; ESI–MS, electrospray ionization-mass spectrometry; HMBC, heteronuclear multiple bond connectivity; HMQC, heteronuclear multiple quantum coherence.

The ceramides are important components in the cell membranes of animals and plants and are emerging as significant second messengers for various cellular processes (14). Many extracellular stresses, such as tumor necrosis factor-α and HIV, have been shown to activate sphingomyelinase, which releases ceramides that inhibit cell growth and induce apotosis (15,16). Because of the important role of ceramides, their chemistry and biology has been an attractive subject of research in recent years (17,18). The acylglycerylgalactosides are also important glycoglycerolipids that exist in both terrestrial plants and in plants of marine origin, such as sponges and algae. Some glycoglycerolipids have shown significant cytotoxic activity against several tumor cell lines (19,20).

EXPERIMENTAL PROCEDURES

Chromatographic and instrumental methods. IR spectra were recorded on a PerkinElmer 577 spectrometer (Überlingen, Germany) with a KBr disk. UV spectra were determined on a Varian Cary 300 Bio spectrometer (Varian, Palo Alto, CA). Optical rotations were measured on a PerkinElmer 341 polarimeter at room temperature. NMR spectra (^{1}H, ^{13}C, and 2-D NMR) were recorded on a Bruker AM-400 instrument (Karlsruhe, Germany) at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR, and tetramethylsilane was used as an internal standard; coupling constants were represented in hertz. EI–MS (ionizing potential, 70 eV) was carried out on a Finnigan MAT 95 mass spectrometer (Thermo Finnigan Mat GmbH, Bremen, Germany). Electrospray ionization (ESI) mass spectra were recorded on a Finnigan LCQ^{DECA} mass spectrometer (Finnigan, San Jose, CA). GC–MS was performed on a Finnigan 4510 gas chromatograph–mass spectrometer in the electron impact (EI) mode (ionizing potential, 70 eV) with an HP-5 capillary column (30 m × 0.25 mm) packed with 5% phenyldimethyl silicone (Hewlett-Packard, Palo Alto, CA). Helium was used as carrier gas, and the column temperature was ramped from 160 to 240°C at 5°C/min.

Materials. Column chromatography (CC) was conducted by using silica gel (230–400 mesh; Qingdao Marine Chemical Ltd., Qingdao, People's Republic of China), RP-18 (Lichroprep®, 40–63 μm; Merck, Darmstadt, Germany), and MCI Gel CHP20P (75–150 μm; Mitsubishi Chemical Industries Ltd., Tokyo, Japan). All solvents used were of analytical grade (Shanghai Chemical Plant, Shanghai, People's Republic of China). TLC analysis was carried out on plates precoated with silica gel F_{254} (Qingdao Marine Chemical Ltd).

The leaves of *P. microphylla* were collected from the Hangzhou district of the Zhejiang province in China in July 2002 and were identified by Prof. Zeng-Tao Wang of the Shanghai University of Traditional Chinese Medicine. A voucher specimen (SIMM-PM-030012) was deposited at the Herbarium of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, (Shanghai, People's Republic of China).

Extraction and isolation. The powder of *P. microphylla* (860 g) was extracted with 95% ethanol (3 L × 3) at room temperature. The crude extract (132 g) was partitioned between H_2O and EtOAc to provide an ethyl acetate-soluble fraction (50 g), which was applied to a silica gel column and eluted first with petroleum ether/acetone (30:1–1:2, by vol) and then with methanol to give five major fractions, 1–5. Fraction 2 (1.10 g) was subjected to silica gel CC by eluting with petroleum ether/EtOAc (4:1, by vol) to yield **3** (45 mg). Fraction 3 (810 mg) was subjected to silica gel CC ($CHCl_3$/CH_3OH 50:1, by vol) to yield **4** (17 mg). Fraction 4 (1.23 g) was separated by silica gel CC ($CHCl_3$/MeOH 30:1–20:1, by vol) to yield **1** (195 mg). For fraction 5 (17.2 g), the column was packed with MCI Gel CHP20P and eluted with MeOH/H_2O (7:3–10:0, by vol) to afford subfractions 5a–5e. Fraction 5b (650 mg) was applied to a silica gel column and eluted with $CHCl_3$/MeOH (8:1, by vol) to yield **7** (13 mg). Compounds **5** (40 mg) and **6** (30 mg) were obtained from fraction 5c (860 mg) by using an RP-18 silica gel column and eluting with MeOH/H_2O (9:1–10:0, by vol). Fraction 5e (710 mg) was applied to a silica gel column and eluted with $CHCl_3$/MeOH/H_2O (3:1:0.1, by vol) to yield **2** (12 mg) (Scheme 1).

*(2*S*,3*S*,4*R*,11*E*)-2-[(2*R*)-2-hydroxytetracosanoylamino]-11-octadecene-1,3,4-triol (**1**).* White amorphous powder; $[\alpha]_D^{20}$ = +10.4° (*c* = 1.0, pyridine). IR (KBr) ν 3334, 3220 (OH), 2918, 2850, 1622 (N–C=O), 1545 (NH), 1468, 1353, 1066, 1022, 723 cm^{-1}; ^1H and ^{13}C NMR (see Table 1); negative ESI–MS *m/z* 680 $[M - 1]^-$; high-resolution (HR)–EI–MS *m/z* 681.6293 $[M]^+$ ($C_{42}H_{83}NO_5$, calcd. 681.6271); EI–MS (70 eV) *m/z* (relative intensity, %): 681 $[M]^+$ (2), 663 $[M - H_2O]^+$ (11), 454 (13), 439 (36), 426 (52), 408 (48), 384 (72), 357 (60), 339 (16), 280 (24), 111 (24), 97 (44), 83 (56), 69 (59), 60 (100).

*Methanolysis of compound **1**.* Nineteen milligrams of **1** was dissolved in 5 mL methanol containing 5% HCl and refluxed for 18 h. The reaction mixture was neutralized with 2N $NaHCO_3$ to pH 7 and diluted with 10 mL water. The aqueous solution was partitioned with chloroform to obtain a mixture that was then purified by CC on a silica gel column eluted with petroleum ether/acetone (10:1, by vol) to give a white solid (5.0 mg); this solid was identified as methyl 2-hydroxytetracosenate by EI–MS and optical rotation. EI–MS (70 eV) *m/z* (relative intensity, %): 398 $[M]^+$ (48), 370 $[M - H_2O]^+$ (35) 339 $[M - COOCH_3]^+$ (53), 113 (70), 97 (88), 83 (78), 69 (72), 55 (100); $[\alpha]_D^{20}$ = +4.3° (*c* = 0.5, $CHCl_3$).

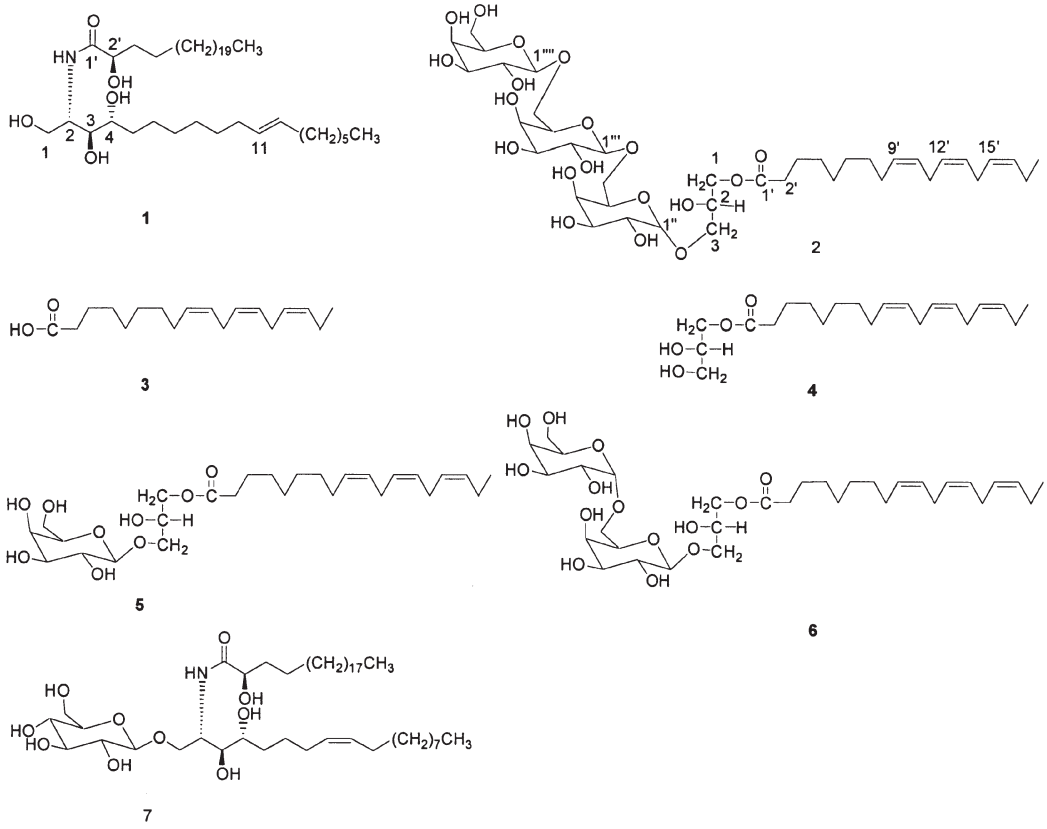
*Ozonolysis of compound **1**.* Ozone was passed into a stirred solution of compound **1** (3 mg) in 5 mL of anhydrous methanol/dichloromethane (1:1) at –78°C until the solution became blue. The reaction mixture was then purged with nitrogen and allowed to return to room temperature. Analysis of the ozonolytic mixture by GC–MS allowed identification of compound **1** as heptaldehyde ($C_7H_{14}O$, at *m/z* 114 $[M]^+$, t_R 6.23 min).

*1-O-(9Z,12Z,15Z-octadecatrienoyl)-3-O-[β-D-galactopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→6)-O-α-D-galactopyranosyl] glycerol (**2**).* White amorphous powder; $[\alpha]_D$ = +28° (*c* = 0.55, CH_3OH). UV (MeOH) λ_{max} 269 nm (log ε 2.6); IR (KBr) ν 3328 (*br*), 3010, 2925, 1724, 1645, 1456, 1139, 1070, 694 cm^{-1}; ^1H and ^{13}C NMR (see Table 2); positive ESI–MS *m/z* 861 $[M + Na]^+$; HR–ESI–MS *m/z* 861.4102 $[M + Na]^+$ $[(C_{39}H_{66}O_{19} + Na)^+$, calcd. 861.4096].

RESULTS AND DISCUSSION

The ethyl acetate-soluble fraction of the leaves of *P. microphylla* was extensively separated by CC to yield two new compounds, **1** and **2**, along with five known isolates (**3–7**) (Scheme 1).

Compound **1** was obtained as a white amorphous powder, $[\alpha]_D$ +10.4° (*c* = 1.0, pyridine). The HR–EI–MS spectrum of **1** at *m/z* 681.6293 $[M]^+$ indicated a molecular formula of $C_{42}H_{83}NO_5$ (calcd. 681.6271). The IR spectrum of **1** revealed the absorption bands of hydroxyls at 3334 and 3220 cm^{-1}, a secondary amide at 1545 and 1622 cm^{-1}, and long aliphatic chains at 723 cm^{-1}. The ^1H NMR spectrum of **1** showed the presence of an amide proton signal at 8.55 (1H, *d*, *J* = 9.1 Hz, NH) and two olefinic protons at δ 5.52 and 5.50 (each 1H, *dt*, *J* = 15.6, 6.0 Hz, H-12 and H-11). One proton signal geminal with the nitrogen at δ 5.07 (1H, *m*, H-2), three proton signals binding to the oxygenated tertiary carbons at δ 4.59 (1H, *dd*, *J* = 7.8, 4.2 Hz, H-2′), 4.32 (1H, *m*, H-3), and 4.27 (1H, *m*, H-4), two proton signals of an oxygenated methylene at δ 4.48 (1H, *dd*, *J* = 10.8, 4.8 Hz, H-1a) and 4.40 (1H, *dd*, *J* = 10.8, 4.8 Hz, H-1b), and two terminal methyls at δ 0.86 (6H, *t*, *J* = 6.8 Hz) were also indicated by the ^1H NMR spectrum. The ^{13}C NMR spectrum of **1** showed the presence of a carbonyl signal at δ 175.2 for an amide, two carbon signals at δ 130.7 and 130.8 for one double bond, three carbon signals at δ 76.8, 72.9, and 72.4 for the secondary alcohols, one carbon signal at δ 62.0 for a primary alcohol, and a nitrogenated methine at δ 52.9. The ^{13}C NMR spectrum of **1** also exhibited multiple carbon signals at δ 29.5–30.3 for the methylene groups and a carbon signal at δ 14.3 for two terminal methyls, suggesting that compound **1** possessed two long alkyl chains. The aforementioned spectral data suggest that compound **1** was likely a phytosphingosine-type ceramide possessing a 2-hydroxy fatty acyl moiety (21,22). Methanolysis of **1** yielded methyl 2-hydroxytetracosenate identified by EI–MS, and the absolute configuration of C-2 was determined to be *R* by comparison of the optical rotation value with data reported in the literature (22,23). The double bond in the amino alcohol part was allocated by chemical degradation *via* ozonolysis. The ozonolysis of **1**, which afforded heptaldehyde, allowed the determination of one Δ^{11} double bond. The planar structure of **1** was thereafter proposed and confirmed by the use of 2-D NMR techniques, especially the heteronuclear multiple bond connectivity (HMBC) spectrum,



SCHEME 1

in which the proton signal at δ 4.59 (H-2′) correlated with the carbon signal at δ 175.2 (C-1′), the proton signal of an amide (NH) at δ 8.55 correlated with the carbon signals at both δ 175.2 (C-1′) and 52.9 (C-2), and the carbon signal at δ 76.8 (C-3) correlated with the proton signals at δ 4.48 (H-1a), 4.40 (H-1b), and 4.27 (H-4).

The large vicinal coupling constants of two olefinic protons ($J_{11,12}$ = 15.6 Hz) clearly indicated that the double bond was an *E* configuration (24). On consideration of biogenetic and steric factors, the chemical shifts of H-1 to H-4 and H-2′, and the chemical shifts of the carbon signals of C-1 to C-4, C-1′, and C-2′ of glucosphingolipids and sphingolipids could be used to determine their absolute stereochemistry. The chemical shifts and coupling constants of H-1 to H-4 and H-2′, and the chemical shifts of the carbon signals of C-1 to C-4, C-1′, and C-2′ of **1** were in good agreement with those of ceramides possessing a 2*S*,3*S*,4*R*,2′*R*-configuration (21,22,25–28). The structure of **1** was thus assigned as (2*S*,3*S*,4*R*,11*E*)-2-[(2*R*)-2-hydroxytetracosanoylamino]-11-octadecene-1,3,4-triol. The complete assignments of proton and carbon signals (Table 1) were achieved by ^{1}H-^{1}H COSY, heteronuclear multiple quantum coherence (HMQC), and HMBC spectra.

Compound **2** was obtained as a white amorphous powder. Its molecular formula was determined to be $C_{39}H_{66}O_{19}$ by positive HR–ESI–MS at *m/z* 861.4102 $[M + Na]^+$ (calcd. 861.4096). Its IR spectrum showed a broad absorption band for the hydroxyl groups (3328 cm^{-1}) and an ester carbonyl (1724 cm^{-1}). The ^{13}C NMR spectrum showed the presence of three anomeric carbons at δ 105.8 (two carbon signals resolved at δ 105.79 and 105.77 before approximation) and 101.3 assignable to the presence of three galactopyranosyls, one carbonyl at δ 176.1 arising from an acyl group, six carbon signals at δ 133.2, 131.6, 129.7 (2 × C), 129.4, and 128.7 belonging to three double bonds, and one terminal methyl at δ 15.1. In the ^{1}H NMR spectrum, three anomeric proton signals at δ 5.06 (1H, *d*, *J* = 3.2 Hz), 4.51 (1H, *d*, *J* = 7.4 Hz), and 4.49 (1H, *d*, *J* = 7.5 Hz) indicated the presence of one α-galactopyranosyl and two β-galactopyranosyls (Table 2). Six olefinic protons at δ 5.54 (6H, *m*) and one methyl at δ 1.16 (3H, *t*, *J* = 7.5 Hz) were also observed in the ^{1}H NMR spectrum. In addition, the characteristic proton signals observed at δ 3.00 (4H, *t*, *J* = 5.7 Hz) and 2.55 (2H, *t*, *J* = 7.4 Hz) were easily assignable to those of methylene proton signals inserted between double bonds and those of methylene proton signals next to a carbonyl, respectively (19). The spectral features of compound **2** were very similar to those of compound **6**, indicating that **2** was also a glyceroglycolipid derivative having the same aglycone as that of **6** and presenting one more galactopyranosyl in **2**. In the ^{13}C NMR spectrum, the carbon signals at δ 70.3 (C-6″) and δ 68.7 (C-6″) were shifted farther downfield compared with the carbon signal at δ 63.1 (C-6‴), indicating that the three sugars likely formed a linear linkage between C-1‴ and C-6‴ and between C-1″ and C-6″ by the formation of ether bonds. A comparison with the data of known compounds possessing a trigalactopyranosyl

TABLE 1
^{1}H and ^{13}C NMR Spectral Data of Compound 1 (C_5D_5N)

Position	δ_H	δ_C	Position	δ_H	δ_C
1a	4.48 (*dd*, 10.8, 4.8)	62.0	16	1.24–1.30	32.1
1b	4.40 (*dd*, 10.8, 4.8)	62.0	17	1.24–1.30	22.9
2	5.07 (*m*)	52.9	18	0.86 (*t*, 6.8)	14.3
3	4.32 (*m*)	76.8	1′		175.2
4	4.27 (*m*)	72.9	2′	4.59 (*dd*, 7.8, 4.2)	72.4
5	2.24 (*m*)	34.1	3′	2.20 (*m*), 2.04 (*m*)	35.7
6	1.70 (*m*)	26.7	4′	1.76 (*m*)	25.8
7–9	1.24–1.30	29.5–30.3	5′–21′	1.24–1.30	29.5–30.3
10	2.00 (*m*)	33.0	22′	1.24–1.30	32.1
11	5.50 (*dt*, 15.6, 6.0)	130.7	23′	1.24–1.30	22.9
12	5.52 (*dt*, 15.6, 6.0)	130.8	24′	0.86 (*t*, 6.8)	14.3
13	2.05 (*m*)	33.3	NH	8.55 (*d*, 9.1)	
14–15	1.24–1.30	29.5–30.3			

TABLE 2
^{1}H and ^{13}C NMR Spectral Data[a] of Compound 2 (CD_3OD)

Position	δ_H	δ_C	Position	δ_H	δ_C
1	4.35 (*m*)	67.2	1″	5.06 (*d*, 3.2)	101.3
2	4.08 (*m*)	70.7	2″	4.15 (*m*)	71.1
3	3.86 (*m*), 4.06 (*m*)	72.6	3″	3.95 (*m*)	71.7
1′		176.1	4″	4.27 (*m*)	71.9
2′	2.55 (*t*, 7.4)	35.5	5″	3.73 (*m*)	73.1[b]
3′	1.85 (*m*)	26.5	6″	4.02 (*m*), 4.18 (*m*)	70.3
4′–7′	1.47–1.56	30.7–31.1	1‴	4.51 (*d*, 7.4)	105.8
8′	2.26 (*m*)	28.7	2‴	3.73 (*m*)	73.0[b]
9′	5.54 (*m*)	131.6	3‴	3.68 (*m*)	75.9
10′	5.54 (*m*)	128.7	4‴	3.92 (*m*)	70.8
11′	3.00 (*t*, 5.7)	27.0[a]	5‴	3.72 (*m*)	75.6
12′	5.54 (*m*)	129.7	6‴	3.92 (*m*), 4.10 (*m*)	68.7
13′	5.54 (*m*)	129.7	1⁗	4.49 (*d*, 7.5)	105.8
14′	3.00 (*t*, 5.7)	26.9[a]	2⁗	3.73 (*m*)	73.1[b]
15′	5.54 (*m*)	129.4	3⁗	3.96 (*m*)	75.1
16′	5.54 (*m*)	133.2	4⁗	4.19 (*m*)	70.2
17′	2.26 (*m*)	22.0	5⁗	3.72 (*m*)	77.2
18′	1.16 (*t*, 7.5)	15.1	6⁗	3.95 (*m*)	63.1

[a]Assignments with the same roman superscript (a,b) may be interchangeable.

linkage (29) indicated that the stereochemistry of the sugar linkage in compound **2** was β-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl, judging from the coupling constants of anomeric protons and the related chemical shifts of carbon signals in the sugars, such as the C-1 and the C-6. The connectivity of the sugar, glycerol, and acyl parts were carried out by HMBC, in which the carbonyl at δ 176.1 (C-1′) showed a cross-peak with the proton signal at δ 4.35 (2H, H-1); the anomeric proton signal at δ 4.49 (H-1⁗) correlated with the carbon signal at δ 68.7 (C-6‴); the anomeric proton signal at δ 4.51 (H-1‴) correlated with the carbon signal at δ 70.3 (C-6″); and the anomeric proton signal at δ 5.06 (H-1″) gave a cross-peak with the carbon signal at δ 72.6 (C-3). The geometry of the double bonds of **2** was determined by comparison with the known compounds **3**–**6**. The structure of **2** was thus determined to be 1-*O*-(9*Z*,12*Z*,15*Z*-octadecatrienoyl)-3-*O*-[β-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl] glycerol. Assignments of the ^{1}H NMR and ^{13}C NMR data of **2** (Table 2) were completely achieved by resolution of the HMQC and HMBC spectra.

On the basis of the spectral data and physical constants, five known compounds (**3**–**7**) were characterized as α-linolenic acid (30), 1-monolinolenin (31), 1-*O*-(9*Z*,12*Z*,15*Z*-octadecatrienoyl)-3-*O*-β-D-galactopyranosyl glycerol (32), gingerglycolipid A (33,34), and 1-*O*-β-D-glucopyranosyl-(2*S*,3*S*,4*R*,8*Z*)-2-[(2*R*)-2- hydroxydocosanoylamino]-8-octadecene-1,3,4-triol (35), respectively. Among them, compound **5**, reported previously as a synthetic compound, was obtained as a natural isolate for the first time.

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