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Effect of cyclic fatty acid monomers on fat absorption and transport depends on their positioning within the ingested triacylglycerols

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Abstract We investigated the intestinal digestion of cyclic fatty acid monomers (CFAM) isolated from heated linseed oil and their effects upon fatty acid lymphatic transport and lipoprotein profile in lymph. These cyclic fatty acid monomers were acylated in specific positions in the glycerol backbone of triacylglycerols (*sn*-1/3 position for the 1C oil, *sn*-2 position for the 2C oil and together in the *sn*-1,2, and 3 positions for the 3C oil) and administered intragastrically to lymph-cannulated rats. Their luminal digestibility was also assessed in vitro using a pancreatic lipase assay. The lipase activity was 1.9 to 6.6 less towards the triacylglycerols acylated with cyclic fatty acids compared to control. The lowest activity was with the 2C oil. In the hydrolytic products, the cyclic fatty acid contents were similar between the experimental groups. When absorbed as 2-monoacyl-*sn*-glycerol (2C oil), cyclic fatty acid monomers were better and unselectively recovered into the lymph than when absorbed as free fatty acids (1C oil). In that latter situation, the bulkier cyclic fatty acids (C6 and *cis* membered-ring CFAM) were transported into the lymph to a lesser extent. The appearance of the lymphatic chylomicrons was delayed in rats fed the 1C oil. Cyclic fatty acid monomers from the 2C oil only increased the lymphatic transport of saturated fatty acids (80%). Cyclic fatty acids from the 3C oil (absorbed as 2-monoacyl-*sn*-glycerol and free fatty acid) usually elicited intermediary effects. **■** We conclude that the effects of cyclic fatty acid monomers upon the intestinal metabolism are greatly influenced by their positioning within the triacylglycerol and that the structure of the cyclic fatty acids influences their lymphatic recovery only when they are absorbed as free fatty acid.—**Martin, J.-C., C. Caselli, S. Broquet, P. Juanéda, M. Nour, J.-L. Sébédio, and A. Bernard.** Effect of cyclic fatty acid monomers on fat absorption and transport depends on their positioning within the ingested triacylglycerols. *J. Lipid Res.* 1997. **38**: 1666–1679.

Supplementary key words intestinal metabolism • triacylglycerol structure • intestinal lipoproteins • pancreatic lipase

Cyclic fatty acid monomers (CFAM) are formed from the unsaturated 18 carbon chain length fatty acids of the edible oils as a result of domestic frying and industrial refining (1). CFAM occurring from 18:1n–9 are

composed of at least 8 different saturated cyclic fatty acids with a C5- or a C6-membered ring (2). Thirteen different monoenoic CFAM are formed from 18:2n–6, containing mostly a C5-membered ring (1, 3). Finally, 18:3n–3 gives rise to 16 identified dienoic CFAM, with a mixture of C5- and C6-membered rings with some bicyclic acids (1, 4, 5). Although they are usually present at low levels in the oils (from 0.01 to 0.66% of the total fatty acids (6, 7)) they have experimentally demonstrated adverse effects in animal models. For instance, they caused a higher death rate when administered orally to mice (8). They decreased the weight gain of weaning rats, increased the liver weight (9, 10) and the death rate of rat pups from mothers fed CFAM, as well as reduced the number of pups per litter (10, 11). They are also incorporated into heart cells in culture (12) where they altered the electrophysiological properties (13). It is not known whether the toxicity is related more to the C5- or to the C6-membered ring CFAM or both equally.

CFAM have elicited an effect on the lipid metabolism in the liver (9, 14), but comparable information is not available for the intestine. For instance, although CFAM displayed a high intestinal absorption rate (87–96%, 14, 15) nothing is known concerning their hydrolysis by lipase, their impact on the intestinal lipoprotein formation/secretion, their individual transport, or the transport of the other fatty acids into lymph. Neverthe-

Abbreviations: CFAM, cyclic fatty acid monomer; BDG, 3-O-benzyl-1,2-diacyl-*rac*-glycerol; DCC, 1,1'-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; TG, triacylglycerol; FFA, free fatty acids; 2MG, 2-monoacyl-*sn*-glycerol; FAME, fatty acid methyl esters; TLC, thin-layer chromatography; DG, diacylglycerol; HPLC, high performance liquid chromatography; GC, gas chromatography; MG, monoacylglycerol.

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less, the intestine is the first step of nutrient delivery to tissues and as such may modulate the bioavailability of the ingested fatty acids including CFAM.

Naturally occurring fatty acids are ingested mainly as triacylglycerols (TG) (16). The action of the gastrointestinal lipases leads to the formation of free fatty acids (FFA) and 2-monoacyl-*sn*-glycerol (2MG) as ultimate hydrolytic products (16, 17). These two lipid products are the only forms generated from TG that can be absorbed by the enterocyte (16, 17). Schematically, because of the regiospecificity of the lipases, fatty acids from the two outer positions (*sn*-1 and 3) of the TG molecule are released and taken up as FFA whereas fatty acids initially present in the central position (*sn*-2) of TG are released and absorbed as 2-monoacyl-*sn*-glycerol (18). The metabolic fate of these two lipid forms in the enterocyte is different (16, 19–21). Thus, the TG structure will govern the metabolic fate of the fatty acid, either in the lumen at the hydrolytic step, or in the enterocyte (partitioning between different metabolic pathways). This could lead, for instance, to different absorption rates for a given fatty acid (22–24), or could modify the absorption rate of the co-ingested fatty acids (25) or fat (24). Therefore, in order to study the lymphatic transport of CFAM as well as their effects on some aspects of the intestinal metabolism, it seems necessary to take into account their positioning within the TG molecule. CFAM acylated into TG either at one of the two outer positions, in the central position, or evenly distributed in the three positions were administered intragastrically to rats. Their lymphatic apparent recovery as well as the lymphatic apparent recovery of the co-ingested fatty acids were measured and the lymphatic lipoprotein profile was determined by electronic micrographs. The activity of the pancreatic lipase, the main lipolytic enzyme, was also determined *in vitro* in order to address whether the lymphatic apparent recovery of CFAM could be correlated with the initial step of hydrolysis in the lumen.

MATERIALS AND METHODS

Chemicals

All chemicals were supplied by Sigma-Aldrich-Supelco (Saint Quentin Fallavier, France), except boron trifluoride (Alltech, Templeuve, France). Solvents were from SDS (Peypin, France). Hexane, chloroform, methanol, acetone, and acetonitrile were distilled before use. The other solvents were of HPLC grade. Linseed oil was from Robbe Hyfran (Compiègne, France). Customized soybean oil with no CFAM was a generous gift from Lesieur (Coudekerque, France). Silica gel plates (20 × 20 cm, 2.5 mm thickness) as well as silicic acid for column

chromatography (Silica Si, particle size 70–200 mesh) were from SDS (Peypin, France). Nylon tubing (7.5 cm o.d.) was from Walter Coles, Co (London, UK).

Animals, surgery and lymph collection

Official French regulations (no. 87848) for the care and use of laboratory animals were followed (no. 03056). Male Wistar rats weighing 180–250 g were obtained from Centre d'élevage Dépré (Saint Doulchard, France). They were housed for 1 week before the study in a controlled environment, with constant temperature and humidity and a dark period from 8.00 PM to 8.00 AM. They were fed the standard nonpurified diet AO4 containing 3.5 g/100 g lipid (UAR Villemoisson sur Orge, Epinay sur Orge, France), and had free access to tap water. A laparotomy was performed under slight ethyl-ether anesthesia on fed rats. Mesenteric lymph duct cannulation was performed using a heparinized polyethylene catheter (no. 1, i.d. 0.3, o.d. 0.7, Biotrol, Paris, France) (26). Immediately after the surgery, rats were placed in restraining cages in an air-conditioned room (25°C). They did not receive solid food but had free access to a water solution containing sodium chloride (7 g/L) and potassium chloride (2 g/L).

Basal lymph from rats that had been fasted for 16 h was collected for 1 h in tubes placed inside Dewar flasks containing ice, immediately before lipid administration. Then, 1 mL of one of the experimental oils was administered intragastrically (27), which corresponds to 800 mg of TG. The lymph was collected separately at 1, 3, 6, 12, and 24 h from the conscious animals. Only animals with a basal lymph flow over 0.4 mL/h were considered. Lymph from animals in which the basal lymph flow did not increase 2 to 3 times during lipid absorption or whose lymph flow was perturbed by clotting was discarded. Then the samples were immediately treated for both morphological study of lipoproteins and lipid extraction.

Microscopic observation of lymph particles

Observation of lymph particles was done as described by Degrace et al. (27). Lymph was defibrinized by gauze filtration and 10 µL of the defibrinized lymph was fixed with 200 µL osmium tetroxide (0.2 g/L) for 1 h at 4°C. One drop of fixed suspension was placed on a collodion carbon grid. After complete evaporation of the fixing solution, the particles were shadowed with gold-palladium at a 50° angle with an Edwards E12E vacuum evaporator (Edwards High Vacuum Ltd, Crawley, UK). Two to three grids of each lymph fraction were examined using an Hitachi HU 11E transmission electron microscope (Hitachi Ltd, Tokyo, Japan). At least 1,000–2,000 shadowed particles for each lymph fraction were counted and classified by size.

TABLE 1. Characteristics and composition of the cyclic fatty acid monomers fraction used for acylation in the model TG

CFAM ^a	Ring Size	Ring Position	Double Bond Positions	Double Bond Configuration	Ring Configuration	% ^b
a	5	11-15	9, 12	Z	<i>trans</i>	10.1
b	5	10-14	12, 15	E	<i>trans</i>	8.8
c	5	11-15	9, 12	E	<i>trans</i>	10.0
d	5	10-14	12, 15	Z	<i>trans</i>	8.7
e	5	10-14	12, 15	E	<i>cis</i>	1.4
f	5	11-15	9, 12	E	<i>cis</i>	4.7
g	5	11-15	9, 12	Z	<i>cis</i>	7.0
h	5	10-14	12, 15	Z	<i>cis</i>	2.9
i	6	10-15	8, 12	E	<i>cis</i>	12.7
j	6	10-15	8, 12	Z	<i>trans</i>	1.9
k	6	10-15	8, 12	E	<i>trans</i>	8.3
l	6	10-15	12, 16	Z	<i>cis</i>	1.5
m	6	10-15	12, 16	E	<i>trans</i>	7.6
n	6	10-15	12, 16	E	<i>cis</i>	10.2
o	6	10-15	12, 16	Z	<i>trans</i>	2.0
p	6	10-15	12, 16	Z	<i>cis</i>	2.5

^aStructures determined by Dobson, Christie, and Sébédio (4), using a combination of silver-ion HPLC and GC-MS methods.

^bIndividual CFAM contents were determined by GC using BPX 70 and DBWax column (see Methods).

Cyclic fatty acid monomers preparation

CFAM were obtained after heating linseed oil for 12 h at 275°C under nitrogen according to Sébédio et al. (28). Briefly, after heating, the oil was saponified with KOH and the recovered FFA were converted to fatty acid methyl esters (FAME) with 1% H₂SO₄ in methanol. The FAME were purified from polar compounds by silicic acid chromatography (28). The CFAM were isolated from the other fatty acids by two consecutive urea fractionations. The CFAM composition is reported in Table 1.

Triacylglycerol synthesis

Tri-cyclic acyl-*sn*-glycerol was synthesized with 1 mol of glycerol esterified with 3 mol of free CFAM solubilized in CH₂Cl₂, and 3.3 mol of 4-dimethylamino-pyridine (DMAP) in the presence of 1,1'-dicyclohexylcarbodiimide (DCC) as adapted from Kodali et al. (29). Molecular sieves (3 Å) (2 g for 3 g of FFA) were added to the mixture. The reaction was carried out overnight under nitrogen with vigorous stirring. After filtration, TG was purified by silicic acid dry column chromatography adapted from the procedure of Loev and Goodman (30): Fifty cm of nylon tubing was filled with 450 g of silicic acid previously impregnated for 20 min with 50 mL of hexane-diethylether 80:20 (v/v). Up to 10 g of sample were applied on top of the column and eluted with the above solvent system. The elution was stopped when the solvent reached the lower extremity of the column. The column portion corresponding to TG (24–36 cm from top) was sliced and the lipid was extracted from silica with a mixture of CHCl₃-CH₃OH 2:1 (v/v).

Solvents were reduced at low pressure. The purity was checked by thin-layer chromatography (TLC) (hexane-diethylether-acetic acid 60:40:4, (v/v)).

Free fatty acids from soybean oil were obtained by saponification with KOH according to the AOCS procedure Ca-6a-40. They were used to synthesize randomized TG following the above instructions.

TG with CFAM at a defined position in the glycerol backbone were prepared from diacylglycerol (DG) as starting material. For TG with CFAM located in the central position, 1,3-diacyl-*sn*-glycerol was used and prepared as follows from the adapted method of Bentley and Mac Crae (31): 1 mol of dihydroxyacetone was acylated with 2 mol of soybean oil FFA for 3 h using the procedure described above for the TG. Completion of the reaction was checked by TLC (*R_f* 0.65 for the 1,3-dihydroxypropan-2-one-1,3 diacyl) with a mixture of CHCl₃-acetone 96:4 (v/v) as developing solvent. After filtration, the solvent was removed under reduced pressure and the residue was redissolved in tetrahydrofuran (50 mL for 3 g of FFA in the starting material). The mixture was stirred under nitrogen and 1 mL of water was added. The solution was chilled in an ice bath and NaBH₄ (0.9 g) was added. After 30 min, the reaction was stopped by adding acetic acid dropwise (300 µL). The resulting 1,3 diacyl-*sn*-glycerol was extracted with chloroform (300 mL) and aqueous NaHCO₃ (2%, 150 mL). The organic phase was washed again with water and then dried over Na₂SO₄. The reaction was monitored by TLC on a borate-impregnated silica gel plate using CHCl₃-acetone 96:4 (v/v) as the running solvent. It revealed unreacted 1,3-dihydroxypropan-one-1,3 diacyl (*R_f* 0.70), 1,3 diacyl-*sn*-glycerol (*R_f* 0.45) and a trace amount of 1,2 diacyl-rac-glycerol (*R_f* 0.25). The 1,3 diacyl-*sn*-glycerol was then purified by silicic acid dry column chromatography from an adapted procedure (30): to 425 g of silica was added 530 mL of a 10% boric acid methanolic solution. The methanol was evaporated under reduced pressure at 70°C. The deactivation of silicic acid was checked and required a grade I on the Brockman scale to achieve a good separation of the DG isomers. The chromatography was then operated as described above for the TG, except that CHCl₃-acetone 96:4 (v/v) was used as the developing solvent. After sampling of silica along the column, the DG isomers were identified by TLC on a silica gel G plate impregnated with boric acid. The column portion corresponding to the 1,3 diacyl-*sn*-glycerol was sliced and the lipid was extracted with CHCl₃. Boric acid was discarded by washing with chilled water and the organic phase was filtered and dried over Na₂SO₄. Chloroform was evaporated at low pressure at room temperature. The purified 1,3 diacyl-*sn*-glycerol was redissolved in a minimum of CHCl₃ and stored at -20°C.

The synthesis of TG with CFAM in one at the two

outer positions started with 1,2 diacyl-*rac*-glycerol. These DG were prepared as follows: isopropylidene-*O*-benzyl-*rac*-glycerol was synthesized from isopropylidene-*rac*-glycerol and chloromethylbenzene as described (32). This was then hydrolyzed (32) to yield 3-*O*-benzyl-*rac*-glycerol. The 3-*O*-benzyl-*rac*-glycerol was then acylated with the soybean oil FFA using DMAP and DCC in CH₂Cl₂ as described above for the dihydroxyacetone. The resulting 3-*O*-benzyl-1,2 diacyl-*rac*-glycerol was extracted with petroleum ether (bp 30°–60°C) and washed with saturated aqueous NaCl to remove most of the impurities, as monitored by TLC with hexane–diethyl ether–acetic acid 60:40:4 (v/v) as developing solvent. The 3-*O*-benzyl-1,2 diacyl-*rac*-glycerol (BDG) was then debenzylated as described by Kodali and Duclos (33) with slight modifications. The BDG dissolved in CH₂Cl₂ was cooled at –20°C in a bath of dry ice and acetone and kept under a nitrogen atmosphere. Three mol/mol of substrate of bromodimethylborane (previously prepared according to Guindon, Yoakim, and Morton (34)) was added dropwise while stirring. After 1.5 h, the reaction was stopped by adding saturated aqueous NaHCO₃. The reaction products were extracted with diethyl ether, washed with H₂O, and dried over Na₂SO₄. The resulting 1,2 diacyl-*rac*-glycerol was purified from the unreacted materials and the trace amount of isomerized 1,3 diacyl-*sn*-glycerol by silicic acid dry column chromatography impregnated with boric acid as described above for the 1,3 diacyl-*sn*-glycerol, except that hexane–diethyl ether–acetic acid 60:40:4 (v/v) was used as the developing solvent.

The CFAM were acylated in the *sn*-2 position of the 1,3 diacyl-*sn*-glycerol or in the *sn*-1 or *sn*-3 position of the 1,2 diacyl-*rac*-glycerol using DMAP and DCC as described above. The resulting triacyl-*sn*-glycerols were then purified by dry column chromatography as described for the randomized soybean oil TG. The composition of the *sn*-2 and *sn*-1/3 positions was ascertained by analysis of the fatty acid composition of the 2 monoacyl-*sn*-glycerol released after pancreatic lipase lipolysis (see below).

Preparation of the experimental oils

The model TG containing the CFAM was mixed to the randomized oil TG in order to achieve 6–7% of CFAM content in the total fatty acid composition (Table 2).

Pancreatic lipase hydrolysis

To check the CFAM composition in the *sn*-2 and in the *sn*-1/3 positions of the synthesized TG, an aliquot of the pure model TG (5 mg) was subjected to pancreatic lipase hydrolysis (E.3.1.1.3, type VI-S) with 5,000 U of enzyme for 10 min in a Tris/HCl buffer containing arabic gum (35, 36). The 2 monoacyl-*sn*-glycerol was iso-

TABLE 2. Fatty acid composition of the experimental oils (%)

Oils ^a	16:0	18:0	18:1	18:2n-6	18:3n-3	CFAM
1C	11.31	3.76	22.88	49.27	5.86	6.90
2C	9.70	2.66	19.56	53.61	7.71	6.76
3C	12.81	4.57	25.19	47.03	4.52	5.90
Control ^b	13.04	5.09	27.80	49.70	4.37	0

^aExperimental oil in which the CFAM are acylated in the *sn*-1/3 position (1C), in the *sn*-2 position (2C), or evenly in the 3 *sn* positions (3C).

^bRandomized soybean oil.

lated by TLC on silica gel G-impregnated plate with hexane–diethyl ether–acetic acid 60:40:4 (v/v) as the running solvent. Analysis by gas chromatography showed that 90% of the CFAM was found in the *sn*-2 position of the 2C oil TG and 10% in the *sn*-1/3 position of the 1C oil TG.

For the lipase assay, closer physiological conditions were established: the standard assay medium, containing 15 mg of TG from each of the experimental oils, was warmed at 37°C. It consisted of 5 mL of 0.01% sodium taurocholate and 0.18% CaCl₂ in a 0.5 M Tris/HCl (pH 7.7) buffer solution (37). Lipolysis was started by adding 20 µg (2,000 U) of pancreatic lipase (E.3.1.1.3, type VI-S) together with 4 µg of colipase under vigorous stirring. The reaction was stopped at selected time points by acidification with diluted HCl. The lipid products were extracted with 2 × 4 mL diethyl ether. A portion aliquot (2/3) was used for the analysis of the total lipid profile after trimethylsilylation (see below). The other part was fractionated by TLC on silica gel G-impregnated plate with hexane–diethyl ether–acetic acid 60:40:4 (v/v) as the running solvent. The separated hydrolysis products were scraped off and converted to FAME prior to gas chromatography (GC) analysis. The lipase specific activity extended to 1 h was calculated from the FFA released during the first 2 min of hydrolysis (i.e., in condition of substrate saturation, see Fig. 10B). The release of FFA was monitored by GC using trimethylsilyl derivatives (see Fig. 10B).

Lipid extraction and derivatization

Total lipids from a 1-mL portion of lymph were diluted with 1 mL of water and extracted with 18 mL of CHCl₃–CH₃OH 2:1 (v/v). The upper phase was discarded and the lower phase was washed again with 4 mL of distilled water. The organic phase was then removed and the solvent was reduced under nitrogen. The lipid residue was redissolved in a minimum of solvent and kept at –20°C until analysis.

The fatty acids from the lymph lipids were converted to FAME with 3 mL of BF₃ (14% in CH₃OH) and 2 mL of benzene, at 100°C for 1.5 h. The fatty acids from the oil lipids and from the oil lipolytic products were derivatized to FAME according to Morrison and Smith (38)

with BF_3 (14% in CH_3OH). FAME were extracted by 2×3 mL of hexane after addition of 2 mL of water and dried over Na_2SO_4 .

An aliquot (6 mg) of the non-fractionated total lipids resulting from the pancreatic lipase hydrolysis was converted to trimethylsilyl derivatives with 50 μL of a 1:1 (v/v) mixture of sylon BFT (N,O-bis-(trimethylsilyl)-trifluoroacetamide:trimethylchlorosilane (99:1, v/v)) and pyridine at 80°C for 30 min. The reagent was evaporated under nitrogen and the lipid residue was extracted with 2×2 mL of hexane after addition of 2 mL of water, dried over Na_2SO_4 , and stored at -20°C until analysis.

High performance liquid chromatography fractionation of the fatty acid methyl esters

FAME from the lymph samples were fractionated by HPLC in order to concentrate CFAM for further GC analysis. FAME were dissolved in 100 μL of acetone, injected in a reversed-phase column Nucleosil C18, 250 mm length, 10 μm i.d. and 5 μm particle size (Shandon, Cergy-Pontoise, France) through a Spectra-physics 8810 HPLC system (Spectra-Physics, La Verpillière, France) and detected with a Waters 410 differential refractometer (Waters, Saint Quentin en Yvelines, France). Acetonitrile was the mobile phase (4 mL/min). CFAM co-eluted with 18:2n-6, 14:0, 15:0, and 16:1. The collected fractions were then taken to dryness at reduced pressure, dissolved in a minimum of solvent, and stored at -20°C prior to GC analysis.

Gas chromatography analysis

FAME analysis from the total lymph lipids was carried out on a Hewlett-Packard gas chromatograph (model 5890, Les Ulis, France) fitted with a flame ionization detector and a split-splitless injector both set at 250°C . Carrier gas was helium (1.1 mL/min) and elution was performed with a BPX 70 column (SGE, Villeneuve-Saint-Georges, France) (50 m length, 0.33 mm i.d., and 0.25 μm film thickness) in program mode. The column was operated at 60°C for 1.1 min and the temperature was raised to 170°C at a rate of $20^\circ\text{C}/\text{min}$, held for 5 min, then increased at $2.5^\circ\text{C}/\text{min}$ to 220°C and held for 15 min. CFAM from the HPLC fractions were similarly analyzed, but with a different temperature program as operated by Dobson, Christie, and Sébédio (4): the temperature was started at 60°C , held for 1.1 min, then raised to 160°C at $20^\circ\text{C}/\text{min}$, held for 30 min, and increased to 220°C at $10^\circ\text{C}/\text{min}$ (held for 10 min). Unresolved CFAM peaks (d and g, Table 1), were separated on a DBWax column (J&W, Courtaboeuf, France) (30 m length, 0.25 mm i.d., and 0.2 μm film thickness) which was operated at 160°C for 5 min, then raised to

180°C at $0.5^\circ\text{C}/\text{min}$, held for 17 min and finally taken to 220°C at $20^\circ\text{C}/\text{min}$ (held for 10 min). Identification of the CFAM peaks was made by comparison with the detailed analysis of the same linseed oil CFAM fraction performed by Dobson et al. (4).

The FFA, MG, DG, and TG from the lipolysis experiment were simultaneously resolved by GC after conversion to trimethylsilyl derivatives (39). The samples were injected in an Intersmat gas chromatograph (Intersmat, Chelle les Coudreaux, France) equipped with an adapted Ros injector and a FID detector. Injector and detector temperatures were set at 350°C . The lipids were eluted with an HP5 column (Hewlett-Packard, Les Ulis, France) (8 m length, 0.32 mm i.d., 0.25 μm film thickness). Helium was the carrier gas (1.1 mL/min). The oven temperature was set at 170°C and immediately after injection was raised to 340°C at $10^\circ\text{C}/\text{min}$, held for 18 min. The lipid classes of the mixture were identified by comparison with the retention times of the TLC-isolated FFA, MG, DG, and TG of a sample. For all GC analysis, quantitation was made by peak integration performed with a Spectra-Physics Chromjet Integrator and a Spectra-Physics Chemstation run by the WOW software (Spectra-Physics, La Verpillière, France) after correction for detector response was made. Results are expressed in g/100 g of fatty acid or in mol % (lipolytic products). In that latter instance, an average mol % was calculated from the fatty acid composition of the lipolytic products.

Fatty acid percentage recovery

The percentage of apparent recovery of CFAM into lymph was calculated from the relative recovery of 18:3n-3 according to the formula:

$$\% \text{ recovery of CFAM} = (18:3n-3_D / 18:3n-3_L) \times (\text{CFAM}_L / \text{CFAM}_D) \times 100$$

where the subscript D refers to diet and the subscript L refers to lymph.

The percentage recovery of the other fatty acids was calculated from the fatty acid profile of the experimental group and control oil groups as follows:

$$\% \text{ recovery of FA} = (\text{FA}_D / \text{FA}_L)_e \times (\text{FA}_L / \text{FA}_D)_c \times 100$$

where D and L refer to diet and lymph for each selected fatty acid, respectively, and e and c refer to the ratios calculated for the experimental oil groups and for the control oil group, respectively.

Statistics

Results were computed with Microsoft Excel and statistics operated with SigmaStat™ (Jandel Scientific, San

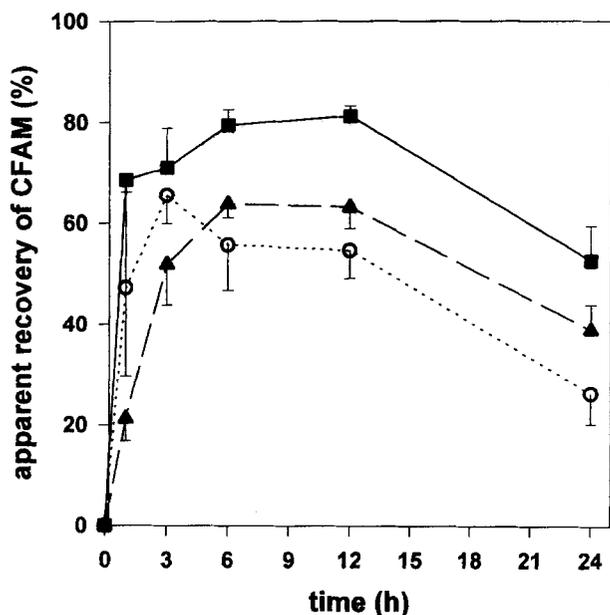


Fig. 1. Relative lymphatic CFAM recovery over 24 h. 1C (Open circle): experimental oil in which the CFAM are located in one of the two outer positions of the oil TG; 2C (filled square): experimental oil in which the CFAM are located in the central position of the TG; 3C (filled triangle): experimental oil in which the CFAM are located evenly in the three positions of the TG. The recovery is statistically different among groups ($P < 0.05$; repeated measures). $n = 4$ rats for the 1C group, $n = 6$ rats for the 2C group, $n = 5$ rats for the 3C group.

Rafael, CA). For the absorption studies, values are expressed as means \pm SEM for $n = 4$ – 6 animals per group. Comparisons were made with the ANOVA for multiple comparisons and the Neuman's Keuls test ($\alpha = 0.05$) as soon as heterogeneity between groups was demonstrated. Comparison for CFAM recovery over 24 h was made by repeated measures. Results were considered as statistically significant for $P < 0.05$.

RESULTS

In vivo experiment

Figure 1 displays the relative % recovery of CFAM in the mesenteric lymph over 24 h. It shows that the CFAM were best recovered when they were primarily acylated in the central position of the ingested TG, then evenly in the 3 positions, and then less when present in only one of the two outer positions ($P < 0.05$, repeated measures). However, the % apparent recovery never exceeded the apparent recovery of 18:3n-3, and reached 80% at 12 h for the 2C diet. The peak of absorption occurred between 6 and 12 h for both the 2C diet and

the 3C diet. It started earlier for the 1C diet (between 6 and 12 h). We then examined the CFAM lymphatic apparent recovery rate according to their structure for the three kinds of TG administered. **Figure 2** reports the C5/C6-membered ring CFAM ratio calculated for the lymph lipids over 24 h. The original ratio (R in Fig. 2) was 1.1 for all diets. This ratio was similar between the dietary lipids and the lymphatic lipids for the 2C diet. Conversely, the C5-membered ring CFAM were better recovered than their C6 counterpart for the 3C diet at the peak of absorption. The ratio was even greater for the 1C diet.

A calculation similar to that for the ring size was made for the ring conformation (*cis*- or *trans*-membered ring). Results are reported in **Fig. 3**. The *cis/trans*-membered ring CFAM ratios were similar for the ingested TG and the lymphatic lipids for the 2C diet. On the other hand, the *cis*-membered ring CFAM were less recovered than their *trans* counterpart for both the 1C diet and the 3C diet, though the differences were only significant at the peak of absorption (between 6 and 12 h).

No other differences among dietary groups were observed for the lymphatic CFAM recovery concerning the ring position along the acyl chain nor the position of the double bond or its conformation (Z or E, data not shown).

We have also determined the total % apparent recovery of the other dietary fatty acids in the lymph for the three experimental oils compared to control oil. **Figure**

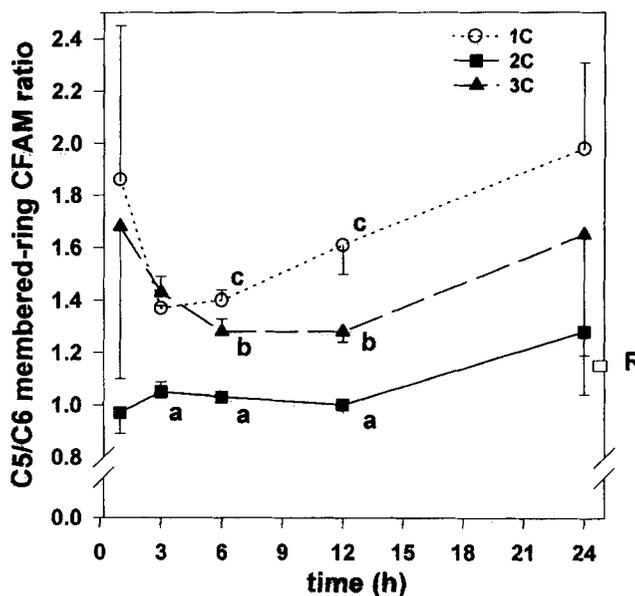


Fig. 2. C5/C6-membered ring CFAM ratios found in lymph over 24 h. For each time-point, symbols with a different letter are statistically different. R: ratio in the ingested oils; see Fig. 1 for legends.

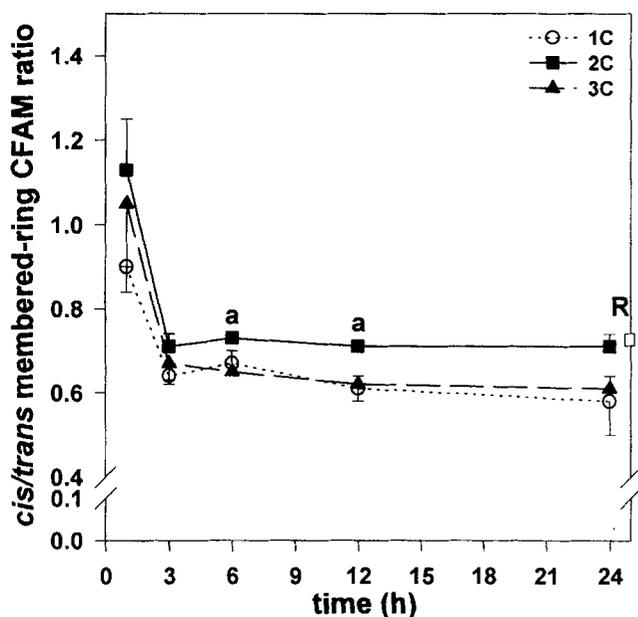


Fig. 3. *Cis/trans*-membered ring CFAM ratios found in lymph over 24 h. For each time-point, symbols with a different letter are statistically different. R: ratio in the ingested oils; see Fig. 1 for legends.

4 represents the results for saturated fatty acids. It shows greater apparent recovery of both 16:0 and 18:0 in lymph but only in the diet in which CFAM were originally located in the *sn*-2 position. For all the other fatty acids, the apparent recovery was similar to the control oil group (100%, data not shown).

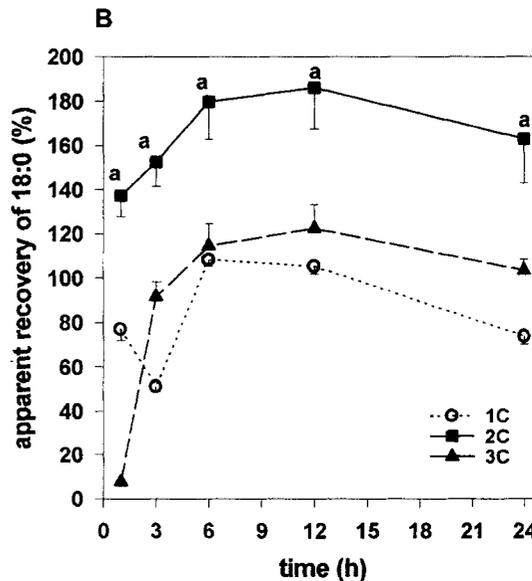
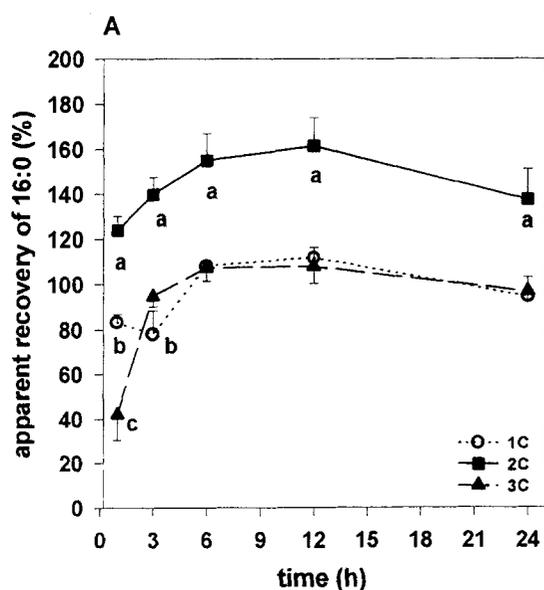


Fig. 4. Relative lymphatic recovery of palmitic acid (panel A) and stearic acid (panel B) for rats administered the experimental oils. For each time-point, symbols with a different letter are statistically different. See Fig. 1 for legends.

Figure 5 presents the electron micrographs of the lymphatic chylomicrons ($\geq 0.07 \mu\text{m}$) and of the lymphatic VLDL ($< 0.07 \mu\text{m}$) for each dietary group at selected times after ingestion. It shows that the relative percentage of lipoproteins seems to differ among the dietary groups. Results for the lipoproteins' profile over the 24 h are presented in Fig. 6. The percentage of chylomicrons was calculated from the total lipoproteins, i.e., VLDL plus chylomicrons. Chylomicrons are large lipoproteins that allow a maximum of lipid transport into lymph. Therefore, the peak of chylomicron secretion corresponds to the maximum of lipid transport from the intestine. The peak of chylomicron secretion occurred between 3 and 12 h for the 2C group and corresponded with the peak of secretion of the CFAM into lymph (Fig. 1). It also corresponded to the lipoprotein profile of the control group (randomized soybean oil). For the two other oil groups, the peak of chylomicron secretion did not correspond as accurately to the peak of CFAM secretion into lymph. The profile of the lymphatic lipoproteins of the 3C oil group was intermediate between those of the 2C and 1C group (Fig. 6).

In vitro experiment

We conducted in vitro experiments to determine whether it was possible to correlate some of our in vivo results to the pancreatic lipase activity towards the TG containing CFAM. According to the regiospecificity of the lipase, CFAM from the 2C diet and primarily acylated in the central position of the oil TG would be re-

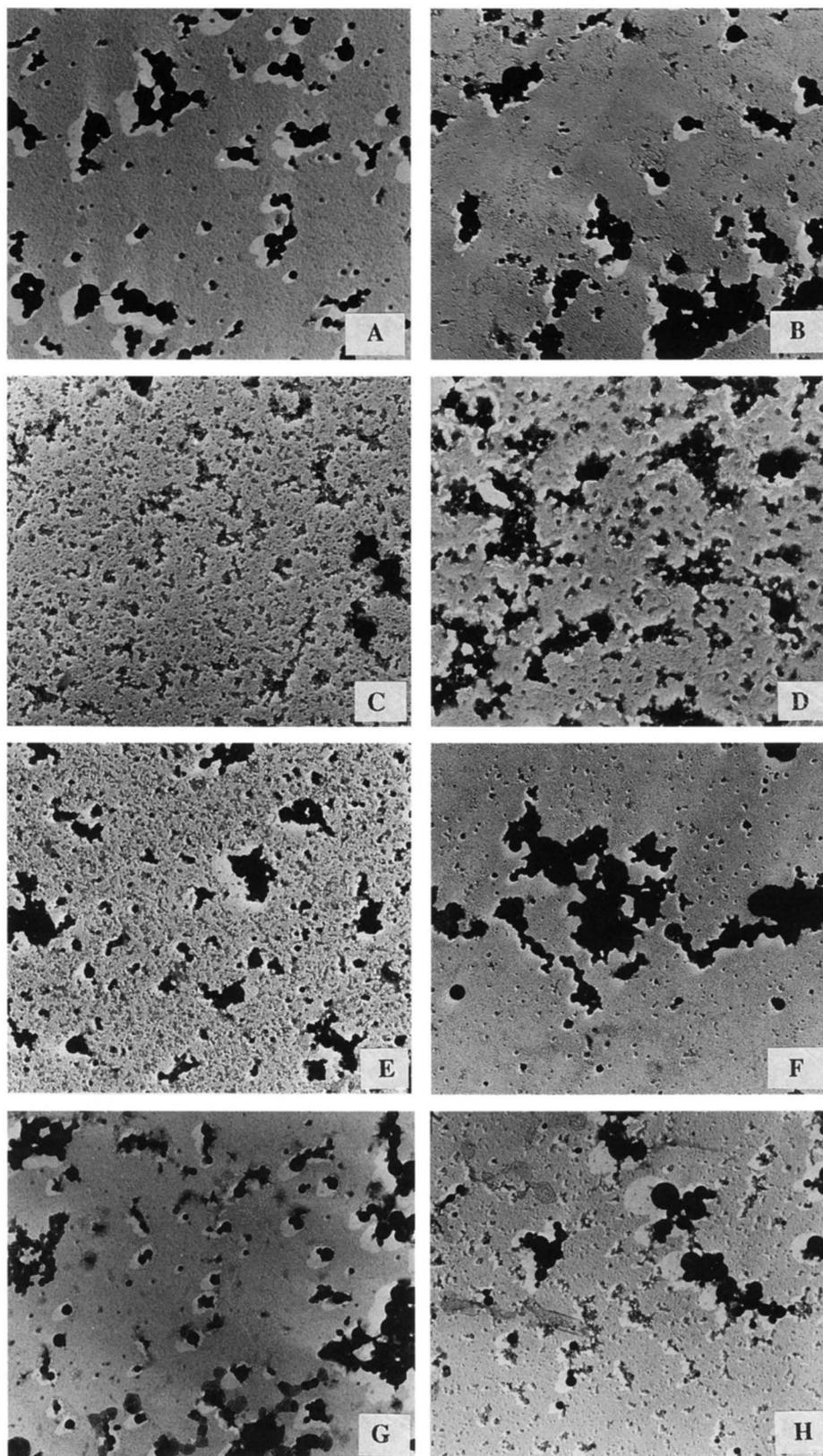


Fig. 5. Electron micrographs of lymph lipoprotein particles of rat administered 1 mL of experimental oil. Panels A and B: control oil (randomized soybean oil); panels C and D: 1C oil (CFAM in the $sn-1/3$ positions); panels E and F: 2C oil (CFAM in the $sn-2$ position); panels G and H: 3C oil (CFAM evenly in the 3 sn positions). A, C, E, G: lymph collected between 1 and 3 h after administration; B, D, F, H: lymph collected between 3 and 6 h (B, F, H) or 6 and 12 h (D). Chylomicrons $\geq 0.07 \mu\text{m}$; VLDL $< 0.07 \mu\text{m}$. $\times 15000$.

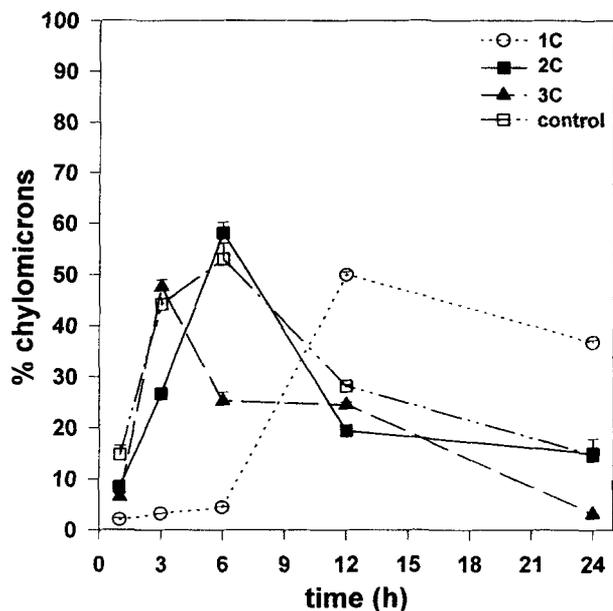


Fig. 6. Lymph lipoprotein profiles over 24 h for rats administered the experimental and control oils. The control oil ($n = 5$ rats) is represented by open squares. Mean of two determinations. See Fig. 1 for the other legends.

leased as 2MG after lipolysis. CFAM from the 1C diet acylated in the two outer positions would be released only as FFA. Finally, CFAM from the 3C would be released both as FFA and 2MG. To determine the CFAM content in the hydrolytic products, we determined the fatty acid content only in the 2MG fraction for both the 2C and the 3C groups, and only in the FFA fraction for both the 1C and 3C groups. **Figure 7** and **Fig. 8** display the C5/C6 and *cis/trans* CFAM-membered ring ratios, respectively, in the above hydrolytic products for the 2C, the 1C, and the 3C oils. All the ratios calculated were similar and similar to the ratio found in the original oils prior to hydrolysis (Fig. 7 and Fig. 8), indicating that no selectivity of the lipase arose with regard to the ring size or the ring configuration. **Figure 9** shows a more general result. We calculated the theoretical content of CFAM relative to the other fatty acids, in the *sn*-2 position for the 2C oil, in the *sn*-1/3 positions for the 1C oil, and in the 3 positions for the 3C oil. This theoretical content was then compared to the experimental content we found in the hydrolytic products (i.e., 2MG for the 2C oil group and the 3C oil group, FFA for the 1C and the 3C oil group) by calculating the experimental/theoretical CFAM ratios. A value of one would indicate that no substrate discrimination occurred during the hydrolysis. A ratio over one would correspond to a better hydrolytic activity towards the TG containing CFAM; a ratio lower than one would mean the reverse. Overall, the lipase did not seem to discriminate very much to-

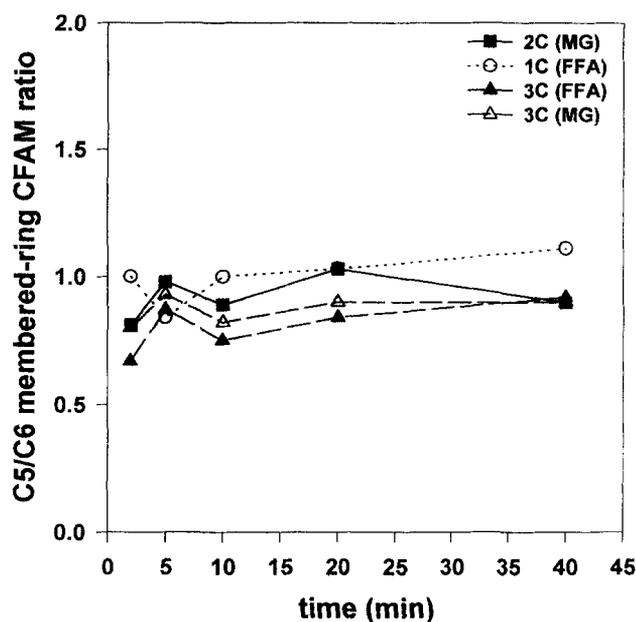


Fig. 7. C5/C6-membered ring CFAM ratio found in selected hydrolytic products of the experimental oils digested with 2000 U of porcine pancreatic lipase. Average of duplicate analysis. The SEM bars usually did not exceed the symbol size. 1C (Open circle): experimental oil in which the CFAM are located in one of the two outer positions of the oil TG; 2C (filled square): experimental oil in which the CFAM are located in the central position of the TG; 3C (filled and open triangles): experimental oil in which the CFAM are located evenly in the 3 positions of the TG. The CFAM were determined in the FFA fraction for the 1C oil, in the 2MG fraction for the 2C oil, and both in the FFA and 2MG fraction for the 3C oil.

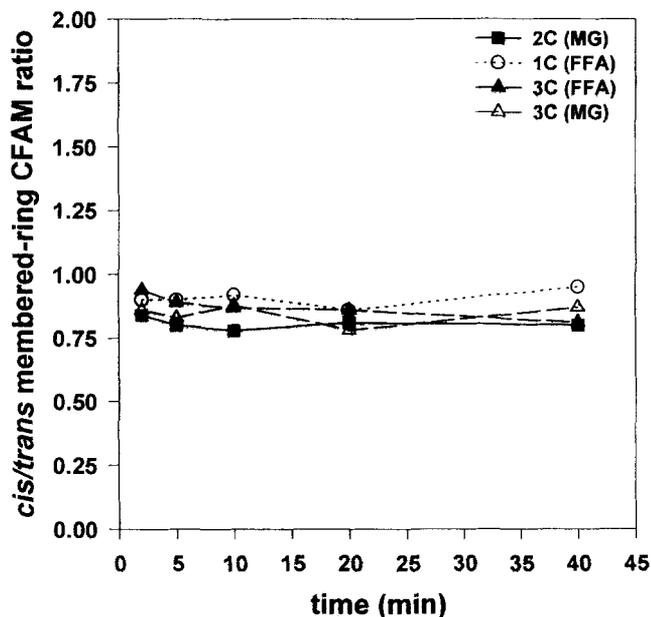


Fig. 8. *Cis/trans*-membered ring CFAM ratio found in selected hydrolytic products of the experimental oils digested with 2000 U of porcine pancreatic lipase. Average of duplicate analysis. The SEM bars usually did not exceed the symbol size. See Fig. 7 for legends.

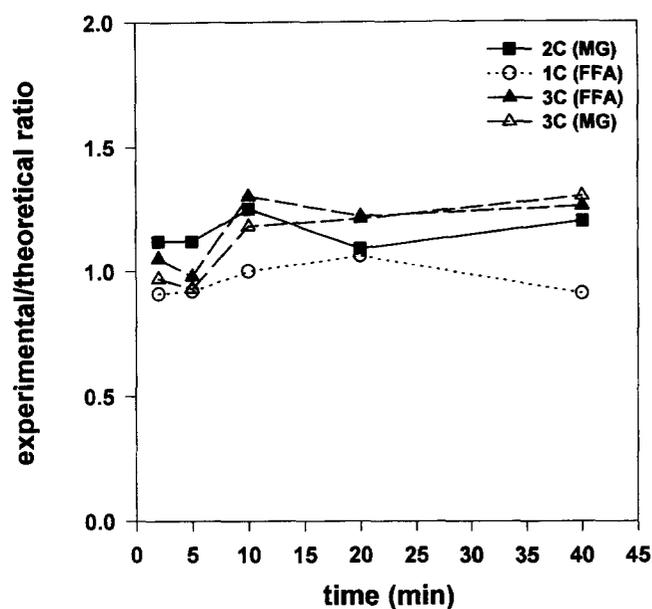


Fig. 9. Experimental/theoretical CFAM ratios found in the selected hydrolytic products of the oil TG digested with 2000 U of porcine pancreatic lipase. Average of duplicate analysis. The SEM bars usually did not exceed the symbol size. See Fig. 7 for legends.

wards the TG with the CFAM, although a slight tendency to a better hydrolysis would appear for the 2C and the 3C oil groups.

Figure 10 monitored the lipid fraction profiles for each experimental oil during lipolysis. Figure 10A displays the TG profile whereas Table 2 presents the specific activity of the lipase in conditions of substrate saturation. Large differences were seen between groups during the initial step of hydrolysis. Specifically, only 40% of the total TG from the 2C group was hydrolyzed during the first 2 min, whereas 58% and 72% of the TG disappeared for the 1C and the 3C group, respectively. The control group underwent the highest rate of lipolysis (88%). Although to a lesser extent, the same trend still occurred at 5 and 10 min (Fig. 10A). No more differences were seen at 20 min. The FFA profile inversely mirrored the TG profile, but the differences observed at the initial time of lipolysis still occurred after 40 min (Fig. 10B). Differently stated, in conditions of substrate saturation, the lipase specific activity was highest with the soybean oil group (1415 versus 214 to 753 for the CFAM oil groups, Table 3). Though different, the 3C group was the closest to control group (Table 3 and Fig. 10A and 10B).

The MG and DG profiles for the experimental oils were different from that of the control oil group, although again closer values were shown between the 3C group and the control group (Fig. 10C and 10D).

DISCUSSION

In this study, to assess the lymphatic transport of CFAM, we have taken into account not only their structural differences but also the molecular form of their TG carrier. We studied the absorption modalities of CFAM occurring from 18:3n-3 because they are representative of the whole CFAM with regard to the ring (contain either a C5- or a C6-membered ring) and because they can be analyzed easily in their non-hydrogenated form. This gives more information when observing the relationship between the chemical structure and the biological effects.

We calculated the apparent lymphatic recovery rate of the CFAM relative to 18:3n-3. We chose this fatty acid as a reference because it was present in high amounts in the diet (> 5%, Table 2) and is not reported to be in the bile phospholipids that also provide fatty acids for the lymph lipids (40). It is also a minor fatty acid in the lymph collected before oil feeding (i.e., baseline time, 1.3%, data not shown), assuming again a low participation of the endogenous pool for this fatty acid. Moreover, the baseline lymphatic fatty acid profile reflects the intestinal endogenous pool of fatty acids whereas the post-ingestion fatty acid profile in lymph involved only the fatty acid originating from the lumen (mainly diet, bile, and sloughing epithelium) without the participation of the enterocyte pool (41). Therefore, the variation of the lymphatic 18:3-3 contents occurring from non-dietary origins that would alter the results was kept to a minimum.

In our in vitro lipase assay, striking differences among groups were seen for the relative amount of hydrolysis products (Fig. 10). These differences were best illustrated by the specific activity of the lipase towards any of the oil groups. Under conditions of substrate saturation, the specific activity was 1.9- to 6.6-fold less towards the TG containing CFAM than control (Table 3), indicating a regulatory role of the CFAM. Van Kuiken and

TABLE 3. Specific activity of the pancreatic lipase towards triacylglycerols containing cyclic fatty acid monomers under conditions of substrate saturation

	Oils ^a			Control ^b
	1C	2C	3C	
	<i>mEq/mg protein/h</i>			
Specific activity	472	214	753	1415

Results are given as the average of two separate determinations. Activity was calculated during the first 2 min of hydrolysis (substrate saturation) and extended to 1 h.

^aExperimental oils in which CFAM are acylated in the *sn*-1/3 position (1C), in the *sn*-2 position (2C), or evenly in the 3 *sn* positions (3C).

^bRandomized soybean oil.

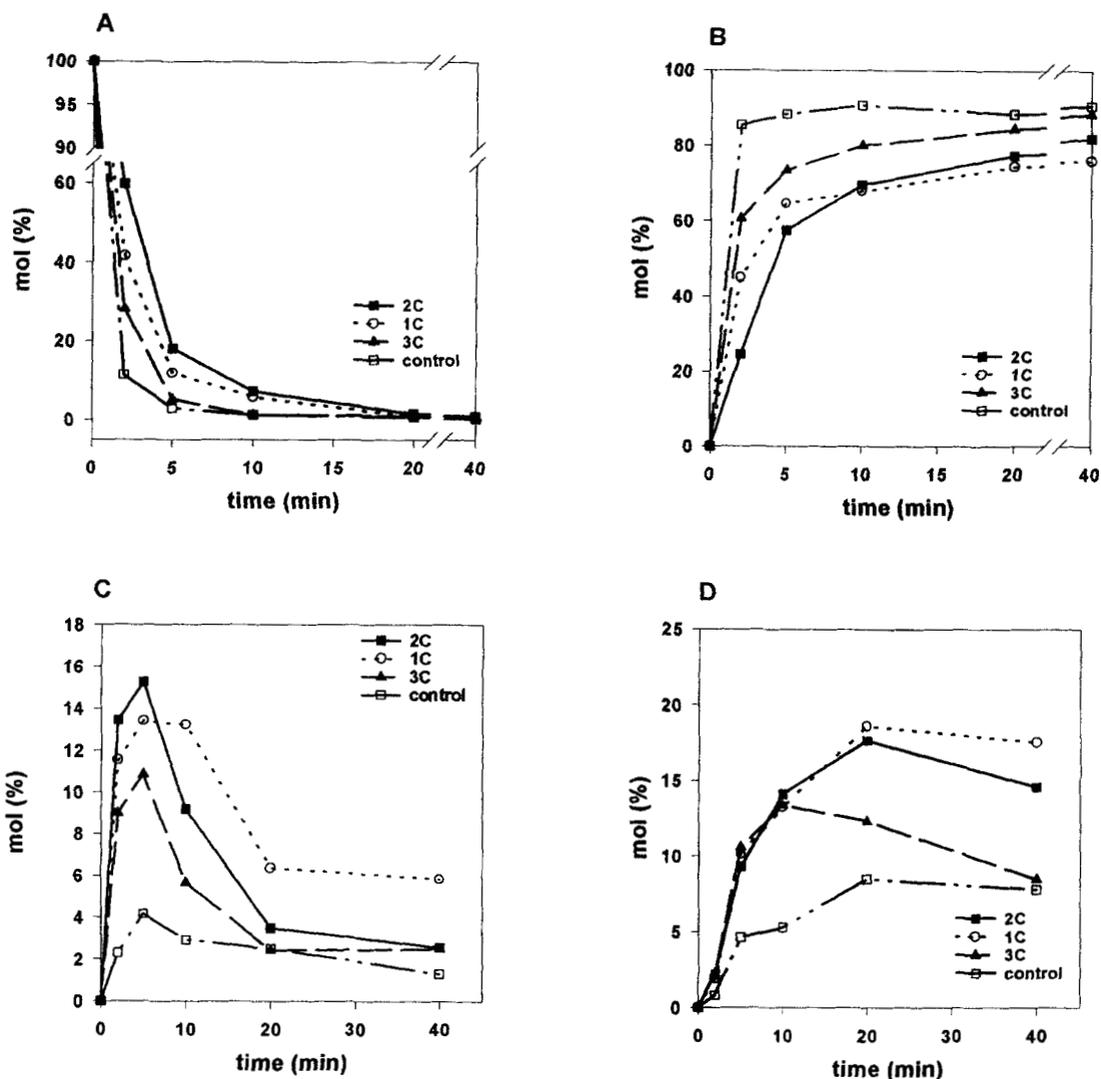


Fig. 10. Lipid profiles over time of the experimental oils digested with 2000 U of porcine pancreatic lipase. Panel A: TG profiles; panel B: FFA profiles; panel C: DG profiles; panel D: MG profiles. Average of duplicate analysis. The SEM bars usually did not exceed the symbol size. See Fig. 7 for legends.

Behnke (42) identified FFA regulatory binding sites on pancreatic lipase. A modulation of the lipase activity only by free CFAM is unlikely as the CFAM released only as 2MG (2C oil) accounted for the lowest lipase activity. Therefore, the lipase activity would also be regulated by the CFAM at a different level than the FFA. The TG species acylated with CFAM accounted for 18% of the TGs in both the 1C and 2C oil and only 6% in the 3C diet. Both the 1C and 2C oils exhibited the highest inhibitory effect against the lipase. This with the above results indicate that the lipase activity would be regulated by the CFAM at the TG level. Rats fed 1 mL of oil correspond by extrapolation to humans fed 350 mL. One may surmise that under our in vivo experimental

conditions, the intestinal absorption steps should be saturated with the absorbable lipids in every oil group. The observed differences in the lipase activity would not be great enough to affect the fatty acid absorption. Whether or not this would be also the case for a lower fat load is not known.

In vivo, after hydrolysis by the gastrointestinal lipases and according to their original distribution in the ingested TG, CFAM are absorbed as 2MG in the 2C diet, FFA in the 1C diet, and both 2MG and FFA in the 3C diet (16). Our in vitro assay also showed that the total CFAM did not accumulate nor were depleted in both the FFA and the MG fractions formed after lipase hydrolysis (Fig. 9). Therefore, it is unlikely that the lipolytic

steps within the lumen modulated the apparent CFAM recovery into the lymph through a better release as 2MG or FFA. The 2MG and the FFA released after the enzymatic digestion are absorbed either passively (2MG) or both through a passive diffusion and a putative active mechanism (FFA) (21). The 2MG taken up directly enter the 2-monoglyceride pathway to be reacylated mainly into TG (43) which are secreted into the lymph as lipoproteins. Conversely, fatty acid binding protein (FAT, FABPpm) (21, 44, 45) may facilitate the transport of fatty acid through the apical membrane of the enterocyte. Once in the cytosol, the fatty acids that were either passively or actively taken up are then reversibly bound to cytosolic FABP. They are then activated in acyl-CoA by an acylcoenzyme A synthetase. Most of these activated fatty acids are acylated into the glycerolipids that are used for lipoprotein structuration. A selectivity for fatty acid binding has been described for all these FABP (21, 45). Therefore, if the lipolytic steps in the lumen are not limiting, one may speculate on a negative selectivity of the FABP towards the CFAM absorbed as FFA and/or a defective reacylation into the glycerolipids. This may explain why the CFAM are better recovered when absorbed as 2MG (2C diet) than as both 2MG and FFA (3C diet) and then as FFA alone (1C diet) (Fig. 1). Another explanation would be a better solubilization of the 2MG released after lipolysis compared to the FFA. The physical properties of the MG cause their localization at the lipid/water interface of the micelles (17). On the other hand, at the pH prevailing in the intestine, all the fatty acids are not ionized and thus remain in the lipid core of the micelles (17). This positioning would allow the MG to be taken up better than the FFA and thereby would enhance the absorption rate and the lymphatic recovery of the CFAM initially present in the *sn*-2 position. Whatever the mechanism involved, the differences in the recovery of CFAM in lymph according to their molecular form of uptake would at least partly explain the results found by Sébédio et al. (11). They found that rats given 10% of the total fatty acids as CFAM during gestation and lactation in the form of a heated linseed oil TG showed a higher mortality for the offspring (51%) and 3-fold more accumulation of the CFAM in the liver than when the same amount of CFAM was fed as ethyl ester. In linseed oil, trilinolenin is the most abundant TG species (46). The CFAM formed from this oil were thereby absorbed as FFA and 2MG whereas the CFAM given as ethyl esters were absorbed only as FFA.

The C5-membered ring CFAM are better recovered in the lymph relative to the C6-membered ring when they are absorbed as FFA (1C oil) (Fig. 2). Such an observation was not noticed for the CFAM absorbed as

2MG (2C diet), where the C5/C6-membered ring ratio was similar to that of the ingested oil (Fig. 2). The CFAM absorbed both as 2MG and FFA (3C diet) gave intermediary results. A trend similar to that for the ring size was observed for the ring conformation (*cis/trans* ring conformation), although to a lesser extent (Fig. 3). On the other hand, both the ring size and the ring conformation were identical for CFAM found in the 2MG and the FFA hydrolytic products (Fig. 7 and 8). This would indicate that the selective lymphatic recovery of the C5 and *trans*-membered ring CFAM did not originate from selective hydrolysis by the pancreatic lipase. In a preliminary experiment using the 3C oil, we did not detect CFAM in the intestinal mucosa lipids 48 h after force-feeding (two animals, data not shown). Thus, a preferential retention of the C6-membered ring CFAM into the mucosa lipids that could explain the CFAM lymphatic profile is unlikely. Ribot et al. (12) studied the incorporation of the same CFAM fraction as ours into lipids of heart cells in culture. The CFAM were given as FFA and therefore bypassed any lipolysis that would be involved in fatty acid cellular uptake. In that situation, a preferential accumulation of the C5-over the C6-membered ring CFAM was also observed. Both intestinal cells and heart cells possess FABP (21). The similarity in the CFAM profile observed in lymphatic and heart lipids strongly suggest a relative selectivity of binding by the cytosolic FABP for the CFAM with a C5 or a *trans*-membered ring. It is noteworthy that the C6 ring is bulkier than the C5 ring and that a *cis* ring configuration gives a non-linear configuration compared to a *trans* ring configuration. The CFAM taken up as 2MG escape the binding step to FABP and therefore would escape any binding discrimination. This would be the reason why the C5/C6 and the *cis/trans*-membered ring CFAM ratios were identical in the dietary lipids and in the lymph lipids when the CFAM are absorbed as 2MG (2C oil group). Together with the results found for the total CFAM recovery and discussed above, one may surmise that the steric features of the CFAM would thus both quantitatively and qualitatively modulate their binding to the FABP.

An intriguing observation was the influence of the positional distribution of the CFAM on the lymphatic apparent recovery rate of the saturated fatty acids. Compared to control, the CFAM absorbed as 2MG (2C oil) were merely associated with a large enhancement of the 16:0 and 18:0 content in the lymphatic lipoproteins. Several studies reported a better absorption of the saturated long-chain fatty acids when they were located in the central position of the ingested TG (23, 25, 47). This cannot be the case in the present study because the fatty acids in all dietary groups have been randomly assigned to each *sn* position but the CFAM. Whatever

the mechanism involved, we do not have a satisfactory explanation to account for this above effect which is specific for the CFAM absorbed as 2MG.

The lipoprotein profiles differed among dietary groups (Fig. 5 and 6). The 2C diet was similar to control, thereby indicating that CFAM absorbed as 2MG did not modify the formation/secretion of the intestinal lipoproteins. On the other hand, although they amounted to only 6% of the total fatty acids in the diet, CFAM delayed the appearance of chylomicrons in lymph when absorbed only as FFA (1C oil). CFAM from the 3C diet were absorbed both as 2MG and FFA and the lipoprotein profile averaged those of both the 2C diet and the 1C diet (Fig. 5). The formation and secretion of the intestinal lipoproteins is a very complex process that involves several steps, including fatty acid uptake, reesterification, apolipoprotein synthesis, lipoprotein assembly and transport within the cell compartments, secretion in intercellular spaces and appearance into lymph (48). Each of these steps that can be regulated affects the overall recovery of lipoproteins into lymph. Our study cannot present an explanation for the different lipoprotein profiles elicited by any of the oil groups. However, the biological activity of the CFAM in the intestinal cells upon the lipoprotein formation/secretion pathways is apparently modulated by their molecular form of transport into the enterocyte, i.e., 2MG or FFA, with an intermediate effect observed for an uptake in both forms.

In conclusion, our results have shown that the positioning of CFAM within the ingested TG is an important factor to take into account in studying the effect of these molecules on intestinal metabolism. It modified *in vitro* the activity of the pancreatic lipase which is the main enzyme of lipid digestion in the lumen. However, under our experimental conditions, our results suggest that the CFAM profile and content in lymph was not determined by the luminal hydrolysis step but rather by a different efficiency of the biochemical pathways within the enterocyte to use CFAM taken up as 2MG or FFA. Moreover, the profile of CFAM in lymph was influenced by their molecular structure only when they were taken up as FFA. These two forms of absorption also differently affected some other biochemical mechanisms that regulate the incorporation of saturated fatty acids within the lymph lipids as well as the formation/secretion pathways of the intestinal lipoproteins. As for the pancreatic lipase in the lumen, the lipoprotein lipase from the capillary bed releases the fatty acid from the circulating TG as FFA and 2MG that are ultimately taken up by the neighboring tissues (49). In that situation, our results also raise the question of the metabolic effect of CFAM on the target organs when they are taken up by cells in either of these two forms. ■

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