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Conjugated linoleic acid isomers in mitochondria: evidence for an alteration of fatty acid oxidation

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Abstract The beneficial effects exerted by low amounts of conjugated linoleic acids (CLA) suggest that CLA are maximally conserved and raise the question about their mitochondrial oxidizability. *Cis-9,trans-11-C_{18:2}* (CLA1) and *trans-10,cis-12-C_{18:2}* (CLA2) were compared to *cis-9,cis-12-C_{18:2}* (linoleic acid; LA) and *cis-9-C_{16:1}* (palmitoleic acid; PA), as substrates for total fatty acid (FA) oxidation and for the enzymatic steps required for the entry of FA into rat liver mitochondria. Oxygen consumption rate was lowest when CLA1 was used as a substrate with that on CLA2 being intermediate between it and the respiration on LA and PA. The order of the radiolabeled FA oxidation rate was PA >> LA > CLA2 > CLA1. Transesterification to acylcarnitines of the octadecadienoic acids were similar, while uptake across inner membranes of CLA1 and, to a lesser extent, of CLA2 was greater than that of LA or PA. Prior oxidation of CLA1 or CLA2 made re-isolated mitochondria much less capable of oxidising PA or LA under carnitine-dependent conditions, but without altering the carnitine-independent oxidation of octanoic acid. Therefore, the CLA studied appeared to be both poorly oxidizable and capable of interfering with the oxidation of usual FA at a step close to the beginning of the β -oxidative cycle.—Demizieux, L., P. Degrace, J. Gresti, O. Loreau, J-P. Noël, J-M. Chardigny, J-L. Sébédio, and P. Clouet. **Conjugated linoleic acid isomers in mitochondria: evidence for an alteration of fatty acid oxidation.** *J. Lipid Res.* 2002, 43: 2112–2122.

Supplementary key words CLA • respiration • acyl-CoA synthetase • carnitine • CAT-I • CAT-II • CACT • inner membrane crossing

Conjugated linoleic acids (CLA) refer to a group of dienoic derivatives of linoleic acid (LA) produced by microbial conversion in the rumen and $\Delta 9$ desaturation of vaccenic acid in the mammary gland (1). CLA are mainly found in ruminant meats and dairy products (2, 3). These derivatives have been shown to exhibit a variety of unique properties such as anti-cancer (4, 5), anti-atherogenic (6),

and immune response enhancing effects (7) in animal models. CLA have also been reported to reduce total body fat content in mice (8, 9), rats, and chickens (10). In mice, the apparent decrease in weight of adipose tissues was associated with a lowered lipoprotein lipase activity and a reduced triacylglycerol (TAG) content, while a greater specific activity of carnitine palmitoyltransferase was found in periepididymal adipose and muscle tissues (8). Conversely, liver weight was increased, due at least in part to lipid accumulation. In rats however, the weight of liver did not change, while the lipid content was increased. In perfused liver, TAG secretion was diminished in association with a greater ketone body production. Surprisingly, no study has yet been devoted extensively to the mitochondrial oxidation of CLA. For example, carnitine acyltransferase I activity was found to be increased in periepididymal adipose tissue of CLA-fed rats, but only with palmitoyl-CoA as a substrate (11). Some related aspects have been studied in vivo (12) or addressed in vitro, but only by oxygen consumption measurement (13). In this study, we investigate whether two CLA isomers are suitable substrates for total mitochondrial fatty acid oxidation and for the enzymatic steps from the activation by CoA to the formation of acylcarnitine and the subsequent entry of acyl moieties into the matrix.

In natural products, CLA are essentially represented by *cis-9,trans-11-C_{18:2}* (3, 14) (CLA1), while the *trans-10,cis-12* homologue (CLA2) is mainly found in synthetic products utilized in most of the feeding studies (15). In CLA-fed rats, either form was recovered in TAG and, to a far lesser extent, in phospholipids of hepatocytes (16). CLA1 has been shown to be one of the most avid fatty acid ligands yet described for PPAR α , CLA2 being less potent (17). However, in contrast to clofibrate, these CLA did not act

Abbreviations: CACT, carnitine acylcarnitine translocase; CAT, carnitine acyltransferase; CLA1, *cis-9,trans-11-C_{18:2}*; CLA2, *trans-10,cis-12-C_{18:2}*; LA, linoleic acid; PA, palmitoleic acid; TAG, triacylglycerols.

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as classic peroxisome proliferators in the rat (17). The retention of these CLA in tissues, even when they were added at low amounts to diets, and the fact that these fatty acids did not induce activities related to peroxisomal fatty acid oxidation prompted us to study the oxidisability of CLA1 and CLA2 in mitochondria, essentially. All the measurements were made by reference to LA, as the natural octadecadienoic acid isomer, and to palmitoleic acid (PA), which, like CLA1, both contain a *cis*-9 double bond; some measurements were achieved by using specifically synthesized [^{14}C]labeled fatty acids. Mitochondria were isolated from liver because this organ plays a key role in whole body lipid metabolism and the fact that CLA added to diets are capable of inducing TAG accumulation (8, 9), but only in certain physiological conditions (16). In the present study, rats were fed a standard diet instead of a CLA-enriched diet to prevent the incorporation of uncommon fatty acids in mitochondrial phospholipids (PL), as this has been shown to alter mitochondrial oxidative activities per se (with *trans*-monounsaturated octadecenoic acid) (18).

MATERIALS AND METHODS

Materials

Cis-9, *trans*-11- and *trans*-10, *cis*-12- $\text{C}_{18:2}$ were supplied by Natural Lipids (Industriveien, Hovdebygda, Norway) and were 92% and 96% pure, respectively. Other fatty acids used were purchased from Sigma Chemical Co. (St. Louis, MO). They were stored in ethyl alcohol at -22°C . Contents of solutions were determined monthly by capillary gas liquid chromatography of fatty acids as methyl esters prepared using the methanol-boron trifluoride method (19), specifically modified for CLA (20). [^{14}C] *cis*-9, *cis*-12- $\text{C}_{18:2}$ (linoleic acid, LA; 60.8 Ci/mol) was from NEN Life Sciences Products (Boston, MA). The preparation of [^{14}C] *cis*-9- $\text{C}_{16:1}$ (palmitoleic acid, PA; 56 Ci/mol) and of the two [^{14}C]CLA isomers (CLA1 and CLA2: 53.4 and 54.3 Ci/mol, respectively) was performed by CEA (Gif-sur-Yvette, France) as described elsewhere (21). Evaporation of ethyl alcohol under nitrogen and saponification of fatty acids with potassium hydroxide (22) were carried out immediately prior to incubations. l -[^3H]Carnitine was provided by Amersham International (Amersham, UK). Unlabeled l -carnitine was given by Dr. C. Cavazza of Sigma-Tau (Pomezia, Italy). Octanoic acid ($\text{C}_{8:0}$) and all other biochemicals were from Sigma Chemical Co. (St. Louis, MO). The bovine serum albumin (BSA) used was prepared from fraction V albumin and was essentially fatty acid-free. Chemicals obtained from Prolabo (Paris, France) and Merck (Darmstadt, Germany) were of analytical grade.

Male Wistar rats were purchased from Dépré (Saint-Doulchard, France). They were kept at 23°C in a light-controlled room (light period fixed between 08:00 and 20:00 h). They had free access to tap water and were fed on standard laboratory chow (ref AO3 from UAR, Epina-sur-Orge, France) containing 58.7% carbohydrate, 17% protein, and 3% fat. They were 8 weeks old (about 300 g) when they were stunned and killed by exsanguination at 08:00 h.

Fatty acid oxidation using liver homogenates

Livers were rapidly removed and cut into very small pieces in ice-cold 0.25 M sucrose medium containing 1 mM EGTA and 10 mM Tris/HCl, pH 7.4, rinsed five times in the same medium,

blotted with absorbent paper, and weighted. Tissues were homogenized and diluted (1:80, w/v) in chilled 0.25 M sucrose containing 2 mM EGTA and 10 mM Tris/HCl, pH 7.4, by manual rotation of a Teflon pestle in a 10-ml Potter-Elvehjem homogenizer maintained in ice throughout. Fatty acid oxidation by tissue homogenates was measured using slight modifications of two incubation media (23). In method A, mitochondrial and peroxisomal activities were allowed to occur; in method B, only peroxisomal activity was measured. The media buffered at pH 7.4 and maintained at 37°C consisted, for A, of 30 mM KCl, 75 mM Tris/HCl, 10 mM KPi , 0.7 mM EGTA, 5 mM MgCl_2 , 1 mM NAD, 5 mM ATP, 100 μM CoA, 0.4 mM l -carnitine, 0.5 mM l -malate, and 25 μM cytochrome c. In B, the medium differed only by the absence of l -malate, cytochrome c, and l -carnitine, and the presence of 73 μM antimycin and 10 μM rotenone to block the respiratory chain. Tissue homogenates (2.5 mg under a 200 μl volume) were added to vials containing 1 ml of each incubation medium A or B. After preincubation for 2 min, the reactions were started by the addition of 120 μM [^{14}C]fatty acid (1.5 Ci/mol) as potassium salt, bound to BSA in a 5:1 molar ratio. Reactions were stopped 30 min later by the addition of 3 ml of 10% (w/v) perchloric acid to precipitate proteins and fatty acids that were still unesterified or esterified to CoA or carnitine. A conical polyethylene tube containing 400 μl of Hyamine (Packard, Meriden, CT) was inserted in the incubation vial before closure to trap the released $^{14}\text{CO}_2$. After 1 h at room temperature, media were filtered on Millipore filters (0.45 μm pore size) under very low pressure and 1 ml of each filtrate containing the acid-soluble products (ASP; short metabolites issued from fatty acid oxidation) was added to 3.5 ml of Ultima Gold XR (Packard, Meriden, CT) for radioactivity measurements. The entire Hyamine solution was sampled and counted according to the same procedure. The radioactivity of (ASP + CO_2) was considered to represent the overall fatty acid oxidation.

Preparation of mitochondrial fractions

Pieces of blotted liver tissue prepared as for liver homogenates were homogenized in 10 vol of 0.25 M sucrose medium containing 10 mM Tris/HCl, pH 7.4 and 1 mM EGTA by only three strokes of a Teflon pestle rotating at 300 rpm in a cooled Potter-Elvehjem homogenizer. The homogenate was centrifuged at 2,000 g for 4 min and the supernatant was immediately centrifuged at 13,000 g for 3 min. The pellet was resuspended with the homogenization mixture and centrifuged under the preceding conditions. The procedure was repeated once and the pellet resuspended in the same mixture was used as the mitochondrial fraction. A rapid protein determination ($\pm 10\%$) was carried out by spectrophotometry on a water-diluted sample of this fraction after absorption at 280 nm and 260 nm, as previously described (22). Activities were later corrected after protein determination by the bicinchoninic acid procedure (24).

Mitochondrial activities

Respiration measurements on CLA. Respiration with fatty acids as substrates was measured polarographically at 30°C with a Clark-type oxygen-electrode (Yellow Springs Instruments, Yellow Springs, OH). The assay medium (1.6 ml) contained 130 mM KCl, 25 mM HEPES, 0.1 mM EGTA, 3 mM MgCl_2 , 10 mM KPi , 1 mM ATP, 50 μM CoA, 0.5 mM dithiothreitol (DTT), 5 mM l -carnitine, and 10 μM cytochrome c. Fatty acids were already present at the indicated concentration in the assay medium prior to the addition of mitochondrial protein (~ 1 mg). Respiratory rates were measured in the presence of either 2 mM malate (to measure rates of flux through β -oxidation and the citric acid cycle) or 10 mM malonate (to measure flux through β -oxidation alone), and of 2

mM ADP or about 0.2 μ M (by titration) carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone (FCCP) to provide state-3 or the uncoupled state, respectively.

Respiration on long- and medium-chain fatty acids in mitochondria reisolated after prior oxidation of the CLA-isomers, LA, or PA. To know whether the CLA-isomers entering the whole fatty acid oxidative pathway were likely to alter the subsequent oxidation of long- and medium-chain fatty acids, a two-step procedure was followed. The first step aimed at oxidising fatty acids added incrementally, causing each time an increase in concentration of 7 μ M or 10 μ M, with an interval of 4 min between successive additions, over a total incubation time of 20 min; the cumulative fatty acid concentrations ranged from 10 μ M to 50 μ M. The medium used in the initial step of the procedure contained 130 mM KCl, 25 mM HEPES, 10 mM KP_1 , 0.1 mM EGTA, 3 mM $MgCl_2$, 1 mM ATP, 50 μ M CoA, 0.5 mM DTT, 2 mM malate, 2 mM ADP, 2 μ M cytochrome c, 5 mM L-carnitine, and fatty acids as indicated, under a total volume of 16 ml buffered at pH 7.4. Fatty acid oxidation was initiated by addition of 10 mg of mitochondrial protein. At the end of the incubation, the media were diluted three times with chilled medium containing 0.25 M sucrose, 1 mM ATP, 50 μ M BSA and 10 mM Tris/HCl, pH 7.4, and were then centrifuged at 15,000 *g* for 8 min to remove intact fatty acids and corresponding CoA- and carnitine-derivatives situated outside mitochondria. Pellets were washed once more in the same volume of chilled buffered 0.25 M sucrose and were used for the second step of the procedure. Oxygen consumption was monitored with 10 μ M PA (carnitine-dependent oxidation) or 200 μ M octanoic acid (carnitine-independent oxidation) as oxidation substrates. The respiration medium was the same as that used for prior incubation, except that ATP, CoA, DTT, and carnitine were either present or absent in the medium containing octanoic acid. In both cases, respiration rates were measured in the presence of ADP added after 2 min of preincubation of the mitochondria. Oxygen consumption was expressed by reference to control assays, in which prior incubation was performed in the absence of any fatty acid.

Fatty acid oxidation measurements. The incubation medium consisted of 20 mM KP_1 , pH 7.4, 50 mM KCl, 4 mM $MgCl_2$, 1 mM ATP, 50 μ M CoA, 0.4 mM L-carnitine, 2 mM L-malate, and 50 μ M [^{14}C]fatty acid (3.5 Ci/mol) as potassium salt, bound to BSA in a molar ratio ranging from 0.4 to 3 to increase the amount of the free form. The reaction was initiated with 0.4 mg of mitochondrial protein in 1 ml of medium maintained at 30°C and was stopped after 8 min by addition of 4 ml of 10% (w/v) perchloric acid. The medium was kept 30 min on ice and was filtered as described for experiments in which fatty acid oxidation by liver homogenates was studied.

Acyl-CoA synthetase activity. Acyl-CoA synthetase (ACS) activity was measured by a fluorometric method using a fluorescent analogue of CoA, the [1, N^6 -etheno]CoA (25). The reaction mixture, kept at 25°C, consisted of 0.2 ml containing 0.35 M Tris/HCl (pH 7.4), 8 mM $MgCl_2$, 5 mM DTT, 10 mM ATP, Triton WR1339 (1 mg/ml), 0.1 mM etheno-CoA, and fatty acids (from 0 to 60 μ M). The reaction was initiated by the addition of 20 μ g of mitochondrial protein and stopped after 2 min with 200 μ l of BSA solution (6.25 mg/ml) and 2 ml of 0.3 M trichloroacetic acid (TCA). After 5 min on ice, the media were centrifuged at 200 *g* for 5 min to remove the unused acid-soluble etheno-CoA. Pellets, that contained acyl-etheno-CoA were resuspended and washed in further 2 ml of 0.3 M TCA. The fluorescence of the final pellets solubilized in 3 ml of 25 mM NaOH was determined by spectrofluorometry (spectrofluorometer PTI Delta-Ram) at 275 nm/410 nm excitation/emission wavelengths and compared with a standard curve constructed using various amounts of etheno-CoA in 25 mM NaOH. Accuracy of measurements was optimized by measur-

ing the fluorescence of the solubilized pellets immediately after the last TCA-precipitation.

Carnitine acyltransferase activity. CAT I (FROM ACS TO CAT I). Incubations were carried out at 30°C in 1 ml of medium containing 80 mM mannitol, 75 mM KCl, 25 mM HEPES, 1 mM EGTA, 4 mM $MgCl_2$, 1 mM ATP, 50 μ M CoA, 50 μ M DTT, 2 mM KCN, and 50 μ M of fatty acid, pH 7.4. Fatty acid-BSA mixtures were prepared in molar ratios ranging from 0.25 to 3.5, so as to obtain an increased availability of unbound fatty acids. After 2 min of preincubation in the presence of 0.25 mg of mitochondrial protein, the reaction was initiated by the addition of 0.4 mM L-[methyl- 3H]carnitine (2.5 Ci/mol). The reaction was stopped after 4 min with 1 ml of 1.2 N HCl. The radioactivity of acyl-[3H]carnitine, extracted with butan-1-ol and mixed with Ultima Gold XR (Packard, Meriden, CT), was determined in a scintillation spectrometer.

CAT II (FROM ACS TO CAT II). Incubations were carried out at 37°C in 1 ml of the same initial medium as for CAT I, but with the further addition of 0.16% Triton X-100 (26) and fatty acids at concentrations ranging from 0 to 50 μ M. After 2 min of preincubation in the presence of 0.25 mg of mitochondrial protein, 2 mM sodium tetrathionate was added to oxidise free CoA to prevent the reverse reaction from occurring. CAT II reaction was initiated by immediate addition of 0.5 mM L-[methyl- 3H]carnitine (2.5 Ci/mol) and was stopped after 4 min. Acyl-[3H]carnitine was then extracted and its radioactivity was estimated as for CAT I. In this assay, acyl-CoA synthesized on the external side of outer membranes was the direct substrate of CAT II after membrane disruption by Triton X-100, causing inactivation of CAT I (26) and loss of carnitine acylcarnitine translocase (CACT) action.

Entry of acyl moieties into the mitochondrial matrix. FROM ACS TO CAT II VIA CACT. The amounts of acylcarnitine crossing the inner membrane via CACT action were estimated in relative terms as the radioactivity of acyl moieties trapped inside mitochondria and mediated by (a) acyl-[3H]carnitines or (b) [^{14}C]acyl chains bound to carnitine or CoA (due to CAT II activity). They were also indirectly estimated (c) as the radioactivity trapped inside mitochondria and exchanged with external palmitoylcarnitine via CACT activity.

a) Intramitochondrial acylcarnitines were measured after incubation in a medium buffered at pH 7.4, kept at 30°C, and containing 130 mM KCl, 25 mM HEPES, 0.1 mM EGTA, 3 mM $MgCl_2$, 1 mM ATP, 50 μ M CoA, 0.5 mM DTT, 5 mM L-[methyl- 3H]carnitine (8 Ci/mol), 2 mM KCN, 50 μ M fatty acid as potassium salt, and 25 μ M BSA. Mitochondria were first exposed to rotenone and antimycin (14 and 110 μ g/g protein, respectively) for 10 min at 0°C, after which an aliquot containing 7.5 mg protein was added to 10 ml of medium. Control assays were performed in the absence of CoA and ATP. The reactions were stopped after 2, 4, or 6 min by cooling of vials on ice and addition of 30 ml of chilled buffered 0.25 M sucrose containing 25 μ M BSA. After centrifugation at 15,000 *g* for 6 min to remove extramitochondrial carnitine and acylcarnitine, the pellet was resuspended with a glass rod in 20 ml of the chilled medium and re-sedimented under the same conditions. Each time, the walls of the tubes were rinsed three times with buffered 0.25 M sucrose, the supernatant being removed after a brief centrifugation (acceleration to 5000 *g* and immediate deceleration). Final pellets were solubilized by successive additions of 1% Triton X-100 (for a total volume of 1 ml), transferred into conical 1.5 ml-tubes to which were added 400 μ l of 10% perchloric acid, and shaken vigorously. The supernatants in tubes centrifuged at 300 *g* for 3 min were removed to discard free labeled carnitine trapped inside mitochondria during the incubation. Tube walls and pellets were rinsed twice with 10% perchloric acid. Pellets were finally solubilized

with 25 mM NaOH and mixed with Ultima Gold XR (Packard, Meriden, CT) to estimate the amounts of acyl-[methyl-³H]carnitine sedimented with the mitochondria at the end of incubation.

b) Acyl-[³H]carnitines recovered with the mitochondrial pellet did not correspond to the overall amount of acyl moieties that had entered into the matrix because a proportion of the acylcarnitines were transesterified to acyl-CoAs in the presence of CAT II. Moreover, some endogenous fatty acids released from phospholipid hydrolysis and activated by CoA could have been transesterified to carnitine by CAT II and could interfere to some extent with the above measurements. Consequently, CLA-isomers were specifically synthesized with a ¹⁴C-labeling on the carboxylic end. The incubation medium was the same as that used for the estimation of acylcarnitines described in (a), except that L-carnitine was unlabeled, the fatty acids were prepared at the specific activity of 2.3 Ci/mol, and DTT was omitted in order not to reduce the mersalyl that was used to inhibit CACT (27). The reactions were stopped after 15 s to 55 s by cooling on ice, followed by addition of cold mersalyl (1 mM) for 10 s, and dilution with buffered 0.25 M sucrose supplemented with 25 μM BSA, as above.

c) Exchange of internal labeled acylcarnitine with external palmitoylcarnitine. These experiments complemented the above measurements; acyl-[³H]carnitines trapped inside mitochondria were exchanged for unlabeled external palmitoylcarnitine and quantified as the radioactivity recovered in the final supernatant. In this procedure, mitochondria were incubated under the same conditions as those described in (a), except that L-carnitine and fatty acids were unlabeled, the duration of incubation was of 10 min, and the whole procedure was stopped after the last mitochondrial wash. When mitochondria were first loaded with [³H]carnitine and then washed and reisolated exactly as (28), they contained acyl-[³H]carnitines owing to the continuous ac-

tion of CAT II. They were immediately used for the assay of CACT activity, which was performed in the presence of 20 μM palmitoylcarnitine (28). Control assays were carried out throughout the procedure to take into account the interference of endogenous fatty acids in the initial incubation and of endogenous labeled carnitine in the CACT assay.

Statistics

Statistical differences in mean values between different fatty acids were tested by one way ANOVA. Significant differences between means were analysed using Student's *t*-test.

RESULTS

Total fatty acid oxidation

Oxidation by liver homogenates. When using tissue homogenates, the oxidative potential due solely to the mitochondrial component was in the order PA > LA > CLA1 > CLA2 (Fig. 1). In contrast, the potential of the peroxisomal component, which was lower than that of mitochondria, displayed relatively few differences between the fatty acids tested.

Oxidation by isolated liver mitochondria. When incubations were performed with increasing fatty acid/BSA molar ratios (Fig. 2), PA was the best substrate for the entire range of ratios tested. With octadecadienoic acid isomers, results were significantly different ($P < 0.01$) for all the ratios tested (1.5 to 3); the order of fatty acid oxidation rate was LA > CLA2 > CLA1. It has to be noted that this order was

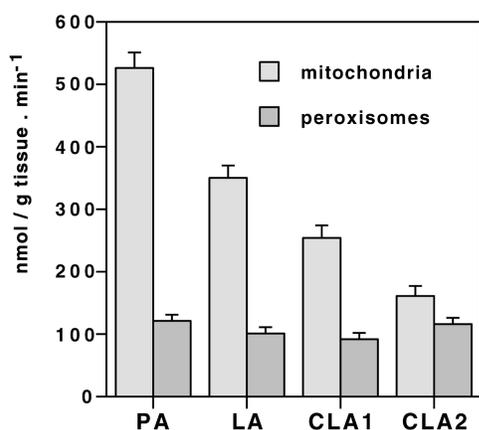


Fig. 1. Capacities of mitochondria and peroxisomes present in liver homogenates to oxidise both conjugated linoleic acids, as compared to linoleic and palmitoleic acids. Values are means \pm SEM ($n = 3$ experiments). Results are expressed as nmol of [¹⁴C]fatty acid, whose oxidation rates were estimated from the sum of labeled acid-soluble products and CO₂. The composition of media used for measuring mitochondrial and peroxisomal activities was described in the Materials and Methods section. Oxidation rates of fatty acids in mitochondria were all significantly different at $P < 0.001$, as determined by ANOVA and Student's *t*-test comparisons. In contrast, no significant difference was found between oxidation rates of these fatty acids in peroxisomes using the same test comparisons. Abbreviations: PA, palmitoleic acid; LA, linoleic acid; CLA1, *cis*-9, *trans*-11-C_{18:2}; CLA2, *trans*-10, *cis*-12-C_{18:2}.

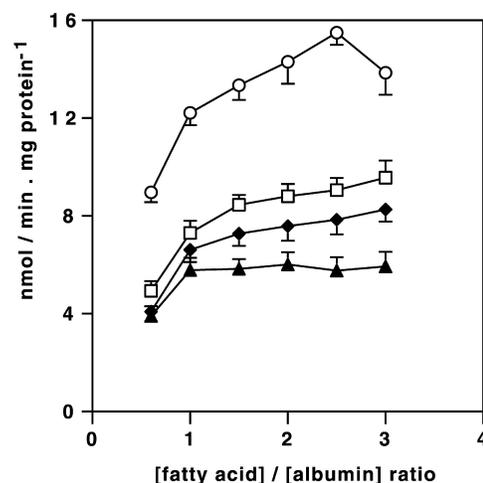


Fig. 2. Mitochondrial capacities to oxidise both conjugated linoleic acids, as compared to linoleic and palmitoleic acids. Values are means \pm SEM ($n = 3$ experiments). Results are expressed as nmol of [¹⁴C]fatty acid, whose oxidation rates were estimated from the sum of labeled acid-soluble products and CO₂. Details of medium composition and treatments of assays were given in the Materials and Methods section. Except for the two lowest fatty acid/BSA molar ratios with linoleic acid isomers, values of the points of the four curves at each fatty acid concentration were significantly different at $P < 0.05$, as determined by ANOVA and Student's *t*-test comparisons. Circle, *cis*-9-C_{16:1}, palmitoleic acid or PA; square, *cis*-9, *cis*-12-C_{18:2}, linoleic acid or LA; triangle, *cis*-9, *trans*-11-C_{18:2}, CLA1; diamond, *trans*-10, *cis*-12-C_{18:2}, CLA2.

TABLE 1. Rates of respiration on linoleic acid isomers and palmitoleic acid in liver mitochondria of normal rats

	<i>cis</i> -9-C _{16:1}	<i>cis</i> -9, <i>cis</i> -12-C _{18:2}	<i>cis</i> -9, <i>trans</i> -11-C _{18:2}	<i>trans</i> -10, <i>cis</i> -12-C _{18:2}
	<i>ng atom of O/min per mg mitochondrial protein</i>			
ADP-stimulated respiration on 10 μ M fatty acid concentration				
Malonate	29 \pm 5 ^a	31 \pm 4 ^a	18 \pm 3 ^b	20 \pm 2 ^b
Malate	96 \pm 6 ^a	89 \pm 5 ^a	47 \pm 4 ^b	78 \pm 4 ^c
FCCP-stimulated respiration on 22 μ M fatty acid concentration				
Malonate	65 \pm 3 ^a	55 \pm 3 ^b	44 \pm 5 ^{b,c}	42 \pm 5 ^c
Malate	185 \pm 5 ^a	176 \pm 6 ^a	128 \pm 4 ^b	135 \pm 3 ^b

Palmitoleic acid, *cis*-9-C_{16:1}; linoleic acid, *cis*-9, *cis*-12-C_{18:2}; CLA1, *cis*-9, *trans*-11-C_{18:2}; and CLA2, *trans*-10, *cis*-12-C_{18:2}. Tabulated values are means \pm SEM (n = 5) and are expressed as ng atom of O/min per mg of mitochondrial protein. Rates of respiration were measured, each time, using the same mitochondrial preparation for all fatty acids and under the indicated experimental conditions. Incubations were carried out in the presence of either 10 mM malonate or 2 mM malate. Further details are given in Materials and Methods.

^{a,b,c}The values within a same line with the same superscript letter were not significantly different at $P < 0.05$, as determined by ANOVA and Student's *t*-test comparisons. All other values were significantly different.

the same, irrespective of whether homogenates or mitochondria were used. However, an inverted order was observed for the CLA isomers. The fact that dietary CLA are recovered predominantly in TAG and PL of hepatic tissue (29) suggests that, in tissue homogenates (see above), CLA2 was mainly diverted to esterification reactions.

Respiration with fatty acids by isolated liver mitochondria. In the assays described above, fatty acid oxidation was estimated as the amount of [¹⁴C]ASP released from acyl chains labeled in the carboxyl group only. When mitochondrial respiration is monitored with fatty acid substrates, oxygen consumption results from β -oxidation of the whole acyl chain; its rate is stimulated by malate and slowed down by malonate, which accelerates and blocks the entry of acetyl moieties into the tricarboxylic acid cycle, respectively. Two ranges of critical fatty acid concentrations, 8–12 or 20–25 μ M, were used to assess the optimal respiration rates by coupled (ADP-stimulated respiration; state-3) or FCCP-uncoupled mitochondria, respectively. **Table 1** shows that, in comparison with respiration using PA at 10 μ M (final concentration), ADP-stimulated respiration in the presence of malate was 45–50% lower with CLA1, intermediate with CLA2 (17–23% lower) and slightly lower with LA (although this was not significant). When fatty acids were used at a concentration of 22 μ M in the presence of malate, oxygen consumption by FCCP-uncoupled mitochondria was maximal with PA, marginally lower with LA, and about 30% lower with both CLA isomers. Respiration with malonate was about 3 times lower than with malate, but gave qualitatively similar results.

Effect of prior oxidation of each octadecadienoic acid isomer or PA on subsequent oxidation of PA. The results in **Fig. 3** are expressed as a percentage of those obtained in control assays performed without fatty acid substrates during the first step of the procedure. They indicate that prior oxidation of LA, at a concentration below 30 μ M, was practically without effect on the subsequent oxidation of PA. Respiration was also unchanged with CLA2 when used below 12 μ M. Above this concentration, oxidation of PA was markedly inhibited. Prior oxidation of CLA1, even at lowest concentrations used, markedly inhibited oxidation of PA. The inhibition increased the higher the CLA concentration used

in the initial incubation. However, the inhibition effect of both CLA isomers on PA-supported oxidation was strongly attenuated if the mitochondrial washing steps after initial centrifugation of the first incubation mixture were performed 1 h later (data not shown).

Effect of prior oxidation of each octadecadienoic acid isomer or PA on the subsequent respiration due to carnitine-independent or -dependent oxidation of octanoic acid. Octanoic acid can diffuse through the mitochondrial inner membranes (30). In the matrix,

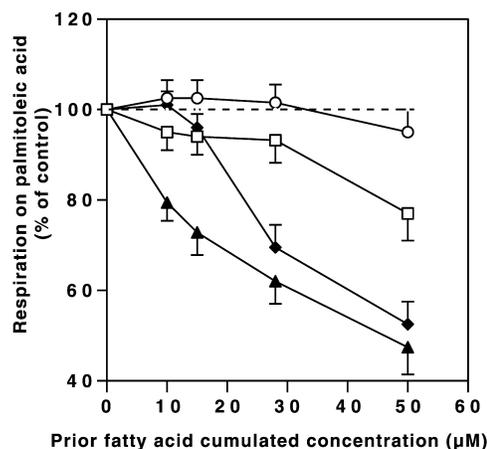


Fig. 3. Effect of oxidation of both CLA, linoleic and palmitoleic acids, on consecutive ADP-stimulated respiration on palmitoleic acid. Values are means \pm SEM (n = 3 experiments). Mitochondria used for these measurements had been previously incubated for 20 min in the presence of CLA1, CLA2, LA, or PA, as oxidative substrates (x axis), and reisolated as indicated in the Materials and Methods section. Oxygen consumption rates were monitored using a medium added with 10 μ M PA (y axis) and were expressed as a percentage of those obtained with mitochondria having never been previously in the presence of any fatty acid (control, dotted line). Values of the points of the curves corresponding to preincubations with LA or PA until 30 μ M were not different from those of control assays ($P < 0.05$), as determined by ANOVA and Student's *t*-test comparisons. The same pattern was observed for preincubations with CLA2, used below 20 μ M only. All greater concentrations markedly reduced subsequent respiration on PA, while CLA1 appeared to be still more inhibiting even at the lowest concentration assayed. For symbol notation, see legend of Fig. 2.

it is esterified to CoA via the action of a medium-chain acyl-CoA synthetase (31, 32) and directly enters the β -oxidative cycle. Under these conditions of carnitine-independent oxidation (Fig. 4B), respiration on octanoic acid was not impaired after prior respiration on the conjugated linoleic acids. This fact strongly suggests that the inhibition of respiration on PA (Fig. 3) was not due to a specific ef-

fect of CLA isomers at any particular level of the β -oxidative cycle. However, when respiration on octanoic acid is performed in a medium containing CoA, ATP, and carnitine, the medium-chain fatty acid is activated by exogenous CoA on the outside of mitochondria (31, 33), while the transesterification of octanoyl-CoA to carnitine is achieved through the carnitine octanoyltransferase activity present in the mitochondrial preparations (33, 34). Consequently, the oxidation of the medium-chain fatty acid becomes carnitine-dependent (cf. long-chain fatty acids). The data in Fig. 4A show that, under these particular conditions, oxidation of octanoic acid was inhibited to the same extent as that of PA (Fig. 3) after prior oxidation of CLA isomers. However, the inhibition was hardly perceptible after oxidation of LA at the lowest concentration used (7 μ M). These observations suggest that the inhibition described with CLA was related to carnitine-dependent reactions.

Enzymatic steps not involved in permeation of the inner membrane

Acyl-CoA synthetase activity versus octadecadienoic acid isomers and PA. In the fluorimetric assay used, preliminary studies showed that substitution of 0.1 mM etheno-CoA for 0.1 mM CoA (35) results in a similar acyl-CoA synthetase activity for comparable amounts of mitochondrial protein and times of incubation (data not shown). Under these conditions, as shown in Fig. 5, LA and PA gave comparable values and appeared to be the best substrates for the

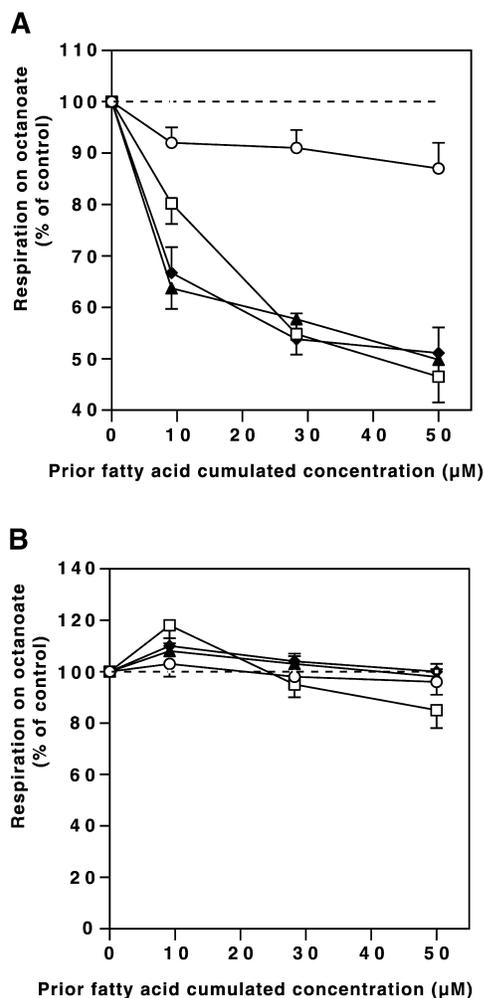


Fig. 4. Effect of oxidation of both CLA, linoleic and palmitoleic acids, on consecutive ADP-stimulated respiration on octanoate. Values are means \pm SEM ($n = 3$ experiments). Prior respiration (x axis), reisololation of mitochondria and expression of results are the same as those given in the legend of Fig. 3. Oxygen consumption rates were monitored using a medium added with 200 μ M octanoate (y axis) and components required for carnitine-dependent (A) or -independent (B) fatty acid oxidation (see Materials and Methods). A: All values of the points of the curves corresponding to preincubation in the presence of various amounts of the octadecadienoic acid isomers studied strongly differed from those of control assays ($P < 0.05$), as determined by ANOVA and Student's t -test comparisons. The inhibiting effect on respiration was minimum when the preincubation was performed in the presence of PA. B: octanoate was oxidised after direct diffusion into the mitochondrial matrix. The corresponding oxygen consumption was very little altered when preincubations had been carried out in the presence of any of the long chain-fatty acids assayed, as determined by ANOVA and Student's t -test comparisons, all values being slightly higher at 10 μ M fatty acid concentration. For symbol notation, see legend of Fig. 2.

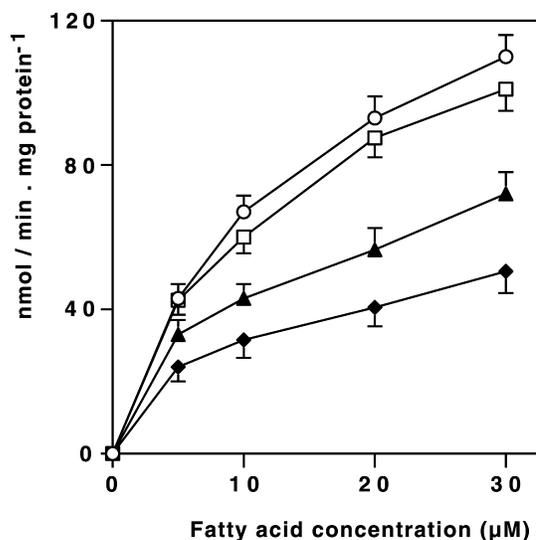


Fig. 5. Acyl-CoA synthetase specific activity versus both CLA isomers as compared to linoleic and palmitoleic acids in rat liver mitochondria. Values are means \pm SEM ($n = 5$ experiments). Results are expressed as nmol of acyl-[etheno-CoA] produced per min per mg mitochondrial protein. For each experiment, the same mitochondrial preparation was used with all fatty acids at the concentrations indicated (see Materials and Methods). Except for the concentration 5 μ M, values of the points of the four curves at each fatty acid concentration were significantly different ($P < 0.05$) as determined by ANOVA and Student's t -test comparisons. For symbol notation, see legend of Fig. 2.

reaction, while activation of CLA1 and CLA2 to CoA-esters was about 30% and 50% lower, respectively.

CAT I activity versus octadecadienoic acid isomers and PA. The data in Fig. 6 show that the three octadecadienoic acid isomers were esterified to carnitine at a similar rate, despite the geometric and positional differences of their double bonds. Therefore, comparable amounts of carnitine derivatives of these isomers are expected to reach the catalytic site of CACT in the inner membrane. PA was however esterified to carnitine at a rate that was 30% greater than that for the other three fatty acids studied.

CAT II activity versus octadecadienoic acid isomers and PA. CAT II activity was measured in the reverse direction utilizing the acyl-CoA previously synthesized by acyl-CoA synthetase present in the mitochondrial fraction. CAT II had access to acyl-CoAs diffusing through membranes permeabilized by Triton X-100. The CAT I and CACT steps were bypassed and were not involved in the reaction. Figure 7 shows that, under the experimental conditions chosen, PA and CLA1 were esterified to carnitine at equivalent rates, whereas the transfer of LA was slightly lower, but not significantly. The transfer of CLA2 was about 30% lower than that of CLA1. However, it is apparent from Fig. 5 that CLA2 was also activated by CoA to a lesser extent (30%) than CLA1. This suggests that the first enzymatic step can directly affect the last reaction. However, *cis*-9 octadecenoic acid (oleic acid), which was far better oxidised *in vivo* than was LA (36), was transesterified to carnitine by CAT II at

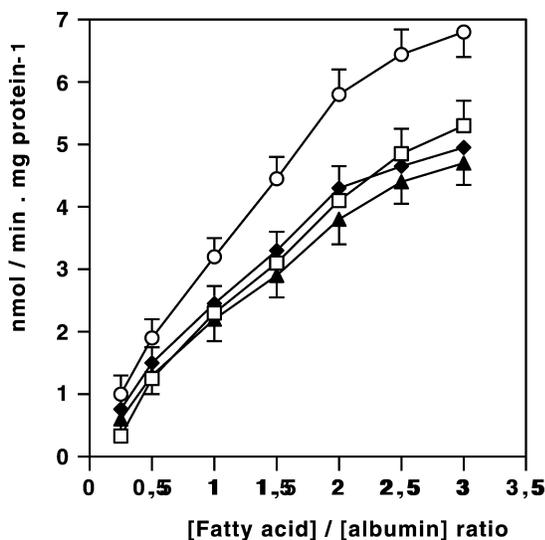


Fig. 6. Carnitine acyltransferase I specific activity versus conjugated linoleic acid isomers as compared to linoleic acid palmitoleic acids in rat liver mitochondria. Values are means \pm SEM ($n = 4$ experiments). Results are expressed as nmol of acylcarnitine produced per min per mg mitochondrial protein. For each experiment, the same mitochondrial preparation was used with all fatty acids. Values of the points of the curves corresponding to LA and its conjugated isomers were not different at $P < 0.05$, as determined by ANOVA and Student's *t*-test comparisons. With PA, all values obtained for ratios above 0.5 were significantly different from those with all the octadecenoic acid isomers assayed. For symbol notation, see legend of Fig. 2.

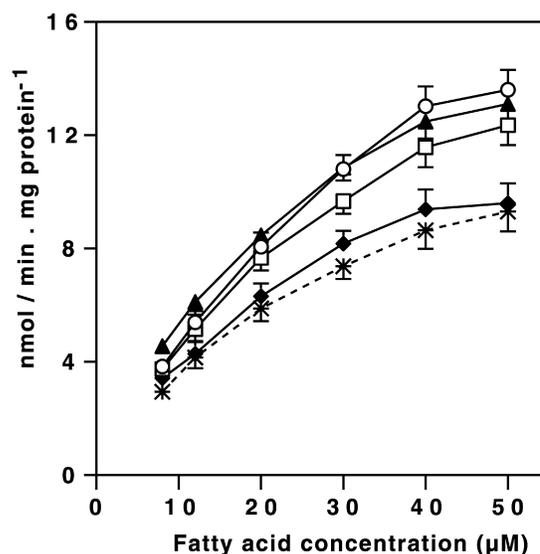


Fig. 7. Carnitine acyltransferase II specific activity versus both CLA isomers as compared to linoleic acid and two *cis*-9 monounsaturated fatty acids. Values are means \pm SEM ($n = 4$ experiments). Results are expressed as nmol of acylcarnitine produced per min per mg mitochondrial protein. For each experiment, the same mitochondrial preparation was used with all fatty acids at the indicated concentrations as described in Materials and Methods. Values of the points of the curves corresponding to CLA1, LA, and PA were not different ($P < 0.05$), as determined by ANOVA and Student's *t*-test comparisons. Values obtained with CLA2 at concentrations above 10 μ M were significantly lower than those with the three preceding fatty acids and comparable to those obtained with *cis*-9 octadecenoic acid (oleic acid). Symbol other than those shown in the legend of Fig. 2. *Oleic acid.

the same rate as CLA2, which indicates that the CAT II reaction cannot be a rate-controlling step for fatty acid oxidation. In the whole, the data show that the transesterification of acylcarnitine to acyl-CoA (and conversely) should be easily achieved from all the fatty acids studied.

Effect of prior oxidation of each octadecadienoic acid isomer or PA on the subsequent CAT I and CAT II activities performed in the presence of palmitoyl-CoA. Further experiments showed that CAT I and CAT II activities were totally unaffected by prior oxidation of PA, LA, or the conjugated isomers (data not shown). Therefore, inhibition of either enzyme by the conjugated fatty acids under any form is not likely.

Inner membrane crossing

Because the octadecadienoic acid isomers were esterified to carnitine by CAT I at equivalent rates, the amount of each of them crossing the inner membrane and accumulating into the matrix was expected to depend on the CACT activity. The equilibrium between acylcarnitines and acyl-CoAs established via CAT II activity will also have affected the radioactive labeling associated with L-carnitine or acyl chains.

Acyl moieties trapped inside mitochondria. When using [3 H]carnitine (Fig. 8A), the radioactivity recovered with mitochondria over a time of 6 min was always highest with CLA1 and lowest with PA. CLA2 accumulated to slightly

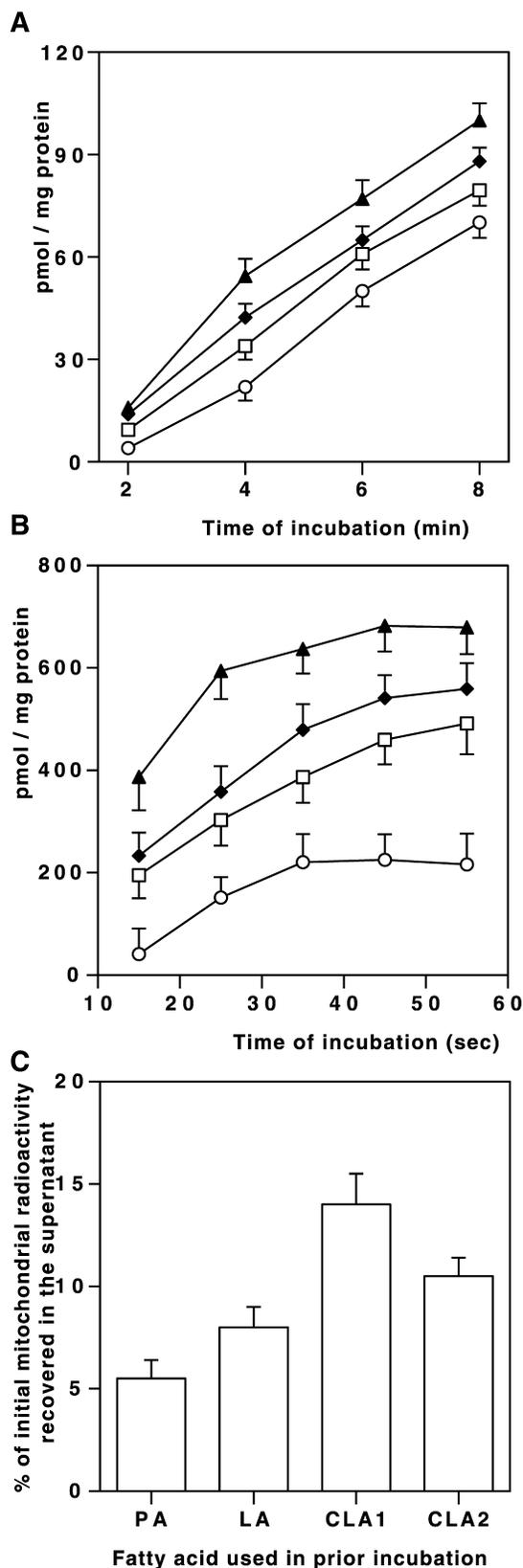


Fig. 8. Amount of acylcarnitine trapped inside mitochondria after incubation in a medium blocking β -oxidative reactions. **A:** Values are means \pm SEM ($n = 4$ experiments). Results are expressed as pmol of acyl- ^3H carnitine coprecipitated with mitochondria per mg protein after the indicated time of incubation. Incubation medium and mitochondrial reisolations were detailed in Materials and Methods. For each time of incubation, the values obtained with all fatty acids significantly differed, except with CLA2 and LA each other, at $P < 0.05$, as determined by ANOVA and Student's t -test comparisons. For symbol signification, see legend of Fig. 2. **B:** Values are means \pm SEM ($n = 3$ experiments). Results are expressed as pmol of ^{14}C acyl moieties esterified with CoA and carnitine, coprecipitated with mitochondria per mg protein over a time of incubation from 15 to 55 sec. Incubation medium and mitochondrial reisolations were detailed in the Materials and Methods section. For each time of incubation, the values obtained with all fatty acids were significantly different, except with CLA2 and LA ($P < 0.05$), as determined by ANOVA and Student's t -test comparisons. For symbol notation, see legend of Fig. 2. **C:** Values are means \pm SEM ($n = 4$ experiments). Results are expressed as pmol of acyl- ^3H carnitine entered into mitochondria during a prior 10-min non-oxidative incubation and exchanged, using 1 mg mitochondrial protein, with unlabeled 20 μM palmitoylcarnitine over a total time of 1 min at 5°C (see Materials and Methods). Control assays were performed with mitochondria incubated, in the first step of the procedure, in the medium devoid of CoA, ATP, and MgCl_2 . The values obtained with the four fatty acids significantly differed except with CLA2 and LA ($P < 0.05$), as determined by ANOVA and Student's t -test comparisons.

higher level than LA (but not significantly), so resulting in values that were intermediate between those obtained with CLA1 and with PA. When using ^{14}C fatty acids (Fig. 8B), the radioactivity recovered with mitochondria over

very short times (15 s to 55 s) gave results similar to those obtained with ^3H carnitine.

Exchange of internal acyl- ^3H carnitine with external palmitoylcarnitine. In order to confirm the intramitochondrial accu-

mulation of acyl-[³H]carnitines during the first incubation under non-oxidative conditions (see details in Materials and Methods section), a procedure of exchange with unlabeled 20 μM palmitoylcarnitine was performed as described in (28). As palmitoylcarnitine translocation is associated with an efflux of labeled carnitine and acylcarnitine (27), the radioactivity associated with the carnitine efflux, obtained after prior incubation of mitochondria in the absence of any fatty acid, has to be subtracted from the total radioactive efflux to assess the amount of each acylcarnitine exchanged. Figure 8C shows that carnitine esters of CLA1 were recovered most abundantly outside mitochondria, contrary to PA-carnitine, which was exchanged to a much lower extent. The amount of CLA2- or LA-carnitine found extra-mitochondrially was about 30% and 50% lower, respectively, than that of CLA1-carnitine.

From the data of Fig. 8, it appears that PA-carnitine was the poorest substrate and CLA1-carnitine the best substrate for the CACT reaction. However, the amount of PA-carnitine translocated into mitochondria was sufficient to allow β-oxidative reactions to proceed at the highest rate, as compared to carnitine esters of the other fatty acids assayed. Therefore, PA could not accumulate as carnitine- and CoA-esters within the matrix. Conversely, the influx of the CLA isomers into mitochondria appeared to be relatively more facilitated at the CACT step. This must cause, at least transitorily in vitro, a concomitant accumulation of carnitine- and CoA-derivatives, all the more because both CLA were poorly oxidized.

DISCUSSION

β-oxidative reactions

The main observation of the present study was the demonstration by complementary approaches, that the two conjugated isomers of linoleic acid so far studied, *cis*-9, *trans*-11-C_{18:2} and *trans*-10, *cis*-12-C_{18:2}, are not only poor substrates for the overall mitochondrial fatty acid oxidation pathway, but also caused changes in the oxidation rate of other fatty acids. However, these fatty acids were shown to be relatively good substrates for each step of the enzymatic sequence aiming at the transfer of carnitine-derivatives into the matrix, carnitine esters of CLA1 and CLA2 being even better substrates than esters of LA and PA for CACT. Since the CLA studied were poorly oxidized, we conclude that CLA-CoA esters may be inefficient substrates for some enzymatic steps of the β-oxidative cycle. The particularity of unsaturated fatty acid oxidation is usually to need auxiliary enzymes, depending on whether the *cis* or *trans* double bond is at even- or odd-position of the acyl chain (37). A same Δ3-*cis*-Δ2-*trans*-enoyl-CoA isomerase is required for the *cis*-9 double bond of CLA1, LA and PA, and its activity should be largely sufficient to allow the high rate of PA oxidation observed. As regards the *cis*-12 double bond, a complex epimerization reaction (38, 39) should affect to some extent the β-oxidation rate of LA and CLA2. However, the *trans*-10 double bond in CLA2 does not require any auxiliary enzyme. In contrast,

when the β-oxidative reactions proceed until the *trans*-11 double bond of CLA1, the Δ3-*trans*-enoyl-CoA newly formed is a very poor substrate for the only available auxiliary enzyme, Δ3-*cis*-Δ2-*trans*-enoyl-CoA isomerase (40), which therefore must limit the β-oxidation rate of CLA1.

In the whole, CLA2 was shown to be always oxidised at a slower rate by mitochondria than was LA, while it was better oxidised than CLA1 (cf. yeast cultures) (41). In liver homogenates, however, CLA2 was found to be less oxidized by mitochondria than was CLA1. Under these conditions, it is possible that, in a medium containing all the cell components, CLA2 was esterified to form various lipid classes through endoplasmic reticulum reactions, which would avoid it entering, at least partly, the β-oxidative pathway. However, when rats were fed [1-¹⁴C]CLA1, CLA2, or LA, the production of expired labeled CO₂ over 24 h was found to be lowest with LA (12). In fact, the total amount of CO₂ released in vivo after oxidation of a labeled fatty acid refers to the labeled form ingested and to the endogenous form present in body lipids. As LA is quantitatively well represented and either CLA practically non-existent in tissue lipids of control rats, the actual amount of CO₂ produced by oxidation of LA must be much more elevated than that of either CLA, meeting in that way the main conclusion of our in vitro study.

Oxidation of CLA and oxidative fate of normal fatty acids

From the experimental data, it is apparent that, as a result of the efficient crossing of the inner membrane by the CLA isomers and the concomitant impairment of β-oxidative reactions, these CLA accumulated as carnitine- and CoA-acyl esters within the mitochondrial matrix. This accumulation was shown to not alter subsequent carnitine-independent oxidation of octanoic acid. As the medium-chain fatty acid is activated by CoA within mitochondria, a lack of internal CoA by overproduction of CLA-CoA was unlikely. This contrasts with the sequestration of CoA as *cis*-13 docosenoyl-CoA (erucoyl-CoA) reported in heart mitochondria during rapeseed oil feeding (42). Further, octanoyl-CoA entering the strict β-oxidative cycle was dehydrogenated through the activity of a medium-chain acyl-CoA dehydrogenase (MCAD) (42, 43), which allowed it to bypass the step devoted to long-chain acyl-CoA dehydrogenation (possibly unsuitable for CLA-CoA). As the oxidation of octanoate, after prior respiration on either CLA, was inhibited under carnitine-dependent conditions, the inhibition must have occurred after the entry of acyl moieties into mitochondria and before the β-oxidative reactions, i.e., at the CAT II-catalysed step. One hour after prior incubation with CLA, the inhibition of the carnitine-dependent respiration on PA or octanoic acid was strongly attenuated, suggesting that the delay allowed accumulated acylcarnitine esters to exit the mitochondrial matrix via the CACT activity. This precluded them from interfering (in the second part of the procedure) with the oxidative fate of another fatty acyl substrate. In contrast, when the intervening period is short, the CAT II activity is occupied by the carnitine- and CoA-esters of CLA, thus blocking the progress of the less easily translocated acyl-

carnitines, such as PA-carnitine, towards β -oxidative reactions. These data are indirectly supported by the fact that CLA feeding increases carnitine palmitoyltransferase activity in the liver of rats (44), which is usually observed in situations of impaired fatty acid oxidation (45).

CLA in overall fatty acid oxidative metabolism

The fact that the CLA studied, as well as LA and PA, were oxidised at comparable (but modest) rates by peroxisomes present in liver homogenates (this study) and that CLA are reported not to induce peroxisomal proliferation (17) emphasizes the significance of the mitochondria in the oxidation of CLA. Single administrations of CLA, as well as longer treatments, are shown to rapidly affect various metabolic parameters in mice (17, 46). The present study carried out with control animals demonstrates for the first time that CLA are oxidised to a marked lesser extent than LA and are capable of accumulating inside the mitochondrial matrix, of altering the oxidation rates of other normal fatty acids and, also possibly, of controlling the tricarboxylic acid cycle activity (47). CLA isomers appear to be able to diffuse, as acylcarnitines, out of mitochondria through translocase activity (48). The resulting acyl moieties transesterified as acyl-CoA are susceptible to enter anabolic pathways, such as triacylglycerol or phospholipid synthesis (29). The effects of CLA could not be related to a simple increase in cell acyl-CoA concentration, which would have induced peroxisome proliferation (49). Because of their inability to be oxidised normally, CLA have marked effects even when administered at low level in vivo due to their special configuration imposed by the geometry and the position of the two double bonds. This makes them a particular class of nuclear mediators, as the CLA studied are already known to be ligands for PPAR α (17). ■

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