Pathway of α -linolenic acid through the mitochondrial outer membrane in the rat liver and influence on the rate of oxidation

Comparison with linoleic and oleic acids

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The movement of α -linolenic acid ($C_{18:3, n-3}$) through the mitochondrial outer membrane to oxidation sites was studied in rat liver and compared with the movement of linoleic acid ($C_{18:2, n-6}$) and oleic acid ($C_{18:1, n-9}$). All differ in the degree of unsaturation, but have the same chain length and the same position of the first double bond when counted from the carboxyl end. The following results were obtained. (1) The overall β -oxidation in total mitochondria was in the order $C_{18:3, n-3} > C_{18:2, n-6} > C_{18:1, n-9}$, independent of the amount of albumin in the medium. (2) The rate of formation of acylcarnitine from acyl-CoA was higher with oleoyl-CoA than with linoleoyl-CoA, and remained very low with α -linolenoyl-CoA for all concentrations studied. (3) When the formation of acylcarnitines originated from fatty acids (as potassium salts) in a medium containing CoA and ATP, the conversion of α -linolenate was greater than that of linoleate, which in turn was greater than that of oleate. (4) Use of a more purified mitochondrial fraction, practically devoid of peroxisomes, did not modify the results obtained with α -linolenate. (5) α -Linolenoyl-CoA did not inhibit oxidation of labelled α -linolenate, whereas the other acyl-CoAs did. (6) Transfer to carnitine of all three fatty acids (as potassium salts) by carnitine palmitoyltransferase-I (CPT-I) was similarly inhibited by increasing concentrations of malonyl-CoA. (7) On using a fraction containing mitochondrial outer membranes, the formation of acylcarnitines from potassium salts of fatty acids was qualitatively and quantitatively similar to that found with whole mitochondria. (8) Our observations show that α -linolenoyl-CoA synthesized other than in the mitochondria cannot be used to any great extent by the mitochondria due to its configuration. However when added as the unactivated form, α -linolenate appears to be very quickly oxidized, but should first be activated by acyl-CoA synthetase in the mitochondrion itself. Then it is rapidly channelled to CPT-I. These enzymic sites are probably close together in the mitochondrial outer membrane. The different behaviour of the α -linolenic group compared with the other acyl groups in the studied pathway can be explained by a different spatial arrangement due to the number and position of the double bonds.

INTRODUCTION

Several $n-3$ fatty acids abundant in fish oils have been reported to have beneficial effects on human health, chiefly by increasing blood fluidity. These acids are known to repress the formation of thromboxane A_2 from arachidonic acid in platelets (Siess *et al.*, 1980), and to inhibit secretion of very low density lipoproteins (Nestel *et al.*, 1984; Von Schacky *et al.*, 1985). Since this last effect does not arise from lipid accumulation in liver, it could be expected that enhanced fatty acid oxidation occurs in this organ when $n-3$ fatty acids are abundant in the diet, as shown by Wong *et al.* (1984). This stimulation may arise on the one hand from induction of hepatomegaly and peroxisomal proliferation which is observed in the livers of rats given diets enriched in $n-3$ fatty acids (Yamazaki *et al.*, 1987); on the other hand, direct effects of these fatty acids may occur at any step in the total oxidative process.

In an attempt to find out how these fatty acids could facilitate fatty acid oxidation, we studied the main steps of the oxidation pathway for α -linolenic acid ($C_{18:3, n-3}$). This essential fatty acid is the precursor of the two most important polyunsaturated $n-3$ fatty acids, eicosapen-

taenoic acid (EPA) and docosahexaenoic acid (DHA). A common oxidation pathway, due perhaps to the $n-3$ position, may exist for these fatty acids, even though α -linolenic acid occurs in low amounts in fish oil. The choice of α -linolenic acid is also favoured by the lesser complexity of a less unsaturated fatty acid of the $n-3$ series, and its higher solubility as a salt. Little is known about its breakdown, which has only been studied *in vivo* (Leyton *et al.*, 1987) or *in vitro* from free fatty acid (Stoffel & Schiefer, 1965; Huxtable & Wakil, 1971) and from acylcarnitine (Osmundsen *et al.*, 1987). The latter study bypassed the regulation step of the carnitine palmitoyltransferase I (CPT-I).

Our present study provides more data on the step involving the binding of carnitine to α -linolenic acid, by comparison with linoleic ($C_{18:2, n-6}$) and oleic ($C_{18:1, n-9}$) acids. All have the same overall chain length and the same position of the first double bond when counting from the carboxyl end. The results strongly support the view that CPT-I is located next to acyl-CoA synthetase in outer mitochondrial membranes, as shown by Murthy & Pande (1987) by a different method, and suggest a possible role of $n-3$ fatty acids on this outer side of the mitochondria.

EXPERIMENTAL

Animals

Male Wistar rats, bred in the laboratory, were housed 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 food. Food was given as commercial pellets (UAR, 91360 Epinay, France) containing 58.7 % carbohydrate, 17 % protein and 3 % fat. Animals weighing 280–300 g were killed by stunning and cervical dislocation at 08:30 h.

Mitochondrial fractions

The liver was immediately removed and cooled to 4 °C in 0.25 M-sucrose medium containing 10 mM-Tris/HCl buffer (pH 7.4) and 1 mM-EGTA. The liver was then chopped, rinsed several times, blotted, weighed and homogenized in 10 vol. of the same mixture containing 1 % (w/v) bovine serum albumin (fraction V; fatty acid-free) by four strokes of a Teflon pestle in a Potter–Elvehjem homogenizer. Added albumin efficiently protects membrane integrity by binding free fatty acids, which are known to induce mitochondrial swelling (Lehninger & Remmert, 1959). Preparation of the mitochondrial fraction M1 was performed as described by Saggerson (1982). Because this fraction still contained a noticeable catalase activity (see Table 1), a purified mitochondrial fraction, which was low in peroxisomes (M4), was prepared by using the procedure of Clouet *et al.* (1986). In this case, the homogenization mixture contained 1 % bovine serum albumin. The procedure was more time-consuming, but peroxisomes were removed more efficiently.

Mitochondrial outer membranes were prepared as described by Parsons *et al.* (1967) from both mitochondrial fractions (M1 and M4) by the following procedure. After mitochondrial swelling in hypo-osmotic medium and shrinking of the internal part in the presence of ATP and Mg^{2+} , the heavier internal part was separated from the outer membrane of lighter density by centrifuging for 10 min at 3 °C and 35000 *g* ($r_{av.} = 7$ cm). Afterwards, the pellet was resuspended and the heavier fraction of outer-membrane-free and non-disrupted mitochondria was separated by gentle centrifugation and discarded. The supernatant was sedimented at a higher speed, giving the crude preparation of outer membranes. Purification was performed

on a discontinuous sucrose gradient of density from 1.11 to 1.18 for 3 h at 3 °C and 80000 *g* ($r_{av.} = 11.8$ cm). The layer with the higher monoamine oxidase specific activity was withdrawn, diluted and then concentrated to give the final outer membrane fraction. Care was taken to exclude other layers containing outer membranes which were still bound to inner membranes (Hackenbrock *et al.*, 1979), or showing activities of a non-mitochondrial source. Table 1 shows enzyme activities in the different mitochondrial fractions used in this study.

Enzyme assays

Mitochondrial outer membrane content was estimated at 37 °C by the activity of monoamine oxidase (EC 1.4.3.4) as described by Weissbach *et al.* (1960). For inner membranes, the activity of cytochrome *c* oxidase was measured as described by Beaufoy *et al.* (1974). The presence of the mitochondrial matrix was assessed by measuring the activity of the glutamate dehydrogenase (Martin & Denton, 1970). Peroxisomal contamination was assessed by measuring the activity of catalase (EC 1.11.1.6) as described by Aebi (1974) and urate oxidase (EC 1.7.3.3) by the method of Leighton *et al.* (1968). The activity of CPT-I was measured using the acyl-CoAs of the unsaturated fatty acids under investigation by using the method described by Bremer (1981) with slight modifications (Clouet *et al.*, 1985). The acyl-[3H]carnitine was extracted with butan-1-ol (Bremer & Norum, 1967) and then assessed for radioactivity in Picofluor 15 (Packard Instrument Co.) using a Packard 300 C scintillation counter. The same procedure was used to estimate the acylcarnitines produced from the corresponding fatty acids. CPT-I activities with the three acyl-CoAs were measured in the presence of phosphatidylcholine and phosphatidylethanolamine (10:3, w/w) as indicated by Sire *et al.* (1983) in order to compensate for an eventual loss of phospholipids during the preparation.

Fatty acid oxidation

The incubation medium for mitochondria consisted of 20 mM-potassium phosphate, pH 7.4, 50 mM-KCl, 4 mM-MgCl₂, 1 mM-ATP, 50 μ M-CoA, 0.2 mM-L-malate and 50 μ M of the potassium salt of [^{14}C]oleate ($C_{18:1, n-9}$), [^{14}C]linoleate ($C_{18:2, n-6}$) or α -[^{14}C]linolenate ($C_{18:3, n-3}$), each bound to bovine serum albumin (fraction

Table 1. Specific activity of some marker enzymes in the isolated subcellular fractions

Values are means $\pm$ S.E.M. ($n = 4$). Activities are expressed as μ mol/min for cytochrome *c* oxidase, glutamate dehydrogenase and catalase, as nmol/min for monoamine oxidase and urate oxidase, and are given per mg of protein of mitochondrial or of outer-membrane fraction. The mitochondrial fraction isolated from the supernatant of the centrifuged liver homogenate is called M1; that obtained from the four-times-washed pellet as indicated in the Experimental section is M4. The OM fraction contains the gradient-purified mitochondrial outer membranes.

Enzyme	Fraction ...	Enzyme activity		
		M1	M4	OM
Monoamine oxidase		12.4 $\pm$ 1.2	12.1 $\pm$ 1.1	186 $\pm$ 10
Cytochrome <i>c</i> oxidase		1.91 $\pm$ 0.21	1.41 $\pm$ 0.18	0.12 $\pm$ 0.05
Glutamate dehydrogenase		2.77 $\pm$ 0.25	3.16 $\pm$ 0.20	0.24 $\pm$ 0.05
Urate oxidase		89.2 $\pm$ 2.9	12.6 $\pm$ 2.5	0
Catalase		369 $\pm$ 8	54 $\pm$ 5	< 1

V; fatty acid-free) in a molar ratio ranging from 0.3 to 3.5. L-Carnitine (1 mM) was added to the medium when indicated.

The reaction was initiated with 0.3–0.4 mg of mitochondrial protein in 1 ml of medium maintained at 37 °C and with gentle shaking. After 8 min, the reaction was stopped by addition of 0.1 ml of 10 M-HCl. The released $^{14}\text{CO}_2$ was trapped in 0.3 ml of Hyamine hydroxide for 2 h and its radioactivity was calculated in a mixture of Permafluor III (Packard) and xylene (1:10, v/v). Then, 6 ml of 10% (w/v) ice-cold HClO_4 was added to precipitate the protein as well as the long-chain fatty acids which were still intact and those bound to CoA or carnitine. These precipitated molecules were then entirely removed by filtering on a Millipore filter (pore size 0.45 μm). The radioactivity of the filtrate, determined in Picofluor 15 (Packard), corresponded to labelled ketone bodies, acetyl-CoA, acetylcarnitine and intermediary products of the tricarboxylic cycle. These molecules originating from β -oxidation were called 'acid-soluble products'. The absence of contaminating [^{14}C]fatty acids in filtrates was checked by the absence of radioactivity in the filtrates of control assays performed without mitochondria. This was also tested in the presence of mitochondria by the absence of labelled fatty acids bound to CoA or carnitine in filtrates. Any possible acyl groups in the filtrates were saponified by 5% (w/v) KOH in methanol at 100 °C for 2 h, then acidified and extracted with chloroform. After methylation, acyl derivatives were separated by g.l.c. (unlabelled fatty acids being added as markers), collected individually on glass wool as described by Bézard *et al.* (1964) and checked for radioactivity. The formation of $^{14}\text{CO}_2$ was very low in the presence of liver mitochondria as already shown (Clouet *et al.*, 1986) and is reported for oxidation of the three fatty acids in the legend to Fig. 1.

Protein determination

The protein content of each mitochondrial preparation was estimated just before incubation by measurement of the absorbance at 280 nm of the water-diluted preparation, a correction being made at 260 nm. This very fast procedure gave values within 10% of the actual ones, which were determined later by a more accurate method. The following formula was applied: $(1.55 \times A_{280} - (1.1 \times A_{260}) \times 60$ (dilution factor). Results are given directly as mg of protein per ml of mitochondrial suspension. The accurate determination of protein was made by the procedure of Smith *et al.* (1985) using bicinchoninic acid.

Materials

Chemicals were obtained from Prolabo (Paris, France) and Merck (Darmstadt, Germany). The biochemicals were purchased from Sigma (St. Louis, MO, U.S.A.); phosphatidylcholine and phosphatidylethanolamine from bovine liver were 99 and 98% pure respectively. L- and D-carnitine were provided by Dr. C. Cavazza of Sigma-Tau (Pomezia, Italy). [^{14}C]Oleic acid ($\text{C}_{18:1, n-9}$) was from CEA (Saclay, France). [^{14}C]Linoleic acid ($\text{C}_{18:2, n-6}$), [^{14}C]linolenic acid ($\text{C}_{18:3, n-3}$) and L-[methyl- ^3H]carnitine were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Labelled fatty acids were diluted with the corresponding unlabelled fatty acids to a specific radioactivity of 2 Ci/mol, and used as potassium salts.

RESULTS

Mitochondrial oxidation of the three octadecenoic acids was found to be strictly carnitine-dependent whatever the amount of albumin added to the medium (Fig. 1). α -Linolenic acid ($\text{C}_{18:3, n-3}$) was the best substrate for the total pathway from CoA activation to the formation of acid-soluble products, all resulting from β -oxidative reactions, up to $^{14}\text{CO}_2$ production (see legend to Fig. 1). Linoleic acid ($\text{C}_{18:2, n-6}$) was oxidized to a slightly greater extent than was oleic acid ($\text{C}_{18:1, n-9}$). Since carnitine is important for these reactions, the conversion into carnitine derivatives of the acyl-CoAs corresponding to the three fatty acids was studied by measuring the activity of CPT-I, the carnitine transferase for long-chain acyl groups (Fig. 2). Surprisingly, oleoyl- and linoleoyl-CoAs were more easily transferred to carnitine than was α -linolenoyl-CoA. α -Linolenoyl-CoA could not easily reach the CPT-I site. The transfer of α -linolenoyl-CoA was not improved by modifying the [acyl-CoA]/[albumin] molar ratio, by using very low amounts of

Fig. 1. Mitochondrial oxidation of octadecenoic acids with different degrees of unsaturation

The incubation conditions are described in the Experimental section. Each fatty acid (as the potassium salt; 50 nmol) and 0.35 mg of mitochondrial protein (M1) were used in 1 ml of medium. Albumin was added to give various molar ratios of [fatty acid]/[albumin] ranging from 0.3 to 3.5. Results are given as nmol of ^{14}C -labelled fatty acids whose radioactivity was recovered as acid-soluble products (ASP) at the end of incubation and are expressed/min per mg of mitochondrial protein. ●, Oleic acid ($n-9$); ▲, linoleic acid ($n-6$); ■, α -linolenic acid ($n-3$). The broken line shows oxidation of the three fatty acids in the absence of exogenous carnitine. $^{14}\text{CO}_2$ production from oxidation of ^{14}C -labelled fatty acids was maximal at a ratio of 3.5, with values as follows (nmol/min per mg): oleic acid, 0.080 ± 0.005 ; linoleic acid, 0.087 ± 0.007 and linolenic acid, 0.159 ± 0.012 . Results are means of four experiments carried out from separate mitochondrial preparations. Bars represent S.E.M.

acyl-CoA, or by reducing the incubation time (in order to avoid a hypothetical higher hydrolase activity to α -linolenoylcarnitine). Assays carried out in the presence of increasing amounts of phospholipid (from 10 to 110 μ g when expressed per mg of mitochondrial protein) did not alter the activity of CPT-I towards oleoyl- and linoleoyl-CoA, and did not improve that towards

Fig. 2. CPT-I activity measured with several octadecenoyl-CoAs in whole mitochondria

The incubation conditions are described in the Experimental section. A total of 5–70 nmol of oleoyl-CoA, (●), linoleoyl-CoA (▲) or α -linolenoyl-CoA (■) were used in the presence of 50 nmol of albumin ([acyl-CoA]/[albumin] molar ratio varying from 0.1 to 1.4). Each assay of 1 ml contained 0.25 mg of mitochondrial protein (M1). Results indicate the acyl-[3 H]carnitine whose radioactivity was recovered in the butanol-extractable products at the end of incubation, and are expressed in nmol/min per mg of mitochondrial protein. Values are means of four experiments carried out with separate mitochondrial preparations. Bars represent S.E.M.

α -linolenoyl-CoA (P. Clouet, I. Niot, P. Bouchard & J. Bézard, unpublished work).

When we examined the conversion of the three fatty acids (as potassium salts) to acylcarnitines, involving both activation by CoA and transfer to carnitine mediated by CPT-I (Fig. 3), α -linolenic acid was the best substrate at the [fatty acid]/[albumin] molar ratios studied. Linoleic acid transferred more effectively to carnitine than did oleic acid. It was evident from the data that CoA was necessary for the reaction.

Fig. 3. CPT-I activity towards octadecenoic acids measured in whole mitochondria

The incubation conditions were the same as for fatty acid oxidation, as described in the Experimental section, but the medium was devoid of L-malate and contained 2 mM-KCN and antimycin (2 μ g per assay) to block the oxidative pathway. [3 H]Carnitine was added to a concentration of 0.4 mM (0.62 mCi/mmol). The reaction was initiated with 0.35 mg of mitochondrial protein (M1). Results are given as nmol of acyl-[3 H]carnitine recovered in the butanol-extractable products/min per mg of mitochondrial protein. ●, Oleic acid; ▲, linoleic acid; ■, α -linolenic acid. The broken line shows values in the absence of exogenous CoA. Results are means of four experiments carried out with separate mitochondrial preparations. Bars represent S.E.M.

Table 2. Effects of mitochondrial inhibitors on oxidation and transfer to carnitine of α -linolenic acid in mitochondrial fractions of different purity

Values are means $\pm$ S.E.M. ($n = 4$). To estimate fatty acid oxidation, results are given as nmol of α -[1- 14 C]linolenic acid whose radioactivity was recovered in the acid-soluble products (ASP)/min per mg of mitochondrial protein. For measurement of the transfer of α -linolenic acid to carnitine, only carnitine was labelled and values are given as nmol of α -linolenoyl-[3 H]carnitine formed/min per mg of mitochondrial protein. The medium contained in 1 ml: 20 mM-phosphate (pH 7.4), 50 mM-KCl, 4 mM-MgCl₂, 1 mM-ATP, 50 μ M-CoA, 0.4 mM-L-carnitine and 50 μ M- α -linolenic acid as the potassium salt, bound to albumin in a molar ratio of 1:1. The following products were added as indicated: 0.2 mM-L-malate, 2 mM-KCN/antimycin (2 μ g/assay), 1 mM-D-decanoylcarnitine and 150 μ M-malonyl-CoA. All reactions were carried out at 37 $^{\circ}$ C for 8 min in the presence of 0.35 mg of mitochondrial protein. M4 refers to the highly purified mitochondrial fraction.

Fraction	Product	Addition...	L-Malate	KCN/ antimycin	D-Decanoyl- carnitine	Malonyl- CoA
M1	ASP		5.81 $\pm$ 0.25	1.01 $\pm$ 0.10	0.39 $\pm$ 0.08	0.34 $\pm$ 0.05
M1	Acylcarnitine		1.13 $\pm$ 0.08	4.15 $\pm$ 0.30	3.25 $\pm$ 0.25	0.23 $\pm$ 0.05
M4	ASP		7.08 $\pm$ 0.35	1.02 $\pm$ 0.09	0.42 $\pm$ 0.07	0.35 $\pm$ 0.04
M4	Acylcarnitine		1.62 $\pm$ 0.11	4.25 $\pm$ 0.35	3.00 $\pm$ 0.28	0.22 $\pm$ 0.05

Fig. 4. Effect of unlabelled acyl-CoAs on the time-course of oxidation of α -[1- 14 C]-linolenic acid

All results are given as percentage increase or decrease in oxidation rate compared with control assays carried out in the absence of added acyl-CoAs (0%). α -[1- 14 C]Linolenic acid (as the potassium salt; 50 nmol) and defatted bovine albumin (50 nmol) were used in 1 ml of the medium described for the oxidation assay in the Experimental section. Each acyl-CoA (12 μ M) was added in a small volume of water, with the same volume of water added to control assays. Measurements were obtained after stopping the incubations each min, up to 8 min. Oxidation was estimated by the sum of radioactivity found in acid-soluble products and in CO_2 . ○, Palmitoyl-CoA; ●, oleoyl-CoA ($n=9$); ▲, linoleoyl-CoA ($n=6$); ■, α -linolenoyl-CoA. The dotted line (0%) shows the oxidation of labelled α -linolenic acid in the absence of added acyl-CoA. Bars represent S.E.M.

Since the mitochondrial fraction M1 contained some peroxisomal activity (Table 1), we assessed the possible role of this contamination in the unexpected fate of α -linolenic acid. The results (Table 2) show that the use of the more purified mitochondrial preparation M4 did not alter the rate of oxidation of α -linolenic acid nor its transfer to carnitine. Moreover, mitochondrial inhibitors were very efficient in the total oxidative pathway by selecting their known target.

Since added α -linolenoyl-CoA did not easily reach the CPT-I site and thus the β -oxidative pathway, it is possible this unlabelled activated compound is not able to compete with oxidation of α -[1- 14 C]linolenate. This was investigated by comparison with other unlabelled acyl-CoAs which are, by contrast, better exogenous substrates for CPT-I, (especially palmitoyl-CoA). The results (Fig. 4) show that the oxidation rate of labelled α -linolenate was not reduced by adding unlabelled α -linolenoyl-CoA. It even increased a little, since the addition of the acyl-CoA probably displaced labelled α -linolenate from albumin, rendering it more available for activation. The other unlabelled acyl-CoAs decreased the oxidation of α -linolenate, probably by competing with the labelled α -linolenoyl-CoA formed in mitochondria from added α -

Fig. 5. Effect of malonyl-CoA concentration on the transfer of octadecenoic acids to carnitine in whole mitochondria

The incubation conditions were those described in the legend to Fig. 3 for CPT-I activity using unactivated fatty acids. Each fatty acid (50 nmol), bound to albumin (34 nmol), and 0.25 mg of mitochondrial protein (M1) was present in 1 ml of medium. Preincubation of mitochondria in the presence of malonyl-CoA was not necessary for obtaining efficient inhibition. Results are given as percentages of the values observed in the absence of inhibitor. ●, Oleic acid; ▲, linoleic acid; ■, α -linolenic acid. Results are means of four experiments carried out with separate mitochondrial preparations. Bars represent S.E.M.

linolenate. The competition is probably greater since, as previously noted, the added acyl-CoAs would displace labelled α -linolenate from albumin, increasing its availability for activation.

To see whether the three different unsaturated fatty acids are channelled by the same route from CoA activation to transfer to carnitine, we studied the formation of acylcarnitines from the respective potassium salts in the presence of increasing malonyl-CoA concentrations. The data in Fig. 5 show that the products of both reactions could be almost entirely suppressed by malonyl-CoA, and that the changes in the degree of inhibition with changing concentration were very similar for all three fatty acids.

Since our results can be explained by the location of CPT-I close to fatty acyl-CoA synthetase in the mitochondrial outer membrane, we estimated the formation of acylcarnitines from fatty acids (added as potassium salts) using the purified fraction of mitochondrial outer membranes. Experiments were performed on a sample containing monoamine oxidase activity corresponding to 1 mg of protein of whole mitochondria. In these conditions (Fig. 6), both reactions gave qualitatively and quantitatively similar results to those obtained in the presence of whole mitochondria.

Fig. 6. CPT-I activity measured with octadecenoic acids in mitochondrial outer membranes

The incubation conditions were exactly the same as for the experiments reported in Fig. 3. The reaction was initiated by adding 20 μ g of protein from mitochondrial outer membranes. Results are given as nmol of acyl-[3 H]carnitine recovered in the butanol-extractable products/min per monoamine oxidase activity corresponding to 1 mg of whole mitochondria (specific activity assumed to be 12 m-units). ●, Oleic acid; ▲, linoleic acid; ■, α -linolenic acid. Results are means of three experiments carried out with separate preparations of outer membranes. Bars represent S.E.M.

DISCUSSION

Our results clearly show that oxidation of α -linolenic acid in liver mitochondria was greater than that of linoleic and oleic acids. Differences in the oxidation rates of the three fatty acids ($C_{18:3, n-3} > C_{18:2, n-6} > C_{18:1, n-9}$) were also previously found by collecting CO_2 from the respiration of liver mitochondria (Stoffel & Schiefer, 1965) and of heart mitochondria (Huxtable & Wakil, 1971). Similar observations were made in rats given [$1-^{14}C$]fatty acids where expired $^{14}CO_2$ was found to be greater from α -linolenic acid than from linoleic and oleic acids (Leyton *et al.*, 1987).

However, few studies in this field refer to peroxisomal contamination, to factors stimulating fatty acid oxidation by peroxisomes or to the integrity of mitochondrial membranes. Osmundsen *et al.* (1987) showed that γ -linolenoyl-CoA was oxidized better than were linoleoyl- and oleoyl-CoAs by peroxisomes. Since α -linolenic ($C_{18:3, n-3}$) and γ -linolenic ($C_{18:3, n-6}$) molecules are similar, the differences observed in fatty acid oxidation by the mitochondrial preparation M1 may be due to its peroxisomal contamination. In fact, experiments performed with a fraction practically devoid of peroxisomal activity (Table 1) did not alter our previous results, which in addition were strictly influenced by inhibitors of mitochondrial activity (Table 2). Albumin was used at high concentrations to protect the integrity

of the membranes during mitochondrial isolation, and also to reduce peroxisomal activities in assays (Mannaerts *et al.*, 1979). These data indicate that the observed differences can be entirely ascribed to mitochondrial activities.

Since oxidation of α -linolenic acid is strictly carnitine-dependent, the activity of CPT-I should have been highest in the presence of added α -linolenoyl-CoA, but this could not be demonstrated experimentally. This added acyl-CoA was found to be a poor substrate for CPT-I, even when phospholipids were added to compensate for a possible loss during mitochondrial isolation. However, when α -linolenic acid was added in the presence of CoA and ATP, acyl-CoA which was then synthesized in the mitochondria was easily transformed by CPT-I into a carnitine derivative (Fig. 3). If this acyl-CoA could escape to outside the mitochondrion, it would be a poor substrate for CPT-I (Fig. 2). Since fatty acid synthetase is located on the external side of the outer membrane (Pande & Blanchear, 1970) and CPT-I on the internal side (Murthy & Pande, 1987), it is probable that these enzymes are linked, so that any newly synthesized α -linolenoyl-CoA is efficiently directed to CPT-I. This view is supported by the improved transfer of α -linolenic acid to carnitine in both whole mitochondria and purified outer membranes.

This scheme might also apply to oleic and linoleic acids, because the same effect was observed on the transfer of all three fatty acids to carnitine when malonyl-CoA was added to mitochondria (Fig. 5). Malonyl-CoA has been reported to bind sites (Bird & Saggerson, 1984) which have been located on the external side of the outer membrane (Murthy & Pande, 1987) and, from there, it would inhibit CPT-I activity. Given the similarity of the malonyl-CoA effect on CPT-I activity when reactions originate from the three fatty acids, an identical channel containing acyl-CoA synthetase and CPT-I can be used by all of the fatty acids.

In this context, differences in transfer to carnitine when starting from the three fatty acids or from their acyl-CoA derivatives could be due to the number of double bonds, all situated in acyl chain beyond the ninth carbon, when counted from carboxyl end. Binding of fatty acids to CoA renders the whole molecule spatially more cumbersome, and an increased number of double bonds enhances the folding of the acyl chain. Therefore, three double bonds seem sufficient to impede entry into the channel of the added α -linolenoyl group when bound to CoA. Besides its inability to enter, this acyl-CoA form did not hinder oxidation of the corresponding free fatty acid form. On the contrary, the other fatty acyl-CoAs studied inhibited oxidation of α -linolenic acid (Fig. 4), because they could compete with labelled α -linolenic acid (either free or bound to CoA or carnitine) first perhaps at the entry into the channel, and then in the β -oxidative pathway. The α -linolenic group, after being activated *in situ* by CoA, is efficiently directed to CPT-I. The mechanism of this transfer has yet to be established.

The optimum passage of α -linolenic acid through the outer membrane could push other less oxidizable fatty acids towards oxidation. This would contribute to the enhanced fatty acid oxidation, which in part may explain the hypolipidaemic effects of diets containing $n-3$ fatty acids (Phillipson *et al.*, 1985).

Our results emphasize the importance of α -linolenic acid for studying the actual movement of long-chain

fatty acids throughout the mitochondrial outer membrane. The mechanisms by which fatty acids enter and cross the mitochondrial outer membrane are yet to be elucidated.

We are grateful to Dr. Jean-Paul Blond for valuable advice and to Dr. Kaija Sebedio for the editing of the English in the manuscript. The assistance of Mr. Patrick Bouchard is greatly appreciated. This work was supported by a grant of 'Groupe Lipides et Nutrition'.

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Received 16 January 1989/30 May 1989; accepted 14 June 1989