A role for 2,4-enoyl-CoA reductase in mitochondrial β -oxidation of polyunsaturated fatty acids

Effects of treatment with clofibrate on oxidation of polyunsaturated acylcarnitines by isolated rat liver mitochondria

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1. β -Oxidation of γ -linolenoylcarnitine, arachidonoylcarnitine and docosahexaenoylcarnitine by isolated rat liver mitochondria is inhibited by uncoupling conditions. Partial re-activation is obtained with added ATP. With mitochondria from clofibrate-treated rats ATP-stimulated rates of β -oxidation of docosahexaenoylcarnitine are higher than ADP-stimulated rates. This is not observed with the β -oxidation of oleoylcarnitine. 2. β -Oxidation of docosahexaenoylcarnitine, in the presence of rotenone, is inhibited by added oxaloacetate, analogous to previous findings with pent-4-enoylcarnitine [see Osmundsen (1978) *FEBS Lett.* **88**, 219–222]. In the absence of rotenone added oxaloacetate stimulates the β -oxidation of docosahexaenoylcarnitine, but has the opposite effect on the β -oxidation of palmitoylcarnitine. 3. β -Oxidation of polyunsaturated acylcarnitines by isolated rat liver mitochondria is selectively increased after treatment of the animals with a low dietary dose (0.2%, w/w) of clofibrate. Treatment with a higher dose of clofibrate (0.5%, w/w) resulted in a general stimulation of β -oxidation. 4. The results presented suggest that long-chain fatty acids possessing a Δ^4 -double bond are not readily β -oxidized unless the 2,4-enoyl-CoA reductase (EC 1.3.1.–) is operating.

Mitochondrial β -oxidation of polyunsaturated fatty acids is established to require the action of Δ^3 -*cis*- Δ^2 -*trans*-enoyl-CoA isomerase (EC 5.3.3.8) as well as of 3-hydroxyacyl-CoA epimerase (EC 5.1.2.–) (Stoffel *et al.*, 1964; Davidoff & Korn, 1965; Stoffel & Caesar, 1965), in addition to the normal β -oxidation enzymes. More recently Kunau & Dommes (1978) have isolated a 2,4-enoyl-CoA reductase (EC 1.3.1.–) from mitochondrial extracts, and suggested that the enzyme might play a significant role during the β -oxidation of unsaturated fatty acids containing double bond(s) at even-numbered carbon atoms, counted from the carboxy end of the molecule. We have subsequently shown that the activity of 2,4-enoyl-CoA reductase is increased at least 4-fold on clofibrate [ethyl α -(*p*-chlorophenoxy)isobutyrate] treatment of the animals (Borrebaek *et al.*, 1980a) and to a somewhat smaller extent with various high-fat diets (Borrebaek *et al.*, 1980b). This induction was proposed to furnish an explanation for the protective action of treatment with clofibrate against the

toxic effects of the hypoglycaemic fatty acid pent-4-enoic acid (Van Hoof *et al.*, 1979; Borrebaek *et al.*, 1980a).

The present work was undertaken to investigate whether treatment of rats with clofibrate causes any specific changes with regard to the β -oxidation of polyunsaturated fatty acids by isolated liver mitochondria. Intramitochondrial NADPH can be formed through the action of the energy-requiring NAD(P)⁺ transhydrogenase (EC 1.6.1.1) (for review see Rydström, 1977). An involvement of 2,4-enoyl-CoA reductase, which is an NADPH-requiring enzyme (Kunau & Dommes, 1978), in the β -oxidation of polyunsaturated fatty acids may therefore be expected to be dependent on mitochondrial energy state, as well as on the supply of NADPH.

Experimental

Animals

Male albino Wistar rats (180–250 g), supplied by

Veterinær Møllegårds Avlsstation, Havdrup, Denmark, were used in these experiments. Animals treated with clofibrate were given standard diets supplemented with 0.2% or 0.5% (w/w) of clofibrate. The animals were allowed food and water *ad libitum*.

Materials

Oleic acid was purchased from Fluka, Buchs, Switzerland. Vaccenic (*cis*-C_{18:1(11)}) acid, *trans*-vaccenic acid, linoleic acid, linoelaidic acid, linolenic acid, elaidolinolenic acid (as methyl ester), γ -linolenic acid, arachidonic acid and docosahexaenoic (C_{22:6(4,7,10,13,16,19)}) acid were purchased from Nu Chek Prep, Elysian, MN, U.S.A. Valinomycin, rotenone (grade II), cytochrome *c* (grade IV), *cis*-oxaloacetic acid (UV grade), EGTA, Mops (4-morpholinepropanesulphonic acid), Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], ADP (grade X) and ATP (grade I) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was obtained from Boehringer Mannheim, West Germany. L-Carnitine was a gift kindly provided by Sigma-Thau s.p.a., Rome, Italy. Acylcarnitine esters were synthesized via the corresponding acylimidazoles as described by Červenka & Osmundsen (1982). The resulting acylcarnitines were found to be chromatographically pure, and contained about 0.5% free fatty acid. The acylcarnitines were stored as methanol solutions in the presence of 0.005% (w/v) butylated hydroxytoluene as antioxidant. The acylcarnitine concentrations of these solutions were measured, as the amount of fatty acid liberated after alkaline hydrolysis, by using g.l.c. (see Červenka & Osmundsen, 1982). Samples were withdrawn before use, and the solvent was evaporated off with a stream of N₂. The acylcarnitine was redissolved in a known volume of water.

Isolation of rat liver mitochondria

Rat liver mitochondria were isolated as described by Osmundsen & Bremer (1977).

Measurements of rates of oxidation of various acylcarnitines by isolated rat liver mitochondria

Polarographic measurements of rates of O₂ uptake were made by using a Clark-type oxygen electrode with an incubation medium containing 130 mM-KCl, 10 mM-Mops, 1 mM-EGTA, 1.5 mM-P_i and 5 mM-malate or 2 mM-malate, pH 7.2. The medium also contained 2 mM-ADP or 0.6 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or 50 μ M-2,4-dinitrophenol.

Rates of β -oxidation were also measured as the acylcarnitine-dependent reduction of ferricyanide (Osmundsen & Bremer, 1977). These assays were

performed as described by Osmundsen (1981), with acylcarnitines at a concentration of 40 μ M.

Determination of mitochondrial protein

Mitochondrial protein concentrations were measured by using the biuret assay (Gornall *et al.*, 1949), with Precimat (Boehringer) protein standards. The assay medium here contained 0.3% (w/v) sodium deoxycholate to ensure solubilization of mitochondrial proteins.

Results

Effects of treatment with clofibrate on acylcarnitine-dependent rates of respiration

Table 1 shows the rates of respiration obtained from various unsaturated acylcarnitines with isolated liver mitochondria from normal rats (Table 1a) and from rats treated with clofibrate (Table 1b). These results show relatively small increase in rates of oleoylcarnitine respiration after clofibrate treatment, in the presence of either malonate or malate. With increasing degree of unsaturation of the acyl group a clofibrate-dependent stimulation becomes marked, being most pronounced with docosahexaenoylcarnitine. With this carnitine ester the treatment with clofibrate led to an almost 300% increase in respiratory rates.

Uncoupler-stimulated rates of respiration are usually similar to, or higher than, ADP-stimulated rates, as was always found with oleoyl- and linoleoyl-carnitine (see Table 1a). This was also the case with linolenoylcarnitine, provided that malate was present in the incubation medium. With arachidonoylcarnitine and docosahexaenoylcarnitine, in contrast, rates of respiration were decreased by about 50% in the presence of uncoupler, with either malonate or malate in the incubation medium (see Table 1a). A similar result was obtained with mitochondria from clofibrate-treated animals, except that uncoupling no longer had any inhibitory effect on the oxidation of arachidonoylcarnitine when malate was included in the incubation medium (see Table 1b).

Effects of ADP and ATP on uncoupler-inhibited respiration on some polyunsaturated acylcarnitines

Fig. 1 shows typical traces showing effects of additions of ADP or ATP on uncoupled respiration on γ -linolenoyl-, arachidonoyl- and docosahexaenoyl-carnitine by normal rat liver mitochondria. These show that the uncoupler-inhibited rate of respiration was markedly more stimulated by added ATP than by added ADP. These results were not significantly altered by the addition of oligomycin (results not shown). With oleoylcarnitine, in contrast, the uncoupler-stimulated rate was the fastest (see Table 1 and Fig. 1), and this rate was not

Table 1. *Effects of uncoupling conditions on rates of respiration from various unsaturated acylcarnitines by isolated rat liver mitochondria*

Rates of oxidation of various unsaturated acylcarnitines ($40\mu\text{M}$) by control mitochondria were measured polarographically (a) in the presence of either 5 mM-malate or 2 mM-malate. In both instances rates of respiration were measured in the presence of 2 mM-ADP or of $50\mu\text{M}$ -2,4-dinitrophenol. Tabulated values represent means derived from the numbers of separate experiments (n) indicated; for values of $n \geq 4$ the s.d. has been given, and with smaller values of n the range has been tabulated. The measurements were made at 25°C , and each assay mixture contained 4–10 mg of mitochondrial protein in a final volume of 2.5 ml. With 2 mM-malate in the incubation medium the amount of protein used was decreased by 50%. Rates of oxidation of the various acylcarnitines ($40\mu\text{M}$) by liver mitochondria isolated from rats treated with clofibrate (0.2%, w/w, in the diet for 12 days) were also measured (b). These measurements were made as described above. Experimental details are otherwise given in Experimental section.

		Rate of respiration (ng-atoms of O/min per mg of protein)			
		Malonate present		Malate present	
Acylcarnitine	No. of expts. (n)	ADP present	Dinitrophenol present	ADP present	Dinitrophenol present
(a) Control					
Oleoylcarnitine	5	38 ± 3	45 ± 10	65 ± 12	94 ± 28
Linoleoylcarnitine	3	27 ± 10	34 ± 5	59 ± 10	100 ± 18
Linolenoylcarnitine	3	30 ± 5	24 ± 8	56 ± 13	78 ± 23
Arachidonoylcarnitine	4	11 ± 3	7 ± 2	47 ± 12	28 ± 15
Docosaehaenoylcarnitine	5	8 ± 3	4 ± 3	41 ± 10	20 ± 4
(b) Clofibrate					
Oleoylcarnitine	5	50 ± 12	66 ± 8	65 ± 19	141 ± 14
Linoleoylcarnitine	3	53 ± 6	53 ± 7	79 ± 24	137 ± 24
Linolenoylcarnitine	2	47 ± 5	47 ± 9	79 ± 12	130 ± 6
Arachidonoylcarnitine	2	25 ± 1	22 ± 6	69 ± 12	82 ± 21
Docosaehaenoylcarnitine	4	21 ± 8	11 ± 2	70 ± 12	64 ± 14

Fig. 1. *Effects of ADP and ATP on mitochondrial respiration on selected unsaturated acylcarnitines in the presence of uncoupler*

Typical polarographic traces showing effects of added ADP or ATP on acylcarnitine-dependent respiration by rat liver mitochondria that had been uncoupled by prior addition of $50\mu\text{M}$ -2,4-dinitrophenol (Dnp-OH, as indicated in the Figure). The point of addition of 2 mM-ADP or 2 mM-ATP is shown, and an unlabelled arrow indicates the time of addition of arachidonoylcarnitine (a), γ -linolenoylcarnitine (b) or docosaehaenoylcarnitine (c). With oleoylcarnitine (d) uncoupler was not added together with ADP or ATP, and addition of ADP or ATP in the presence of uncoupler did not cause any further increase in respiratory rate (results not shown). The numbers in parentheses represent rates of respiration, expressed as ng-atoms of O/min per mg of protein. These measurements were made with 5 mM-malate present in the incubation medium. Experimental details are otherwise given in the legend to Table 1 and in the Experimental section.

significantly increased by additions of ATP (results not shown).

Additions of valinomycin (30 ng/ml) (in the absence of uncoupler) stimulated respiration of docosahexaenoylcarnitine to about the same extent as did ADP. Also, valinomycin-stimulated respiration was markedly inhibited by the prior addition of uncoupler (results not shown).

Effects of various concentrations of added ADP or ATP on coupled rates of respiration of oleoylcarnitine or docosahexaenoylcarnitine

Fig. 2 shows results obtained from measurements of respiratory rates of docosahexaenoylcarnitine or oleoylcarnitine with increasing concentrations of added ADP or ATP, with mitochondria isolated from normal rats or from rats treated with clofibrate. With oleoylcarnitine higher rates of respiration were always obtained with ADP. With control mitochondria docosahexaenoylcarnitine-dependent rates were low, and were not stimulated by increasing concentrations of ADP or ATP. With mitochondria from rats treated with clofibrate higher rates were obtained with ATP, provided that the concentration of ATP was higher than 1 mM, and the ATP-stimulated rates were never markedly less than those obtained with ADP (see Fig. 2).

Measurements of rates of oxidation of unsaturated acylcarnitines by spectrophotometric measurements of ferricyanide reduction

Table 2 shows the initial rates of ferricyanide reduction with various unsaturated acylcarnitines,

with mitochondria isolated from normal rats and from clofibrate-treated rats. This assay has been shown to give a selective measure of the flux through the acyl-CoA dehydrogenases (Osmundsen & Bremer, 1977). It provides selective measurement of the rotenone-insensitive component of β -oxidation ('pre-oxaloacetate' rates in Table 2) and of the rotenone-sensitive rate (that obtained on removal of accumulated NADH by addition of 10 mM-oxaloacetate, 'post-oxaloacetate' rates in Table 2). The results presented in Table 2 indicate that the rotenone-insensitive rate increased relative to the rotenone-sensitive rate with increasing degree of unsaturation. This may be caused by re-oxidation of NADH, mediated through NADPH-dependent reduction of double bonds (see Scheme 1). For oleoylcarnitine the ratio of the rotenone-insensitive rate to the rotenone-sensitive rate was about 5:1, and this decreased to about 1:1 for docosahexaenoylcarnitine (see Table 2). Clofibrate treatment of the animals tended to increase the rotenone-sensitive rate proportionally more (see Table 2). Elaidolinenoylcarnitine gave a zero rotenone-insensitive rate with control mitochondria (i.e. no stimulation of the endogenous rate on addition of acylcarnitine). Clofibrate treatment, however, also resulted in a small rotenone-insensitive rate with this acylcarnitine (see Table 2).

These effects were qualitatively identical irrespective of whether rats had been fed on diets containing 0.2% or 0.5% (w/w) of clofibrate. Increasing the amount of clofibrate in the diet, however, led to significantly increased rates of

Fig. 2. Effects of various concentrations of added ADP or ATP on oleoylcarnitine- or docosahexaenoylcarnitine-dependent respiration by liver mitochondria isolated from normal rats and from rats treated with clofibrate

Rates of respiration obtained from 40 μ M-oleoylcarnitine (○ and ●) or 40 μ M-docosahexaenoylcarnitine (□ and ■), in the presence of the various concentrations of ADP (● and ■) or of ATP (○ and □), were measured polarographically in the presence of 5 mM-malonate. Experiments were performed with mitochondria isolated from normal rats (a) and with mitochondria isolated from rats treated with clofibrate for 12 days (b). Experimental details and otherwise given in the legend to Table 1 and in the Experimental section.

Table 2. *Effects of various treatments with dietary clofibrate on rates of β -oxidation of various unsaturated acylcarnitines by isolated rat liver mitochondria*

Rates of β -oxidation of the various acylcarnitines shown were measured spectrophotometrically as initial rates of reduction of ferricyanide. Rats were treated with clofibrate (0.2% or 0.5%, w/w, in the diet) for 12 days before experimental use. In the Table are presented rates of rotenone-insensitive β -oxidation [i.e. the rate obtained before addition of oxaloacetate ('pre-oxaloacetate' rate)], as well as rotenone-sensitive rates [i.e. rates obtained after the rotenone-imposed block of β -oxidation has been removed by addition of oxaloacetate ('post-oxaloacetate' rate)]. For normal rat liver mitochondria tabulated rates represent means $\pm$ s.d. for five separate experiments. Tabulated values for mitochondria from animals treated with clofibrate represent means for two separate experiments with ranges indicated. The various acylcarnitines were used at a concentration of 40 μ M, and oxaloacetate was added to a concentration of 10 mM. The assays were performed at 30°C. Each assay mixture contained 2–4 mg of mitochondrial protein in a final volume of 2.5 ml. Experimental details are otherwise given in Experimental section.

Acylcarnitine	Rate of β -oxidation (nmol/min per mg of protein)					
	Mitochondria from normal rats		Mitochondria from 0.2%-(w/w)-clofibrate-treated-rats		Mitochondria from 0.5%-(w/w)-clofibrate-treated rats	
	Pre-oxaloacetate rate	Post-oxaloacetate rate	Pre-oxaloacetate rate	Post-oxaloacetate rate	Pre-oxaloacetate rate	Post-oxaloacetate rate
Oleoylcarnitine	12 $\pm$ 2	55 $\pm$ 5	11 $\pm$ 1	57 $\pm$ 3	16 $\pm$ 1	85 $\pm$ 2
Vaccenoylcarnitine	14 $\pm$ 3	48 $\pm$ 4	10 $\pm$ 1	61 $\pm$ 2	16 $\pm$ 1	109 $\pm$ 6
<i>trans</i> -Vaccenoylcarnitine	11 $\pm$ 2	42 $\pm$ 2	9 $\pm$ 1	52 $\pm$ 6	13 $\pm$ 1	83 $\pm$ 5
Linoleoylcarnitine	16 $\pm$ 2	42 $\pm$ 3	13 $\pm$ 1	54 $\pm$ 1	23 $\pm$ 2	96 $\pm$ 5
Linoelaidoylcarnitine	13 $\pm$ 3	26 $\pm$ 5	19 $\pm$ 5	43 $\pm$ 2	15 $\pm$ 4	61 $\pm$ 3
Linolenoylcarnitine	19 $\pm$ 3	44 $\pm$ 2	21 $\pm$ 2	68 $\pm$ 3	31 $\pm$ 3	102 $\pm$ 5
Elaidolinolenoylcarnitine	0 $\pm$ 0	20 $\pm$ 1	7 $\pm$ 1	22 $\pm$ 4	—	—
Arachidonoylcarnitine	15 $\pm$ 3	32 $\pm$ 6	26 $\pm$ 2	52 $\pm$ 2	48 $\pm$ 9	92 $\pm$ 10
γ -Linolenoylcarnitine	20 $\pm$ 2	42 $\pm$ 1	29 $\pm$ 6	71 $\pm$ 5	49 $\pm$ 7	108 $\pm$ 6
Docosahexaenoylcarnitine	14 $\pm$ 1	16 $\pm$ 2	37 $\pm$ 2	59 $\pm$ 1	60 $\pm$ 6	87 $\pm$ 7

β -oxidation for all substrates (see Table 2). Although we have previously reported that dietary clofibrate (0.3%, w/w) has no marked effect on rates of oxidation of long-chain acylcarnitines (Christiansen *et al.*, 1978), the present results show this to be dependent on drug dosage.

With mitochondria isolated from control animals, γ -linolenoylcarnitine, arachidonoylcarnitine and in particular docosahexaenoylcarnitine showed non-linear progress curves, with rates of ferricyanide reduction decreasing with increasing times of reaction. This is illustrated in Fig. 3, which shows typical recorder tracings obtained with docosahexaenoylcarnitine. Here it can be seen that the rotenone-insensitive rate was linear (also for much longer times of reaction than that shown in the Figure). On the addition of oxaloacetate, a small transient stimulation of β -oxidation was usually observed. The final rate of β -oxidation, however, was not significantly different from the endogenous rate (see Fig. 3b). When oxaloacetate was added before the docosahexaenoylcarnitine, only a very small transient stimulation of the rate of ferricyanide reduction was observed, which rapidly decreased towards the endogenous rate of reduction (see Fig. 3c). With mitochondria from clofibrate-treated rats, however,

a relatively high rate of rotenone-insensitive β -oxidation was found. This was further increased on the addition of 10 mM-oxaloacetate (see Fig. 3a). Also, with these mitochondria rates of oxidation decreased with increasing times of incubation. The reaction, however, had to run for about 3 min before this effect became apparent.

Effects of rotenone and oxaloacetate on rates of respiration obtained with docosahexaenoylcarnitine

Fig. 4 presents typical polarographic traces showing that rates of respiration obtained with docosahexaenoylcarnitine were slightly stimulated after the inclusion of rotenone in the incubation mixture. With palmitoylcarnitine, in contrast, powerful inhibition resulted, as would be expected (see Fig. 4). Additions of oxaloacetate were stimulatory to docosahexaenoylcarnitine-dependent respiration, in the absence of rotenone. With rotenone present, additions of oxaloacetate were inhibitory, in line with results obtained when β -oxidation was measured as final rates of reduction of ferricyanide (see Fig. 3). With palmitoylcarnitine as substrate the effects of oxaloacetate were opposite to those obtained with docosahexaenoylcarnitine, i.e. inhibition of O_2 uptake in the absence of rotenone, and stimulation of

Fig. 3. Effects of oxaloacetate on docosahexaenoylcarnitine-dependent reduction of ferricyanide by liver mitochondria isolated from normal rats and from rats treated with clofibrate

Typical recorder traces showing docosahexaenoylcarnitine-dependent reduction of ferricyanide are presented. Points of addition of $40\mu\text{M}$ -docosahexaenoylcarnitine are indicated by an unlabelled arrow. Additions of 10mM -oxaloacetate (OxAc) are also indicated. Trace (a) was obtained with mitochondria from clofibrate-treated rats, trace (b) with normal mitochondria, and trace (c) also with normal mitochondria but with prior addition of oxaloacetate to the incubation mixture. These measurements were made with an Aminco DW-2 spectrophotometer. Each assay mixture contained $2\text{--}3\text{mg}$ of protein in a final volume of 2.5ml , and was thermostatically maintained at 30°C . Experimental details are otherwise given in the Experimental section. The numbers in parentheses represent rates of β -oxidation, expressed as $\text{nmol of Fe(CN)}_6^{3-}$ reduced/min per mg of protein.

Fig. 4. Effects of rotenone and oxaloacetate on docosahexaenoylcarnitine-dependent respiration by isolated liver mitochondria

Typical polarographic traces showing the stimulatory effect of rotenone on docosahexaenoylcarnitine-dependent respiration are presented (a). When present, rotenone was added before acylcarnitine. Additions of 10mM -oxaloacetate (OxAc) are also indicated. The point of addition of acylcarnitine is indicated by an unlabelled arrow. These experiments were performed with 5mM -malonate present in the incubation mixture. The numbers in parentheses represent rates of respiration, expressed as $\text{ng-atoms of O/min per mg}$ of protein. Effects of rotenone and oxaloacetate on palmitoylcarnitine-dependent respiration are shown in (b) for comparison. In the absence of rotenone, docosahexaenoylcarnitine added $5\text{--}6\text{min}$ after addition of oxaloacetate was oxidized at a rate of about 70% of that obtained before addition of oxaloacetate (results not shown).

respiration in the presence of rotenone (see Fig. 4). These latter effects of oxaloacetate would normally be expected, in view of the ability of oxaloacetate to mediate re-oxidation of NADH (Bremer & Wojtczak, 1972).

Discussion

Functional characteristics of β -oxidation of some polyunsaturated fatty acids, suggesting a requirement for 2,4-enoyl-CoA reductase during their oxidation

The uncoupler-dependent inhibition of rates of oxidation of some polyunsaturated acylcarnitines, notably arachidonoylcarnitine and docosahexaenoylcarnitine (see Table 1), clearly suggests a requirement for an energy-dependent function to obtain optimal rates of oxidation of these carnitine esters. This uncoupler-dependent inhibition was also observed with γ -linolenoylcarnitine (see Fig. 1), and the inhibited rate could be stimulated by added ATP. Added ADP, in contrast, had little stimulatory effect (see Fig. 1). Also, in the coupled state rates of oxidation of docosahexaenoylcarnitine were stimulated by added ATP, although this now was only marked with mitochondria isolated from rats treated with clofibrate (see Fig. 2). With such mitochondria higher rates of respiration could be obtained in the presence of ATP as compared with rates obtained in the presence of an identical concentration of ADP (see Fig. 2). None of these effects was observed with oleoylcarnitine or linoleoylcarnitine (see Table 1 and Figs. 1 and 2), suggesting that the requirement for an energy-linked function for optimal rates of oxidation is exclusive to some polyunsaturated fatty acids.

These unusual β -oxidative properties may be explained in terms of a requirement for 2,4-enoyl-CoA reductase activity for optimal rates of oxidation of some polyunsaturated fatty acids. This enzyme is strictly dependent on NADPH as a source of reducing equivalents (Kunau & Dommes, 1978). With the conditions of incubation used in the present investigation, in particular with malonate present, it is likely that the enzyme will be dependent on the NAD(P)⁺ transhydrogenase activity for its NADPH supply, as outlined in Scheme 1.

NAD(P)⁺ transhydrogenase activity, in the direction of NADPH formation, is greatly favoured by energy, derived from substrate oxidation, or as ATP (see Rydström, 1977). Hence the effects of mitochondrial energy state on rates of oxidation may primarily be due to effects on NAD(P)⁺ transhydrogenase activity. The observed effects, i.e. inhibition by uncoupling conditions and stimulation by added ATP, are in agreement with the known properties of NAD(P)⁺ transhydrogenase. The transhydrogenase-mediated reduction of NADP⁺

Scheme 1. Diagrammatic presentation of the reactions involved during utilization of NADH in the reduction of Δ^4 -double bond encountered during β -oxidation of an unsaturated fatty acid

NADH, which has been generated during β -oxidation, is re-oxidized by NAD(P)⁺ transhydrogenase (EC 1.6.1.1), forming NADPH. This NADPH is subsequently re-oxidized by the 2,4-dienoyl-CoA reductase (EC 1.3.1.-), which in the process converts a 2,4-dienoyl-CoA β -oxidation intermediate into a 2-enoyl-CoA intermediate, which is subjected to further β -oxidation.

has been shown to be inhibited by uncoupler (Krebs, 1954), and to be activated by added ATP, also in the presence of uncoupler (Danielson & Ernster, 1963; Estabrook *et al.*, 1963). In the presence of valinomycin the transhydrogenase activity has also been shown to be stimulated by a K⁺ gradient (Conover, 1971). This may be related to the stimulatory effect of valinomycin on the oxidation of docosahexaenoylcarnitine. The observed inhibitory effect of added oxaloacetate on docosahexaenoylcarnitine-dependent reduction of ferricyanide (see Fig. 3) is strictly analogous to the effect of oxaloacetate on pent-4-enoylcarnitine-dependent reduction of ferricyanide previously observed (Osmundsen, 1978). The only structural similarity between these two fatty acids is the double bond in the Δ^4 -position. These findings therefore suggest that fatty acids with a Δ^4 -double bond are slowly oxidized by isolated liver

mitochondria, under conditions that give optimal rates of oxidation for other fatty acids. Both γ -linolenoylcarnitine and arachidonoylcarnitine showed similar, although much less marked, characteristics. With these fatty acids, however, a Δ^4 -double bond is encountered after one and two cycles of β -oxidation respectively. This may therefore tend to diminish the inhibitory effect of oxaloacetate.

In line with these results are the findings by Lawson & Holman (1981) that, of various positional isomers of the $C_{18:1}$ acid, the Δ^4 -isomer was consistently oxidized at the lower rate.

Added oxaloacetate is known to cause re-oxidation of intramitochondrial NADH (Bremer & Wojtczak, 1972). This is presumably inhibitory to oxidation of fatty acids containing Δ^4 -double bonds, because the substrate for $NAD(P)^+$ transhydrogenase is thereby removed.

The absence of inhibition by rotenone on docosahexaenoylcarnitine-dependent respiration (see Fig. 4) can also be understood in this context. NADH accumulated as a result of the rotenone-induced block of NADH dehydrogenase (EC 1.6.99.3) can now be utilized as substrate for $NAD(P)^+$ transhydrogenase. Hence both the direct block on β -oxidation due to accumulated NADH, and that due to limited 2,4-enoyl-CoA reductase activity, are removed. The inhibitory effect of oxaloacetate, in the presence of rotenone, is obviously due to re-oxidation of NADH. The stimulatory effect observed with oxaloacetate in absence of rotenone, however, is most probably caused by NADPH generation due to $NADP^+$ -dependent isocitrate dehydrogenase (EC 1.1.1.42) activity. With these conditions the addition of oxaloacetate will lead to citrate formation, as a consequence of its function as acceptor for acetyl groups.

Effects of treatment with clofibrate on rates of oxidation of unsaturated fatty acids

The selective increase in rates of oxidation of polyunsaturated fatty acids after treatment of the animals with the lower dose of clofibrate (0.2%, w/w) is, in the light of the findings discussed above, readily explained by the clofibrate-dependent induction of 2,4-enoyl-CoA reductase (Borrebaek *et al.*, 1980a) (see Table 1). This was in particular marked with docosahexaenoylcarnitine, as is shown by the very marked increase in ATP (and ADP)-stimulated rates of respiration (see Fig. 2) after treatment of the animals with clofibrate. Also, the traces obtained when the docosahexaenoylcarnitine-dependent ferricyanide reduction was monitored became more similar to those obtained with most other acylcarnitines, i.e. addition of oxaloacetate led to extensive further stimulation of β -oxidation. The 4–5-fold increase in 2,4-enoyl-CoA reductase ac-

tivity (Borrebaek *et al.*, 1980a) would be expected to render this activity a more effective drain on reducing equivalents.

Clofibrate also has a general stimulatory effect on fatty acid oxidation, although in the present study this was expressed to a significant extent only with the higher dietary dose (0.5%, w/w) (see Table 2). In line with this are the findings by Furuta *et al.* (1981) of induction of acyl-CoA dehydrogenase activity (EC 1.3.99.3) and electron-transferring flavoprotein after treatment of the animals with clofibrate or di-(2-ethylhexyl) phthalate.

Rates of oxidation of linoelaidoylcarnitine and elaidolinolenoylcarnitine by normal mitochondria were about 50% of the rates obtained with the corresponding *cis*-isomers (linoleoylcarnitine and linolenoylcarnitine respectively) (see Table 2). This was not markedly altered by clofibrate treatment of the animals, although the rate obtained with elaidolinolenoylcarnitine decreased to about one-third of the rate given by the *cis*-isomers (see Table 2). This certainly shows that *trans*-isomers of polyunsaturated fatty acids are likely to be poor substrates for β -oxidation, and that their oxidation may not necessarily be appreciably aided by induction of additional 2,4-enoyl-CoA reductase activity. Like the Δ^3 -*cis*- Δ^2 -*trans*-enoyl-CoA isomerase, which is markedly more active towards the Δ^3 -*cis*-double bond as compared with the Δ^3 -*trans*-double bond (Struijk & Beerthius, 1966), it is also possible that the 2,4-enoyl-CoA reductase is more active towards the Δ^4 -*cis*-double bond.

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