Factors influencing palmitoyl-CoA oxidation by rat liver peroxisomal fractions

SUBSTRATE CONCENTRATION, ORGANELLE INTEGRITY AND ATP

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(Received 14 December 1979)

1. The first dehydrogenation step of peroxisomal β -oxidation involves the reduction of O_2 to H_2O_2 . Production rates of H_2O_2 and acetyl units by purified rat liver peroxisomes oxidizing palmitoyl-CoA were equal, indicating that H_2O_2 production is a reliable index for the release of acetyl units during peroxisomal fatty-acid oxidation. 2. Measurements of H_2O_2 and acid-soluble oxidation products during $[1-^{14}C]$ palmitoyl-CoA oxidation by purified peroxisomes revealed that the number of acetyl units released per molecule of palmitoyl-CoA oxidized rapidly decreased with increasing unbound palmitoyl-CoA concentrations. Structural damage to the peroxisomes caused by detergents or other treatments also decreased the number of acetyl units released. Under conditions where oxidation proceeded linearly with time the theoretical maximum of 5 acetyl units released per molecule of palmitoyl-CoA oxidized [Lazarow (1978) *J. Biol. Chem.* **253**, 1522–1528] was never reached. 3. Expressed in terms of acetyl units produced and measured at low unbound-palmitoyl-CoA concentrations, mitochondrial oxidation was 10–20-fold higher than peroxisomal oxidation. 4. ATP stimulated peroxisomal palmitoyl-CoA oxidation approx. 2-fold. The ATP effect required the presence of Mg^{2+} and was lost when peroxisomal membranes were disrupted by Triton X-100 or high concentrations of unbound palmitoyl-CoA. 5. Disruption of peroxisomes by detergents, freeze-thawing, osmotic or mechanical treatment did not stimulate palmitoyl-CoA oxidation in the presence of ATP, indicating that peroxisomal fatty-acyl-CoA oxidation was not latent. In the absence of ATP, Triton X-100 stimulated peroxisomal palmitoyl-CoA oxidation approx. 2-fold.

Mitochondria as well as peroxisomes of rat liver catalyse the β -oxidation of long-chain fatty-acyl-CoA esters. The capacity of the peroxisomes to oxidize fatty acids can be increased by approximately one order of magnitude by treatment of rats with clofibrate and other compounds known to induce peroxisomal proliferation in rat liver (Lazarow & de Duve, 1976; Lazarow, 1977; Osumi & Hashimoto, 1978a; Mannaerts *et al.*, 1979; Inestrosa *et al.*, 1979a). Fatty-acid oxidation by both organelles generates acetyl-CoA through successive steps of dehydrogenation, hydration, dehydrogenation and thiolitic cleavage (Lazarow, 1978; Osumi & Hashimoto, 1979). Despite these similarities, peroxisomal oxidation of fatty acids differs from mitochondrial oxidation in several aspects. (1) The

first dehydrogenation step of peroxisomal β -oxidation involves the reduction of O_2 to H_2O_2 . The catalysing enzyme, fatty acyl-CoA oxidase, has been partially purified (Osumi & Hashimoto, 1978b; Inestrosa *et al.*, 1979b). It is FAD-dependent and functions as an oxidase rather than as a dehydrogenase. (2) The peroxisomal oxidation sequence is not coupled to a phosphorylating system; hence its insensitivity towards inhibitors of mitochondrial phosphorylation and respiration (Lazarow & de Duve, 1976; Mannaerts *et al.*, 1979). (3) Peroxisomal oxidation is not stimulated by carnitine and is not inhibited by inhibition of carnitine palmitoyltransferase (Mannaerts *et al.*, 1979). (4) Fatty-acid oxidation by peroxisomes does not produce ketone bodies, suggesting the absence from peroxisomes of the enzymes of the 3-hydroxy-3-methylglutaryl-CoA cycle (Mannaerts *et al.*,

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1979). (5) Peroxisomal fatty-acid degradation does not go to completion. Under conditions leading to complete substrate utilization only 5 acetyl units are formed per equivalent of palmitoyl-CoA oxidized (Lazarow, 1978), apparently as a result of the fact that the peroxisomal fatty acyl-CoA oxidase does not act on fatty-acyl-CoA esters shorter than octanoyl-CoA (Osumi & Hashimoto, 1978b; Hryb & Hogg, 1979; Inestrosa *et al.*, 1979a).

The physiological significance of peroxisomal fatty-acid oxidation remains to be established. Its contribution to palmitate (C_{16}) and oleate ($C_{18:1}$) oxidation in isolated hepatocytes from control and clofibrate-treated rats has been estimated at less than 10% (Mannaerts *et al.*, 1979). Comparisons of palmitate and erucate ($C_{22:1}$) metabolism in isolated hepatocytes from control and clofibrate-treated rats led Christiansen (1978) to suggest that the β -oxidation system of peroxisomes might primarily handle very-long-chain fatty acids. Clearly, a detailed understanding of the properties of the system and of its physiological function requires further study.

Since peroxisomal fatty-acid oxidation is incomplete, labelled acid-soluble oxidation products liberated during degradation of palmitoyl-CoA labelled in $C_{(1)}$ (Mannaerts *et al.*, 1979) do not provide an exact measure of the number of acetyl units released by the peroxisomal system. In the first part of the present study it is demonstrated that acetyl flux through the peroxisomal system can be measured accurately by following H_2O_2 production. This flux can then validly be compared with the mitochondrial flux.

Whether the peroxisomal membrane is intact after preparation of purified peroxisomal fractions and whether this membrane is freely permeable to substrate and cofactors for peroxisomal fatty-acid oxidation remain open questions. In the second part of the present study, it is shown that available homogenization and centrifugation techniques result in considerable damage to the peroxisomes and that various treatments, deleterious to the peroxisomal membrane, do not stimulate palmitoyl-CoA oxidation provided ATP is present. Further results show that an energy-requiring step other than activation is involved in peroxisomal fatty-acid oxidation.

Experimental

Animals

Male Wistar rats maintained on a stock laboratory diet were used. The clofibrate treatment consisted of a daily subcutaneous injection of 200 mg/kg body wt. for 14 consecutive days. Control rats received a sham injection. Initial weight of the rats was approx. 100 g. At the end of the treatment they weighed 180–200 g. There was no

difference in body weight between control and treated rats, but liver weight was increased by 15–20% in the treated group.

Preparation of liver homogenates and cell fractionation

Liver homogenates (25%, w/v) in 0.25 M-sucrose, containing 0.1% ethanol to prevent the formation of the inactive catalase compound II (Leighton *et al.*, 1968), were prepared in a Dounce homogenizer (Kontes, Vineland, NJ, U.S.A.) with six strokes each of a loose and a tight plunger. The homogenates were fractionated by the method of Leighton *et al.* (1968) in a ν fraction, containing the nuclei and most of the mitochondria, a λ fraction, mostly enriched in peroxisomes and lysosomes, and a ψ fraction, made up mostly of 'microsomes' and soluble components.

Enzyme measurements

Catalase (EC 1.11.1.6), a marker enzyme for the peroxisomal matrix, was assayed by the method of Baudhuin *et al.* (1964). Total catalase activity was measured after pretreatment of the enzyme source with 10 mg of Triton X-100 per ml for 2 min to disrupt the peroxisomal membranes. Free catalase activity refers to the activity measured in 0.25 M-sucrose in the absence of detergent. Urate oxidase (EC 1.7.3.3), a marker enzyme for the peroxisomal core, was assayed as described by Leighton *et al.* (1968). Enzyme activities are expressed in the units used by the authors mentioned. Protein was determined by the method of Lowry *et al.* (1951), with bovine albumin as standard.

Measurement of acid-soluble labelled oxidation products

Mitochondrial or peroxisomal oxidation of [$1-^{14}C$]palmitoyl-CoA was measured in liver homogenates or λ fractions as described (Mannaerts *et al.*, 1979). Peroxisomal oxidation was stimulated by approx. 10% of FAD concentrations from 25 to 100 μ M. Because of its limited effect, FAD was not routinely included in the assay mixture.

Determination of acetyl-CoA, acetylcarnitine and acetate

Acetyl-CoA, acetylcarnitine and acetate production was measured after incubation of λ fractions in the reaction mixture for peroxisomal [$1-^{14}C$]palmitoyl-CoA oxidation, except that unlabelled palmitoyl-CoA (0.2 mM) was used as substrate. Incubation time was 10 min at 37°C. Reactions were terminated with $HClO_4$ and care was taken not to exceed pH 5 during neutralization to avoid hydrolysis of acetyl-CoA and acetylcarnitine. Acetyl-CoA, acetylcarnitine and acetate were sequentially measured in these neutralized $HClO_4$ extracts. Acetyl-CoA was determined by means of the citrate

synthase reaction coupled to the malate dehydrogenase reaction with reduction of NAD^+ , using a slight modification of the method described by Decker (1970). The final reaction volume of 2 ml contained triethanolamine/HCl (20 mM), pH 7.8, EDTA (1.25 mM), CoA (1 mM), dithiothreitol (5 mM), ATP (10 mM), MgCl_2 (25 mM), L-malate (10 mM), NAD^+ (0.5 mM) and 1 ml of the neutralized HClO_4 extract. The reaction was run at 25°C.

The sequential increase in A_{340} on addition of 60 units of malate dehydrogenase and 2.2 units of citrate synthase served to calculate the amount of acetyl-CoA present, taking into account the non-linear stoichiometry between acetyl-CoA reacted and NAD^+ reduced (Decker, 1970). Then the successive increase in A_{340} on introduction to the same cuvette of first 4 units of carnitine acetyltransferase (which converts acetylcarnitine into acetyl-CoA) and then 0.15 unit of acetyl-CoA synthetase (which converts acetate into acetyl-CoA) served to calculate the amount of acetylcarnitine and acetate present. Appropriate blanks and standards were always carried through the HClO_4 treatment and subsequent assay procedures. The recovery of acetylcarnitine and acetate was essentially 100%, and that of acetyl-CoA was $85 \pm 3\%$ ($n = 10$).

H₂O₂ measurement

H_2O_2 production by purified peroxisomal fractions oxidizing palmitoyl-CoA was measured indirectly as the rate of formaldehyde production from methanol during the peroxidatic decomposition of H_2O_2 by catalase (Keilin & Hartree, 1945; Mannaerts *et al.*, 1979). λ fractions were incubated in the assay mixture for peroxisomal oxidation containing unlabelled palmitoyl-CoA (0.2 mM), methanol (50 mM), 4-methylpyrazole (1 mM), as an inhibitor of alcohol dehydrogenase (Theorell *et al.*, 1972), and semicarbazide (10 mM), as an aldehyde-trapping agent. KCN, which inhibits catalase, was omitted from these reaction mixtures. Addition of rotenone (4 $\mu\text{g}/\text{ml}$) plus antimycin (20 $\mu\text{g}/\text{ml}$), which block mitochondrial oxidation, did not affect palmitoyl-CoA-dependent H_2O_2 production, showing that the H_2O_2 was formed by the peroxisomes. Incubation time was 5 or 10 min at 37°C. Incubations were stopped with trichloroacetic acid (final concentration, 7.5%, w/v) and formaldehyde was measured by the method of Nash (1953) with a standard curve obtained by the addition of known amounts of formaldehyde to complete reaction mixtures.

A recovery factor of 0.83 ± 0.03 ($n = 18$) for the formaldehyde production rates was obtained from incubations where uric acid (0.2 mM) instead of palmitoyl-CoA was the H_2O_2 -generating substrate. In addition to the components listed above, these incubations contained an inhibitor of urate oxidase,

2,6,8-trichloropurine (0.2–0.5 mM), to keep the H_2O_2 -production rates within the range observed during palmitoyl-CoA oxidation.

Application of this recovery factor to the formaldehyde-production rates during palmitoyl-CoA oxidation allowed for the correction of the proportion of catalatic H_2O_2 decomposition as well as for the correction of possible incomplete recoveries of the formaldehyde production during peroxidatic H_2O_2 decomposition. This recovery problem has been discussed in detail by Mannaerts *et al.* (1979).

Structural integrity of the peroxisomes

To have some estimation of the structural integrity of the peroxisomes during incubation, λ fractions were incubated under various experimental conditions in the assay mixture for peroxisomal palmitoyl-CoA oxidation. Incubation time was 5 or 10 min at 37°C. At the end of the incubation period, a portion of the reaction mixture was taken for the measurement of free and total catalase activity as described above. KCN, which inhibits catalase, was omitted from these reaction mixtures.

Since catalase is a latent enzyme (Adams & Burgess, 1957), free catalase activity measured after incubation of the λ fractions and expressed as the percentage of total catalase activity present would seem a good index for the degree of structural damage to the peroxisomal membranes. However, one should bear in mind that approx. 40% of the total catalase activity present in a crude rat liver homogenate does not sediment during centrifugation, and is recovered in the high-speed supernatant (Leighton *et al.*, 1968; Mannaerts *et al.*, 1979). All of this catalase activity can be measured as free catalase. In contrast, urate oxidase, a marker enzyme for the peroxisomal core, completely sediments. If one then assumes that all catalase and urate oxidase were originally confined to the peroxisomes, the centrifugation data indicate that purified peroxisomal fractions may contain considerably more damaged peroxisomes than might be deduced from free catalase measurements in these fractions. We therefore measured total catalase as well as urate oxidase in freshly prepared λ fractions before incubation and calculated for each enzyme the percentage of total homogenate activity present in these fractions. If no catalase leaks from the peroxisomes during homogenization and centrifugation, which appears not to be the case, one would expect identical percentages of total homogenate catalase and urate oxidase in the λ fractions so that the ratio of the percentages of catalase to urate oxidase would equal unity. The mean catalase/urate oxidase ratios found were 0.57 ± 0.01 and 0.58 ± 0.03 in λ fractions of livers from control ($n = 8$) and clofibrate-treated ($n = 6$) rats respec-

tively, again indicating that approx. 40% of the catalase originally present in the peroxisomes was no longer associated with these organelles in the λ fractions. To have a better index for the structural damage to the peroxisomes than the free catalase activity measured after incubation, which would not include the loss of catalase caused by homogenization and centrifugation, the term 'corrected' free catalase was introduced. This term is defined as the sum of (a) the percentage of catalase activity that had already leaked from the peroxisomes during preparation of the λ fractions $\{[1 - (\text{catalase/urate oxidase ratio})] \times 100\}$ and (b) free catalase activity measured after incubation of these λ fractions expressed as the percentage of total catalase activity present during incubation. Total catalase activity itself was expressed as the percentage that had not been released during homogenization and centrifugation, i.e. $(\text{catalase/urate oxidase ratio}) \times 100$.

As mentioned above, the correction of free catalase activity is based on the assumption that in the liver cell all catalase activity is confined to the peroxisomes and that none of the activity exists as the soluble enzyme. Since the occurrence of soluble catalase activity in rat liver is still a matter of debate (Leighton *et al.*, 1975) our 'corrected' free catalase activities necessarily reflect a maximal estimate of peroxisomal damage.

Materials

Bovine albumin (fraction V, powder) and 2,6,8-trichloropurine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. The fraction V albumin was defatted by the method of Chen (1967), dialysed in the cold for 48 h against water and freeze-dried. $[1-^{14}\text{C}]$ Palmitoyl-CoA was from The Radiochemical Centre, Amersham, Bucks., U.K. Coenzyme A, acetyl-CoA and unlabelled palmitoyl-CoA were from P.-L. Biochemicals Inc., Mil-

waukee, WI, U.S.A. NAD⁺, FAD, adenine nucleotides, dithiothreitol, enzymes and coenzymes used for various determinations were purchased from Boehringer G.m.b.H., Mannheim, Germany. Triton X-100 was from BDH Chemicals, Poole, Dorset, U.K.; sodium deoxycholate, digitonin, uric acid and semicarbazide were from E. Merck, Darmstadt, Germany. 4-Methylpyrazole was from Research Plus Laboratories Inc., Denville, NJ, U.S.A. L-(–)-Carnitine and L-(–)-acetylcarnitine were kindly donated by Otsuka Pharmaceutical Factory Inc., Naruto, Tokushima, Japan. Clofibrate was generously provided by I.C.I., Macclesfield, Cheshire, U.K.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Significance of differences between means was established by Student's *t* test.

Results

H₂O₂ production as an index for the release of acetyl units during peroxisomal palmitoyl-CoA oxidation

The first dehydrogenation step of the peroxisomal β -oxidation sequence, catalysed by acyl-CoA oxidase, involves reduction of O₂ to H₂O₂. Since this reaction appears to be rate-limiting for the whole oxidation sequence (Osumi & Hashimoto, 1979; Hryb & Hogg, 1979; Inestrosa *et al.*, 1979a), the amount of H₂O₂ produced during peroxisomal fatty-acid oxidation could be a reliable index for the amount of acetyl units released. To test this assumption, H₂O₂ production as well as the release of acetyl units were measured during the oxidation of palmitoyl-CoA by purified peroxisomes of livers from clofibrate-treated rats (Table 1).

In the absence of albumin (high unbound-pal-

Table 1. Production rates of H₂O₂ and acetyl units (acetyl-CoA, acetylcarnitine and acetate) during palmitoyl-CoA oxidation by purified peroxisomes of livers from clofibrate-treated rats

λ fractions were incubated for 10 min at 37°C either in the standard reaction mixture for peroxisomal oxidation (for the measurement of acetyl units) or in the assay mixture for the measurement of H₂O₂ as described in the Experimental section. H₂O₂ production was measured indirectly as formaldehyde production from methanol during the peroxidatic decomposition of H₂O₂. H₂O₂-production rates were calculated from the formaldehyde-production rates taking into account the recovery factor obtained with uric acid as the H₂O₂-generating substrate. The term 'Acetyl units' represents the sum of acetyl-CoA, acetylcarnitine and acetate. Results are corrected for the formation of H₂O₂ or acetyl units in the absence of palmitoyl-CoA and represent means $\pm$ S.E.M. for three experiments. At $\nu = \infty$, no albumin was present; at $\nu = 1.67$, the palmitoyl-CoA/albumin ratio was 1.67.

Incubation conditions	Production rates ($\mu\text{mol/min per g of liver}$) of:					B/A
	H ₂ O ₂ (A)	Acetyl-CoA	Acetyl- carnitine	Acetate	Acetyl units (B)	
$\nu = \infty$; CoA (0.5 mM), no carnitine	1.18 $\pm$ 0.17	1.20 $\pm$ 0.12	—	—	1.20 $\pm$ 0.12	1.02
$\nu = 1.67$; CoA (0.5 mM), no carnitine	0.27 $\pm$ 0.04	0.07 $\pm$ 0.01	—	0.11 $\pm$ 0.03	0.18 $\pm$ 0.04	0.67
$\nu = 1.67$; no CoA, carnitine (1.0 mM)	0.31 $\pm$ 0.06	0.04 $\pm$ 0.01	0.26 $\pm$ 0.05	0.03 $\pm$ 0.01	0.32 $\pm$ 0.06	1.03

mitoyl-CoA concentration) rates of H_2O_2 production and release of acetyl units were equal, indicating that under this experimental condition H_2O_2 production is an excellent index for the formation of acetyl units. The end product was acetyl-CoA, a finding consistent with the data obtained by Lazarow (1978). However, when peroxisomal fatty-acid oxidation was measured at low unbound-palmitoyl-CoA concentration, i.e. at a palmitoyl-CoA/albumin ratio of 1.67, only 0.67 acetyl unit per equivalent of H_2O_2 could be detected suggesting that one-third of the acetyl units was metabolized further or that one-third of the oxidation cycles did not go to completion. In addition, more than 60% of the acetyl units were recovered as acetate, suggesting the presence of an acetyl-CoA deacylase either in the peroxisomes or in contaminating organelles. Since no acetate was formed in the absence of albumin and since acetate production at a molar palmitoyl-CoA/albumin ratio of 1.67 was potently suppressed in the presence of 0.5 mg of Triton X-100 per ml (16% acetate; 84% acetyl-CoA; mean for three experiments, results not shown), the putative acetyl-CoA deacylase may be sensitive to detergents (Triton X-100 or palmitoyl-CoA). Alternatively, the deacylase may be located in the peroxisomes and released on treatment with detergents so that the solubilized enzyme would become less accessible for acetyl-CoA produced in the peroxisomes.

Next, 1 mM-carnitine was added to the reaction mixture containing albumin in an attempt to trap acetyl-CoA as acetylcarnitine through the action of the peroxisomal carnitine acetyltransferase. The activity of this enzyme is severalfold increased after clofibrate treatment (Kähönen, 1976; Markwell *et al.*, 1977). Since in purified peroxisomal fractions as in homogenates (Mannaerts *et al.*, 1979) peroxisomal palmitoyl-CoA oxidation was only slightly increased by the addition of exogenous CoA, it was omitted from the reaction mixture to displace the equilibrium of the reaction towards acetyl-carnitine formation. As can be seen from Table 1 the bulk of acetyl units was now in the form of acetylcarnitine and, more interestingly, rates of acetyl production equalled rates of H_2O_2 formation. These results indicate that partial-oxidation cycles do not occur during peroxisomal fatty-acid oxidation and that H_2O_2 production is a good index for the release of acetyl units, both at low and high unbound-palmitoyl-CoA concentrations. In this respect it should be mentioned that while this manuscript was in preparation Inestrosa *et al.* (1979a) measured the activity of the acyl-CoA oxidase by a similar detection method for H_2O_2 . These authors did not measure the release of acetyl units but found a 1:1 stoichiometry for H_2O_2 generation and NAD^+ reduction, occurring at the second dehydrogenation step of β -oxidation. Our

results further show that, in the presence of carnitine, acetylcarnitine can be formed by the peroxisomal carnitine acetyltransferase, a process that may be important for possible further metabolism of the acetyl units in the mitochondria. Finally, the fact that one-third of the acetyl units released at a molar palmitoyl-CoA/albumin ratio of 1.67 could not be detected in the absence of carnitine suggests that they were partly metabolized. Conversion into ketone bodies or CO_2 by contaminating mitochondria seems unlikely since no labelled CO_2 or labelled ketones could be detected when $[1-^{14}C]$ -palmitoyl-CoA oxidation was followed under identical conditions. Whether contaminating organelles or perhaps the peroxisome itself might be able to convert acetyl-CoA into some other compound remains to be determined.

Stoichiometry of peroxisomal β -oxidation as a function of the molar palmitoyl-CoA/albumin ratio

λ fractions of livers from clofibrate-treated rats were used to determine the number of acetyl units released per molecule of palmitoyl-CoA oxidized (C_2/C_{16} ratio) as a function of increasing unbound-palmitoyl-CoA concentrations (increasing molar palmitoyl-CoA/albumin ratios). The substrate was $[1-^{14}C]$ palmitoyl-CoA. The generation of acid-soluble labelled oxidation products served to calculate the number of substrate molecules that entered β -oxidation, and the production of H_2O_2 was taken as a measure of the corresponding release of acetyl units. The formation of labelled acid-soluble oxidation products was always measured in the presence of methanol, 4-methylpyrazole and semicarbazide. These agents, required for the measurement of H_2O_2 , did not affect the production rates of acid-soluble material. The results (Table 2) show that, at a molar palmitoyl-CoA/albumin ratio of 1.67, approx. 2.5 acetyl units were released per acyl-CoA oxidized. Unexpectedly, the number of acetyl units released per palmitoyl-CoA equivalent decreased rapidly with increasing molar palmitoyl-CoA/albumin ratios to reach a value of 1 at a ratio of 6.68, indicating that palmitoyl-CoA was less extensively degraded at increased unbound concentrations. Nevertheless, the total acetyl flux through the system rose with increasing palmitoyl-CoA/albumin ratios.

Comparison of the capacity of the mitochondrial and peroxisomal fatty-acid oxidizing systems in liver homogenates as a function of the molar palmitoyl-CoA/albumin ratio

Since peroxisomes, unlike mitochondria, do not degrade palmitoyl-CoA completely (see the introduction), comparison of production rates of labelled oxidation products from $[1-^{14}C]$ palmitoyl-CoA by both systems is inaccurate (Mannaerts *et al.*, 1979).

However, use of the stoichiometry values shown in Table 2 allows for the calculation of the peroxisomal acetyl flux and for exact comparison with mitochondria. Fig. 1 shows rates of mitochondrial and peroxisomal [1-¹⁴C]palmitoyl-CoA oxidation in whole-liver homogenates from control (*a* and *b*) and from clofibrate-treated rats (*c* and *d*). The oxidation rates were measured at the same molar palmitoyl-CoA/albumin ratios as those presented in Table 2. In (*a*) and (*c*) rates are plotted as μmol of [1-¹⁴C]palmitoyl-CoA oxidized, computed from the

formation of acid-soluble labelled oxidation products, whereas in (*b*) and (*d*) the same oxidation rates are expressed in terms of acetyl units. Comparison of the capacity of the mitochondrial and peroxisomal fatty-acid oxidizing systems has no physiological meaning at high palmitoyl-CoA/albumin ratios because of the prominent detergent effects of the substrate under these conditions. At low molar palmitoyl-CoA/albumin ratios, e.g. at a ratio of 1.67, the ratio of mitochondrial acetyl flux to peroxisomal acetyl flux was 21.5 and 12.3 in liver

Table 2. Effect of increasing molar palmitoyl-CoA/albumin ratios on the formation of H₂O₂ and acid-soluble labelled oxidation products during [1-¹⁴C]palmitoyl-CoA oxidation by purified peroxisomes of livers from clofibrate-treated rats H₂O₂ production was measured indirectly as formaldehyde production from methanol during the peroxidatic decomposition of H₂O₂. λ fractions were incubated for 5 min at 37°C. For the calculation of H₂O₂-production rates, see Table 1. Results represent means $\pm$ S.E.M. for the numbers of experiments indicated in parentheses.

Molar palmitoyl-CoA/ albumin ratio	Formation of acid-soluble labelled oxidation products (B)		
	H ₂ O ₂ production (A) ($\mu\text{mol}/\text{min}$ per g of liver)	(μmol of palmitoyl-CoA oxidized/ min per g of liver)	C ₂ /C ₁₆ ratio (A/B)
1.67 (8)	0.37 $\pm$ 0.04	0.15 $\pm$ 0.01	2.46 $\pm$ 0.16
3.33 (7)	0.65 $\pm$ 0.12	0.34 $\pm$ 0.04	1.94 $\pm$ 0.29
6.68 (4)	1.45 $\pm$ 0.19	1.43 $\pm$ 0.15	1.02 $\pm$ 0.15
13.36 (4)	1.82 $\pm$ 0.21	1.87 $\pm$ 0.11	0.97 $\pm$ 0.09
25.05 (4)	1.94 $\pm$ 0.23	1.88 $\pm$ 0.14	1.03 $\pm$ 0.06
∞ (5)	1.62 $\pm$ 0.08	1.66 $\pm$ 0.08	0.98 $\pm$ 0.06

Fig. 1. Mitochondrial and peroxisomal oxidation rates of [1-¹⁴C]palmitoyl-CoA in liver homogenates from control (*a* and *b*) and clofibrate-treated (*c* and *d*) rats versus increasing palmitoyl-CoA/albumin ratios Symbols: O, and ●, mitochondrial oxidation; □ and ■, peroxisomal oxidation. The curves depicted in (*a*) and (*c*) represent the rates of formation of acid-soluble labelled oxidation products expressed in terms of palmitate equivalents oxidized. Incubation time was 5 min at 37°C. (*b*) and (*d*) represent production rates of acetyl (C₂) units obtained by multiplying the rates of [1-¹⁴C]palmitoyl-CoA oxidation (*a* and *c*) by 8 (mitochondrial oxidation) or by the corresponding C₂/C₁₆ ratios represented in Table 2 (peroxisomal oxidation).

homogenates from control and clofibrate-treated rats respectively. Thus, at low unbound-substrate concentrations, a condition likely to mimic the situation *in vivo*, peroxisomal oxidation only represented 5% of mitochondrial oxidation in liver homogenates from control rats and only 8% in homogenates from clofibrate-treated rats. These low percentages are in excellent agreement with our previous estimation of the activity of the peroxisomal pathway in the intact liver cell based on H_2O_2 measurements during palmitate and oleate oxidation by isolated hepatocytes (Mannaerts *et al.*, 1979).

Structural integrity of peroxisomes and peroxisomal fatty-acid oxidation

In the preceding paragraph it was assumed that the gradual increase of peroxisomal palmitoyl-CoA oxidation with increasing palmitoyl-CoA/albumin ratios was due to a simple substrate concentration effect. However, the increased rates of oxidation at increasing unbound palmitoyl-CoA concentrations might be due to a combination of a substrate concentration effect with the known detergent action of palmitoyl-CoA, inferring latency of peroxisomal palmitoyl-CoA oxidation. Similarly, the decrease in the ratio of H_2O_2 produced to palmitoyl-CoA oxidized with increasing substrate/albumin ratios (Table 2) in purified peroxisomal fractions might be the result of either a substrate concentration effect or of the combination of substrate concentration and the detergent effect of palmitoyl-CoA. Indeed, if one assumes that the peroxisomal membrane is readily permeable for palmitoyl-CoA, increasing unbound palmitoyl-CoA concentrations might gradually compete more easily with full-cycle intermediates (C_{14} fatty-acyl-CoA, C_{12} fatty-acyl-CoA, etc.) formed during β -oxidation. The result would be that more palmitoyl-CoA molecules would enter β -oxidation, but that they would be degraded less extensively. Alternatively it is possible that the substrate permeability of the peroxisomal membrane is restricted. At low substrate/albumin ratios, palmitoyl-CoA would

proceed through multiple consecutive β -oxidation cycles, but at high ratios loss of structural integrity of the peroxisomes by the detergent action of the substrate might release the full-cycle intermediates from their sites of oxidation and favour the access of more palmitoyl-CoA molecules to these sites.

Experiments were designed to assess the structural integrity of the peroxisomes in purified peroxisomal fractions and to study the effects of decreased structural integrity on palmitoyl-CoA oxidation. Structural integrity of the peroxisomal population in λ fractions was estimated by assaying the latency of catalase. Free catalase in freshly prepared λ fractions of livers from control ($n = 7$) and clofibrate-treated rats ($n = 5$) was $11.5 \pm 2.6\%$ and $15.3 \pm 2.0\%$ of total catalase activity respectively. 'Corrected' free catalase values (see the Experimental section) were $49.2 \pm 1.7\%$ and $49.4 \pm 3.9\%$ respectively, suggesting that nearly half of the peroxisomes in freshly prepared λ fractions were damaged. Table 3 shows the effect of increasing palmitoyl-CoA/albumin ratios on free catalase in λ fractions of livers from clofibrate-treated rats oxidizing palmitoyl-CoA under peroxisomal conditions. The results indicate that incubation alone, carried out in the absence of palmitoyl-CoA, was responsible for some further damage to the peroxisomes. At molar palmitoyl-CoA/albumin ratios of 1.67 and 3.33, palmitoyl-CoA had little or no additional effect on the structural integrity of the peroxisomes, at least as judged from free catalase measurements. However, at ratios of 6.68 and above, the detergent action of the substrate appeared to severely damage the peroxisomal membrane since no latency of catalase could be demonstrated under these conditions.

The effects on palmitoyl-CoA oxidation of several treatments, known to decrease the structural integrity of peroxisomes, were also examined. Triton X-100 at a concentration of 0.5 mg/ml, which completely abolished the latency of catalase, stimulated the formation of acid-soluble oxidation pro-

Table 3. *Effect of increasing molar palmitoyl-CoA/albumin ratios on free catalase activity in purified peroxisomal fractions of livers from clofibrate-treated rats*

λ fractions were incubated for 5 min at 37°C in the reaction mixture used for the measurement of H_2O_2 production as described in the Experimental section. Results represent means for two experiments. Values given in parentheses refer to free catalase activity in the presence of 7.2 mg of albumin/ml of incubation mixture (the amount present in the incubations at $\bar{v} = 1.67$), but in the absence of palmitoyl-CoA.

	Molar palmitoyl-CoA/albumin ratio ($\bar{v}$)	Free catalase activity (% of total)				
		1.67	3.33	6.68	13.36	25.05
Uncorrected	...	28 (30)	36	100	100	104
Corrected*	...	59 (61)	64	100	100	102

* For the calculation of 'corrected' free catalase activity, see the Experimental section.

ducts approx. 1.5-fold (Table 4). This stimulation closely paralleled the 1.6-fold increase in 'corrected' free catalase activity observed in these experiments. However, the detergent did not affect the rate of H_2O_2 production indicating that the increased formation of acid-soluble labelled oxidation products in the presence of Triton X-100 was not due to latency of peroxisomal palmitoyl-CoA oxidation but apparently to the fact that more palmitoyl-CoA molecules went through less β -oxidation cycles.

Digitonin (0.4 mg/ml), deoxycholate (0.4 mg/ml), freeze-thawing, osmotic shock (resulting from dilution of a λ fraction, suspended in 0.8 M-sucrose, to 0.36 M-sucrose) and mechanical disruption [by homogenization of a λ fraction for 2 min with an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany)] resulted in various degrees of increase of free catalase but all treatments caused an inhibition of H_2O_2 production (results not shown) so that these experiments did not offer any indication of latency of peroxisomal fatty-acid oxidation. In addition, all these treatments, including the addition of Triton X-100 (Table 4), caused a decrease in C_2/C_{16} ratio.

Effect of ATP on peroxisomal palmitoyl-CoA oxidation

Preliminary experiments with whole-liver homogenates had shown that ATP had a stimulatory effect on peroxisomal oxidation of fatty-acyl-CoA esters. Therefore, all experiments presented thus far were carried out in the presence of 4 mM-ATP. Since the peroxisomal β -oxidation sequence is thought not to be coupled to a phosphorylating system (Lazarow & de Duve, 1976; Mannaerts *et al.*, 1979), it was unlikely that the stimulatory effect of ATP could be explained by the formation of ADP that would then serve as phosphate acceptor. We therefore wanted to characterize this phenomenon

more precisely and thus studied the effects of ATP and other adenine nucleotides on palmitoyl-CoA oxidation by purified peroxisomal fractions.

Table 5 shows the effects of ATP and ADP on H_2O_2 production and formation of acid-soluble labelled oxidation products during [$1-^{14}C$]palmitoyl-CoA oxidation by λ fractions of livers from clofibrate-treated rats. At a molar substrate/albumin ratio of 1.67, ATP (4 mM) stimulated H_2O_2 production and formation of acid-soluble labelled oxidation products about 2-fold. Interestingly, this stimulatory action of ATP disappeared in the presence of Triton X-100 or when palmitoyl-CoA oxidation was assayed in the absence of albumin ($\bar{v} = \infty$, where $\bar{v}$ is the molar substrate/albumin ratio), two conditions in which the structural integrity of the peroxisomes is seriously compromised (Tables 3 and 4). ADP (4 mM) and AMP (4 mM, results not shown) had no effect on palmitoyl-CoA oxidation, confirming the absence of a coupled phosphorylating system in peroxisomes. Inclusion of ATP or ADP had no effect on the C_2/C_{16} ratio under the different conditions tested, indicating that the number of β -oxidation cycles through which palmitoyl-CoA was degraded did not change.

Table 5 further demonstrates that the effects of Triton X-100 on peroxisomal oxidation of palmitoyl-CoA assayed at a molar palmitoyl-CoA/albumin ratio of 1.67 were completely different in the absence or presence of ATP. In the absence of ATP, Triton stimulated both the formation of H_2O_2 and acid-soluble labelled oxidation products 2.5–3-fold. In contrast, in the presence of ATP the detergent no longer stimulated the production of H_2O_2 , a finding consistent with the results shown in Table 4. In addition, the stimulatory effect of Triton on the formation of acid-soluble labelled oxidation products was less pronounced.

The effect of ATP on peroxisomal [$1-^{14}C$]pal-

Table 4. *Effect of Triton X-100 on the formation of H_2O_2 and acid-soluble labelled oxidation products during [$1-^{14}C$]palmitoyl-CoA oxidation by purified peroxisomes of livers from clofibrate-treated rats*

H_2O_2 production was measured indirectly as formaldehyde production from methanol during the peroxidatic decomposition of H_2O_2 . λ fractions were incubated for 5 min at 37°C. The molar palmitoyl-CoA/albumin ratio was 1.67. For the calculation of H_2O_2 -production rates, see Table 1. Results represent means $\pm$ S.E.M. for the numbers of experiments indicated in parentheses.

Incubation period (min)	Additions	Formation of acid-soluble labelled oxidation products (B)		
		H_2O_2 production (A) (μ mol/min per g of liver)	(μ mol of palmitoyl-CoA oxidized/ min per g of liver)	C_2/C_{16} ratio (A/B)
5	None (3)	0.41 ± 0.10	0.16 ± 0.02	2.54 ± 0.27
	Triton X-100 (0.5 mg/ml) (3)	0.39 ± 0.15	$0.24 \pm 0.03^*$	$1.56 \pm 0.41^*$
10	None (7)	0.30 ± 0.06	0.09 ± 0.01	3.06 ± 0.18
	Triton X-100 (0.5 mg/ml) (7)	0.29 ± 0.07	0.14 ± 0.03	$2.06 \pm 0.09^*$

* Significantly different from respective control, $P < 0.05$.

Table 5. Effect of ATP and ADP on the formation of H_2O_2 and acid-soluble labelled oxidation products during $[1-^{14}C]$ palmitoyl-CoA oxidation by purified peroxisomes of livers from clofibrate-treated rats

H_2O_2 production was measured indirectly as formaldehyde production from methanol during the peroxidatic decomposition of H_2O_2 . λ fractions were incubated for 5 min at 37°C in the absence or presence of ATP (4 mM) or ADP (4 mM). Peroxisomal palmitoyl-CoA oxidation was measured at a molar substrate/albumin ratio ($\bar{\nu}$) of 1.67 or in the absence of albumin ($\bar{\nu} = \infty$). H_2O_2 -production rates were calculated as described in Table 1. Results represent means $\pm$ S.E.M. for four experiments.

Incubation conditions		H_2O_2 production (A) ($\mu\text{mol}/\text{min}$ per g of liver)	Formation of acid-soluble labelled oxidation products (B) (μmol of palmitoyl-CoA oxidized/ min per g of liver)	C_2/C_{16} ratio (A/B)
$\bar{\nu} = 1.67$	ATP and ADP absent	0.13 ± 0.05	0.06 ± 0.01	2.36 ± 0.35
	ATP (4 mM)	$0.29 \pm 0.07^*$	$0.12 \pm 0.01^*$	2.57 ± 0.25
	ADP (4 mM)	0.14 ± 0.03	0.05 ± 0.01	2.63 ± 0.21
$\bar{\nu} = 1.67$ [+ Triton X-100 (0.5 mg/ml)]	ATP and ADP absent	0.33 ± 0.05	0.17 ± 0.04	2.10 ± 0.33
	ATP (4 mM)	0.30 ± 0.09	0.15 ± 0.04	1.97 ± 0.38
	ADP (4 mM)	0.32 ± 0.07	0.16 ± 0.04	1.98 ± 0.23
$\bar{\nu} = \infty$	ATP absent	1.67 ± 0.52	1.22 ± 0.47	1.45 ± 0.13
	ATP (4 mM)	1.66 ± 0.49	1.14 ± 0.48	1.62 ± 0.21

* Significantly different from control, $P < 0.05$.

Fig. 2. Effect of ATP and Mg^{2+} on $[1-^{14}C]$ palmitoyl-CoA oxidation by purified peroxisomal fractions of livers from clofibrate-treated rats

Peroxisomal oxidation was measured at a molar palmitoyl-CoA/albumin ratio of 1.67. Results represent rates of formation of acid-soluble labelled oxidation products expressed in terms of palmitate equivalents oxidized and are means for two experiments. Incubation time was 5 min at 37°C. Symbols: $\bullet$, Mg^{2+} omitted; $\bullet$, 1.25 mM- Mg^{2+} ; $\circ$, 5 mM- Mg^{2+} ; Δ , presence of Triton X-100 (0.5 mg/ml). The curves in the presence of Triton X-100 were not affected by the Mg^{2+} concentration and are represented as one curve.

mitoyl-CoA oxidation (at a palmitoyl-CoA/albumin ratio of 1.67) was also studied at different Mg^{2+} concentrations. It is clear from the data in Fig. 2 that the stimulatory effect of ATP on the formation of

acid-soluble labelled oxidation products was completely lost when Mg^{2+} was omitted from the incubation medium. At 1.25 mM- Mg^{2+} , the concentration present in the modified Krebs–Henseleit bicarbonate buffer, a pronounced stimulation was already seen with 0.4 mM-ATP. A plateau was reached at a concentration of 0.8 mM-ATP. Further increases in the Mg^{2+} concentration in the incubation medium to 5 mM did not significantly alter the shape of the curve.

The possibility that extensive palmitoyl-CoA hydrolysis during incubation of λ fractions might seriously decrease the palmitoyl-CoA concentration and that the stimulatory effect of ATP on peroxisomal palmitoyl-CoA oxidation was due to reactivation of the palmitate formed was also considered. However, rates of palmitoyl-CoA hydrolysis measured during incubation of λ fractions did not differ in the absence or presence of increasing ATP concentrations. Moreover, at the end of a 5 min incubation period maximally 5% of the palmitoyl-CoA initially present could be recovered as palmitate. Since palmitate formation was about equal in the presence or absence of a λ fraction, palmitoyl-CoA hydrolysis appeared to be a non-enzymic process (results not shown).

Discussion

Our experiments have shown that the production of H_2O_2 during palmitoyl-CoA oxidation by purified peroxisomal fractions is an excellent index for the release of acetyl units (Table 1). At low unbound palmitoyl-CoA concentrations the capacity of the peroxisomal fatty-acid oxidizing system, expressed

in terms of total acetyl flux, was less than 10% of the capacity of the mitochondria, both in homogenates from control and clofibrate-treated rats (Fig. 1). This value closely agrees with our previous estimation of the contribution of the peroxisomes to overall fatty-acid oxidation in isolated hepatocytes (Mannaerts *et al.*, 1979).

The H_2O_2 measurements revealed that the number of acetyl units released per molecule of palmitoyl-CoA oxidized by purified peroxisomal fractions is strongly dependent on the incubation conditions, e.g. unbound substrate concentration, structural integrity of the peroxisomes (Tables 2 and 4). The overall picture that emerges from these experiments is that palmitoyl-CoA seems to be able to compete for oxidation with its full-cycle intermediates, a process that appears to be favoured by structural damage to the peroxisomes.

The theoretical maximum of 5 acetyl units released per molecule of palmitoyl-CoA entering peroxisomal β -oxidation (see the introduction) was never reached in our experiments, but some of our observations suggest that it may be attained *in vivo*. Indeed, all treatments that damaged the peroxisomes, caused a decrease in the C_2/C_{16} ratio. As suggested by the 'corrected' free catalase determinations, roughly 50% of the peroxisomes present in our incubations may have been damaged (Table 3). Thus, *in vivo*, fatty-acyl-CoA esters may well be degraded more efficiently than in partially damaged peroxisomes *in vitro*.

Finally, in the presence of ATP, we could not find any indication for latency of peroxisomal palmitoyl-CoA oxidation (Table 5). In contrast, when ATP was omitted Triton X-100 increased both the number of palmitoyl-CoA molecules entering peroxisomal β -oxidation and total acetyl flux through the system 2–3-fold. The effect of ATP on peroxisomal fatty-acyl-CoA oxidation was neither due to the generation of ADP nor due to the reactivation of palmitate formed by hydrolysis of the substrate. The facts that the effect of ATP is lost when the peroxisomal membranes are disrupted by the use of Triton X-100 and that the stimulatory effects of ATP and of Triton X-100 (in the absence of ATP) are essentially the same suggest that ATP may facilitate the transport of palmitoyl-CoA, or possibly one of the cofactors required for its oxidation, through the peroxisomal membrane. The Mg^{2+} dependency (Fig. 2) of the stimulatory effect suggests that the molecular basis may involve a kinase reaction or an Mg^{2+} -dependent ATPase activity. Further studies are required to characterize

the exact mechanism through which ATP stimulates peroxisomal palmitoyl-CoA oxidation.

We thank Mrs. G. Verhoeven-Vandebroek and Mr. L. Govaert for expert technical assistance and Miss M. Bateau for dedicated secretarial help. This work was supported by Grant 3.0031.79 from the Fonds voor Geneeskundig Wetenschappelijk Onderzoek and Grant OT/V/40 from the Onderzoeksfonds van de Katholieke Universiteit Leuven.

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