Biochem. J. (1979) **182**, 779–788
Printed in Great Britain

Detection of Peroxisomal Fatty Acyl-Coenzyme A Oxidase Activity

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(*Received* 30 *April* 1979)

It has been postulated that the peroxisomal fatty acid-oxidizing system [Lazarow & de Duve (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2043–2046; Lazarow (1978) *J. Biol. Chem.* **253**, 1522–1528] resembles that of mitochondria, except for the first oxidative reaction. In this step, O_2 would be directly reduced to H_2O_2 by an oxidase. Two specific procedures developed to detect the activity of the characteristic enzyme fatty acyl-CoA oxidase are presented, namely polarographic detection of palmitoyl-CoA-dependent cyanide-insensitive O_2 consumption and palmitoyl-CoA-dependent H_2O_2 generation coupled to the peroxidation of methanol in an antimycin A-insensitive reaction. Fatty acyl-CoA oxidase activity is stimulated by FAD, which supports the flavoprotein nature postulated for this enzyme. Its activity increases 7-fold per g wet wt. of liver in rats treated with nafenopin, a hypolipidaemic drug. Subcellular fractionation of livers from normal and nafenopin-treated animals provides evidence for its peroxisomal localization. The stoicheiometry for palmitoyl-CoA-dependent O_2 consumption, H_2O_2 generation and NAD^+ reduction is 1 : 1 : 1. This suggests that fatty acyl-CoA oxidase is the rate-limiting enzyme of the peroxisomal fatty acid-oxidizing system.

Rat liver peroxisomes posses a cyanide-insensitive fatty acyl-CoA-oxidizing system (Lazarow & de Duve, 1976) that is assumed to be homologous with the β-oxidation system in glyoxysomes (plant peroxisomes) from castor-bean endosperm described by Cooper & Beevers (1969). Both systems are characterized by their apparent independence from an electron-transport chain and by the direct transfer of electrons to O_2 with the production of H_2O_2. Lazarow (1978) has reported the occurrence, in peroxisomes from clofibrate-treated rats, of each of the classical β-oxidation reactions leading from the enoyl-CoA derivative to the release of acetyl-CoA and a shortened fatty acyl-CoA. The reaction responsible for the dehydrogenation of the fatty acyl-CoA has been attributed to a flavoprotein, by analogy with the mitochondrial system. In peroxisomes the reoxidation of the prosthetic group would be made directly by O_2 and would lead to the generation of H_2O_2. No quantitative determination, which is suitable for use with liver homogenates or crude subcellular fractions, has been developed for the first H_2O_2-generating oxidative step.

Quantitative studies of the peroxisomal fatty acid β-oxidation rely on the reduction of NAD^+ by a cyanide-insensitive fatty acyl-CoA-dependent reaction (Lazarow, 1978; Kawamoto *et al.*, 1978; Osumi & Hashimoto, 1978*a*). This indirect procedure has limitations, since, *in vitro*, mitochondrial as well as peroxisomal enoyl-CoA hydratase (EC 4.2.1.17) and β-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) participate.

We have developed quantitative procedures to determine fatty acyl-CoA-dependent H_2O_2 production and the cyanide-insensitive O_2 uptake by the postulated peroxisomal fatty acyl-CoA oxidase. Its activity increased 7-fold after treatment with nafenopin and the enzyme was shown to be localized in peroxisome-rich subcellular fractions. In addition, our observations support the role of FAD as the prosthetic group. Fatty acyl-CoA oxidase is apparently the rate-limiting enzyme, and would be responsible for the specificity of the system. Preliminary communications of these results have been made (Inestrosa *et al.*, 1977, 1978; Bronfman *et al.*, 1978).

Experimental and Results

Preparative procedures

Male Sprague–Dawley rats, 170–240g body wt., kept at 18–20°C with 12h daily cycles of artificial illumination, were fed on either a standard diet or on one that contained nafenopin {2-methyl-2-[4-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionic acid}. The drug was added 0.1 % (w/w) to standard pelleted rat food as a 1 % (w/v) solution in ethanol, allowing at least 24h for the solvent to evaporate, and was administered for 6–8 days. The animals, without previous food deprivation, were killed by decapitation and the livers were homogenized

(25–33 %, w/v) in cold 0.25M-sucrose with Teflon-pestle glass-vessel homogenizers.

Palmitoyl-CoA, acetyl-CoA and CoA were purchased from P–L Biochemicals, Milwaukee, WI, U.S.A. or Sigma Chemical Co., St. Louis, MO, U.S.A. L-Carnitine chloride was a gift from Dr. Yuko Kawashima, Otsuka Pharmaceutical Factory,Tokushima, Japan. NAD^+, $NADP^+$, ATP, FAD, cytochrome *c*, antimycin A, bovine serum albumin (fraction V), nicotinamide, sodium palmitate, 2-glycerophosphate, Triton X-100 and Tris were also obtained from Sigma Chemical Co. All other chemicals were analytical grade when commercially available. Nafenopin was a gift from Dr. Ernold Granzer, Farbwerke Hoechst A.G., Frankfurt am Main, Germany.

Fatty acyl-CoA-dependent H_2O_2 generation in liver homogenates

Methanol peroxidation to formaldehyde and H_2O_2, a reaction mediated by catalase (EC 1.11.1.6) (Keilin & Hartree, 1936), was used to detect the generation of H_2O_2. Catalase was not added to the homogenates, since the activity of endogenous catalase is enough to catalyse peroxidation reactions at the rates observed (Portwich & Aebi, 1960).

The efficiency of the system for the detection of H_2O_2 generation was checked from the activity of two well-characterized peroxisomal oxidases, L-α-hydroxy acid oxidase (EC 1.1.3.1) and urate oxidase (EC 1.7.3.3). The values obtained (Table 1) agree with the determinations made by direct spectrophotometry of perfused liver (Oshino *et al.*, 1973).

When sodium palmitate (1.0mM) was incubated with liver homogenates, 20mg of tissue in 1.5ml of 100mM-Tris/HCl, pH8.3, at 37°C for 60min, it produced only minor changes in the production of formaldehyde (Table 2). Formaldehyde was detected in the supernatant by the procedure of Nash (1953) after the addition of 80μl of 3M-trichloroacetic acid. Supplementation with sodium palmitate plus 0.1mM-CoA, 2.5mM-ATP and 5mM-$MgCl_2$ led to a 5-fold increase in the production of formaldehyde. This result is considered to be evidence that palmitate must be activated to the acyl-CoA derivative, by endogenous acyl-CoA synthetase (EC 6.2.1.3). In fact, we have detected a peroxisomal acyl-CoA synthetase in rat liver (M. Bronfman, N. C. Inestrosa & F. Leighton, unpublished results). In all the determinations of the rate of formaldehyde generation, a 5min preincubation in the absence of palmitate was required to exhaust endogenous substrates for H_2O_2-generating reactions.

If the incubation is carried out in the absence of palmitate for the entire 60min, a moderate basal H_2O_2 generation is detected, as shown in Tables 1 and 2. The basal values observed in nafenopin-treated rats are 6–8 times higher. These values probably represent the activity of the peroxisomal fatty acid-oxidizing system on endogenous fatty

Table 1. *Detection of H_2O_2 production by peroxisomal oxidases in liver homogenates*

Measurements were made by incubating liver homogenate from control rats (20mg wet wt. of tissue per test) in the incubation media described. Formaldehyde was measured after 60min at 37°C as described in the Experimental and Results section. All results are expressed as means±S.D. and the numbers of independent determinations are shown in parentheses.

Incubation medium	H_2O_2 generation (nmol of formaldehyde/min per g wet wt. of liver)
Basal: 100mM-Tris/HCl buffer, pH8.3,+100mM-methanol	25± 6 (7)
Basal +1.2mM-sodium glycollate	443±20 (5)
Basal +1.2mM-sodium urate	731±47 (5)

Table 2. *Minimal cofactor requirements for palmitate-induced H_2O_2 generation in liver homogenates*

Measurements were made by incubating liver homogenate from control rats (20mg wet wt. of tissue per test) in the incubation mixtures described. Formaldehyde was measured after 60min at 37°C as described in the Experimental and Results section. All results are expressed as means ± S.D. and the numbers of independent determinations are shown in parentheses. Significant differences between the basal value and those from the other incubation mixtures were calculated by using Student's *t* test; N.S., not significant.

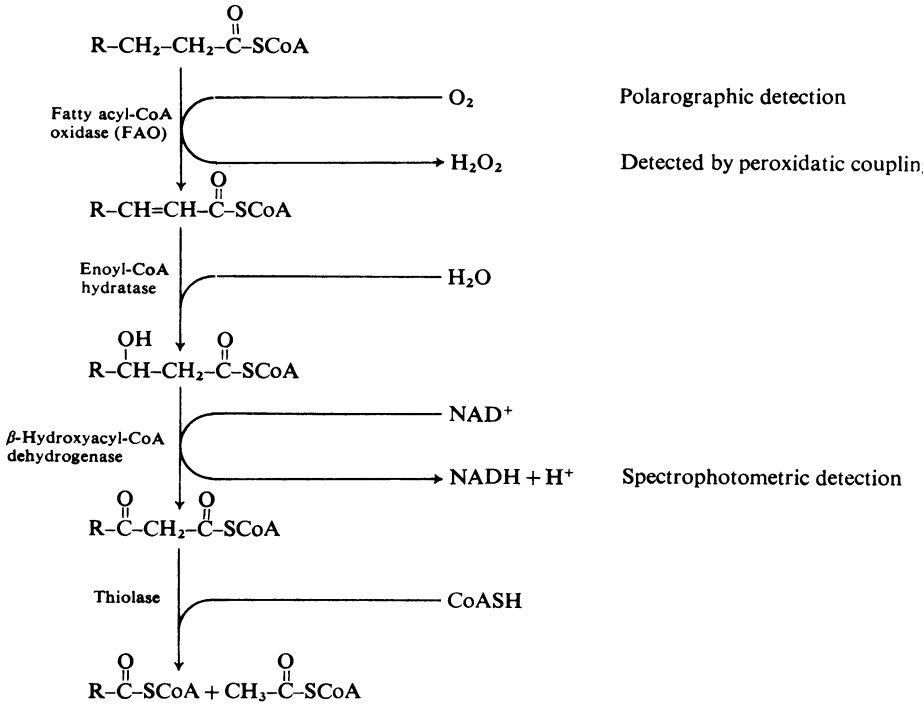
Incubation mixture	H_2O_2 generation (nmol of formaldehyde/min per g wet wt. of liver)	
Basal: 100mM-Tris/HCl buffer, pH8.3, +100mM-methanol	26± 7 (7)	
Basal +1.0mM-sodium palmitate	42±10 (3)	$P<0.05$
Basal +0.1mM-CoA	32± 7 (4)	N.S.
Basal +2.5mM-ATP+5mM-$MgCl_2$	47±17 (9)	$P<0.01$
Basal +sodium palmitate, CoA, ATP and $MgCl_2$	129± 7 (5)	$P<0.001$

acids, since the relative increment after nafenopin treatment parallels that of the total activity observed with palmitate. The activity of other peroxisomal oxidases are either strongly decreased or abolished after treatment with nafenopin (Leighton *et al.*, 1975), rendering any contribution from them unlikely.

The addition of 50μM-FAD to the incubation mixture containing palmitate-activation cofactors increases the activity of fatty acyl-CoA oxidase in liver homogenates from both normal and nafenopin-treated rats by 72 and 42% respectively. In fact, Cooper & Beevers (1969) postulated that the H_2O_2-generating step of the peroxisomal fatty acid-oxidizing system is mediated by a flavoprotein.

NAD^+ is required as a cofactor for the peroxisomal fatty acid-oxidizing system, as shown in Scheme 1. When NAD^+ was added to the homogenate, in the presence of nicotinamide as protecting agent, plus palmitate, ATP, CoA and $MgCl_2$ at the concentrations already indicated, the rate of H_2O_2 generation, estimated from formaldehyde production, was $4.63 \pm 0.26$$\mu$mol/min per g wet wt. of liver from a nafenopin-treated rat. When NAD^+ and nicotinamide were omitted, the rate decreased by 40% to $2.79 \pm 0.16$$\mu$mol/min per g, and a further decrease to $1.40 \pm 0.26$$\mu$mol/min per g was observed when bovine serum albumin was also omitted. These are average values $\pm$S.D., each one corresponding to 12 separate determinations. Under the three sets of conditions, a linear response of activity was observed at tissue concentrations ranging from 0.3 to 2.6mg wet wt. of liver per assay. The effect of albumin probably reflects solubilization of palmitate.

Before the description of the peroxisomal fatty acid-oxidizing system in rat liver by Lazarow & de Duve (1976), Oshino *et al.* (1973), from liver-perfusion experiments, claimed that octanoate and other fatty acids induce H_2O_2 production in mitochondria by a mechanism that is partially blocked with antimycin A. To check if the palmitate-dependent H_2O_2 generation we observe could be ascribed, at least in part, to production by mitochondria, the effect of antimycin A was studied in liver homogenates from control and nafenopin-treated rats. As shown in Table 3, only a slight inhibition was observed in



Scheme 1. *Reaction sequence for fatty acyl-CoA oxidation in glyoxysomes (Cooper & Beevers, 1969) and peroxisomes (Lazarow, 1978)*

Procedures for the measurement of the activity of the fatty acyl-CoA oxidase from either O_2 uptake or H_2O_2 production are described in the text and are compared with the already established method for detecting the activity of the system from fatty acyl-CoA-dependent NAD^+ reduction at the β-hydroxyacyl-CoA dehydrogenase step.

control rats and none in nafenopin-treated animals, suggesting that mitochondria contribute only slightly or not at all to H_2O_2 generation under the present assay conditions.

Formaldehyde uptake by subcellular fractions enriched in mitochondria has been reported in studies of microsomal *N*-demethylation reactions (Denk *et al.*, 1976; Leighton *et al.*, 1977). The process is partially prevented in the presence of semicarbazide.

After the main requirements had been established for the detection of fatty acyl-CoA oxidase coupled to the peroxidation of methanol, 6.6mM-semicarbazide was tried and found to give an approximately 2-fold increase in accumulated formaldehyde (Table 4). The addition of semicarbazide to the assay mixture does not interfere with the linearity of the assay at tissue concentrations ranging from 0.3 to 2.7mg wet wt. of liver per test from either control or nafenopin-treated rats.

Fatty acyl-CoA-dependent cyanide-insensitive O_2 *uptake*

Polarographic detection of O_2 consumption was made with a YSI Clark-type electrode (Yellow Springs Instruments Co.) connected to a stabilized power supply and operational amplifier (Philbrick Researches) as described by Freebrey & White (1966), or to a YSI model 53 oxygen monitor. The d.c. output was registered with a strip chart potentiometric recorder (E-VOM-11, Houston Instruments Div., Bausch & Lomb) with large offset capacity, allowing a full-scale calibration for an O_2 content of 100–75%. The electrode was placed in a thermostatically controlled glass chamber of 1.6ml volume with a capillary inlet suitable for microsyringes and equipped with a magnetic stirrer. Measurements were made in the dark to avoid photochemical consumption of O_2 by FAD. The optimum composition for the assay was 100mM-Tris/HCl, pH8.3, 1mM-KCN, 200μM-NAD^+, 33mM-nicotinamide, 170μM-CoA,

Table 3. *Effect of antimycin A on palmitate-induced* H_2O_2 *generation by liver homogenates*

Measurements were made by incubating 20mg wet wt. of liver from one control rat or 2.5mg wet wt. of liver from one nafenopin-treated rat in the presence of palmitate activation cofactors, as described in Table 2. Antimycin A, solubilized in aq. 60% (v/v) dimethyl sulphoxide, was added in 4μl to an 8μM final concentration. This amount of dimethyl sulphoxide did not affect the measurement. All results are expressed as means±S.D. and the numbers of determinations are shown in parentheses. The statistical significance of the differences observed in the presence of antimycin A were calculated by using Student's *t* test; N.S., not significant.

Assay conditions	H_2O_2 generation (nmol of formaldehyde/min per g wet wt. of liver)	
Control rat liver	91±4 (3)	
plus antimycin A	74±3 (3)	$P<0.005$
Nafenopin-treated rat liver	656±6 (4)	
plus antimycin A	647±6 (4)	N.S.

Table 4. *Effect of semicarbazide addition on the detection of* H_2O_2 *production by methanol peroxidation*

Measurements were made by incubating liver homogenate from one nafenopin-treated rat (2.5mg wet wt. of tissue per test) in 1.5ml of the incubation mixture described. Formaldehyde was measured after 15min at 37°C as described in the Experimental and Results section. Semicarbazide was added at the medium pH, 6.6mM final concentration. These assay conditions, including semicarbazide, were adopted for measuring the activity of the fatty acyl-CoA oxidase from H_2O_2 production. All results are expressed as means±S.D. and the numbers of determinations are shown in parentheses.

Incubation mixture	Formaldehyde produced (μmol/min per g wet wt. of liver)
Complete: 100mM-Tris/HCl buffer, pH8.3, 100mM-methanol, 1mM-sodium palmitate, 0.1mM-CoA, 2.5mM-ATP, 5mM-$MgCl_2$, 200μM-NAD^+, 33mM-nicotinamide, 0.9mg of bovine serum albumin/ml and 0.01% (w/v) Triton X-100	2.27±0.15 (4)
Complete + 6.6mM-semicarbazide	5.04±0.38 (12)

50μM-FAD, 0.6mg of bovine serum albumin/ml and 0.01 % (w/v) Triton X-100. After addition of the enzyme, the basal O_2 uptake was recorded before the reaction was started with 35μM-palmitoyl-CoA. This substrate concentration produced maximal initial velocities.

The polarographic recordings obtained are illustrated in Fig. 1. Upon addition of the homogenate, a basal O_2 uptake was observed that was 43 % lower in the presence of CN^-. The CN^--insensitive basal O_2 consumption was taken as the blank to calculate net O_2 uptake after substrate addition. Expressed as μmol/min per g wet wt. of liver, it amounted to 0.047 ± 0.015 (±S.D.) in three separate determinations for control livers and 0.297 ± 0.021 (±S.D.) in three separate determinations for liver from nafenopin-treated rats. These basal values are equivalent to those obtained for H_2O_2 generation, i.e. practically all the O_2 consumed was reduced to H_2O_2. As shown in Fig. 1, the addition of palmitoyl-CoA in the presence of CN^- led to twice the O_2 consumption observed in the absence of CN^-. This result further supports the hypothesis that O_2 is reduced to H_2O_2 by the fatty acyl-CoA oxidase, since the inhibition of endogenous catalase by CN^- prevents the regeneration of O_2 from H_2O_2. Sodium palmitate induced O_2 uptake only in the presence of 100μM-CoA and 2.5mM-ATP, confirming that an acyl-CoA synthetase is present. The buffer and pH employed were selected from the pH curves (Fig. 2), which show maximal O_2 uptake with palmitate or palmitoyl-CoA at pH8.3. Bovine serum albumin was added, since it induces higher O_2-uptake rates in the presence of palmitate. Crystallized fatty acid-poor albumin did not modify the reaction rate.

NAD^+ and nicotinamide increased the O_2 uptake by liver homogenates. In a set of experiments in which FAD as well as NAD^+ and nicotinamide were omitted and a high-speed supernatant from nafenopin-treated rat liver homogenized in a Waring blender was used, the activity observed with palmitoyl-CoA was 1.67 ± 0.18 units/g of liver. Addition of 200μM-NAD^+ increased the O_2 uptake to 2.70 ± 0.24. Addition of 33mM-nicotinamide alone gave a value of 3.72 ± 0.17 units/g of liver, and add-

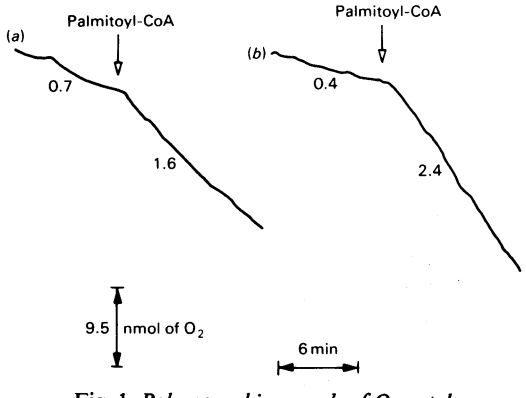


Fig. 1. *Polarographic records of O_2 uptake*
(*a*) Assay medium without cyanide. The addition of palmitoyl-CoA elicits an increase in O_2 uptake. Under these conditions catalatic decomposition of H_2O_2 is expected. Homogenate containing 1.8mg wet wt. of liver from a control rat in 10μl was added before the recording was started. Other assay conditions were as described in the Experimental and Results section. The numbers on the curves correspond to rates of O_2 consumption (nmol/min) in the 1.6ml incubation cell, before and after addition of palmitoyl-CoA. (*b*) Assay medium with 1mM-KCN. Other conditions as in (*a*) with homogenates from the same liver. The O_2 consumption due to endogenous substrates decreased, whereas that resulting from the addition of palmitoyl-CoA increased, since H_2O_2 is not decomposed. This results in approximately a doubling of the net O_2-uptake rate.

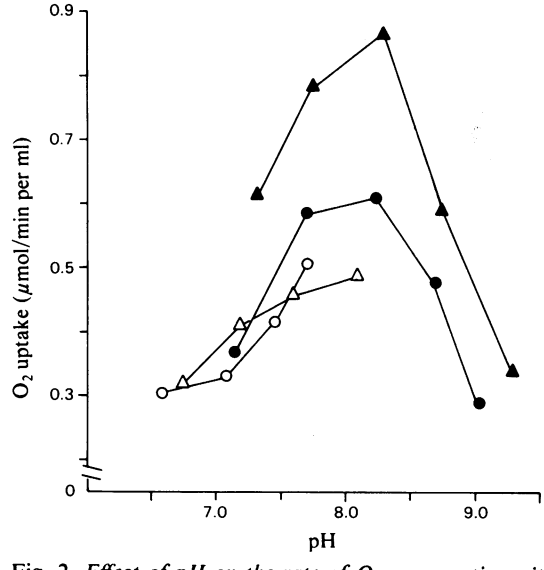


Fig. 2. *Effect of pH on the rate of O_2 consumption with palmitate and palmitoyl-CoA as substrates*
Liver homogenate from a nafenopin-treated rat, 3.3mg wet wt. per test. ○ and ●, Palmitate and activation cofactors, at the same concentrations as shown in Table 4, were used for polarographic measurements of O_2 uptake. △ and ▲, Palmitoyl-CoA was used as substrate in polarographic determinations of O_2 uptake made as described in the Experimental and Results section except for the buffer. ▲, ●, 100mM-Tris/HCl buffer; △, ○, 100mM-sodium phosphate buffer.

ition of both NAD^+ and nicotinamide gave a value of 6.80 ± 0.20 units/g of liver. In all cases the values correspond to the mean±S.D. for three separate determinations from the same liver and are expressed as μmol of O_2 taken up/min per g of wet wt. liver. The mechanism responsible for this effect is not clear. It is always present in liver homogenates or crude extracts in control and nafenopin-treated rats. It could correspond to preferential reoxidation of the acyl-CoA residues after thiolytic cleavage of acetyl-CoA, or result from the removal of inhibitory intermediates accumulated in the absence of β-hydroxyacyl-CoA dehydrogenase activity. Moreover, the possibility of a CN^--insensitive NADH oxidase activity cannot be ruled out. In fact, addition of 1mM-NADH to the standard assay mixture without palmitoyl-CoA induces a small increase in O_2 uptake, which is less than 5% of the increase observed after palmitoyl-CoA addition.

FAD(50 μM) was routinely, used since the fatty acyl-CoA oxidase activity was stimulated by the presence of FAD to a variable but reproducible degree in different subcellular fractions. Omission of FAD led to deficient recoveries in the subcellular fractions obtained from homogenates. In a peroxisome-rich λ fraction (Leighton *et al.*, 1968) prepared from a control rat liver, enzyme activity was 0.51 ± 0.01 (±S.D.) unit/g of liver when FAD was omitted and 1.40 ± 0.02 (±S.D.) units/g of liver in the presence of FAD. Each value corresponds to five separate determinations with the same fraction. The linearity of the response of the assay with FAD was maintained at enzyme concentrations corresponding to 0.3–6.0mg wet wt. of control liver per test or 0.1–3.0mg wet wt. of liver from nafenopin-treated rats.

The two methods developed to measure the oxidation of palmitoyl-CoA at the level of the fatty acyl-CoA oxidase or the procedure of Lazarow & de Duve (1976) which detects palmitoyl-CoA-dependent NAD^+ reduction gave the same values for normal liver homogenate and were equally enhanced by treatment with nafenopin (Table 5).

Fatty acyl-CoA specificity of the fatty acyl-CoA oxidase

In polarographic studies of O_2 uptake, the specificity of the enzyme from normal and nafenopin-treated rats was checked by replacing palmitoyl-CoA by butyryl-CoA, octanoyl-CoA, lauroyl-CoA or oleoyl-CoA at the same concentration. The results for saturated fatty acids indicate that the enzyme has a broad specificity for long-chain acyl-CoA derivatives (Fig. 3). In fact, the specificity measured at the level of the oxidase was the same as already detected for the peroxisomal fatty-acid oxidizing system by Lazarow (1978) when measuring at the β-hydroxyacyl-CoA dehydrogenase step. This indicates that the chain specificity for the system depends on the oxidase and not on the other enzymes involved. In the case of oleoyl-CoA, the specific activity was 40% of that with palmitoyl-CoA.

Subcellular distribution of the fatty acyl-CoA oxidase and of the peroxisomal fatty acid-oxidizing system

The procedures developed for the detection of the fatty acyl-CoA oxidase, and for the detection of the peroxisomal fatty acid-oxidizing system by palmitoyl-CoA-dependent NAD^+ reduction, were applied to the study of the subcellular distribution of these activities, except that in the methanol-peroxidation test for H_2O_2 generation, palmitate and ATP were omitted and 35 μM-palmitoyl-CoA added, in order to avoid the requirement for an acyl-CoA synthetase. Liver homogenates prepared in 0.25M-sucrose were fractionated into subcellular fractions as described

Table 5. *Detection of the peroxisomal fatty acid-oxidizing system by measuring at the fatty acyl-CoA oxidase or β-hydroxyacyl-CoA dehydrogenase steps*

Independent measurements were made with liver homogenates from control or nafenopin-treated rats. For fatty acyl-CoA oxidase, determinations based on O_2 uptake were made as described in the Experimental and Results section, by using palmitoyl-CoA, and determinations based on H_2O_2 generation as described in Table 4. For palmitoyl-CoA-dependent NAD^+ reduction by the β-hydroxyacyl-CoA-dependent dehydrogenase, the determinations were made as described in the Experimental and Results section. All these conditions were selected to give maximal activities. The results are expressed as means±S.D. and the numbers of determinations are shown in parentheses. The differences observed for each group of rats among the three assay procedures were found to be statistically not significant ($P > 0.05$) by using Student's *t* test.

Assay	Activity (μmol/min per g wet wt. of liver)	
	Control	Nafenopin-treated
Fatty acyl-CoA oxidase (O_2 consumption)	0.81 ± 0.07 (7)	6.38 ± 0.55 (7)
Fatty acyl-CoA oxidase (H_2O_2 production)	0.82 ± 0.05 (3)	5.70 ± 0.50 (4)
β-Hydroxyacyl-CoA dehydrogenase (NAD^+ reduction)	0.81 ± 0.05 (7)	6.50 ± 1.00 (5)

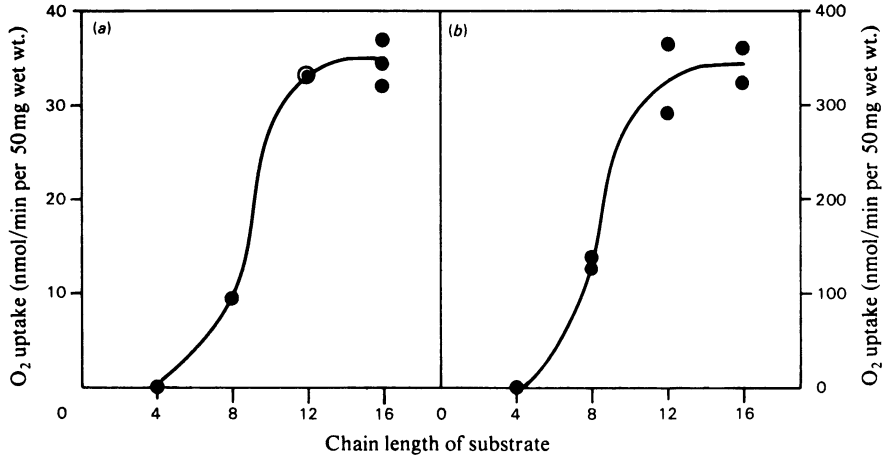


Fig 3. *Chain-length specificity of the fatty acyl-CoA oxidase from control (a) and nafenopin-treated (b) rats* Points correspond to nmol of O_2 utilized/min by 50mg wet wt. of liver from a control rat or a nafenopin-treated rat. Polarographic O_2-uptake measurements were made as described in the Experimental and Results section. Saturated fatty acyl-CoA derivatives were used at a concentration of 31 μM.

by de Duve *et al.* (1955). NADPH–cytochrome *c* reductase, which was used as a marker for the endoplasmic reticulum, was determined as described by Beaufay *et al.* (1974). Carnitine acetyltransferase was measured by a modification of the procedure of Fritz & Schultz (1965); the subcellular fractions were suspended in 10mM-sodium pyrophosphate, pH8.1, containing 0.1% sodium deoxycholate, to extract the enzyme. The remaining markers were determined as described by Leighton *et al.* (1968). For the detection of the peroxisomal fatty acid-oxidizing system at the β-hydroxyacyl-CoA dehydrogenase step, the assay medium contained, at pH8.3, 60mM-Tris/HCl, 24μM-palmitoyl-CoA, 50μM-CoA, 120μM-FAD, 370μM-NAD^+, 94mM-nicotinamide, 2.8mM-dithiothreitol, 2mM-KCN and bovine serum albumin (0.15mg/ml). To extract the enzymes, 1% (w/v) Triton X-100 was added to the samples, of which 5–20μl was used for each test.

The distribution found for the peroxisomal fatty acid-oxidizing system was similar to that of the fatty acyl-CoA oxidase with a peak in the light-mitochondria fraction (L), but with less activity in the supernatant (S) (Fig. 4*a*). In nafenopin-treated rats (Fig. 4*b*), apparently because of leakage, most of the catalase was found in the soluble fraction, as previously described (Leighton *et al.*, 1975), with a minor peak in the peroxisome-rich light-mitochondria fraction. As in control rats, maximal relative specific activities for the oxidase and for the fatty acid-oxidizing system were found in the peroxisome-rich light-mitochondria fraction.

The subcellular distribution data support in general the specificity of the detection procedures for the fatty acyl-CoA oxidase, and confirm the subcellular localization of the CN^--insensitive fatty acid-oxidizing system. Further subfractionation (Bronfman *et al.*, 1978) establishes that the particle-bound activity of the oxidase is indeed peroxisomal. The oxidase, as well as the entire CN^--insensitive fatty acid-oxidizing system appear to be weakly bound to peroxisomes, particularly to proliferated peroxisomes, as for catalase (Leighton *et al.*, 1975).

Nafenopin induces an increase in the activity of carnitine acetyltransferase from 1.35 to 26.5 units (μmol of CoA released/min per g wet wt. of liver; each value is the average from the two separate subcellular-fractionation experiments shown in each of Fig. 4*a* and Fig. 4*b*), and a change in its subcellular distribution to a mainly mitochondrial pattern. Similar data have been reported with other drugs also capable of inducing peroxisome proliferation (Kähönen, 1976; Markwell *et al.*, 1977). The protein content of the light-mitochondria fraction increases after treatment of the animals with nafenopin, reflecting the increase in peroxisomes (Leighton *et al.*, 1975; Stäubli *et al.*, 1977).

Discussion

We have developed two procedures for the quantitative detection of a fatty acyl-CoA oxidase activity in homogenates and subcellular fractions of liver. Fatty acyl-CoA-dependent O_2 uptake in the presence of cyanide is thought to be a specific measurement for the following reasons. Cyanide inhibits the

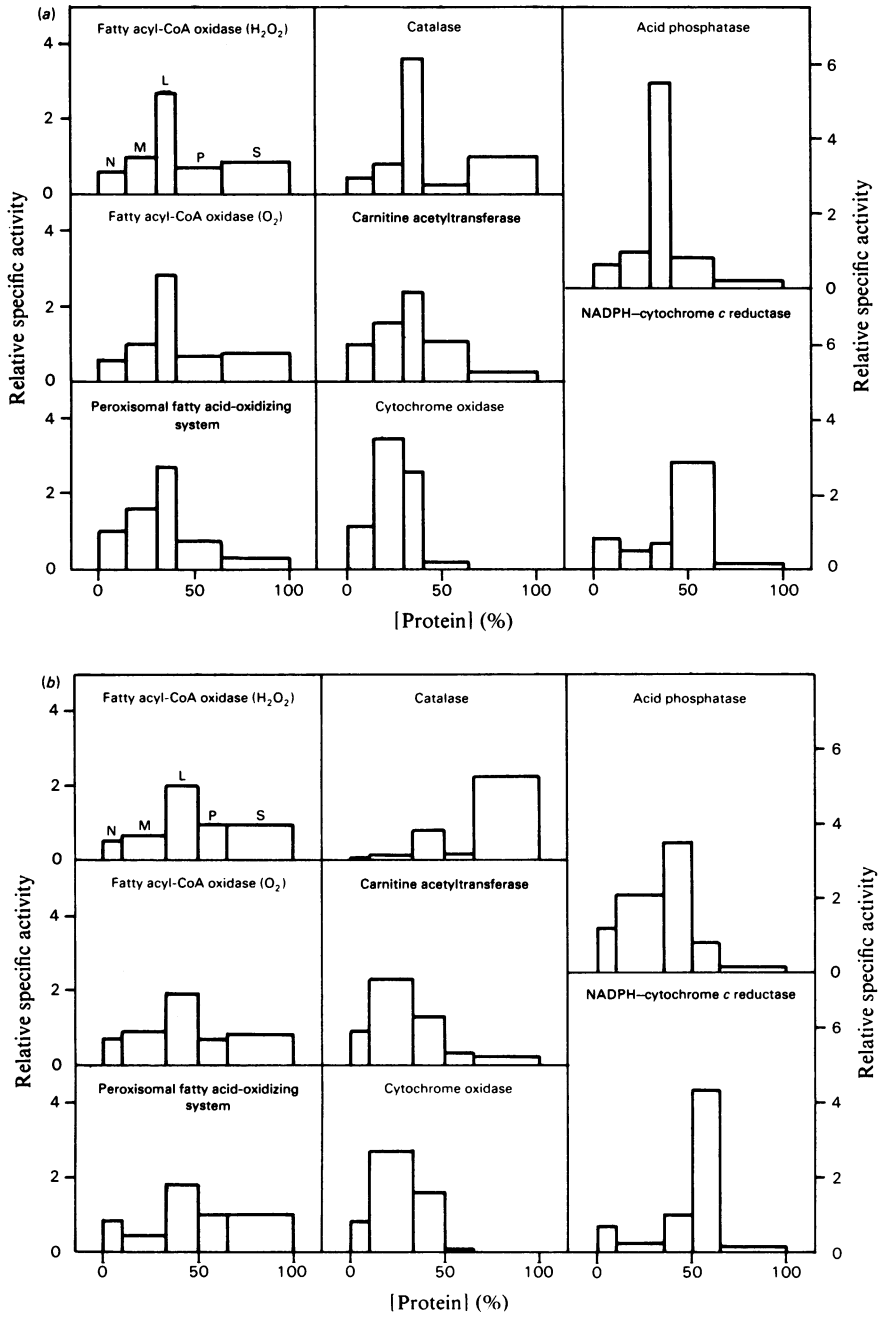


Fig. 4. *Subcellular fractionation of liver from control (a) or nafenopin-treated (b) rats*
The homogenates were fractionated as described by de Duve *et al.* (1955) into nuclear (N), heavy mitochondria (M), light mitochondria (L), microsomal (P) and supernatant (S) fractions. For each distribution pattern, the abscissa represents the protein content of the fraction as a percentage of the total protein of the liver. The ordinate represents relative specific activity, i.e. percentage, in the fraction, of the liver content of the marker enzyme over the percentage of liver protein in that fraction. Each distribution pattern corresponds to the average of two independent fractionation experiments, made from different rats, giving practically the same result.

mitochondrial electron-transport chain, as well as the O_2-consuming reaction mediated by the microsomal fatty acyl-CoA desaturase (Joshi *et al.*, 1977). The reduced long-chain acyl-CoA dehydrogenase from mitochondria cannot be oxidized by O_2 in the presence of substrate (Beinert, 1957). The fatty acyl-CoA oxidase displays a peroxisomal pattern of subcellular distribution, and the O_2 taken up is apparently reduced to H_2O_2 as shown by the 1:1 stoicheiometry and the decrease by one-half of the uptake of O_2 when measured in the absence of cyanide. The specificity of the fatty acyl-CoA-dependent methanol-peroxidation reaction is supported by the coincidence with the results obtained by the cyanide-insensitive O_2-uptake method.

Both detection procedures measure increments over basal activities after the addition of substrate. However, the basal values are probably due to oxidation of endogenous fatty acids. We observed that nafenopin-induced increases in fatty acyl-CoA oxidase activity per g of liver are accompanied by a parallel increase in basal activities. A similar observation was made by Hassinen & Kähönen (1974) for clofibrate-treated rats. These observations, which further support the postulated involvement of liver peroxisomes in fatty acid oxidation, indicate that the oxidation is limited by the activity of the enzymic system, and not by substrate availability.

The 7-fold increase in peroxisomal fatty acid oxidation induced by nafenopin, measured both at the level of the fatty acyl-CoA oxidase and of palmitoyl-CoA-dependent NAD^+ reduction, agrees with the measurements made by NAD^+ reduction after administration of clofibrate (Lazarow & de Duve, 1976), tibric acid and Wy-14643 (Lazarow, 1977), and di(ethylhexyl) phthalate (Osumi & Hashimoto, 1978*a*).

Purified peroxisomes oxidize palmitoyl-CoA to acetyl-CoA and NADH with a 1:5:5 stoicheiometry (Lazarow, 1978). These data, together with our results, imply that peroxisomes oxidize palmitoyl-CoA to acetyl-CoA, produce H_2O_2 and NADH and consume O_2 in the proportions 1:5:5:5:5 when regeneration of O_2 from H_2O_2 is prevented.

The stimulation by FAD of the fatty acyl-CoA oxidase suggests that it is a flavoprotein. This was postulated, by analogy to the mitochondrial β-oxidation system, when the existence of the enzyme was initially proposed (Cooper & Beevers, 1969). After partial purification of the enzyme, we have shown that it is indeed a flavoprotein in which FAD is weakly bound and cannot be replaced by FMN (N. C. Inestrosa, M. Bronfman & F. Leighton, unpublished results). It appears then that an increased amount of fatty acyl-CoA oxidase could account for the 40% increase in liver FAD, with no change in FMN, detected by Hassinen & Kähönen (1974) in rats treated with clofibrate.

The substrate specificity of the peroxisomal fatty acid-oxidizing system is given by the fatty acyl-CoA oxidase, since the results we obtained for the enzyme are the same as those obtained by Lazarow (1978) from acyl-CoA-dependent NAD^+ reduction. A similar conclusion can be also reached from the observations of Osumi & Hashimoto (1978*b*) with a partially purified fatty acyl-CoA oxidase.

The conclusion that the fatty acyl-CoA oxidase is rate-limiting for fatty acyl-CoA-dependent NAD^+ reduction is valid for homogenates and crude subcellular fractions, but maybe not for intact cells. The addition of purified fatty acyl-CoA oxidase to liver homogenates showed that the oxidase can be increased in normal liver up to 30-fold before the rest of the enzymes involved in NADH generation become limiting (N. C. Inestrosa, M. Bronfman & F. Leighton, unpublished results). However, in this reaction mitochondrial as well as peroxisomal enoyl-CoA hydratase and β-hydroxyacyl-CoA dehydrogenase are involved. If Lazarow's (1978) claim that these two enzymes are equally distributed among peroxisomes and mitochondria applies to our experimental conditions, the fatty acyl-CoA oxidase must clearly be rate-limiting; however, the theory of equal distribution is derived from analysis of a small (2.8%), and not necessarily representative, sample of mitochondria.

The relative roles played by mitochondria and peroxisomes in fatty acid oxidation *in vivo* is perhaps the main question raised by the description of the peroxisomal fatty acid-oxidizing system. β-Oxidative cleavage of long-chain fatty acids has also been described in high-speed supernatants from rat liver homogenates (Fiecchi *et al.*, 1973), but we interpret those results as being due to leakage from the peroxisomes of enzymes of the fatty acyl-CoA-oxidation system.

The maximal capacity of mitochondria to oxidize palmitate *in vitro* can be calculated from the specific activities reported for β-oxidation in coupled mitochondria, with palmitoylcarnitine as substrate in the presence of malonate (Bremer & Wojtczak, 1972; Holland & Sherratt, 1973; Osmundsen & Bremer, 1977; Mackerer, 1977). These values, together with our data, allow us to calculate that about 50% of the palmitate would be channelled to peroxisomes in control rats and 80% in nafenopin-treated rats. The measurements by Krahling *et al.* (1978), after correction for particle breakage, support our estimate for normal rats. The contribution of mitochondria would be larger for shorter-chain fatty acids. In fact, the role of peroxisomes appears to be restricted to long-chain saturated fatty acids, with maximal activities for $C_{12:0}$–$C_{18:0}$. There is little information on the mechanism of control of the transport of fatty acids and related metabolites into and out of peroxisomes. Furthermore, apart from preliminary

data for oleate, the role of peroxisomes in unsaturated fatty acid metabolism remains to be established.

This work was supported by the Dirección de Investigaciones de la Universidad Católica de Chile, project 212/77, and by the American States Organization. The skilful participation of Cecilia Necochea and María Nelly Morales is gratefully acknowledged.

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