Detection of Peroxisomal Fatty Acyl-Coenzyme A Oxidase Activity

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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, O2 would be directly reduced to H2O2 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 O2 consumption and palmitoyl-CoA-dependent H2O2 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 stoichiometry for palmitoyl-CoA-dependent O2 consumption, H2O2 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 O2 with the production of H2O2. 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 O2 and would lead to the generation of H2O2. No quantitative determination, which is suitable for use with liver homogenates or crude subcellular fractions, has been developed for the first H2O2-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, 1978a). 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 H2O2 production and the cyanide-insensitive O2 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)phenoy]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, Tokyo-shima, 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 H₂O₂ generation in liver homogenates

Meethanol peroxidation to formaldehyde and H₂O₂, a reaction mediated by catalase (EC 1.1.1.6) (Keilin & Hartree, 1936), was used to detect the generation of H₂O₂. 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 H₂O₂ 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-MgCl₂ 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 H₂O₂-generating reactions.

If the incubation is carried out in the absence of palmitate for the entire 60min, a moderate basal H₂O₂ generation is detected, as shown in Tables 1 and 2. The basal values observed in nafenopintreated 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 H₂O₂ production by peroxisomal oxidases in liver homogenates

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>H₂O₂ generation (nmol of formaldehyde/min per g wet wt. of liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal: 100mm-Tris/HCl buffer, pH8.3, +100mm-methanol</td>
<td>25 ± 6 (7)</td>
</tr>
<tr>
<td>Basal +1.2mm-sodium glycollate</td>
<td>443 ± 20 (5)</td>
</tr>
<tr>
<td>Basal +1.2mm-sodium urate</td>
<td>731 ± 47 (5)</td>
</tr>
</tbody>
</table>

Table 2. Minimal cofactor requirements for palmitate-induced H₂O₂ generation 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. 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.

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>H₂O₂ generation (nmol of formaldehyde/min per g wet wt. of liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal: 100mm-Tris/HCl buffer, pH8.3, +100mm-methanol</td>
<td>26 ± 7 (7)</td>
</tr>
<tr>
<td>Basal +1.0mm-sodium palmitate</td>
<td>42 ± 10 (3)  P&lt;0.05</td>
</tr>
<tr>
<td>Basal +0.1mm-CoA</td>
<td>32 ± 7 (4)  N.S.</td>
</tr>
<tr>
<td>Basal +2.5mm-ATP +5mm-MgCl₂</td>
<td>47 ± 17 (9)  P&lt;0.01</td>
</tr>
<tr>
<td>Basal +sodium palmitate, CoA, ATP and MgCl₂</td>
<td>129 ± 7 (5)  P&lt;0.001</td>
</tr>
</tbody>
</table>
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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 & Bevers (1969) postulated that the H₂O₂-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 MgCl₂ at the concentrations already indicated, the rate of H₂O₂ generation, estimated from formaldehyde production, was 4.63±0.26μmol/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±0.16μmol/min per g, and a further decrease to 1.40±0.26μmol/min per g was observed when bovine serum albumin was also omitted. These are average values ± 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 H₂O₂ production in mitochondria by a mechanism that is partially blocked with antimycin A. To check if the palmitate-dependent H₂O₂ 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

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

Procedures for the measurement of the activity of the fatty acyl-CoA oxidase from either O₂ uptake or H₂O₂ 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.

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control rats and none in nafenopin-treated animals, suggesting that mitochondria contribute only slightly or not at all to H₂O₂ 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, 6.6 mM-emicarbazide 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 2.7 mg wet wt. of liver per test from either control or nafenopin-treated rats.

### Table 3. Effect of antimycin A on palmitate-induced H₂O₂ generation by liver homogenates

<table>
<thead>
<tr>
<th>Assay conditions</th>
<th>H₂O₂ generation (nmol of formaldehyde/min per g wet wt. of liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rat liver</td>
<td>91 ± 4 (3)</td>
</tr>
<tr>
<td>plus antimycin A</td>
<td>74 ± 3 (3)</td>
</tr>
<tr>
<td>Nafenopin-treated rat liver</td>
<td>656 ± 6 (4)</td>
</tr>
<tr>
<td>plus antimycin A</td>
<td>647 ± 6 (4)</td>
</tr>
</tbody>
</table>

### Table 4. Effect of semicarbazide addition on the detection of H₂O₂ production by methanol peroxidation

Measurements were made by incubating 20 mg wet wt. of liver from one control rat or 2.5 mg 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 0.6% (v/v) dimethyl sulphoxide, was added in 4 μl to an 8-μl 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.

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Formaldehyde produced (μmol/min per g wet wt. of liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete: 100 mM-Tris/HCl buffer, pH 8.3, 100 mM-methanol, 1 mM-sodium palmitate, 0.1 mM-CoA, 2.5 mM-ATP, 5 mM-MgCl₂, 200 μM-NAD⁺, 33 mM-nicotinamide, 0.9 mg of bovine serum albumin/ml and 0.01% (w/v) Triton X-100</td>
<td>2.27 ± 0.15 (4)</td>
</tr>
<tr>
<td>Complete + 6.6 mM-semicarbazide</td>
<td>5.04 ± 0.38 (12)</td>
</tr>
</tbody>
</table>
The polarographic recordings obtained are illustrated in Fig. 1. Upon addition of the homogenate, a basal O₂ uptake was observed that was 43% lower in the presence of CN⁻. The CN⁻-insensitive basal O₂ consumption was taken as the blank to calculate net O₂ 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 H₂O₂ generation, i.e. practically all the O₂ consumed was reduced to H₂O₂. As shown in Fig. 1, the addition of palmitoyl-CoA in the presence of CN⁻ led to twice the O₂ consumption observed in the absence of CN⁻. This result further supports the hypothesis that O₂ is reduced to H₂O₂ by the fatty acyl-CoA oxidase, since the inhibition of endogenous catalase by CN⁻ prevents the regeneration of O₂ from H₂O₂. Sodium palmitate induced O₂ 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 O₂ uptake with palmitate or palmitoyl-CoA at pH8.3. Bovine serum albumin was added, since it induces higher O₂-uptake rates in the presence of palmitate. Crystallized fatty acid-poor albumin did not modify the reaction rate.

NAD⁺ and nicotinamide increased the O₂ 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 O₂ uptake to 2.70±0.24. Addition of 33mM-nicotinamide alone gave a value of 3.72±0.17 units/g of liver, and addi-

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Fig. 1. Polarographic records of O₂ uptake
(a) Assay medium without cyanide. The addition of palmitoyl-CoA elicits an increase in O₂ uptake. Under these conditions catalytic decomposition of H₂O₂ 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 O₂ 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 O₂ consumption due to endogenous substrates decreased, whereas that resulting from the addition of palmitoyl-CoA increased, since H₂O₂ is not decomposed. This results in approximately a doubling of the net O₂-uptake rate.

Fig. 2. Effect of pH on the rate of O₂ 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 O₂ uptake. ○ and △, Palmitoyl-CoA was used as substrate in polarographic determinations of O₂ uptake made as described in the Experimental and Results section except for the buffer. ▲, 100mm-Tris/HCl buffer; △, ○, 100mm-sodium phosphate buffer.
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...tion 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 $\mu$mol 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 thiolic cleavage of acetyl-CoA, or result from the removal of inhibitory intermediates accumulated in the absence of $\beta$-hydroxyacyl-CoA dehydrogenase activity. Moreover, the possibility of a CN$^-$-insensitive NADH oxidase activity cannot be ruled out. In fact, addition of 1 mm-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 $\mu$m) 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 peroxosome-rich $\lambda$ 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 0.3–6.0 mg wet wt. of control liver per test or 0.1–3.0 mg 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 $\beta$-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$_2$O$_2$ generation, palmitate and ATP were omitted and 35 $\mu$m-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 in parentheses.

### Table 5. Detection of the peroxisomal fatty acid-oxidizing system by measuring at the fatty acyl-CoA oxidase or $\beta$-hydroxyacyl-CoA dehydrogenase steps

<table>
<thead>
<tr>
<th>Assay</th>
<th>Activity ($\mu$mol/min per g wet wt. of liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Fatty acyl-CoA oxidase (O$_2$ consumption)</td>
<td>0.81±0.07 (7)</td>
</tr>
<tr>
<td>Fatty acyl-CoA oxidase (H$_2$O$_2$ production)</td>
<td>0.82±0.05 (3)</td>
</tr>
<tr>
<td>$\beta$-Hydroxyacyl-CoA dehydrogenase (NAD$^+$ reduction)</td>
<td>0.81±0.05 (7)</td>
</tr>
</tbody>
</table>

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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 10 mm-sodium pyrophosphate, pH 8.1, 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 pH 8.3, 60 mm-Tris/HCl, 24 μM-palmitoyl-CoA, 50 μM-CoA, 120 μM-FAD, 370 μM-NAD⁺, 94 mm-nicotinamide, 2.8 mm-dithiothreitol, 2 mm-KCN and bovine serum albumin (0.15 mg/ml). To extract the enzymes, 1% (w/v) Triton X-100 was added to the samples, of which 5–20 μl 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. 4a). In nafenopin-treated rats (Fig. 4b), 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 ofFig. 4a and Fig. 4b), 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 O₂ 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 O₂-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 O₂ in the presence of substrate (Beinert, 1957). The fatty acyl-CoA oxidase displays a peroxisomal pattern of subcellular distribution, and the O₂ taken up is apparently reduced to H₂O₂ as shown by the 1:1 stoichiometry and the decrease by one-half of the uptake of O₂ 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 O₂-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, 1978a).

Purified peroxisomes oxidize palmitoyl-CoA to acetyl-CoA and NADH with a 1:5:5 stoichiometry (Lazarow, 1978). These data, together with our results, imply that peroxisomes oxidize palmitoyl-CoA to acetyl-CoA, produce H₂O₂ and NADH and consume O₂ in the proportions 1:5:5:5 when regeneration of O₂ from H₂O₂ 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 (1978b) 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 (Fiechi et al., 1973), but we interpret these 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 Krahlng 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 C₁₂:0–C₁₈:0. 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
fatty acid metabolism remains to be established.

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