papers and notes on methodology

Fluorometric assay for rat liver peroxisomal fatty acyl-coenzyme A oxidase activity

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Abstract These studies report the development of a simple, specific, and highly sensitive fluorometric assay for rat liver peroxisomal fatty acyl-CoA oxidase activity. In this in vitro procedure fatty acyl-CoA-dependent H_2O_2 production was coupled in a peroxidase-catalyzed reaction to the oxidation of scopoletin (6-methoxy-7-hydroxycoumarin), a highly fluorescent compound, to a nonfluorescent product. Enzyme-catalyzed reaction rates as low as 5 pmol of H_2O_2 produced per minute could readily be detected. The reaction was studied in liver homogenates from normal rats with respect to absolute activity, time course, protein concentration dependence, substrate concentration dependence, pH optimum, substrate specificity, and cofactor requirements. The properties of the enzyme activity as assessed by the fluorometric assay agree well with those determined by other investigators using other assay methods. After subcellular fractionation of liver homogenates by differential centrifugation, the fatty acyl-CoA oxidase activity distributed like known peroxisomal marker enzymes. These results demonstrate that the fluorometric assay of fatty acyl-CoA oxidase should be useful in studying the distribution, properties, and subcellular localization of the enzyme, particularly in enzyme sources of low activity or in situations when only small amounts of material are available.—**Walusimbi-Kisitu, M., and E. H. Harrison.** Fluorometric assay for rat liver peroxisomal fatty acyl-coenzyme A oxidase activity. *J. Lipid Res.* 1983. **24:** 1077–1084.

Supplementary key words peroxisomes • palmitoyl-CoA • fatty acid oxidation • β-oxidation • subcellular fractionation

It is now well established that rat liver peroxisomes contain enzymes that catalyze the β-oxidation of fatty acids, a major pathway of lipid metabolism previously thought to occur only in mitochondria (1–5). A major difference between the two pathways is that the mitochondrial pathway is initiated by a fatty acyl-CoA dehydrogenase, while the first step in the peroxisomal pathway is catalyzed by a fatty acyl-CoA oxidase (FAO) that transfers electrons directly to O_2 to form H_2O_2 (1, 5, 6). Another difference between the mitochondrial and peroxisomal β-oxidation systems is in their chain length specificities for fatty acyl-CoAs, with the mitochondrial system being more active towards short chain substrates and the peroxisomal system utilizing exclusively medium to long chain fatty acyl-CoAs (2, 3, 5–7). Finally, the activity of peroxisomal fatty acyl-CoA oxidase and the peroxisomal β-oxidation pathway is increased several-fold by various structurally unrelated hypolipidemic drugs that also induce morphologically detectable peroxisome proliferation (1–5, 8).

Peroxisomal β-oxidation in liver homogenates was first assayed by monitoring the cyanide-insensitive, palmitoyl-CoA-dependent reduction of NAD to NADH at the β-hydroxy acyl-CoA dehydrogenase step of the pathway (1). Other assay methods in use include the measurement of $^{14}CO_2$ produced from 1-^{14}C-labeled fatty acid or fatty acyl-CoA (4), or fatty acyl-CoA-dependent oxygen consumption (3). In all of these assays, cyanide must be added to inhibit the mitochondrial oxidation of the substrate. More recently a procedure was developed to detect palmitoyl-CoA-dependent H_2O_2 production by coupling H_2O_2 production to the peroxidation of methanol to formaldehyde (3). The latter reaction is catalyzed by endogenous catalase in the liver homogenate and the formaldehyde produced is measured with the Nash reagent. Spectrophotometric assays based on measuring H_2O_2 by the peroxidase-coupled oxidation of a chromogen have also been used to detect FAO activity (7, 9).

Abbreviations: FAO, fatty acyl-coenzyme A oxidase; E, cytoplasmic extract; M, mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, high speed supernatant; N, nuclear fraction.

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In order to study the possible occurrence, properties, and role of FAO in fatty acid metabolism in tissues other than the liver, we sought to develop a simple and more sensitive assay procedure for the enzyme. This report describes the development of such an assay. In this procedure, palmitoyl-CoA-dependent H_2O_2 production is coupled in a peroxidase-catalyzed reaction to the oxidation of scopoletin, a highly fluorescent compound, to a nonfluorescent product (10). The fluorometric assay is more sensitive than most previously reported methods, and of comparable sensitivity to the spectrophotometric assay of Small, Brolly, and Connock (9). The results reported here for rat liver homogenates show that the absolute activity, enzyme properties, and subcellular distribution of the oxidase as assayed with the fluorometric procedure agree well with those obtained with other assay methods. We have also shown that FAO activity is present in rat kidney and heart tissue.

EXPERIMENTAL PROCEDURES

Animals and materials

Male Sprague-Dawley rats were purchased from Harlan Industries, Indianapolis, IN. The animals were fed ad libitum a commercial pelleted diet and had free access to water. Most experiments were conducted with adult rats weighing 200–300 g.

Palmitoyl-coenzyme A was purchased from P-L Biochemicals, Milwaukee, WI. Other fatty acyl-CoA esters, horse radish peroxidase, scopoletin, cofactors, and enzyme substrates were obtained from Sigma Chemical Co., St. Louis, MO.

Preparation of homogenates

All homogenates were prepared in 0.25 M sucrose containing 1 mM EDTA and 0.1% ethanol (SVE medium). The ethanol contained in this medium served to prevent the conversion of catalase to the inactive compound II. Fed rats were killed by ether overdose and the tissues were quickly removed and immersed in iced-cold SVE medium. For most experiments, minced tissue was mixed with 2–3 volumes of SVE medium and homogenized with a Tekmar Tissumizer®. The resulting homogenate was diluted with SVE medium and used as the enzyme source. For the subcellular fractionation experiments, 6 g of liver mince was mixed with 18 ml of SVE medium and homogenized with one stroke in a Potter-Elvehjem homogenizer as described by de Duve et al. (11).

Subcellular fractionation

Homogenates of rat liver were prepared and fractionated by differential centrifugation according to the method of de Duve et al. (11). In this procedure as used, a crude pellet was prepared by low-speed centrifugation and washed (i.e., resuspended and recentrifuged) two times. The resulting pellet was resuspended to yield the "nuclear" or N fraction. The postnuclear supernatants were pooled to yield the cytoplasmic extract or E fraction. The cytoplasmic extract was then further fractionated to obtain the mitochondrial fraction (M), the light mitochondrial fraction (L), and the microsomal fraction (P). The M and L fractions were washed two times before final resuspension. The P fraction was not washed. The final high-speed supernatant is referred to as the S fraction.

Fatty acyl-CoA oxidase assay

For the in vitro assay of fatty acyl-CoA oxidase, fatty acyl-CoA-dependent H_2O_2 production is coupled in a peroxidase-catalyzed reaction to the oxidation of scopoletin. Standard reaction mixtures in a final volume of 1 ml contained Tris-HCl (60 mM), pH 8.3, fatty acyl-CoA (35 μM), FAD (50 μM), NAD (200 μM), nicotinamide (33 mM), coenzyme A (170 μM), scopoletin (1 μM), peroxidase (10 μg, 3 units), bovine serum albumin (0.6 mg), Triton X-100 (0.01%), and enzyme. In later experiments NAD, nicotinamide, and CoA were omitted as it was found that they did not stimulate enzyme activity (see Results). Incubations were conducted under subdued light at 37°C with shaking. Reactions were terminated by adding 4 ml of 0.1 M borate buffer, pH 10. The fluorescence emission at 470 nm of the resulting solutions was measured at room temperature in an Aminco-Bowman Spectrophotofluorometer with excitation at 395 nm. Appropriate controls without enzyme and/or substrate were included in each assay.

Assays for marker enzymes and other constituents

The following enzymes were assayed by the indicated published procedures as markers for the various subcellular organelles: cytochrome oxidase (mitochondria) (12), N-acetyl-β-glucosaminidase (lysosomes) (13), NADPH-cytochrome C reductase (endoplasmic reticulum) (14), and catalase (15), and uricase (16) (peroxisomes).

Protein was determined by the method of Lowry et al. (17), using bovine serum albumin as a standard.

RESULTS

Quantitation of H_2O_2 production by peroxidase-coupled oxidation of scopoletin

In order to relate changes in the fluorescence of scopoletin to the quantity of H_2O_2 produced, a standard curve was constructed by adding known amounts of

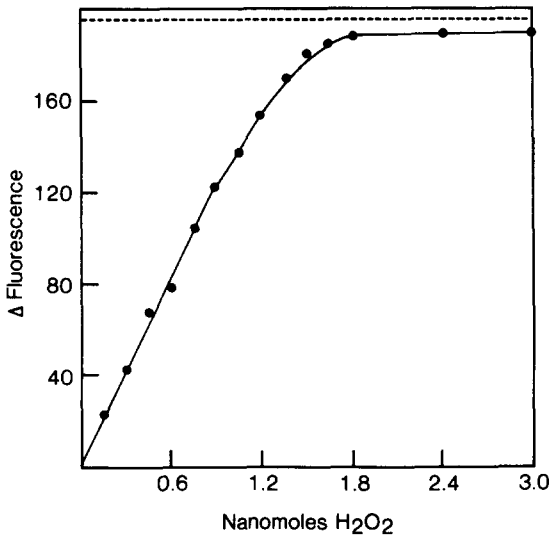

Fig. 1. Standard curve relating quantity of H_2O_2 to the decrease in fluorescence of scopoletin. Reaction mixtures in a final volume of 1 ml contained Tris-HCl (60 mM), pH 8.3, FAD (50 μM), peroxidase (10 μg, 3 units), scopoletin (1 μM), and the indicated amounts of H_2O_2. Incubations were carried out at 37°C for 20 min. Fluorescence measurements were made as described in the text. Broken line indicates the total amount of scopoletin (1 nmol) present in the reaction mixture.

H_2O_2 to mock reaction mixtures containing all components except the liver homogenate. As shown in **Fig. 1,** the fluorescence of scopoletin varies linearly with H_2O_2 concentration up to about 1 nmol of H_2O_2 per reaction mixture. As previously reported (10), about 2–3 nmol of H_2O_2 are required to completely oxidize the 1 nmol of scopoletin present in the reaction mixture.

Using the assay system described, fluorescence changes of 10 units can be easily detected, corresponding to about 0.1 nmol of H_2O_2. Thus, using a standard incubation time of 20 min, reaction rates as low as 5 pmol H_2O_2 produced per min can be measured.

Characteristics of FAO as determined by the fluorometric assay

Studies were conducted to determine the total activity of FAO in liver and the effects of enzyme concentration, times of incubation, pH, and substrate concentration on enzyme activity. **Fig. 2** shows the dependence of enzyme activity on the volume of homogenate added and on the incubation time. Activity was proportional to the amount of homogenate added up to levels corresponding to about 0.08 mg wet weight of liver (i.e., 50 μl in the experiment shown). Rate of product formation was linear with incubation time up to 20 min. From these data and those shown in Fig. 1, it can be calculated that the activity of FAO in liver is about 0.3 units/g liver (1 unit = 1 μmol H_2O_2 produced/min). Other investigators have reported similar values for total activity (range 0.1–1.0 units/g) using a variety of assay methods (1, 3, 4, 8, 9, 18–23).

As shown in **Fig. 3,** the pH optimum for FAO activity was near pH 8. It should be noted that above pH 8 there was a small, but measurable "endogenous" activity in the absence of added palmitoyl-CoA (about 10–20% of that in the presence of added palmitoyl-CoA). The activity observed in the absence of palmitoyl-CoA

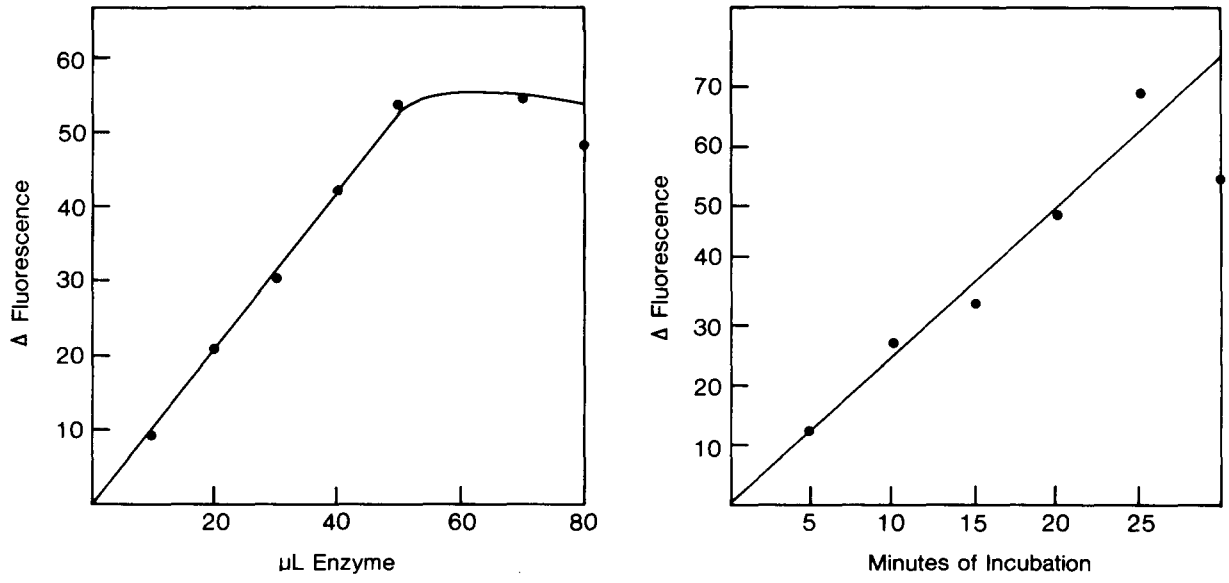

Fig. 2. Effects of amount of liver homogenate and incubation time on FAO activity. Left: Incubations were conducted at 37°C for 20 min and contained Tris-HCl (60 mM), pH 8.3, palmitoyl-CoA (35 μM), FAD (50 μM), NAD (200 μM), CoA (170 μM), nicotinamide (33 mM), peroxidase (10 μg, 3 units), BSA (0.6 mg/ml), Triton X-100 (0.01%), scopoletin (1 μM), and various amounts of a whole liver homogenate (1.55 mg wet weight/ml) in a final volume of 1 ml. Right: Incubations were conducted as above for various times and contained 50 μl of whole liver homogenate (1.55 mg wet weight/ml) as a source of enzyme. Enzyme activity is plotted on the ordinate as the change in fluorescence of scopoletin.

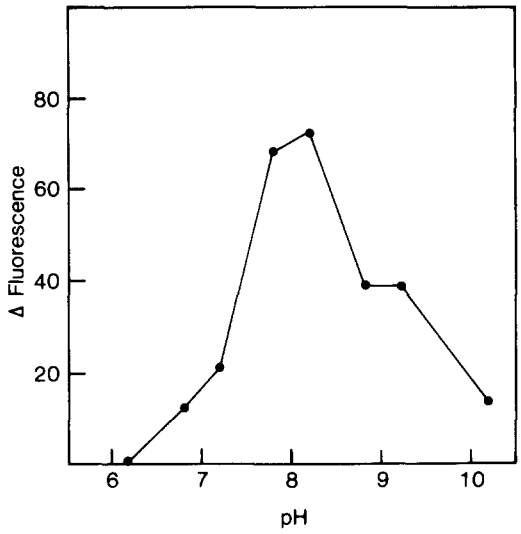

Fig. 3. Effect of pH on FAO activity. At each pH, 20-min incubations were carried out with 50 μl of whole liver homogenate (7.3 mg wet weight/ml) and contained palmitoyl-CoA (35 μM), FAD (50 μM), NAD (200 μM), CoA (170 μM), nicotinamide (33 mM), peroxidase (10 μg, 3 units), BSA (0.6 mg/ml), Triton X-100 (0.01%), and scopoletin (1 μM) in a final volume of 1 ml. Buffers included 100 mM sodium acetate for pH 6.2 and 6.8; 100 mM Tris-HCl for pH 7.2, 7.8, 8.2, 8.8, and 9.2; and 100 mM glycine-NaOH for pH 10.2. Enzyme activity is plotted on the ordinate as the change in fluorescence of scopoletin.

was subtracted from that in its presence in calculating and plotting the final values.

FAO activity increased as the concentration of palmitoyl-CoA was increased from 0 to 70 μM; at higher concentrations the activity decreased (**Fig. 4**). This type of behavior has been previously reported for peroxisomal fatty acyl-CoA oxidase activity acting on long chain substrates (21) and for other enzymes that utilize long chain fatty acyl-CoAs as substrates (24–28).

Effect of cofactors and other components of the reaction mixture

In order to further define the optimal conditions for the fluorometric assay of FAO and to study the enzyme's properties, reactions were conducted in the presence or absence of various components of the complete reaction mixture. As shown in **Table 1,** omission of FAD decreased enzyme activity by more than 50%, consistent with the flavoprotein nature of the oxidase. Likewise, omission of the detergent, Triton X-100 also led to a decrease in enzyme activity. Removing bovine serum albumin from the reaction mixture caused a relatively small decrease in activity. Somewhat unexpectedly, omission of either CoA or NAD had almost no effect on the rate of FAO as assayed by the fluorometric procedure.

In some of the other assay procedures for FAO, particularly when applied to liver homogenates from rats treated with peroxisomal proliferating agents, a significant stimulation of activity was observed upon addition of CoA and/or NAD (1, 3, 4). Thus, experiments were conducted to study in more detail the effects of various concentrations of these cofactors and of nicotinamide on the enzyme activity as measured with our assay (data not shown). A relatively small, but reproducible inhibition of activity was observed when CoA or NAD were added in concentrations of 0.1–0.8 mM. Maximal inhibition of about 20% was found for both cofactors at

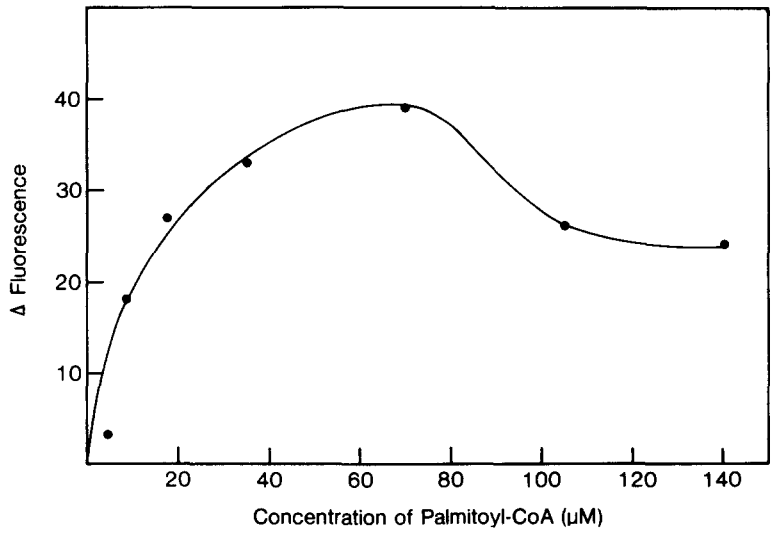

Fig. 4. Effects of increasing concentration of palmitoyl-CoA on FAO activity. All 20-min incubations contained Tris-HCl (60 mM), pH 8.3, FAD (50 μM), NAD (200 μM), CoA (170 μM), nicotinamide (33 mM), peroxidase (10 μg, 3 units), BSA (0.6 μg/ml), Triton X-100 (0.01%), scopoletin (1 μM), and 80 μl of whole liver homogenate (3.1 mg wet weight/ml). Enzyme activity is plotted on the ordinate as the change in fluorescence of scopoletin.

TABLE 1. FAO activity in liver homogenates in the absence of various additions

	Percent of Control
Complete reaction mixture[a]	100
Minus FAD	45
Minus COA	91
Minus NAD	105
Minus BSA	80
Minus Triton X-100	63
Minus peroxidase	0

[a] The complete reaction mixture contained Tris-HCl (60 μM), pH 8.3, palmitoyl-COA (35 μM), FAD (50 μM), NAD (200 μM), COA (170 μM), nicotinamide (33 mM), BSA (0.6 mg/ml), Triton X-100 (0.01%), peroxidase (10 μg, 3 units), scopoletin (1 μM), and 50 μl of whole liver homogenate (2.4 mg wet weight/ml) in a final volume of 1 ml. Incubations were conducted for 20 min at 37°C.

0.8 mM, the highest concentration tested. In contrast, addition of nicotinamide in concentrations up to 32 mM had almost no effect on enzyme activity.

Chain length specificity

The substrate specificity of FAO was checked by replacing palmitoyl-CoA with other fatty acyl-CoAs of various chain lengths; all substrates were tested at 35 μM. As shown in **Fig. 5,** FAO was most active on medium to long chain fatty acyl-CoAs with maximal activity on lauroyl-CoA and decanoyl-CoA. Very low activity was observed with hexanoyl-CoA and no activity was found with butyryl-CoA. The same pattern of activity was observed when fatty acyl-CoAs were tested at a concentration of 56 μM. These results are consistent with the contention that the fluorometric assay is detecting the peroxisomal fatty acyl-CoA oxidase.

Subcellular localization

The distributions of FAO and various marker enzymes after fractionation of rat liver homogenates by differential centrifugation are shown in **Fig. 6,** where they are presented in the form of histograms (11, 29). The distributions of the various marker enzymes agree well with previous reports (11, 29). Thus, mitochondrial cytochrome oxidase was mostly recovered in the M fraction with the remainder in the L fraction. Lysosomes (N-acetyl-β-glucosaminidase) were also mainly recovered in these two fractions but relatively more was in L than in M. Expectedly, most of the microsomal marker, NADPH-cytochrome c reductase, was recovered in the P fraction. The distributions of enzyme markers for peroxisomes were distinctly different from those for the other cell organelles. Uricase, localized in the peroxisomal crystalloid core (30), was mostly recovered in the L fraction; about 30% of the activity was associated with the low speed N fraction. Catalase, a soluble enzyme of the peroxisomal matrix (30) showed a peak in the L fraction but about half the activity was recovered in the soluble, high-speed supernatant as also observed previously by others (29). When the distribution of sedimentable catalase was plotted, it was nearly identical to that of uricase. Finally, FAO activity measured by our assay clearly distributed in a way very much like uricase and sedimentable catalase, thus confirming the association of the enzyme with peroxisomes.

Tissue distribution

Having validated the fluorometric assay for FAO using liver homogenates, we applied it to study the enzyme content of a number of other rat tissues. Whole homogenates were assayed using palmitoyl-CoA (35 μM) as substrate. Activity was detected in kidney (75–100 mU/g) and heart (3–15 mU/g) in addition to liver (300–400 mU/g). It should be pointed out that kidney and heart homogenates contain substantial endogenous activity (i.e., H_2O_2 production occurs in the absence of added fatty acyl-CoA). Thus, homogenates must be carefully diluted and always assayed with controls lacking substrate in order to demonstrate a fatty acyl-CoA-dependent activity. No FAO activity was observed in whole homogenates of brain, skeletal muscle, small intestine, lung, testis, spleen, thymus, and lymph node.

DISCUSSION

The studies reported here demonstrate that peroxisomal fatty acyl-coenzyme A oxidase can be assayed with

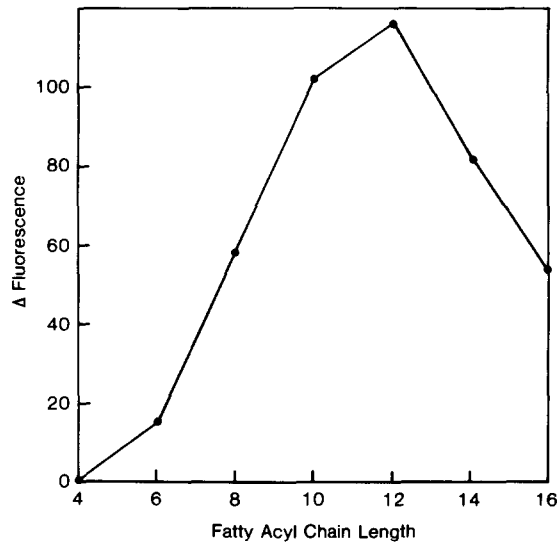

Fig. 5. Substrate specificity of FAO activity. Incubations were conducted using 35 μM concentrations of each saturated fatty acyl-CoA. All 15-min incubations were carried out with 80 μl of a whole liver homogenate (1.64 mg wet weight/ml) and contained Tris-HCl (60 mM), pH 8.3, FAD (50 μM) peroxidase (10 μg, 3 units), BSA (0.6 mg/ml), Triton X-100 (0.01%), and scopoletin (1 μM) in a final volume of 1 ml. Enzyme activity is plotted on the ordinate as the change in fluorescence of scopoletin.

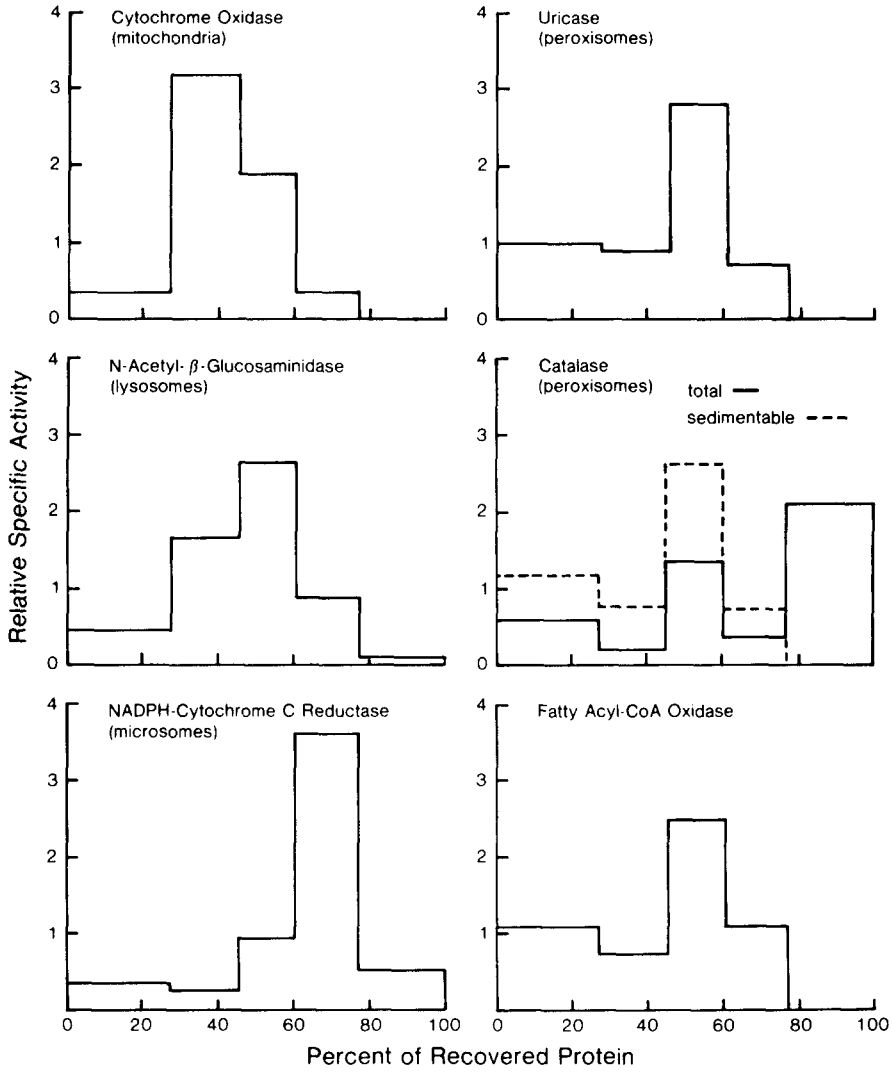

Fig. 6. Distribution of enzyme activities in subcellular fractions of rat liver homogenates. Fractions are plotted in decreasing order of the average coefficient of sedimentation of their subcellular components, i.e., from left to right: N, M, L, P, and S. Each fraction is represented separately on the ordinate by the relative specific activity for the indicated enzyme (percentage of total recovered activity/percentage of total protein recovered in that fraction). On the abscissa each fraction is represented cumulatively by the percentage of the total protein recovered in the fraction, compared to the sum of the amounts recovered in all five fractions. The total recoveries of the constituents in the five subcellular fractions, compared to the whole homogenate were (in %): protein, 82; cytochrome oxidase, 82; N-acetyl-β-glucosaminidase, 124; NADPH-cytochrome c reductase, 91; uricase, 84; catalase, 103; and fatty acyl-CoA oxidase, 74.

high specificity and sensitivity in crude tissue homogenates using a simple fluorometric procedure. The most sensitive previously reported assay for FAO is that of Small et al. (9) in which H_2O_2 production is coupled to the oxidation of leuco-2,7-dichlorofluorescein to produce a dye with a molar extinction coefficient of 91,000 at 502 nm. Thus, in this spectrophotometric assay, a change in absorbance of 0.001/min corresponds to a rate of H_2O_2 production of about 10 pmol/min. As shown above, the fluorometric assay can detect rates as low as 5 pmol/min, making it at least as sensitive as the spectrophotometric procedure and about 100 times more sensitive than O_2 uptake methods.

Our results with rat liver homogenates show that the absolute activity, pH dependence, substrate concentration dependence, and cofactor requirements of the enzyme activity as assessed by the fluorometric assay agree well with previous determinations of the enzymic properties using other methods (1, 3, 4, 5, 7–9, 18–22, 31). In particular, these studies have confirmed that the enzyme shows marked substrate inhibition by palmitoyl-coenzyme A (21, 32). Similar kinetic behavior has been

observed for other enzymes utilizing long chain fatty acyl-CoAs as substrates (24–28) and for enzymes with other soluble amphipaths as substrates (33). The theoretical, physicochemical basis for this behavior has been presented (33). From a practical point of view, it may explain in part the rather wide variation among various determinations of total FAO enzyme activity present in normal rat liver, which range from about 0.1 to 1.0 units/g wet weight (1, 3, 4, 5, 7–9, 18, 22, 31).

A number of reports have shown that the peroxisomal fatty acyl-CoA oxidase cannot utilize butyryl-CoA as a substrate (2, 3, 6, 7), but rather shows a marked preference for medium to long chain fatty acyl-CoAs. Studies using whole homogenates (3), purified peroxisomes (2), and partially purified (25-fold) fatty acyl-CoA oxidase (6) from the livers of rats treated with peroxisomal-proliferating agents have shown that even chain, saturated fatty acyl-CoAs from C_{12}–C_{16} are all about equally good as substrates for the oxidase with C_6–C_{10} substrates giving somewhat less activity. Some authors have observed a similar pattern in homogenates from normal rats (3). In contrast, Hyrb and Hogg (7) found a somewhat different pattern for the chain length specificity of peroxisomal β-oxidation and fatty acyl-CoA oxidase in peroxisomal fractions from normal rat liver. These authors also found that butyryl CoA was not a substrate but that lauroyl-CoA was the best fatty acyl-CoA substrate, giving about two times the activity of palmitoyl-CoA. Our own results, showing a definite peak in activity with C_{10}–C_{12} fatty acyl-CoAs, are in good agreement with these latter results. They also agree with the results of substrate specificity studies on peroxisomal oxidation of fatty acyl-CoAs in brown fat tissue from rats that also showed a definite peak at C_{12} (32). The differences observed by various investigators in the chain length specificity of fatty acyl-CoA oxidase can likely be explained by two factors. First, it is possible that the activity of the enzyme induced by peroxisomal-proliferating agents shows a different substrate specificity than that in normal rats. Consistent with this suggestion are Osmundsen's (34) observations that palmitoyl-CoA is clearly the best substrate for the peroxisomal system in preparations from rats fed a high fat diet to induce the system, while in normally fed rats myristoyl-CoA is a better substrate than the palmitate ester. Second, as shown by Hashimoto's work (21), the pattern of substrate specificity for FAO can depend critically on the substrate concentration used in the experiment due to the marked substrate inhibition by longer chain fatty acyl-CoAs. For example, his data show that at 10 μM $C_8 < C_{10} = C_{12} = C_{14} < C_{16}$, while at 50 μM $C_{16} < C_8 < C_{14} < C_{12} < C_{10}$.

Regardless of the details of the substrate specificity of FAO, it is clear that the fluorometric procedure used here is specific for the peroxisomal fatty acyl-CoA oxidase. This is shown unequivocally by the results of subcellular fractionation experiments. Thus, after differential centrifugation of rat liver homogenates, the FAO activity distributed like the peroxisomal markers, uricase and sedimentable catalase.

The high sensitivity, specificity, and simplicity of the fluorometric procedure make it useful in studying the occurrence, subcellular localization, and properties of fatty acyl-CoA oxidase in cell types and tissues other than rat liver. Preliminary results reported here demonstrate that the enzyme is also present in rat kidney and heart. The enzymic properties of the activity in these two tissues were similar to those reported above for liver. The amounts of enzyme (mU/g wet weight) in these tissues are about 25% and 3%, respectively, of that in liver. Interestingly, the same relative amounts of catalase are found in these three tissues.[2] Other investigators have recently detected FAO activity in brown adipose tissue in rats (32) and in several guinea pig tissues (9). In the latter report evidence is presented suggesting that the enzyme in small intestinal mucosa is localized in the catalase-containing microperoxisomes. Further investigations are required to define the subcellular localization and properties of fatty acyl-CoA oxidase in other tissues.

[2] Harrison, E. H. Unpublished observations.

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