Mitochondrial metabolism of 12- and 15-hydroxyeicosatetraenoic acids

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Abstract We have previously demonstrated that peroxisomal-deficient human skin fibroblasts and mutant Chinese hamster ovary cells do not convert 12- and 15-hydroxyeicosatetraenoic acids (HETEs) to chain-shortened, polar metabolites, suggesting that peroxisomes are the intracellular location for β-oxidation of these compounds. This implies that mitochondria do not β-oxidize HETEs. To test this hypothesis we incubated highly purified rat liver mitochondria with [^{3}H]12-(S)- and [^{3}H]15-(S)-HETE in the presence of carnitine and an acylcoenzyme A-generating system. Extracts obtained from these incubations were analyzed for radiolabeled polar metabolites. Both HETEs were converted to apparent products of β-oxidation, although the 12-HETE compound was a markedly better substrate. The presence of 50 μM 2-tetradecyloxirane carboxylate, a potent inhibitor of carnitine palmitoyl transferase, completely blocked 12- and 15-HETE conversion to these metabolites as did omission of carnitine from the medium. These data demonstrate carnitine-dependent β-oxidation of HETEs in isolated mitochondria and suggest that mitochondria are competent to carry out this metabolic process in eukaryoic cells. Prevailing metabolic conditions in subcellular compartments may have precluded observation of mitochondrial activity in our earlier work with cultured cells. Alternatively, transport mechanisms may exist in the cell types studied that distribute 12-(S)- and 15-(S)-HETEs specifically to peroxisomes.—**Gordon, J. A., K. M. Broekemeier, A. A. Spector, and D. R. Pfeiffer.** Mitochondrial metabolism of 12- and 15-hydroxyeicosatetraenoic acids. *J. Lipid Res.* 1994. **35:** 698–708.

Supplementary key words mitochondria • β-oxidation • hepatocytes • peroxisomes • carnitine

12-(S)-HETE and 15-(S)-HETE are monohydroxylated lipoxygenase derivatives of arachidonic acid, the 20-carbon n-6 (omega-6) polyunsaturated fatty acid (see ref. 1 for review). HETEs are synthesized by activated inflammatory cells (e.g., macrophages, neutrophils, platelets) as unstable hydroperoxides and are reduced, either spontaneously or through the action of peroxidases, to the corresponding monohydroxylated derivative (1). 12-(S)-HETE and 15-(S)-HETE are chemotactic and chemokinetic factors (2), they inhibit prostaglandin formation in cultured endothelial and renal epithelial cells (3, 4), and they are mitogenic for bovine aortic endothelial cells (5). In addition, 15-(S)-HETE may interfere with cellular signal transduction via its incorporation into inositol phospholipids and diacylglycerol in bovine aortic endothelial cells (6), while 12-(S)-HETE is bound stereospecifically in carcinoma cells (7) and enhances metastatic potential (8).

In view of these activities it is important to determine how the production and degradation of HETEs are controlled. Such studies are complicated at present because it is not clear whether the initial products of the lipoxygenase reaction are the active agents, or whether activity requires conversion to metabolites such as the chain-shortened and more polar products that can arise from β-oxidation. The intracellular location(s) of HETE metabolism are also uncertain. In peroxisomal competent cells, 12-(S)-HETE and 15-(S)-HETE are converted to metabolites that are released into the extracellular medium. For example, renal epithelial cells, vascular smooth muscle cells, and murine cerebral vascular endothelium have been shown to convert 12-(S)-HETE to a 16-carbon metabolite identified as 8-hydroxyhexadecatrienoic acid [16:3(8-OH)] (9–11). Normal human skin fibroblasts and wild type Chinese hamster ovary cells also convert 12-(S)-HETE to a polar metabolite that is chromatographically identical to 16:3(8-OH) (12, 13). Endothelial cells convert 15-(S)-HETE to 11-hydroxyhexadecatrienoic acid [16:3(11-OH)] (14). Likewise, normal human skin fibroblasts and wild type Chinese hamster ovary cells also convert 15-(S)-HETE to a polar metabolite that appears to be the same compound produced by the endothelial cells.

Abbreviations: CCP, carbonyl cyanide *p*-chlorophenylhydrazone; HETE, hydroxyeicosatetraenoic acid; HPLC, high performance liquid chromatography; 16:3(8-OH), 8-hydroxyhexadecatrienoic acid; 16:3(11-OH), 11-hydroxyhexadecatrienoic acid.

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In contrast, we have recently reported that peroxisome-deficient human skin fibroblasts and mutant Chinese hamster ovary cells fail to convert 12-(S)-HETE and 15-(S)-HETE to polar metabolites (12, 13). These results suggest that peroxisomal β-oxidation is responsible for hydroxyeicosanoid chain-shortening, a hypothesis that is consistent with the understanding that long chain and highly unsaturated fatty acids are β-oxidized preferentially by peroxisomes (15–18). This hypothesis is also supported by a recent report that shows that highly purified rat liver and kidney peroxisomes produce 16:3(8-OH) as an apparent end product of 12-(S)-HETE β-oxidation (19). On the other hand, mitochondria β-oxidize fatty acids that are saturated at carbons 2–4 and process *cis* double bonds in the C_5 and/or more distal positions (e.g., 20, 21). Furthermore, the rates of these mitochondrial activities seem sufficient to explain HETE oxidation (20, 21), considering that hour-long time scales are used when investigating HETE oxidation by cells (20, 21). Because 12-(S)-HETE and 15-(S)-HETE present the same initial structures to the enzymes of β-oxidation, with *cis*-unsaturated bonds at C_5 and C_8, mitochondria should be able to oxidize these eicosanoids, unless they cannot be activated and/or transported into the matrix space. To evaluate this question, we have investigated the oxidation of 12-(S)- and 15-(S)-HETE in highly purified preparations of rat liver mitochondria that are essentially free of contaminating peroxisomes. The results show that both compounds are β-oxidized, but that the rate is substantially higher with 12-(S)-HETE. Portions of these data have appeared in abstract form (22).

EXPERIMENTAL PROCEDURES

Chemical and reagents

Unlabeled 12-(S)- and 15-(S)-HETE were obtained from Cayman Chemical Co., Ann Arbor, MI. 12-(S)-hydroxy [5,6,8,9,11,12,14,15,(n)-^{3}H]eicosatetraenoic acid (119 Ci/mmol) and 15-(S)-hydroxy[5,6,8,9,11,12,15,(n)-^{3}H]eicosatetaenoic acid (213 Ci/mmol) were purchased from Amersham Corp., Arlington Heights, IL. 2-Tetradecyloxirane carboxylate (methyl palmoxirate) was a generous gift from the R. W. Johnson Pharmaceutical Research Institute. Other reagents were purchased from Sigma Chemical Co., St. Louis, MO. Reagent and HPLC grade solvents were obtained from Fisher Scientific, Fair Lawn, NJ.

Preparation of hepatocytes

Suspension cultures of rat liver hepatocytes were prepared by collagenase perfusion of rat liver according to the method of Fariss et al. (23), with modifications (24). Briefly, male Sprague-Dawley rats weighing 170–190 g were anesthetized with pentobarbital; the abdomen was opened, and heparin was injected through the diaphragm into the heart. The liver was perfused through the portal vein with oxygenated Krebs-Henseleit buffer and subsequently removed from the abdomen. A 15-min recirculating perfusion was begun with 75 mg of collagenase dissolved in the same buffer; after this, the disrupted liver was passed through a fine nylon mesh to aid separation into individual cells. The cells were washed several times with Fisher's media containing 2.5 mM Ca^{2+}, suspended in this medium at a density of $\sim 1 \times 10^7$ cells/ml, and maintained briefly at 37°C until use. Cell counts and the assessment of viability (trypan blue exclusion) were conducted in a hemocytometer. Initial viability was 90% or greater with a reduction of less than 10% during a subsequent 3-h period.

Preparation and purification of mitochondria

Standard preparations of rat liver mitochondria were obtained by differential centrifugation using male Sprague-Dawley rats that weighed approximately 250 g (25). The homogenizing medium contained 0.23 M mannitol, 0.07 M sucrose, 3 mM HEPES (Na^+), pH 7.4, 0.5 mM EGTA (Na^+), plus bovine serum albumin at 2 mg/ml. Washing was conducted in the same medium except that EGTA and BSA were omitted. Preparations obtained by the standard procedure were further purified by Percoll density gradient centrifugation (26–28). One ml of the standard preparation, containing 30–40 mg of mitochondrial protein, was layered onto 23 ml of 30% Percoll that also contained 0.23 M mannitol, 0.07 M sucrose, and 3 mM HEPES (Na^+), pH 7.4. After centrifugation at 50,000 *g* for 30 min in a Beckman Ti 60 rotor (0–4°C), mitochondria were recovered from the bottom of the tube, and the supernatant containing contaminating structures was discarded. The mitochondrial fraction was diluted to approximately 30 ml with the mannitol, sucrose, HEPES washing medium, and centrifuged at 10,000 *g* for 10 min (0–4°C). The resulting pellet was washed three times using the same conditions, and the resulting preparation was suspended in washing medium at 30–40 mg protein/ml. Protein was determined according to the biuret method, using bovine serum albumin as the standard.

Earlier comparisons of standard and Percoll gradient-purified mitochondria have shown both preparations to be generally intact by morphological, biochemical, and bioenergetic criteria (27, 28). However, the degree of intactness is particularly important in the present study because the apparent failure of mitochondria to β-oxidize HETEs in intact cells could arise from impeded access to the matrix space. Were that true, the presence of disrupted mitochondria in the present work would be potentially misleading. Accordingly, gradient-purified mitochondria were examined by electron microscopy using methods described before (29). Functional activities that reflect inner membrane integrity were also examined, with the

conditions used described in the legend to Table 2. The distribution of rotenone-insensitive cytochrome C reductase activity and acylcoenzyme A:1-acyllysophospholipid acyltransferase activity have shown that microsomal contamination accounts for ~ 5% of the protein in standard preparations (27, 28). Percoll density gradient purification reduces this contaminant 7- to 8-fold (28). The efficiency of Percoll gradients in removing contaminating peroxisomes was determined from measurements of catalase activity (30).

Incubation conditions and the extraction of HETE metabolites

Mitochondrial incubations were conducted at 37°C and at 1.0 mg protein/ml in mannitol, sucrose, HEPES (as described above) containing 2.5 μM [^{3}H]12-(S)- or -15-(S)-HETE. The HETE specific activity was 19,000–33,000 dpm/pmol. DL-carnitine (50 μM) and an acylcoenzyme A-generating system (2 mM $MgCl_2$, 2 mM ATP, 50 μM coenzyme A) were also present to allow activation and transport of fatty acids into the matrix space. In addition, the medium contained oligomycin (1.0 nmol/mg protein) and cyclosporin A (0.5 nmol/mg protein). The former agent was present to prevent hydrolysis of ATP by the F_1F_0 ATPase, whereas cyclosporin A was used to prevent the permeability transition (see ref. 31 for review). Occurrence of the transition was an issue because we noticed that Percoll gradient-purified mitochondria are prone to this phenomenon when incubated in the absence of an high activity oxidizable substrate.[2] Mitochondria having undergone the transition were considered unsuitable for the present study because opening of the cyclosporin A-sensitive pore could bypass the requirement for acylcarnitine transferase activity when oxidizing acylcoenzyme A generated in the extramatrix space (31). At appropriate times, 0.5-ml aliquots of the incubations were taken and the mitochondria were rapidly sedimented in a microcentrifuge (13,000 *g* for 2 min). The supernatants were transferred to siliconized vials, acidified with 12 μl of concentrated HCl, and [^{3}H]HETE metabolites were extracted 3 times with 3 volumes of ethyl acetate as previously described (4, 13). The pooled extracts were taken to dryness under a N_2 stream and the residual material was dissolved in 500 μl of HPLC grade acetonitrile.

Hepatocytes were incubated at 37°C and 1×10^6 cells/ml in Fisher's medium containing 2.5 mM $CaCl_2$, and 2.5 μM of either radiolabeled HETE, analogous to the conditions used with mitochondria. At appropriate times, cells were sedimented and the supernatants were extracted using the methods described above.

HPLC analysis of HETE metabolites

Radioactivity in 25-μl aliquots of the acetonitrile solutions was determined by liquid scintillation counting to assess the total recovery of exogenous substrates. To determine the composition of products, additional aliquots containing at least 10,000 dpm were transferred to siliconized vials, dried under N_2, and then resuspended in 30% acetonitrile and 70% water acidified to pH 3.4 with phosphoric acid. HPLC was performed on a Beckman 332 chromatograph equipped with a 4.6 × 250 mm Beckman column having C_{18} reverse-phase spherical packing (5 μm). The solvent gradient consisted of phosphoric acid-acidified water and acetonitrile with the latter component increasing linearly from 30 to 100% over the 45-min runtime (4, 13). Radioactivity was detected by mixing the column effluent with Budget-Solve scintillation solution which then passed through a flow detector (Radiomatic Flo-One Beta; Canberra Corp., Meriden, CT).

RESULTS

Metabolism of HETES in hepatocytes

The cell types used in our earlier studies of HETE metabolism were not available in sufficient quantities to allow the isolation of highly purified subcellular fractions by existing techniques. Accordingly, rat liver was used as the starting material in the present work. Prior to investigating the metabolism of 12-(S)- and 15-(S)-HETE in isolated mitochondria, we determined whether the metabolic pathways for HETE degradation in suspension cultures of rat hepatocytes were similar to what we observed previously in renal epithelial cells and normal human skin fibroblasts (9, 12). [^{3}H]12-(S)-HETE was readily converted to several more prominent polar metabolites when incubated with the hepatocytes for 30 min (**Fig. 1, top**). These extracellular products represented 10% of the radioactivity originally added as 12-(S)-HETE, which is similar to what we observed with other cells (9, 12). One of the major metabolites eluted at 17.6 min, identical to the retention time of 16:3(8-OH). This compound is the major metabolite of 12-(S)-HETE produced by renal tubular epithelial cells and normal human fibroblasts (9, 12). After 60 min (Fig. 1, bottom), these components largely disappeared and a number of new peaks with retention times between 2 and 9 min became apparent. At either the 30- or 60-min time points, no unmetabolized 12-(S)-HETE could be detected in the medium. Analogous incubations, without hepatocytes, demonstrated that no non-enzymatic conversion of 12-(S)-HETE to other compounds occurred (data not shown).

[2]The increased susceptibility of Percoll gradient-purified mitochondria to the permeability transition apparently reflects a reduced content of endogenous substrates within these preparations (as indicated by O_2 consumption studies), compared to mitochondria prepared by differential centrifugation alone. The absence of a fully developed proton motive force is well known to facilitate the transition (29, 32). No differences in susceptibility to the transition were noted between the two preparations when the medium contained excess succinate (data not shown).

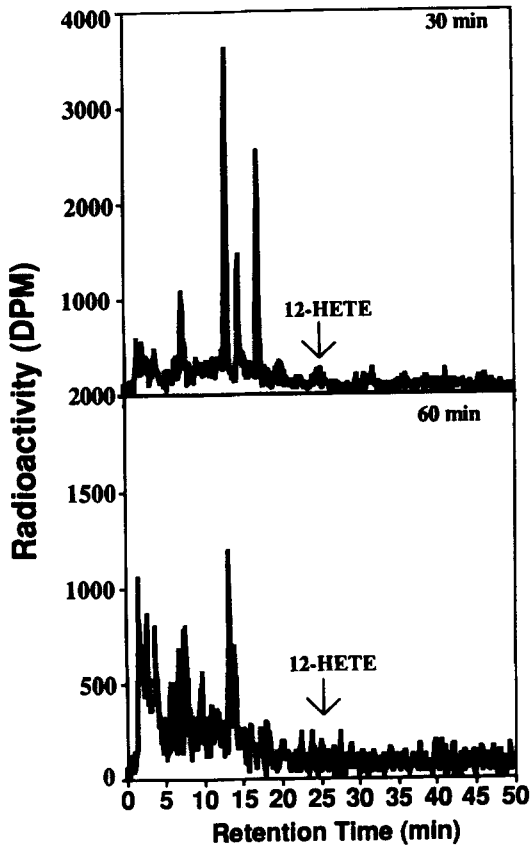

Fig. 1. Profiles of 12-(S)-HETE radioactive metabolites released into the medium from collagenase-dispersed hepatocytes. Rat liver hepatocytes (1×10^6 cells/ml) were incubated at 37°C with 2.5 μM [^{3}H]12-(S)-HETE, in serum-free Fischer's medium as described in Experimental Procedures. After 30 (top) or 60 (bottom) min, the medium was recovered and the lipids were extracted into ethyl acetate as described in Experimental Procedures. After solvent removal under N_2 and suspending the resultant sample in acetonitrile, an aliquot containing ~ 10,000 dpm, was analyzed by reverse-phase HPLC. Radioactivity was determined with an on-line, flow detector (see Experimental Procedures). 12-(S)-HETE has a retention time of 25.1 min under the conditions used.

The oxidation of 15-(S)-HETE by hepatocytes was also investigated. Previous work indicated that endothelial cells oxidize this compound to 16:3(11-OH) (14), as do normal human fibroblasts (12) and wild type Chinese hamster ovary cells (13). When incubated for 30 min with suspension cultures of hepatocytes, 15-(S)-HETE was also converted to polar metabolites, which in total comprised 17% of the added radioactivity. One of the prominent metabolites had a retention time of 11.6 min (**Fig. 2, top**), consistent with that of 16:3 (11-OH) (14). After 60 min of incubation, this component was much less evident (Fig. 2, bottom). As in the case of 12-(S)-HETE, no unmetabolized 15-(S)-HETE was detected in the medium at either the 30- or 60-min time point, and none of these products were formed in incubations without cells. Taken together, these data indicate that the metabolism of 12-(S)- and 15-(S)-HETE by rat hepatocytes is rapid and that they are converted to more polar metabolites similar to those previously observed in other cells.

Metabolism of HETES in isolated mitochondria

Initial O_2 consumption experiments using standard and Percoll gradient-purified mitochondria were consistent with 12-(S)-HETE acting as a substrate for β-oxidation. However, the rates involved were low. This fact, together with the presence of endogenous oxidizable substrates, precluded the use of oxygen consumption measurements for quantitative studies (data not shown). By contrast, a facile conversion of 12-(S)-HETE to chain-shortened polar metabolites was easily demonstrated using the radiolabel approach. The data shown in **Fig. 3** were obtained using Percoll gradient-purified mitochondria at 1.0 mg protein/ml. They show that 2.5 μM 12-(S)-HETE is converted to several more polar products within 10 min, while no unmetabolized 12-(S)-HETE remained in the medium (Fig. 3, top). In total, the metabolites com-

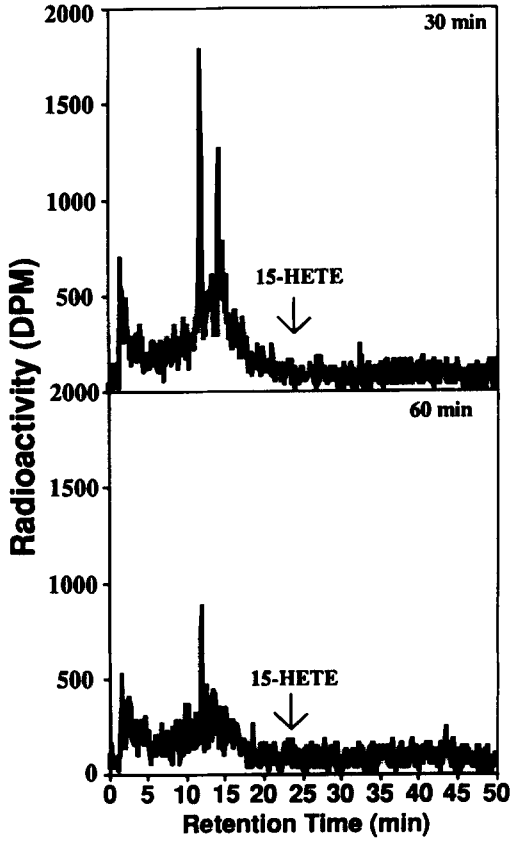

Fig. 2. Profiles of 15-(S)-HETE radioactive metabolites released into the medium from collagenase-dispersed hepatocytes. Rat liver hepatocytes (1×10^6 cells/ml) were incubated at 37°C with 2.5 μM [^{3}H]15-(S)-HETE, in serum-free Fischer's medium as described in Experimental Procedures. After 30 (top) or 60 (bottom) min, the medium was recovered, the lipids were extracted, and HPLC profiles were obtained as described in Experimental Procedures and the legend to Fig. 1. 15-(S)-HETE has a retention of 23.6 min under the conditions used.

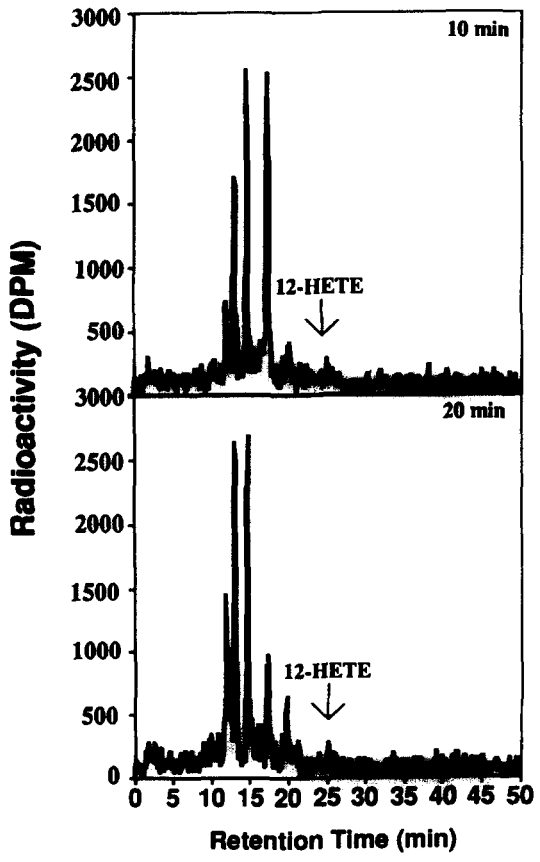

Fig. 3. Profiles of 12-(S)-HETE radioactive metabolites released into the medium from mitochondria. Percoll gradient-purified rat liver mitochondria (1 mg protein/ml) were incubated at 37°C in a medium of mannitol, sucrose, and HEPES, pH 7.4, containing carnitine, an acyl-CoA generating system, and 2.5 μM [^{3}H]12-(S)-HETE, as described in Experimental Procedures. After 10 (top) or 20 (bottom) min, the medium was recovered, the lipids were extracted, and HPLC profiles were obtained as described in Experimental Procedures and in the legend to Fig. 1. 12-(S)-HETE has a retention of 25.1 min. under the conditions used.

prised 58% of the radioactivity originally added as 12-(S)-HETE. Metabolism continued during a further 10-min period, as indicated by reduction of the component eluting at ~17 min associated with an increase in a component appearing at ~13 min (Fig. 3, bottom). A comparison of Fig. 1 with Fig. 3 shows that most products produced by hepatocytes were also generated by isolated mitochondria.

Although actual rates of 12-(S)-HETE oxidation were not determined, HPLC profiles obtained with standard mitochondria and Percoll gradient-purified mitochondria were very similar regardless of whether 10- or 20-min incubation times were compared (data not shown). The relative contamination of these two preparations with peroxisomes, as indicated by catalase activity, is shown in **Table 1.** The standard mitochondrial preparation contained a significant peroxisome contamination (~ 5% of the catalase activity present in the initial homogenate). However, this was dramatically reduced upon further purification by Percoll density gradient centrifugation. Considering the specific activity data in Table 1, 98% of this contaminant in the standard mitochondrial preparation was removed on the gradient. This finding, together with the maintenance of HETE catabolic activity, strongly indicates that mitochondria β-oxidize 12-(S)-HETE.

If a substantial fraction of the gradient purified mitochondria were disrupted, it could be argued that the present data are not referable to in situ conditions because the normal requirements for acyl group transport into the matrix had been bypassed. However, **Fig. 4** shows that ultrastructure is not changed during purification, while the expected reduction in contaminating structures is apparent. **Table 2** shows that the respiratory control ratios obtained with uncoupler, Ca^{2+}, and ADP are the same for standard and purified mitochondria. These

TABLE 1. Loss of catalase activity (peroxisomes) during purification of mitochondria[a]

Sample	Activity	
	% of Total	nmols/min/mg Protein
Homogenate	100 ± 19.5	
Low speed supernatant	68.8 ± 14.9	
Standard mitochondria	5.41 ± 1.01	396 ± 126
Percoll gradient-purified mitochondria	0.065 ± 0.029	7.90 ± 2.40

[a]Values presented are means ± SD obtained from assay of four separate preparations. Activity was assayed by the UV absorbance method (30), using a H_2O_2 concentration of 10 mM. The total homogenate activity is defined as 100% with the variability arising from factors such as liver size, blood content, and animal-to-animal variability in peroxisome prevalence. The low speed supernatant sample contained mitochondria, peroxisomes, and less dense cellular fractions. Standard mitochondria and gradient-purified mitochondria were obtained from this sample as described in Experimental Procedures.

TABLE 2. Respiratory control ratios of standard and Percoll gradient-purified rat liver mitochondria[a]

	Uncoupler (CCP)	Ca^{2+}	ADP
Standard mitochondria	9.0	6.3	5.0
Purified mitochondria	9.4	6.1	5.2

[a]Values are means of duplicate determinations and are the ratios of oxygen consumption rate after, compared to before, addition of the reagent shown. Assays were performed at 25°C using a protein concentration of 1.0 mg/ml. The medium contained 0.23 M mannitol, 0.07 M sucrose, 3 mM HEPES (Na^+) (pH 7.4), 10 mM succinate (Na^+), and rotenone (0.5 nmol/mg protein). With carbonyl cyanide *p*-chlorophenylhydrazone (CCP) (3 nmol/mg protein) and ADP (100 μM), the medium also contains 3.3 mM sodium phosphate. With Ca^{2+} (100 μM as $CaCl_2$) the sodium phosphate concentration was 250 μM.

quantities are sensitive indicators of inner membrane structural integrity and further indicate that normal permeability barriers are retained during Percoll gradient purification.

The data in **Fig. 5** bear further upon the question of mitochondrial intactness. The absence of carnitine (Fig. 5B) or the presence of methyl palmoxirate (Fig. 5C), essentially eliminates the metabolism of 12-(S)-HETE in the Percoll gradient-purified preparation. The former compound is well known to be required for transport of fatty acid into mitochondria (33, 34) but not peroxisomes (see ref. 35 for review). The latter agent is an inhibitor of carnitine-palmitoyltransferase activity (36) which is also required for mitochondrial oxidation of exogenous fatty acids (33, 34). Thus, it seems clear that neither disrupted mitochondria nor residual peroxisomes, if present, can account for the conversion of 12-(S)-HETE to the more polar metabolites. Notice, however, that 4-pentenoic acid, which inhibits β-oxidation at the level of β-ketothiolase (37–39), fails to prevent the conversion of 12-(S)-HETE to more polar compounds (Fig. 5D). This inhibitor also fails to prevent metabolism of HETEs in human skin fibroblasts and other cells (9, 12). The latter observations have been taken to indicate that peroxisomes are the sole intracellular site of HETE β-oxidation (9, 12, 13).

In contrast to 12-(S)-HETE, 15-(S)-HETE is utilized relatively poorly by Percoll gradient-purified mitochondria (**Fig. 6**). Comparing the 10-min data in Figs. 3 and 6 suggests that the rate differential is in excess of 20-fold. The activity that does exist when using 15-(S)-HETE, however, was again lost or greatly diminished upon excluding carnitine or adding methyl palmoxirate, respectively, to the medium (data not shown). Thus, the conversion of 15-(S)-HETE to chain-shortened, more polar metabolites apparently is also an authentic activity of rat liver mitochondria.

DISCUSSION

Previous studies have shown that HETEs are not β-oxidized by cells that lack functional peroxisomes, even though these cells remain capable of oxidizing shorter chain saturated fatty acids and appear to have normal mitochondria by a number of other criteria (12, 13). It has also been shown that highly purified peroxisomes from liver and kidney β-oxidize 12-(S)-HETE to 16:3(8-OH) (19), a product that is prevalent among those released from peroxisomal competent cells during incubation with 12-HETE. Taken together, these data would seem to demonstrate that peroxisomes are the sole site at which HETEs are metabolized to shorter chain products. However, the present data indicate that mitochondria are competent to β-oxidize HETEs, although in Fig. 5, panel D it is seen that 4-pentenoic acid fails to inhibit the metabolism of 12-(S)-HETE. 4-Pentenoic acid inhibits β-ketothiolase activity (37–39) and so it might be argued that the present results do not represent authentic β-oxidation. This point is of further significance because 4-pentenoic acid also fails to inhibit metabolism of 12-(S)-HETEs in fibroblasts and renal epithelial cells, observations that were taken earlier as supporting noninvolvement of mitochondria (9, 12). Such interpretations are seen to be questionable, however, when approximate rates of HETE oxidation are compared to the potential activity of the mitochondrial pathway. O_2 consumption studies (not shown) indicate that β-oxidation of palmitic acid is at least 10-fold faster than that of 12-(S)-HETE under the conditions used here. This allows that β-ketothiolase inhibition would have to approach completion before the residual activity became insufficient to support further utilization of 12-(S)-HETE. 4-Pentenoic acid inhibition of thiolase activity can be substantially less than complete (40), and we suspect that this fact explains the present result obtained with mitochondria and, possibly, related aspects of the previous results obtained with the intact cells.

The absence of HETE β-oxidation in peroxisomal-deficient cells must then be reconciled with the apparent presence of an active mitochondrial HETE oxidation pathway. Several factors can be considered as potential explanations. Considering first 15-(S)-HETE, this compound is poorly utilized by mitochondria, compared to 12-(S)-HETE, as seen by contrasting Figs. 3 and 6. Approximately 60% of total radioactivity was recovered in the ethyl acetate extracts of acidified mitochondrial media, regardless of which HETE was used. When the extent of recovery is considered together with the fact that the acid extraction procedure is not expected to result in hydrolysis of acylCoAs, the prevalence of 15-(S)-HETE in Fig. 6 compared to the absence of 12-(S)-HETE in Fig. 3 suggests that the former compound is a poor substrate

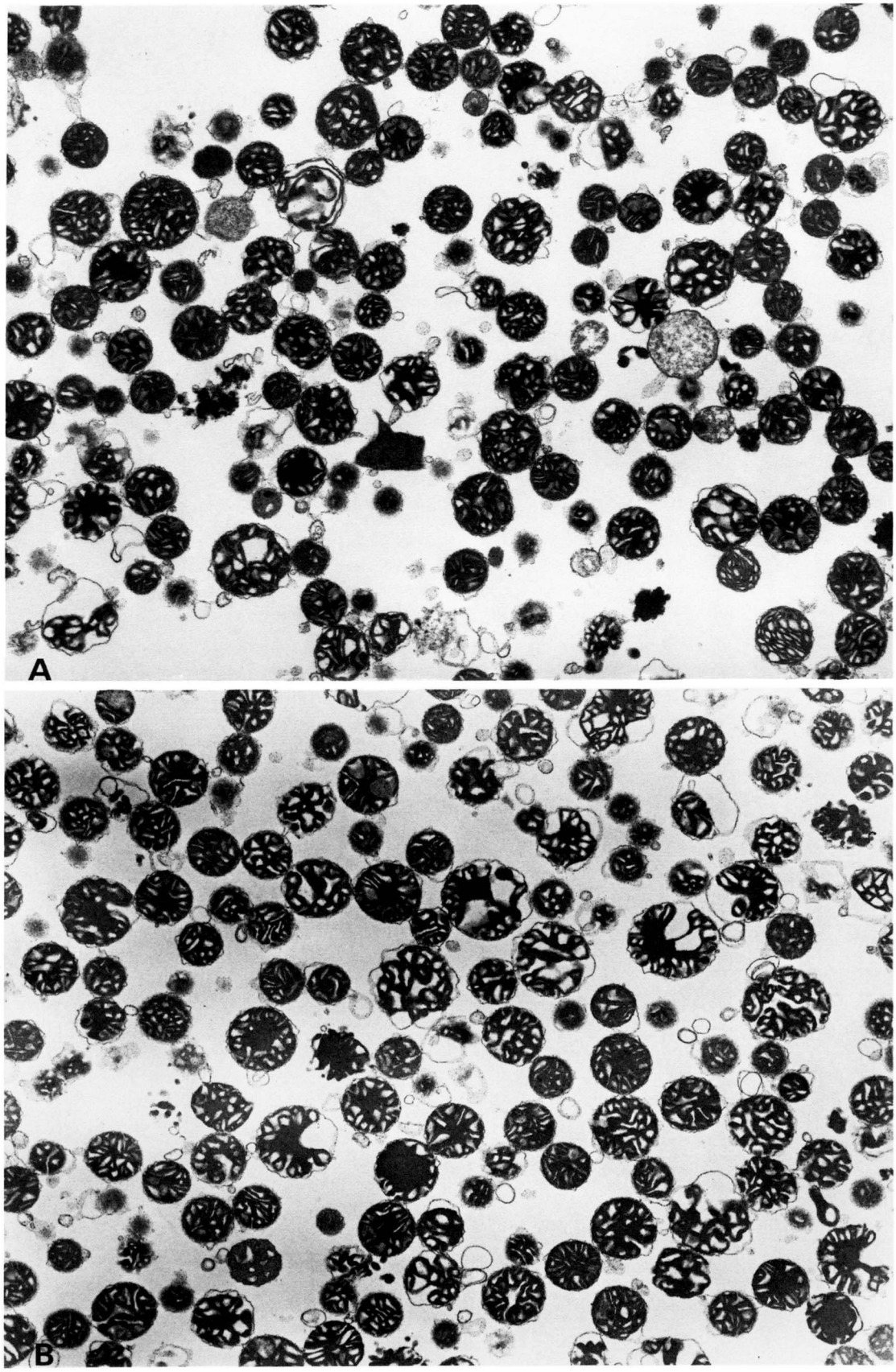

Fig. 4. The ultrastructural characteristics of standard (panel A) and Percoll gradient-purified (panel B) rat liver mitochondria. The procedures used are described in reference 29. Total magnification is 10,200.

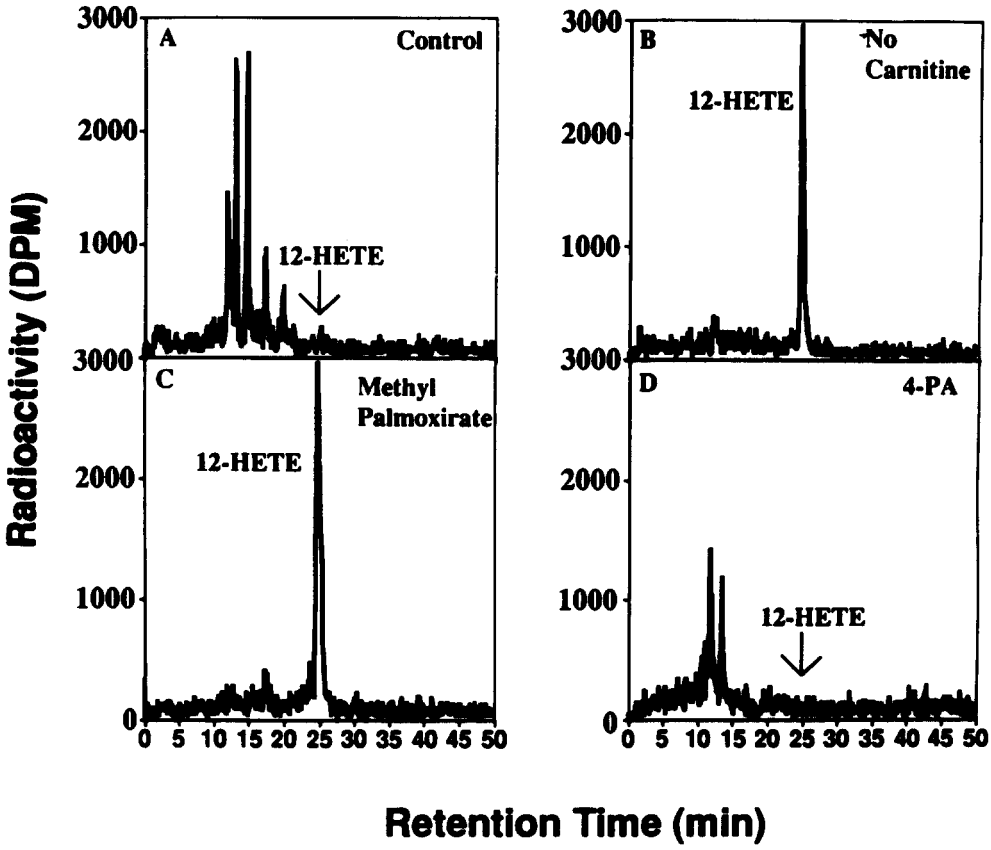

Fig. 5. The effect of metabolic inhibitors and carnitine deletion on the oxidation of 12-(S)-HETE in mitochondria. Panel A: the experiment was conducted as described in Experimental Procedures and the legend to Fig. 3, using a 20-min incubation time. Panel B: same as panel A, except that carnitine was not added to the medium. Panel C: same as panel A, except that 50 μM methylpalmoxirate was present from the beginning of the incubation. Panel D: same as panel A, except that 0.1 mM 4-pentenoic acid was present from the beginning of the incubation.

for mitochondrial acyl-CoA synthetases (or that 15-(S)-HETECoA is used preferentially by mitochondrial acyl-CoA hydrolase). Under in situ conditions, problems arising from poor activation activity may be amplified by the presence of other fatty acids competing with 15-(S)-HETE at the acyl-CoA synthetase level. Thus, poor activation of 15-(S)-HETE by mitochondria could potentially explain why it is not β-oxidized in the absence of peroxisomes. Although 12-(S)-HETE appears to be activated by mitochondria more readily than 15-(S)-HETE, competition from other fatty acids could also be a factor in its poor utilization by mitochondria in situ, with the competition expressed at the level of acyl-CoA synthetase, during subsequent transfer to carnitine or during translocation of the carnitine derivative.

Another factor to consider is the probable redox poise in the mitochondrial matrix, compared to the peroxisome interior. HETE oxidation in normal and peroxisomal-deficient cells was investigated in the presence of excess and varied oxidizable substrates (i.e., serum), adequate O_2 availability, and in the absence of conditions where the requirements for ATP synthesis would be high. Under such conditions, liver cells, for example, have much higher NADH/NAD^+ ratios in the mitochondrial matrix compared to the cytoplasmic space (e.g., ref. 41). The peroxisome-limiting membrane appears to be permeable to pyridine nucleotides (42) and so pyridine nucleotide ratios within the peroxisome are likely to be equilibrated with those of the cytoplasm. It is then possible that inhibition of long chain 3-hydroxyacyl-CoA dehydrogenase by NADH in mitochondria but not in peroxisomes is a factor that favors the latter structure as the site of HETE oxidation. Where this is the case, the oxidation site might change with the state of cell activation (energy load), types of substrates available, etc. Presumably, conditions that differentially affect acetyl-CoA/CoASH ratios in mitochondria and cytoplasm, the reduction state of FAD, or the level of malonyl-CoA could also influence the rate of HETE oxidation by mitochondria relative to peroxisomes. This is because all of these factors can potentially

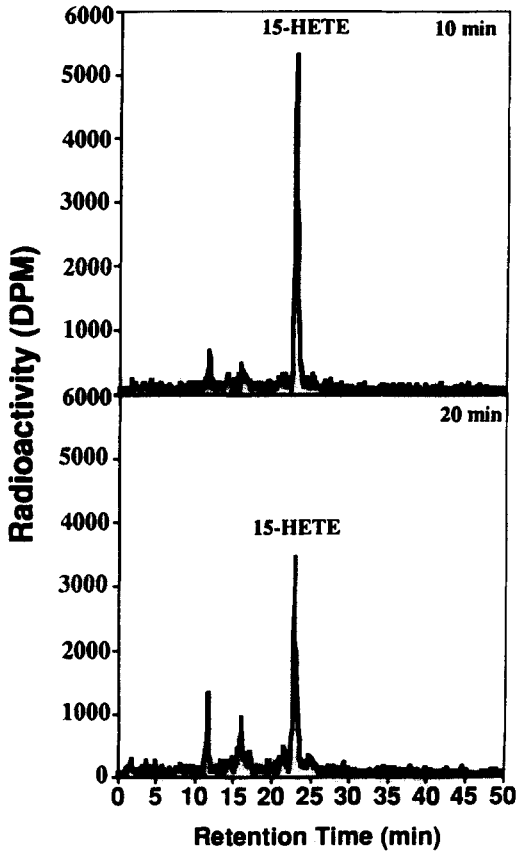

Fig. 6. Profiles of 15-(S)-HETE radioactive metabolites released into the medium from mitochondria. Percoll gradient-purified rat liver mitochondria (1 mg protein/ml) were incubated at 37°C in a medium of mannitol, sucrose, and HEPES, pH 7.4, containing carnitine, an acyl-CoA generating system, and 2.5 μM [^{3}H]15-(S)-HETE, as described in Experimental Procedures. After 10 (top) or 20 (bottom) min, the medium was recovered, the lipids were extracted, and HPLC profiles were obtained as described in Experimental Procedures and the legend to Fig. 1. 15-(S)-HETE has a retention of 23.6 min under the conditions used.

exert regulatory influences on β-oxidation (43 and references therein), and these may be expressed to greater or lesser extents in different subcellular compartments, depending upon the metabolic state.

The potential explanations for the failure of mitochondria to β-oxidize HETEs in peroxisomal-deficient cells, as considered above, are kinetic in nature. It is difficult to judge whether such factors would be sufficient to completely preclude the utilization of HETEs by mitochondria, as apparently occurs in these cells. Furthermore, peroxisomal-deficient cells retain some capacity to β-oxidize other fatty acids which are normally oxidized in the peroxisome and which would be generally subject to the same kinetic limitations with respect to their utilization by mitochondria (13). Thus, it is possible that the normal oxidation site of HETEs is established by more specific mechanisms. It might be, for example, that intracellular fatty acid-binding proteins pick up HETEs as they enter the cell and direct them to peroxisomes through a specific docking interaction. Alternatively, as HETEs are rapidly incorporated into cellular phospholipids (4, 12), it could be that they pass through phospholipid pools prior to β-oxidation. In that event, the site of β-oxidation could become subject to the specific mechanisms that control phospholipid trafficking and turnover. Neither of these mechanisms would be manifest at the level of isolated organelles.

HETEs are hydroxylated fatty acids, derived from arachidonic acid, that are established as lipid mediators of a number of biological phenomena (1). Other biologically active hydroxylated fatty acids are also known, such as 9- and 13-hydroxyoctadecadienoic acids formed from linoleic acid through either oxygenase reactions or lipid peroxidation (44–47). These latter compounds are probably metabolized in a fashion similar to HETEs. Numerous competitive and synergistic relationships within the metabolism can be envisioned. It is then possible that the overall pattern of hydroxylated fatty acid metabolism leading to formation and degradation of biologically active species will prove to be quite complex. The present results show that mitochondria remain as a potential site of hydroxylated fatty acid β-oxidation. In view of the potential role of β-oxidation in modulating the biological actions of hydroxylated fatty acids, it seems important to better define the intracellular sites involved, over a range of metabolic conditions, and the mechanisms that are responsible for site localization.

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REFERENCES

1. Spector, A. A., J. A. Gordon, and S. A. Moore. 1988. Hydroxyeicosatetrenoic acids (HETEs). *Prog. Lipid Res.* **27:** 271–323.
2. Goetzl, E., J. M. Woods, and R. R. Gorman. 1977. Stimulation of human eosinophil and neutrophil polymorphonuclear leukocyte chemotaxis and random migration to 12-L-5, 8,10,14-eicosatetraenoic acid. *J. Clin. Invest.* **59:** 179–183.
3. Hadjiagapiou, C., and A. A. Spector. 1986. 12-Hydroxyeicosatetraenoic acid reduces prostacyclin production by endothelial cells. *Prostaglandins.* **31:** 1135–1144.

4. Gordon, J. A., and A. A. Spector. 1987. Effects of 12-HETE on renal tubular epithelial cells. *Am. J. Physiol.* **253:** C277–C285.
5. Yamaja Setty, B., J. E. Graeber, and M. J. Stuart. 1987. The mitogenic effect of 15- and 12-hydroxyeicosatetraenoic acid on endothelial cells may be mediated via diacylglycerol kinase. *J. Biol. Chem.* **262:** 17613–17622.
6. Legrand, A. B., J. A. Lawson, B. O. Meyrick, I. A. Blair, and J. A. Oates. 1991. Substitution of 15-hydroxyeicosatetraenoic acid in the phosphoinositide signaling pathway. *J. Biol. Chem.* **266:** 7570–7577.
7. Herbertsson, H., and S. Hammarström. 1992. High-affinity binding sites for 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid (12(S)-HETE) in carcinoma cells. *FEBS Lett.* **298:** 249–252.
8. Grossi, I. M., L. A. Fitzgerald, L. A. Umbarger, K. K. Nelson, C. A. Diglio, J. D. Taylor, and K. V. Honn. 1989. Bidirectional control of membrane expression and/or activation of the tumor cell IRGpIIb/IIIa receptor and tumor cell adhesion by lipoxygenase products of arachidonic acid and linoleic acid. *Cancer Res.* **49:** 1029–1037.
9. Gordon, J. A., P. H. Figard, and A. A. Spector. 1989. Identification of the major metabolite of 12-HETE produced by renal tubular epithelial cells. *J. Lipid Res.* **30:** 731–738.
10. Hadjiagapiou, C., H. Sprecher, T. L. Kaduce, P. H. Figard, and A. A. Spector. 1987. Formation of 8-hydroxyhexadecatrienoic acid by vascular smooth muscle cells. *Prostaglandins.* **34:** 579–589.
11. Moore, S. A., L. J. Prokuski, P. H. Figard, A. A. Spector, and M. N. Hart. 1988. Murine cerebral microvascular endothelium incorporate and metabolize 12-hydroxyeicosatetraenoic acid. *J. Cell Physiol.* **137:** 75–85.
12. Gordon, J. A., P. H. Figard, and A. A. Spector. 1990. HETE metabolism in cultured human skin fibroblasts: evidence for peroxisomal β-oxidation. *J. Clin. Invest.* **85:** 1173–1181.
13. Gordon, J. A., R. A. Zoeller, and A. A. Spector. 1991. Hydroxyeicosatetraenoic acid oxidation in Chinese hamster ovary cells: a peroxisomal metabolic pathway. *Biochim. Biophys. Acta.* **1085:** 21–28.
14. Shen, X-Y., P. H. Figard, T. L. Kaduce, and A. A. Spector. 1988. Conversion of 15-hydroxyeicosatetraenoic acid to 11-hydroxyhexadecatrienoic acid by endothelial cells. *Biochemistry.* **27:** 996–1004.
15. Bremer, J., and K. R. Norum. 1982. Metabolism of very long chain monounsaturated fatty acids (C22:1) and the adaptation to their presence in the diet. *J. Lipid Res.* **23:** 243–256.
16. Schulz, H. 1991. Beta-oxidation of fatty acids. *Biochim. Biophys. Acta.* **1081:** 109–120.
17. Osmundsen, H., C. E. Neat, and K. R. Norum. 1979. Peroxisomal oxidation of long-chain fatty acids. *FEBS Lett.* **99:** 292–296.
18. Lazarow, P. B. 1978. Rat liver peroxisomes catalyze the beta-oxidation of fatty acids. *J. Biol. Chem.* **253:** 1522–1528.
19. Wigren, J., H. Herbertsson, Ö. Tollbom, and S. Hammarström. 1993. Metabolism of 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid by kidney and liver peroxisomes. *J. Lipid Res.* **34:** 625–631.
20. Tserng, K-Y., and S-J. Jin. 1990. NADPH-dependent reductive metabolism of *cis*-5 unsaturated fatty acids: a revised pathway for the β-oxidation of oleic acid. *J. Biol. Chem.* **266:** 11614–11620.
21. Smeland, T. E., M. Nada, D. Cuebas, and H. Schulz. 1992. NADPH-dependent β-oxidation of unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms. *Proc. Natl. Acad. Sci. USA.* **89:** 6673–6677.
22. Gordon, J. A., C. J. Heath, A. A. Spector, and D. R. Pfeiffer. 1992. Conversion of hydroxyeicosatetraenoic acids (HETEs) to polar metabolites by rat liver mitochondria. *FASEB J.* **6:** A966.
23. Fariss, M. W., M. K. Brown, J. A. Schmitz, and D. J. Reed. 1985. Mechanism of chemical-induced toxicity. I. Use of a rapid centrifugation technique for the separation of viable and nonviable hepatocytes. *Toxicol. Appl. Pharmacol.* **79:** 283–295.
24. Schmid, P. C., S. B. Johnson, and H. H. O. Schmid. 1991. Remodeling of rat hepatocyte phospholipids by selective acyl turnover. *J. Biol. Chem.* **266:** 13690–13697.
25. Broekemeier, K. M., P. C. Schmid, H. H. O. Schmid, and D. R. Pfeiffer. 1985. Effects of phospholipase A_2 inhibitors on ruthenium red-induced Ca^{2+} release from mitochondria. *J. Biol. Chem.* **260:** 105–113.
26. Mickelson, J. R., M. L. Greaser, and B. B. Marsh. 1980. Purification of skeletal-muscle mitochondria by density-gradient centrifugation with Percoll. *Anal. Biochem.* **109:** 255–260.
27. Neuburger, M., E-P. Journet, R. Bligny, J-P. Carde, and R. Douce. 1982. Purification of plant mitochondria by isopycnic centrifugation in density gradients of Percoll. *Arch. Biochem. Biophys.* **217:** 312–323.
28. Broekemeier, K. M., P. C. Schmid, M. E. Dempsey, and D. R. Pfeiffer. 1991. Generation of the mitochondrial permeability transition does not involve inhibition of lysophospholipid acylation. *J. Biol. Chem.* **266:** 20700–20708.
29. Igbavboa, U., and D. R. Pfeiffer. 1988. EGTA inhibits reverse uniport-dependent Ca^{2+} release from uncoupled mitochondria. *J. Biol. Chem.* **263:** 1405–1412.
30. Aebi, H. 1974. Catalase. *In* Methods of Enzymatic Analysis. Vol. 2. H. U. Bergmeyer, ed. Academic Press, New York. 673–684.
31. Gunter, T. E., and D. R. Pfeiffer. 1990. The mechanisms by which mitochondria transport calcium. *Am. J. Physiol.* **258:** C755–C786.
32. Bernardi, P. 1992. Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by the proton electrochemical gradient: evidence that the pore can be opened by membrane depolarization. *J. Biol. Chem.* **267:** 8834–8839.
33. Pande, S. V. 1975. A mitochondrial carnitine acylcarnitine translocase. *Proc. Natl. Acad. Sci. USA.* **72:** 883–887.
34. Pande, S. V., and R. Parvin. 1976. Characterization of carnitine acylcarnitine translocase system of heart mitochondria. *J. Biol. Chem.* **251:** 6683–6691.
35. Tolbert, N. E. 1981. Metabolic pathways in peroxisomes and glyoxysomes. *Annu. Rev. Biochem.* **50:** 133–157.
36. Schultz, H. 1987. Inhibitors of fatty acid oxidation. *Life Sci.* **40:** 1443–1449.
37. Holland, P. C., A. E. Senior, and H. S. A. Sherratt. 1973. Biochemical effects of the hypoglycaemic compound pent-4-enoic acid and related non-hypoglycaemic fatty acids. *Biochem. J.* **136:** 173–184.
38. Hiltunen, J. K., and E. J. Davis. 1981. Metabolism of pent-4-enoate in rat liver: reduction of the double bond. *Biochem. J.* **194:** 427–432.
39. Schulz, H. 1983. Metabolism of 4-pentenoic acid and inhibition of thiolase by metabolites of 4-pentenoic acid. *Biochemistry.* **22:** 1827–1832.
40. Kunz, W. S. 1991. Application of the theory of steady-state

flux control to mitochondrial β-oxidation. *Biomed. Biochim. Acta.* **50:** 1143–1157.

41. Williamson, D. H., P. Lund, and H. A. Krebs. 1967. The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. *Biochem. J.* **103:** 514–527.
42. Van Veldhoven, P., L. J. Debeer, and G. P. Mannaerts. 1983. Water- and solute-accessible spaces of purified peroxisomes. *Biochem. J.* **210:** 685–693.
43. Wang, H-Y., C. F. Baxter, Jr., and H. Schulz. 1991. Regulation of fatty acid β-oxidation in rat heart mitochondria. *Arch. Biochem. Biophys.* **289:** 274–280.
44. Buchanan, M. R., T. A. Haas, M. Lagarde, and M. Guichardant. 1985. 13-Hydroxyoctadecadienoic acid is the vessel wall chemorepellant factor, LOX. *J. Biol. Chem.* **260:** 16056–16059.
45. Claeys, M., G. A. A. Kivits, E. Christ-Hazelhof, and D. H. Nugteren. 1985. Metabolic profile of linoleic acid in porcine leukocytes through the lipoxygenase pathway. *Biochim. Biophys. Acta.* **837:** 35–51.
46. Engels, F., G. C. Kessels, A. J. Schreurs, and F. P. Nijkamp. 1991. Production of arachidonic acid and linoleic acid metabolites by human bronchoalveolar lavage cells. *Prostaglandins.* **42:** 441–450.
47. Lenz, M. L., H. Hughes, J. R. Mitchell, D. P. Via, J. R. Guyton, A. A. Taylor, A. M. Gotto, Jr., and C. V. Smith. 1990. Lipid hydroperoxy and hydroxy derivatives in copper-catalyzed oxidation of low density lipoprotein. *J. Lipid Res.* **31:** 1043–1050.