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## Metabolism of Eicosapentaenoic Acid in the Liver of Rats: Participation of the Peroxisomal $\beta$ -Oxidation System in Chain-Shortening

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Many studies on eicosapentaenoic acid (EPA) have confirmed its metabolic transformation to prostaglandins (PGs). However, it is not yet clear whether or not a large part of the EPA contained in foods is metabolized and degraded through some pathway other than transformation to PGs. The purpose of this study was to clarify whether the peroxisomal  $\beta$ -oxidation system participates in the chain-shortening of EPA.

EPA-CoA in a reaction mixture for  $\beta$ -oxidation assay was incubated with rat liver peroxisomes obtained by sucrose density gradient centrifugation and the reaction products were analyzed by gas liquid chromatography. Metabolites having chain lengths reduced by 2 and 4 carbon units from EPA were detected. Furthermore, in order to examine the contribution of the peroxisomal and mitochondrial  $\beta$ -oxidation systems, the activities of purified fatty acyl-CoA oxidase and fatty acyl-CoA dehydrogenase toward EPA were determined and extrapolated to obtain the activities in the rat liver. The activities in the normal rat were 139 U/g liver for peroxisomal  $\beta$ -oxidation and 549 U/g liver for in mitochondrial  $\beta$ -oxidation. On treatment with diethylhexylphthalate (DEHP), a peroxisomal proliferator, the activities in the two organelles were induced to similar extents.

From these findings it is concluded that hepatic peroxisomes participate in the chain-shortening of EPA together with mitochondria.

**Keywords**—eicosapentaenoic acid; peroxisome;  $\beta$ -oxidation; chain-shortening; rat liver

Subcellular organelles, peroxisomes, contain an enzyme system catalyzing the activation and  $\beta$ -oxidation of fatty acids, and the properties of this system differing in some properties from those the corresponding enzymes of mitochondria.<sup>1-4)</sup> The capacity of fatty acid oxidation in peroxisomes is enhanced in the liver of rats treated with certain hypolipidemic agents<sup>5-7)</sup> and under various physiological and pathological conditions.<sup>8-13)</sup> Recently, it has been found that peroxisomes have an ability to degrade very long chain trans-monounsaturated fatty acids,<sup>14)</sup> dicarboxylic acids,<sup>15)</sup> trihydroxycholestanic acid<sup>16,17)</sup> and cholesterol<sup>17)</sup> by the  $\beta$ -oxidation pathway. Furthermore, we have already reported that peroxisomal  $\beta$ -oxidation participates in the chain-shortening of xenobiotic acyl compounds.<sup>18,19)</sup>

On the other hand, eicosapentaenoic acid (EPA) is present in fish oil, and many studies on the biological effects of EPA have confirmed its metabolic transformation to prostaglandins (PGs).<sup>20)</sup> Furthermore, EPA may have inhibitory action on platelet coagulation.<sup>21)</sup> However, because PGs are present in biological systems in only small amounts, a large part of EPA in foods must be metabolized and degraded through some pathway other than transformation to PGs.

The purpose of the present study was to clarify whether the peroxisomal  $\beta$ -oxidation system participates in the chain-shortening of EPA in the liver.

### Materials and Methods

Palmitoyl-coenzyme A (CoA), CoA, reduced nicotinamide adenine dinucleotide (NADH) and adenosine triphosphate (ATP) were purchased from Sigma. Ethyleicosapentaenoate (EPAE) and [U-<sup>14</sup>C]EPAE were kindly donated by Mochida Pharmaceutical Industry Co., Japan. All other reagents were of analytical grade. Sodium eicosapentaenoate (EPA-Na) was prepared by overnight hydrolysis of EPAE with use of equimolar NaOH.<sup>22)</sup> EPA-CoA was prepared from EPA and CoA by the acid chloride method.<sup>23)</sup>

**Treatment of Animals**—Male Wistar rats, weighing about 250 g were fed with laboratory chow containing 2% diethylhexylphthalate (DEHP) for 2 weeks. Animals were sacrificed by decapitation and the livers were quickly removed and homogenized in 0.25 M sucrose–20 mM glycylglycine–HCl (pH 7.4). The homogenates were used for the determination of enzyme activities and for the following experiments including subfractionation.

**Isolation of Hepatocytes**—Hepatocytes were prepared from untreated rats by collagenase perfusion according to Moldeus *et al.*<sup>24)</sup> and the preparations, showing more than 90% cell viability in terms of lactate dehydrogenase latency and Trypan blue exclusion were used.

**Assay of the Metabolites Derived from EPA in Isolated Hepatocyte**—The reaction mixture (1 ml) containing  $2 \times 10^6$  hepatocytes/ml 2% (w/w) bovine serum albumin, Krebs–Henseleit buffer (pH 7.4) and 1 mM [U-<sup>14</sup>C]EPA-Na (0.14  $\mu$ Ci/ $\mu$ mol) was incubated at 37 °C for various intervals. Then, the reaction was stopped by the addition of 1 ml of 55% trichloroacetic acid, and the mixture was simultaneously acidified; CO<sub>2</sub> produced in the reaction was trapped with phenethylamine.<sup>25)</sup> After CO<sub>2</sub> trapping, a 1 ml aliquot was withdrawn and lipids were extracted by the method of Folch *et al.*<sup>26)</sup> The chloroform layer was evaporated and the residue was taken up in 0.5 ml of CHCl<sub>3</sub>–methanol (2:1). An aliquot of 50  $\mu$ l was subjected to thin-layer chromatography (TLC) to separate lipids. The solvent systems were as follows: isopropyl ether–acetic acid (24:1, v/v) in the 1st dimension, petroleum ether–diethyl ether–acetic acid (9:10:1, v/v) in the 2nd dimension. After development, the spots corresponding to lipids were scraped off and put in scintillation cocktail, Aquasol-2, and then the radioactivity was counted in a liquid scintillation counter, Aloka 903.

**Subcellular Fractionation and Sucrose Density Gradient Centrifugation**—The homogenate (10% DEHP treated rat liver) was centrifuged at  $5300 \times g$  for 10 min and subsequently at  $12500 \times g$  for 20 min at 4 °C. The resulting pellet (light mitochondrial fraction, LM fraction of de Duve *et al.*<sup>27)</sup>) was suspended in the homogenizing medium, and 3 ml was layered over a 49 ml discontinuous sucrose density gradient composed of 10 ml of 55.0% (w/w) sucrose in 20 mM glycylglycine (pH 7.4), 17 ml of 45.8%, 12 ml of 38.2% and 10 ml of 23.3% from bottom to top. Centrifugation was carried out at 24000 rpm for 2.5 h in a Hitachi RPS-25-2 rotor at 4 °C. The fractions (each 4 ml) were collected from the bottom with a micropump.

**Analysis of the Metabolite Derived from EPA-CoA after Incubation with Peroxisomes**—EPA-CoA was incubated with peroxisome (fraction 3) prepared by sucrose density gradient centrifugation of the LM fraction from DEHP-treated rat liver. The reaction mixture (10 ml) composed of 30 mM potassium phosphate buffer (pH 7.4), 0.15 mg/ml bovine serum albumin (fatty acid free), 0.01% (w/w) Triton X-100, 1 mM KCN, 50  $\mu$ M CoA, 0.2 mM NAD, 77  $\mu$ M [U-<sup>14</sup>C]EPA-CoA (3.9  $\mu$ Ci/ $\mu$ mol) and 1 ml of peroxisomal fraction (fraction 3) was incubated at 37 °C for 140 min, then 1.63 ml of 2 N NaOH was added and the whole was incubated again in order to stop the reaction and hydrolyze the CoA derivative to the free acid. After neutralization by adding 2.67 ml of 3 N HCl, the metabolites were extracted 3 times with an appropriate volume of diethyl ether–petroleum ether (1:1). The combined organic phase was evaporated under a nitrogen stream, and methylated with diazomethane, then the methylated metabolites were analyzed by radio gas chromatography.

**Purification of Fatty Acyl-CoA Oxidase (FAO) and Fatty Acyl-CoA Dehydrogenase (FADH)**—FAO and FADH were partially purified from 40 g of liver of DEHP-treated rats by the method of Osumi *et al.*<sup>28,29)</sup> and that of Furuta *et al.*<sup>30,31)</sup> and Ikeda *et al.*<sup>31)</sup> respectively.

**Enzyme and Protein Assay**—The activity of catalase was determined by the method of Luck.<sup>32)</sup> One unit of the activity was defined as the amount of the enzyme showing  $k=1$  where  $k$  is the rate constant. The activity of the cyanide-insensitive fatty acyl-CoA oxidizing system (FAOS) was determined by the method of Lazarow and de Duve<sup>1)</sup> using EPA-CoA or palmitoyl-CoA as a substrate. The activity of carnitine acyltransferase (CAT) was determined by the method of Markwell *et al.*<sup>33)</sup> using EPA-CoA, palmitoyl-CoA and acetyl-CoA as substrates. The activities of FAO and FADH were determined by the methods of Horie *et al.*<sup>13)</sup> and Hryb and Hogg,<sup>34)</sup> respectively, using EPA-CoA or palmitoyl-CoA as a substrate. The activity of glutamate dehydrogenase (GDH) was determined by the method of de Duve.<sup>35)</sup> Unless otherwise stated, 1 unit of all enzyme activities was defined as the amount of the enzyme that produced 1 nmol of reaction product/min. Protein content was determined by the method of Lowry *et al.*<sup>36)</sup> using bovine serum albumin as a standard.

### Results and Discussion

The metabolism of EPA in rat hepatocytes was examined by using [U-<sup>14</sup>C]EPA-Na. As shown in Fig. 1, the radioactivity derived from labeled EPA was incorporated into

triglyceride, acid soluble products which were mainly composed of ketone bodies, and phospholipid fraction: about 30%, 20%, and 5%, respectively, of the original radioactivity after a 2 h incubation. The amount of ketone bodies is regarded as a measure of  $\beta$ -oxidation. Only small amounts of radioactivity were found in the diglyceride, monoglyceride, cholesterol and  $\text{CO}_2$  fractions. These results show that during a 2 h incubation, about 70% of EPA-Na was metabolized, of which about 30% and 70% were metabolized through  $\beta$ -

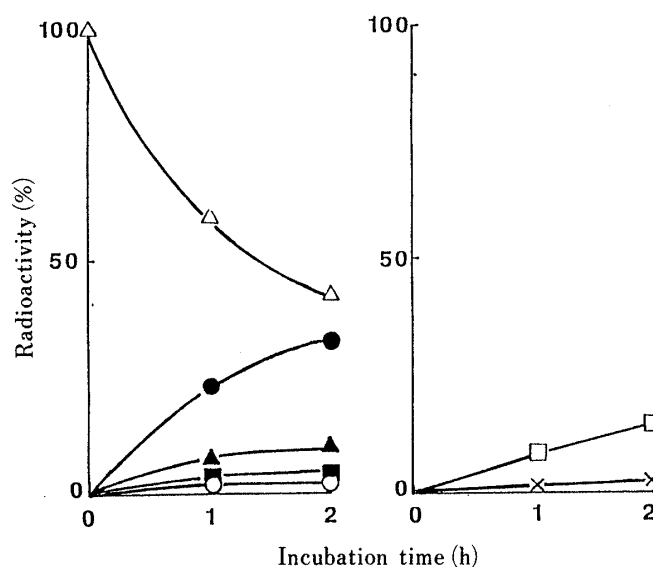


Fig. 1. Incorporation of Radioactivity from  $[\text{U-}^{14}\text{C}]\text{EPA-Na}$  into Lipids and  $\beta$ -Oxidation Products in Isolated Hepatocytes from the Rat

$\Delta$ , fatty acid;  $\bullet$ , triglyceride;  $\blacktriangle$ , phospholipid;  $\blacksquare$ , monoglyceride;  $\circ$ , diglyceride plus cholesterol;  $\square$ , acid soluble products (mainly ketone bodies);  $\times$ ,  $\text{CO}_2$ .

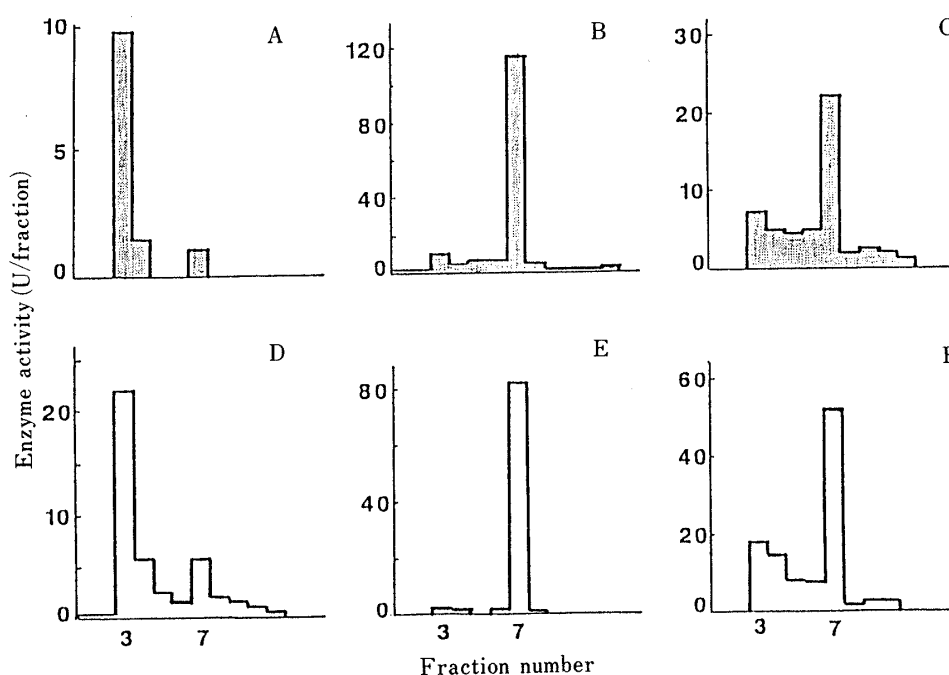


Fig. 2. Sucrose Density Gradient Centrifugation of the Light Mitochondrial Fraction of Rat Liver

A, FAOS (for EPA-CoA); B, CAT (for EPA-CoA); C, FADH (for EPA-CoA); D, FAOS (for palmitoyl-CoA); E, CAT (for palmitoyl-CoA); F, FADH (for palmitoyl-CoA).

oxidation and esterification, respectively. To confirm the subcellular distribution of the activities of  $\beta$ -oxidation for EPA, the LM fraction was fractionated by sucrose density gradient centrifugation. Catalase activity of peroxisomes and GDH activity of mitochondria were mainly located in fraction 3 and fraction 7, respectively (data not shown). The distribution patterns showed that most of the activity of FAOS toward EPA-CoA or palmitoyl-CoA was present in fraction 3, whereas most of the activities of CAT and FADH toward EPA-CoA were present in fraction 7 (Fig. 2). The activities of FAOS and FADH toward EPA-CoA were less than those toward palmitoyl-CoA as a substrate. However, the activity of CAT (which catalyzes the transfer of fatty acids through the mitochondrial membrane) toward EPA-CoA was higher than that toward palmitoyl-CoA. These findings suggest that in the mitochondrial  $\beta$ -oxidation of EPA the reaction of FADH might be rate-limiting. To confirm the chain-shortening of EPA-CoA by peroxisomal  $\beta$ -oxidation, the activity of FAO (a rate-limiting enzyme of peroxisomal  $\beta$ -oxidation) toward EPA-CoA was examined (Table I). The results showed that EPA-CoA could be a substrate of peroxisomal FAO and on treatment with DEHP, a peroxisome proliferator, the FAO activities toward EPA-CoA and palmitoyl-CoA used as references were significantly increased (about 18-fold),

TABLE I. Effects of Diethylhexylphthalate Treatment on Peroxisomal FAO Activity in Rat Liver

Substrate	FAO activity ( $\mu\text{mol}/\text{min}/\text{g}$ liver)	
	Control	DEHP
EPA-CoA	$188 \pm 31$	$3512 \pm 397^a$
Palmitoyl-CoA	$626 \pm 94$	$11768 \pm 951^a$

Treatment: 2% DEHP in the diet, 2 weeks. *a*) Statistical significance:  $p < 0.001$ .

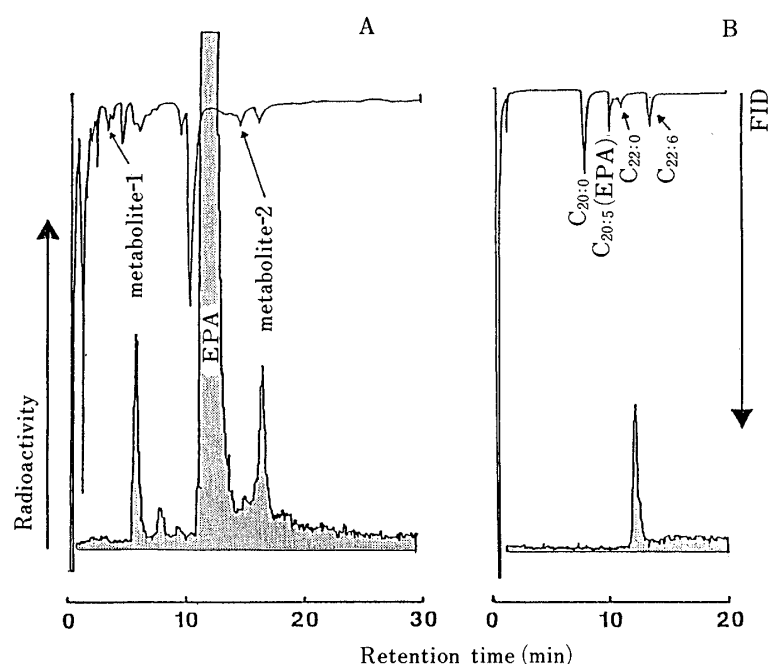


Fig. 3. Radio Gas Chromatographic Analysis of the Incubation Products

A glass column (3 mm internal diameter) packed with 2% LAC-2R-446 on Chromosorb W (AW-DMCS) was used. The column temperature was 145–200°C (5°C/min). A, the complete system (140 min incubation); B, fatty acid standards.

indicating that EPA-CoA is chain-shortened by the peroxisomal  $\beta$ -oxidation pathway. For this to occur in the liver, the formation of EPA-CoA from EPA by acyl-CoA synthetase (ACS) would be essential. The activity of ACS in the liver toward EPA was  $7.3 \mu\text{mol}/\text{min}/\text{g}$  liver, which is sufficiently high to supply the CoA derivative to the  $\beta$ -oxidation pathway. Figure 3 shows the radio gas chromatographic analysis of the metabolite when  $[\text{U-}^{14}\text{C}]\text{EPA}$  was incubated with peroxisomes purified by sucrose density gradient centrifugation. Figure 3B shows the pattern of standard fatty acids and labeled EPA. The retention time of EPA was around 12 min. After 140 min incubation with peroxisomes, two radioactive peaks (metabolite-1 and metabolite-2) in addition to that of EPA were found at retention times of 5 and 16 min, respectively. In order to identify the carbon chain length of metabolite-1, an aliquot of the reaction mixture was subjected to hydrogenation and analyzed by radio gas chromatography again. As shown in Fig. 4A, two radioactive peaks (reduced metabolite-1 and reduced EPA) were found at retention times of about 4 and 10 min, corresponding to those of palmitic acid and arachidonic acid. Furthermore, gas chromatography-mass spectrum analysis of the

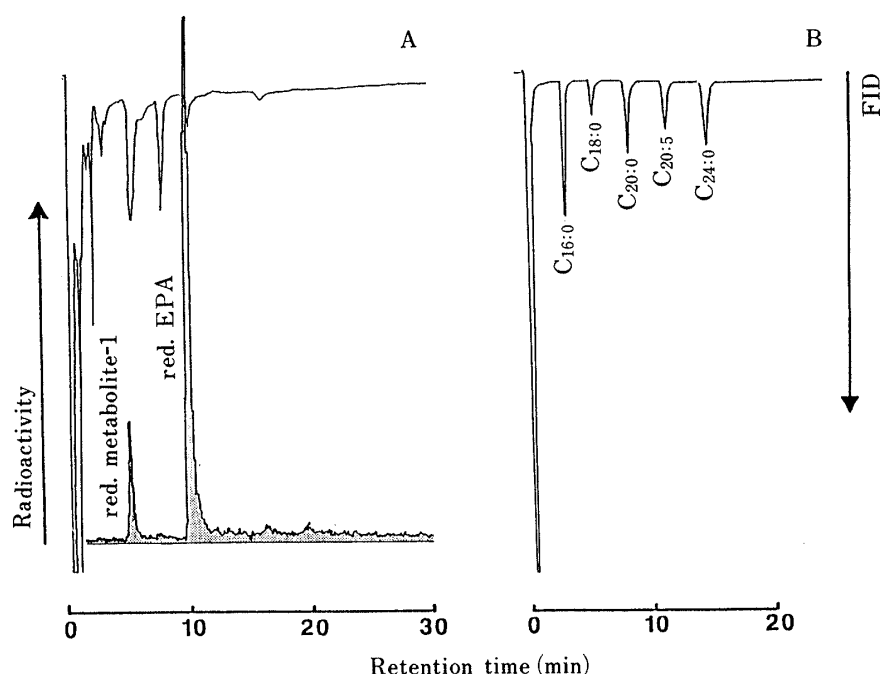


Fig. 4. Radio Gas Chromatographic Analysis of the Reduced Incubation Products

The reaction products were reduced by the method of Mozingo<sup>37)</sup> and then analyzed. Peroxisomal  $\beta$ -oxidation products from cold EPA-CoA were dissolved in 0.5 ml of methanol, 2 mg of palladium carbon was added, and hydrogen gas was introduced with stirring overnight at room temperature, then 5 ml of  $\text{CHCl}_3$  was added. After filtration of the mixture through a silica gel column, the reduced peroxisomal  $\beta$ -oxidation products were analyzed as described previously. A, after reduction of the reaction products. B, fatty acid standards.

TABLE II. Kinetic Constants of FAO and FADH for EPA-CoA

Substrate	FAO		FADH	
	$K_m$	$V_{\max}$	$K_m$	$V_{\max}$
EPA-CoA	5.6	0.56	6.8	1.21
Palmitoyl-CoA	14.6	3.16	6.3	1.86

$K_m$  in  $\mu\text{M}$ ,  $V_{\max}$  in  $\mu\text{mol}/\text{min}/\text{mg}$  protein.

metabolites after incubation of cold EPA-CoA with peroxisomes showed metabolite-1 to be hexadecatetraenoic acid ( $C_{16:4}$ ). Although the structure of metabolite-2 has not yet been clarified, it seems to be a 3-hydroxy intermediate of  $\beta$ -oxidation reaction on the basis of the following reasons. 1) The retention time of metabolite-2 is larger than that of EPA, suggesting that it is not be octadecapentaenoic acid ( $C_{18:5}$ ). 2) Under these reaction conditions, chain elongation could not be performed. 3) The formation of metabolite-2 should not be an artifact, because the corresponding peak could not be found in the starting materials. 4) The retention time of the 3-hydroxy form of a fatty acid is larger than that of the corresponding fatty acid. The disappearance of metabolite-2 after reduction might be explained by overlap with reduced EPA. From these experimental results, we conclude that under these conditions, the EPA-CoA chain was shortened by 4 carbon units and furthermore it is suggested that, at the step of the transformation of octadecapentaenoic acid to hexadecatetraenoic acid, participation of *cis*, *trans*-3,2-enoyl-CoA isomerase is required.

The kinetic parameters of FAO and FADH, which considered to be rate-limiting enzymes of  $\beta$ -oxidation of EPA-CoA in peroxisomes and mitochondria, respectively, are shown in Table II. Although the  $K_m$  values of both enzymes for EPA-CoA were similar,  $V_{max}$  of FAO was about 46% of that of FADH. Based on these values and the amount of enzyme protein finally obtained, the activities of  $\beta$ -oxidation of EPA in peroxisomes and mitochondria were calculated as 139 and 542 U/g liver, respectively, suggesting the contribution of peroxisomal  $\beta$ -oxidation to EPA chain-shortening is about 20%.

From these results, it was concluded that a large part of EPA that is taken up in foods is metabolized and degraded to chain-shortened products through some pathway other than transformation to PGs, resulting in the production of acetyl-CoA, with participation of peroxisomal and mitochondrial  $\beta$ -oxidation.

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