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## Metabolism of Ethyl Eicosapentaenoate (EPA-E) in Rats and Effect of Its Metabolites on Ellagic Acid-Induced Thrombus Formation in the Stenosed Femoral Artery of Rabbits

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The metabolism of ethyl eicosapentaenoate (EPA-E) was investigated in rats *in vivo* and *in vitro* with uniformly labelled  $^{14}\text{C}$ -EPA-E, and the antithrombotic properties of major metabolites of EPA-E in rabbits were examined.

When  $^{14}\text{C}$ -EPA-E was administered orally to rats, eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) were identified in the liver as metabolites of EPA-E. These metabolites were incorporated mainly into phospholipids and triglycerides, and were found at the C-2 position of phospholipids and the C-1 and/or C-3 positions of triglycerides.

When  $^{14}\text{C}$ -EPA coenzyme A ( $^{14}\text{C}$ -EPA-CoA) was incubated with intact mitochondria,  $^{14}\text{CO}_2$  was released and chain-shortened metabolites were detected by radio-high performance liquid chromatography. When  $^{14}\text{C}$ -EPA-CoA was incubated with intact peroxisomes, chain-shortened metabolites were detected as in the case of mitochondria.

When potassium eicosapentaenoate ( $^{14}\text{C}$ -EPA·K) was incubated with microsomes, DPA and DHA were produced as a result of chain elongation and subsequent desaturation reactions. When  $^{14}\text{C}$ -EPA-CoA was incubated with intact peroxisomes, DPA was produced in the presence of malonyl-CoA as a cofactor.

EPA-E significantly decreased the incidence of thrombus formation and also decreased the length of thrombi in rabbits. Major metabolites of EPA-E, such as EPA, DPA and DHA, moderately decreased the incidence of thrombus formation.

**Keywords**—ethyl eicosapentaenoate; metabolism; antithrombotic activity; rat; rabbit

### Introduction

Ethyl eicosapentaenoate (ethyl all-*cis*-5,8,11,14,17-eicosapentaenoate, EPA-E) is the ethyl ester of a long-chain polyunsaturated fatty acid present in fish oil. Yamaguchi *et al.*<sup>1)</sup> and Mizota *et al.*<sup>2)</sup> have shown that EPA-E has an inhibitory action on thrombus formation, and possesses a hypolipidemic activity and preventive effect against peripheral gangrene.

We have previously demonstrated that, after oral administration of  $^{14}\text{C}$ -EPA-E to rats, absorbed radioactivity was transported to the systemic circulation mainly through the lymphatic system and that the increases in the concentrations of radioactivity in the brain, heart, liver and adipose tissues were greater after repeated administration.<sup>3)</sup> However, it remains unknown whether these increased radioactivities were attributable to  $^{14}\text{C}$ -EPA-E or its metabolites. In the present study, we analyzed the metabolites in rat liver after oral administration of  $^{14}\text{C}$ -EPA-E, and detailed *in vitro* experiments were also carried out. We also examined the antithrombotic properties of major metabolites of EPA-E detected in rat liver, such as eicosapentaenoic acid ( $\text{C}_{20:5}$ , EPA), docosapentaenoic acid ( $\text{C}_{22:5}$ , DPA) and docosahexaenoic acid ( $\text{C}_{22:6}$ , DHA), on ellagic acid-induced thrombus formation in the stenosed femoral artery of rabbits, in comparison with the effect of EPA-E.

## Experimental

**Chemicals**—EPA-E, EPA, DPA and DHA (90.5%, 91.3%, 95.4% and 89.0% purity, respectively) were obtained from Nippon Suisan Kaisha. Uniformly labelled  $^{14}\text{C}$ -EPA-E (Fig. 1), specific radioactivity 463.4 mCi/mmol, radiochemical purity 97%, was purchased from Daiichi Pure Chemicals.<sup>3)</sup>  $^{14}\text{C}$ -EPA-E was hydrolyzed to obtain  $^{14}\text{C}$ -EPA, from which  $^{14}\text{C}$ -EPA coenzyme A-thioester ( $^{14}\text{C}$ -EPA-CoA) was prepared by the method of Seubert.<sup>4)</sup> The chemical structure of  $^{14}\text{C}$ -EPA-CoA was confirmed by fast atom bombardment mass spectrometry (FAB-MS), and its radiochemical purity determined by radio-high performance liquid chromatography (radio-HPLC; described below) was 95.6%. The potassium salt of  $^{14}\text{C}$ -EPA ( $^{14}\text{C}$ -EPA·K) was prepared by hydrolysis of  $^{14}\text{C}$ -EPA-E with an ethanolic solution of potassium hydroxide, and its radiochemical purity was 99% by thin layer chromatography (TLC), described below. Coenzyme A (CoA-SH) was purchased from Kohjin, reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), phospholipase  $\text{A}_2$  and lipase from Boehringer Mannheim, malonyl coenzyme A (malonyl-CoA), adenosine triphosphate (ATP) and L-carnitine hydrochloride from Sigma Chemical Co., ellagic acid from Aldrich Chemical, saturated fatty acid methyl esters used as authentic samples in HPLC from Gasukuro Kogyo Inc., and other chemicals from Wako Pure Chemicals.

**Animals**—In the *in vivo* experiments, 6-week-old male Wistar rats were purchased from Shizuoka Laboratory Animal Center (SLC) and fed a standard diet (CE-2, Clea Japan) for 2 weeks before use. In the *in vitro* experiments, 6-week-old male Wistar rats from SLC were fed a fish oil-free diet (F-1, Funabashi Farms) for 2 weeks before use. Rats used in microsomal experiments further received a high-carbohydrate diet.<sup>5)</sup> Male Japanese white rabbits weighing 2.36–3.35 kg were purchased from Funabashi Farms.

**Administration**— $^{14}\text{C}$ -EPA-E was diluted with EPA-E to adjust the radioactivity as required, and emulsified in 5% arabic gum solution for oral administration (30 mg/50  $\mu\text{Ci}$ /3 ml/kg) to rats which had been fasted overnight. In the case of rabbits, EPA-E, EPA, DPA or DHA emulsified in 5% arabic gum solution was orally administered (300 mg/1 ml/kg) without fasting.

**Preparation**—Feces and urine excreted within 24 h after administration were separately collected from rats in a closed metabolic cage with a device to collect expired air, and the liver was removed after 24 h. Lymph was collected from the thoracic duct in a 9 h period after the administration. Total lipids were extracted from liver and lymph according to the procedure of Folch *et al.*<sup>6)</sup> In order to prevent the oxidation of lipids, the solvent was supplemented with 0.2% 2,6-di-*tert*-butyl-4-methylphenol. Triglycerides (TG) and phospholipids (PL) were fractionated from total lipids obtained from liver and lymph by column chromatography. The lipid fractions were identified by comparison with authentic samples by TLC. Mitochondria, peroxisomes and microsomes were obtained according to the methods of Oda<sup>7)</sup> and Kϕlvraa and Gregersen<sup>8)</sup> with some modifications. Briefly, rat liver homogenate was centrifuged at  $50 \times g$  for 7 min to sediment nuclei and cell debris, and 25 ml of the supernatant was overlaid on 25 ml of 0.35 M sucrose solution and centrifuged at  $700 \times g$  for 10 min to remove red blood cells. The supernatant (25 ml) was further centrifuged at  $5300 \times g$  for 10 min and a fraction enriched in mitochondria and peroxisomes was obtained. A sample of this fraction, corresponding to about 4.0 g of liver tissue, was then overlaid on 40 ml of 37.5% Percol<sup>®</sup>, made isotonic with sucrose, and centrifuged at  $32500 \times g$  for 30 min at 4°C. Mitochondria, at the bottom, and peroxisomes, at the upper layer of the gradient, were collected and suspended in 0.01 M potassium phosphate buffer (pH 7.4) containing 0.3 M mannitol. The mitochondrial and peroxisomal fractions were characterized by measuring the activities of marker enzymes, cytochrome c oxidase, urate oxidase and catalase; the relative activities between mitochondrial and peroxisomal fractions were 1:0.04, <0.01:1 and 0.04:1, respectively. The supernatant at  $5300 \times g$  was centrifuged again at  $8500 \times g$  for 10 min, and the supernatant was centrifuged again at  $105000 \times g$  for 60 min to recover microsomes. The resultant pellet was resuspended in 100 mM phosphate buffer (pH 7.4). These organelle were used without further purification, and their protein contents were determined according to Lowry *et al.*<sup>9)</sup> using bovine serum albumin as the standard.

**Analysis of Metabolites**—The acyl groups of lipids extracted from rat liver were converted to methyl esters by refluxing with 14%  $\text{BF}_3$  methanol solution and then subjected to radio-HPLC to separate radioactive fractions. These fractions were subjected to gas chromatography-mass spectrometry (GC-MS) as described below, and both the retention time and the mass spectrum were compared with those of authentic samples. Radioactive substances excreted into feces and urine were extracted with *n*-hexane, then with chloroform-methanol (2:1 v/v) and subjected to radio-HPLC after methylation.

**Determination of Positional Distribution of Metabolites in PL and TG**—TG separated from lymph or liver was hydrolyzed with porcine pancreas lipase as follows.<sup>10)</sup> A mixture of TG (1.3–1.4 mg), 0.05% bile salt (0.25 ml), 2.2%  $\text{CaCl}_2$  solution (0.1 ml) and 1 M Tris-HCl buffer (pH 8.0, 1 ml) was shaken for 1 min at 40°C and then lipase (1 mg) was added. This mixture was shaken for 30 min at 40°C and extracted with ether after addition of ethanol (1 ml) and 6 N HCl (1 ml). PL separated from lymph or liver were hydrolyzed with snake venom phospholipase  $\text{A}_2$  as follows.<sup>11)</sup> A mixture of PL (1.6–7.0 mg), ether (1.5 ml), 0.2 M borate buffer (pH 7.0, 1 ml) and 4 mM  $\text{CaCl}_2$ –0.1 M Tris-HCl buffer (pH 7.4, 0.2 ml) containing phospholipase  $\text{A}_2$  (8 U) was shaken for 1 h at 37°C. The ether layer and the organic layer obtained after extraction of the aqueous layer with chloroform-methanol (2:1) were pooled. The extracts

obtained above were subjected to TLC analysis as described below.

**$\beta$ -Oxidation by Mitochondria**— $^{14}\text{C}$ -EPA-CoA (115 nmol) was incubated with a mixture of mitochondria (10 or 20 mg protein), *L*-carnitine hydrochloride (1.5  $\mu\text{mol}$ ) and oxalic acid (1.0  $\mu\text{mol}$ ) in 10 mM potassium phosphate buffer containing 0.3 M mannitol (pH 7.4, final volume 2 ml) at 37°C for 30 min in a cuvet, in which a piece (1.6  $\times$  6 cm) of filter paper moistened with 0.2 ml of Hyamine 10X<sup>®</sup> was suspended. After the incubation, 0.1 ml of 0.1 N  $\text{H}_2\text{SO}_4$  was added to the mixture through the rubber cap and incubation was continued for a further 30 min, then the radioactivity on the filter paper was counted. The incubation mixture before adding  $\text{H}_2\text{SO}_4$  was saponified by adding 0.25 ml of 4 M aqueous NaOH and 5 ml of methanol, then acidified with 0.25 ml of 9 M HCl and extracted with chloroform. The lipids were converted to methyl esters as described above and subjected to radio-HPLC (described below).

**$\beta$ -Oxidation by Peroxisomes**— $^{14}\text{C}$ -EPA-CoA (115 nmol) was incubated with peroxisomes (10 or 20 mg protein) in 10 mM potassium phosphate buffer containing 0.3 M mannitol (pH 7.4, final volume 2 ml) at 37°C for 30 min. The resulting reaction mixture was processed by the same method as in the case of the mitochondrial experiment except for adding  $\text{H}_2\text{SO}_4$ .

**Elongation and Desaturation or  $^{14}\text{C}$ -EPA  $\cdot$  K by Microsomes**— $^{14}\text{C}$ -EPA  $\cdot$  K (100 nmol) was incubated with a mixture of microsomes (10 mg protein),  $\text{MgCl}_2$  (10  $\mu\text{mol}$ ), ATP (10  $\mu\text{mol}$ ), NADPH (2  $\mu\text{mol}$ ), NADH (2  $\mu\text{mol}$ ), malonyl-CoA (1  $\mu\text{mol}$ ) and CoA-SH (0.3  $\mu\text{mol}$ ) in 100 mM potassium phosphate buffer (pH 7.4, final volume 1.5 ml) at 37°C for 6 min and the resulting reaction mixture was subjected to analysis as described above.

**Elongation of  $^{14}\text{C}$ -EPA-CoA by Peroxisomes**— $^{14}\text{C}$ -EPA-CoA (100 nmol) was incubated with a mixture of peroxisomes (10 mg protein), NADPH (2  $\mu\text{mol}$ ), NADH (2  $\mu\text{mol}$ ) and malonyl-CoA (1  $\mu\text{mol}$ ) in 10 mM potassium phosphate buffer containing 0.3 M mannitol (pH 7.4, final volume 1.5 ml) at 37°C for 6 min. The resulting reaction mixture was subjected to analysis as described above.

**Effects on Ellagic Acid-Induced Thrombus Formation in Rabbits**—Experiments were carried out according to the method of Maekawa *et al.*<sup>12)</sup> The incidence of thrombus formation and the total length of thrombi formed in the stenosed artery were measured 24 h after the injection of ellagic acid. Test drugs dissolved in 5% arabic gum solution were orally administered to animals 3 h before the injection of ellagic acid.

**TLC**—Silica gel 60F<sub>254</sub> (Merck) was used as a carrier. As a developing solvent, chloroform was used for the assay of radiochemical purity of  $^{14}\text{C}$ -EPA, and for the identification of lipids, ether-petroleum ether-acetic acid (60:40:1) or chloroform-methanol-acetone-acetic acid-water (50:10:20:10:5) was employed. Radioactivity was detected by using a TLC scanner (Aloka, JTC-601).

**HPLC**—An HPLC apparatus (Erma, ERC-8710) equipped with a radio-analyzer (Aloka, RLC-551) or a high-sensitivity RI detector (JASCO, RID-300) was operated under the following conditions: column, ERC-ODS-1171; mobile phase, acetonitrile or acetonitrile-water (9:1); flow rate, 1 ml/min or 0.6 ml/min.

**GC-MS**—Mass spectra were obtained using a GC-MS apparatus (JEOL, JMS-DX303) equipped with a gas chromatograph (Hewlett Packard, 5890A) and a data analysis system (JEOL, JMA-DA5500) under the following conditions: column, Hewlett-Packard Ultra #2 (fused silica, 50 m  $\times$  0.2 mm); column temperature, 200°C; carrier gas, helium (1 ml/min); ionization voltage, 23 eV; ionization current, 300  $\mu\text{A}$ ; ionization chamber temperature, 200°C; separator temperature, 250°C.

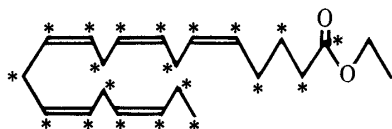


Fig. 1. Chemical Structure of [ $\text{U-}^{14}\text{C}$ ]Ethyl Eicosapentaenoate

## Results

### Structures of Metabolites of EPA-E in Liver

Radio-HPLC analysis of the radioactive substances extracted from the liver showed three peaks named P1, P2, and P3 (Fig. 2). The retention times of P1, P2 and P3 coincided with those of the methyl ester derivatives of EPA, DHA and DPA, respectively. The retention times and mass spectra of the radioactive substances in P1, P2 and P3 were identical with those of the methyl ester derivatives of EPA, DHA and DPA, respectively, in GC-MS (Figs. 3, 4 and 5). Thus, the metabolites in peaks P1, P2, and P3 were identified as EPA, DHA and DPA, respectively.

### Metabolites in Expired Air, Urine and Feces

The radioactive substance excreted into expired air of rats after dosing of  $^{14}\text{C}$ -EPA-E was identified as  $^{14}\text{CO}_2$  because it was captured in ethanolamine and precipitated by  $\text{Ba}(\text{OH})_2$ .

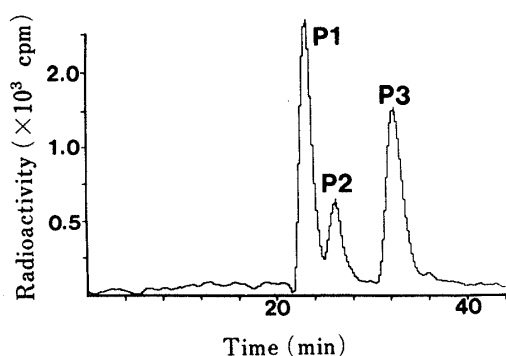


Fig. 2. Radio-High Performance Liquid Chromatogram of  $^{14}\text{C}$ -EPA-E Metabolites Formed in Rat Liver after Oral Administration of  $^{14}\text{C}$ -EPA-E

TABLE I. Urinary, Fecal, and Respiratory Metabolites in Male Rats within 24 h after Oral Administration of  $^{14}\text{C}$ -EPA-E

Metabolite	Urine	Feces	Expired air
EPA-E	N.D.	$2.6 \pm 0.2$	
EPA	N.D.	$7.8 \pm 0.9$	
DPA	N.D.	N.D.	
DHA	N.D.	N.D.	
$\text{CO}_2$			$39.6 \pm 2.7$
Others	$2.0 \pm 0.1$	$4.4 \pm 0.7$	
Total	$2.0 \pm 0.1$	$14.8 \pm 0.9$	$39.6 \pm 2.7$

Results are given as the means (% of dose)  $\pm$  S.E. of three animals. N.D.: not detected.

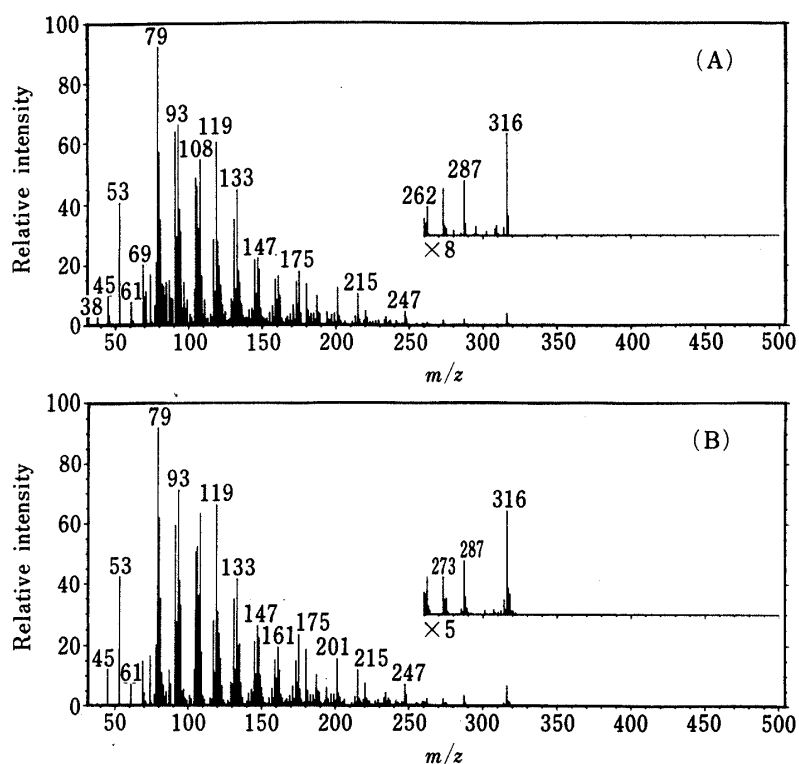


Fig. 3. Mass Spectra of P1 (A) and EPA Methyl ester (B)

solution (Table I).

About 14.8% of the administered radioactivity was excreted into feces within 24 h after dosing. EPA-E and EPA were detected but DPA and DHA were not (Table I).

Only 2.0% of the administered radioactivity was excreted into urine within 24 h after dosing, and EPA-E, EPA, DPA and DHA were not detected (Table I).

#### Determination of Positional Distribution of Metabolites in PL and TG

TLC analysis of C-1 and C-3 selective lipase-treated radioactive TG from lymph showed that 90.7% and 8.8% of the radioactivity were detected in free fatty acid (FFA) and monoglyceride (MG) fractions, respectively. In the case of TG from liver, 86.6% and 5.9% of the radioactivity were likewise detected (Table II).

When radioactive PL derived from lymph was hydrolyzed with phospholipase  $\text{A}_2$ , which

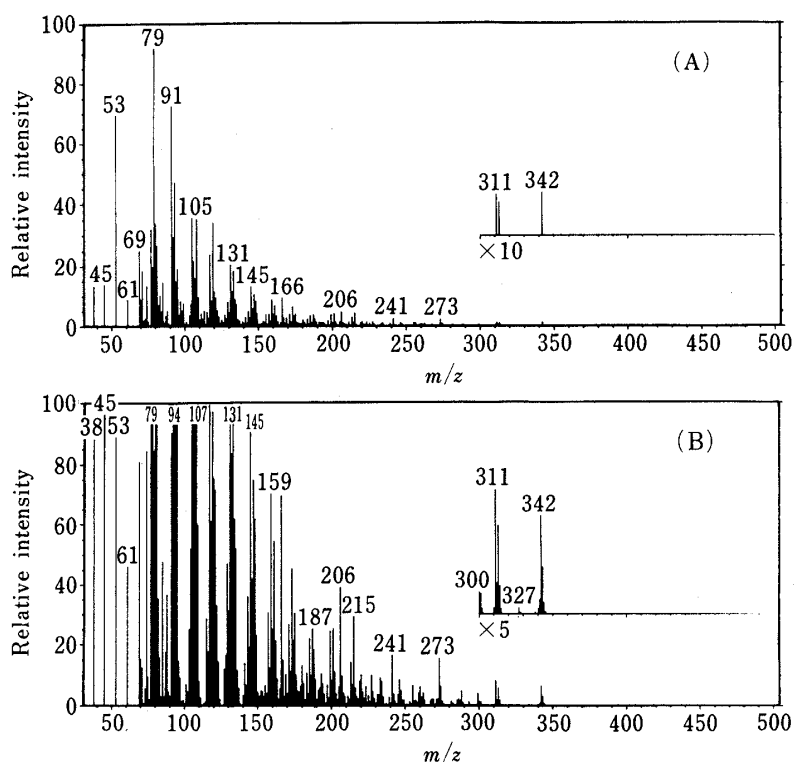


Fig. 4. Mass Spectra of P2 (A) and DHA Methyleneester (B)

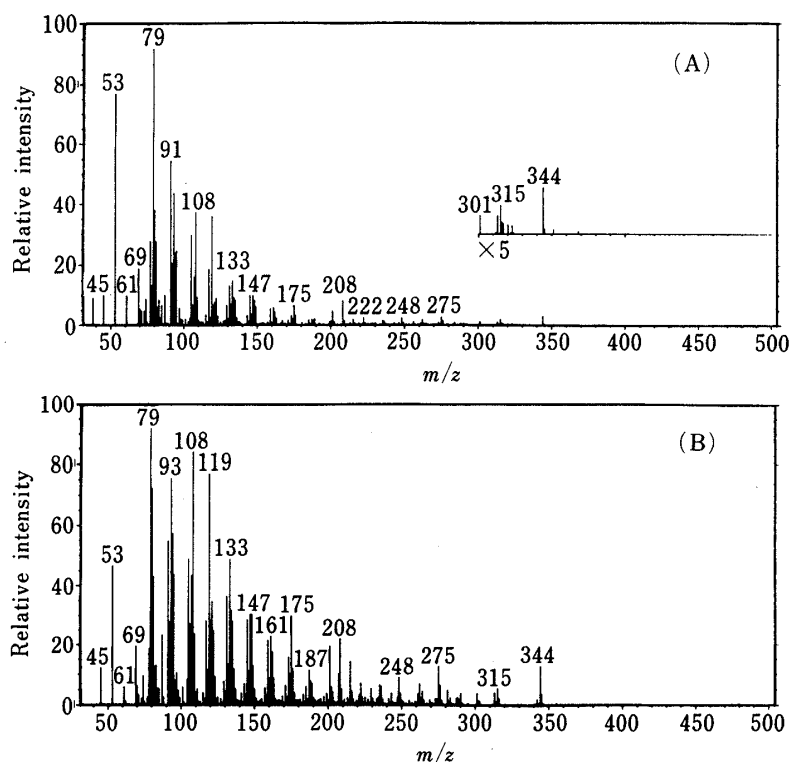


Fig. 5. Mass Spectra of P3 (A) and DPA Methyleneester (B)

releases acyl groups selectively from the C-2 position of PL, and subjected to TLC, 94.3% of the radioactivity was detected in FFA fraction. In the case of phosphatidylethanolamine (PE)-rich PL (PE\*) and phosphatidylcholine (PC)-rich PL (PC\*) from liver, 91.9% and 95.5%

TABLE II. Percent of Radioactivity in Each Fraction on TLC after Hydrolysis of TG with Lipase

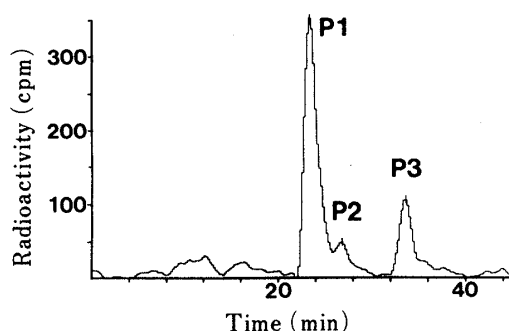
Origin	Subject	% of radioactivity				Recovery <sup>a)</sup> of radioactivity (%)
		TG	FFA	DG	MG	
Lymph	Material	100.0	0.0	0.0	0.0	
	H.P.	0.0	90.7	0.0	8.8	98.3
Liver	Material	94.9	3.7	0.0	0.0	
	H.P.	0.0	86.6	0.0	5.9	98.1

H.P.: hydrolysis product. TG: triglycerides. FFA: free fatty acid. DG: diglycerides. MG: monoglycerides. <sup>a)</sup> Recovery of radioactivity in the organic layer after hydrolysis of TG with lipase.

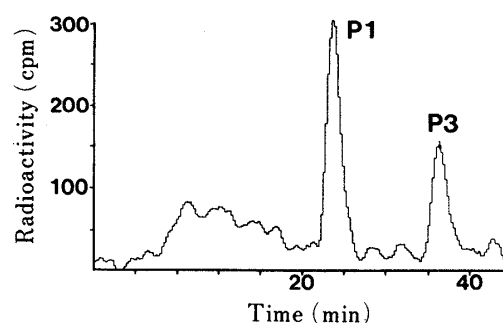
TABLE III. Percent of Radioactivity in Each Fraction on TLC after Hydrolysis of PL with Phospholipase A<sub>2</sub>

Origin	PL	Subject	% of radioactivity			Recovery <sup>a)</sup> of radioactivity (%)
			FFA	PL	LysoPL	
Lymph	PL	Material	0.0	95.5	0.0	
		H.P.	94.3	0.0	0.0	97.9
Liver	PE*	Material	0.0	96.7	0.0	
		H.P.	91.9	0.0	0.0	99.3
	PC*	Material	0.0	96.6	2.5	
		H.P.	95.5	0.0	0.0	99.5

FFA: free fatty acid. PL: phospholipids. PE\*: phosphatidyl-ethanolamine (PE)-rich PL. PC\*: phosphatidylcholine (PC)-rich PL. H.P.: hydrolysis product. <sup>a)</sup> Recovery of radioactivity in the organic layer after hydrolysis of PL with phospholipase A<sub>2</sub>.

Fig. 6. Radio-High Performance Liquid Chromatogram of Methylester of Extract after Incubation of <sup>14</sup>C-EPA-K with Rat Liver Microsomes

P1, EPA methylester; P2, DHA methylester; P3, DPA methylester.

Fig. 7. Radio-High Performance Liquid Chromatogram of Methylester of Extract after Incubation of <sup>14</sup>C-EPA-CoA with Rat Liver Peroxisomes under Reductive Condition

P1, EPA methylester; P3, DPA methylester.

of the radioactivity were detected in FFA fraction, respectively (Table III). No radioactivity was detected in lysoPL fraction in any case.

### Elongation and Desaturation of EPA by Microsomes and Peroxisomes

The chain elongation and desaturation experiments were carried out using microsomes from rats on a high-carbohydrate diet, since the use of microsomes from rats raised on a diet containing fish oil failed to yield DHA (unpublished results), and it was reported<sup>5)</sup> that the microsomal desaturation activity was high in rats on a high-carbohydrate diet. When microsomes were incubated with <sup>14</sup>C-EPA-K, the elongation product of EPA, *i.e.* DPA, and the desaturation product of DPA, *i.e.* DHA, were produced (Fig. 6, Table IV).

Incubation of intact peroxisomes and <sup>14</sup>C-EPA-CoA with malonyl-CoA as a co-factor to supply the C-2 unit resulted in the formation of DPA (Fig. 7, Table IV).

### β-Oxidation by Mitochondria and Peroxisomes

Incubation of <sup>14</sup>C-EPA-CoA with intact mitochondria resulted in the release of <sup>14</sup>CO<sub>2</sub>. Other radioactive substances formed during the incubation were examined by radio-HPLC, and at least 5 species named M1, M2, M3, M4 and M5 were detected (Fig. 8) and thought to be C<sub>10:2</sub>, C<sub>12:3</sub>, C<sub>14:4</sub>, C<sub>16:4</sub>, C<sub>18:5</sub>, respectively, based on the retention times. These products

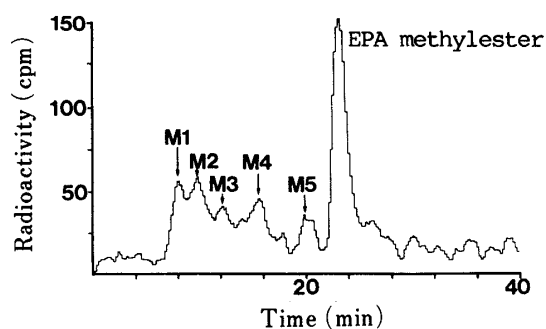


Fig. 8. Radio-High Performance Liquid Chromatogram of Methylester of Extract after Incubation of  $^{14}\text{C}$ -EPA-CoA with Rat Liver Mitochondria

TABLE IV. Percent of Radioactivity after Incubation of  $^{14}\text{C}$ -EPA-K or  $^{14}\text{C}$ -EPA-CoA with Rat Liver Microsomes or Peroxisomes

Substrate	Cell organelle	EPA	DPA	DHA	Other
$^{14}\text{C}$ -EPA-K	Microsomes	60.0 $\pm 2.6$	19.4 $\pm 0.6$	8.5 $\pm 1.1$	12.1 $\pm 2.3$
$^{14}\text{C}$ -EPA-CoA	Peroxisomes	41.4 $\pm 5.2$	19.6 $\pm 1.8$	N.D.	39.0 $\pm 3.5$

N.D.: not detected.

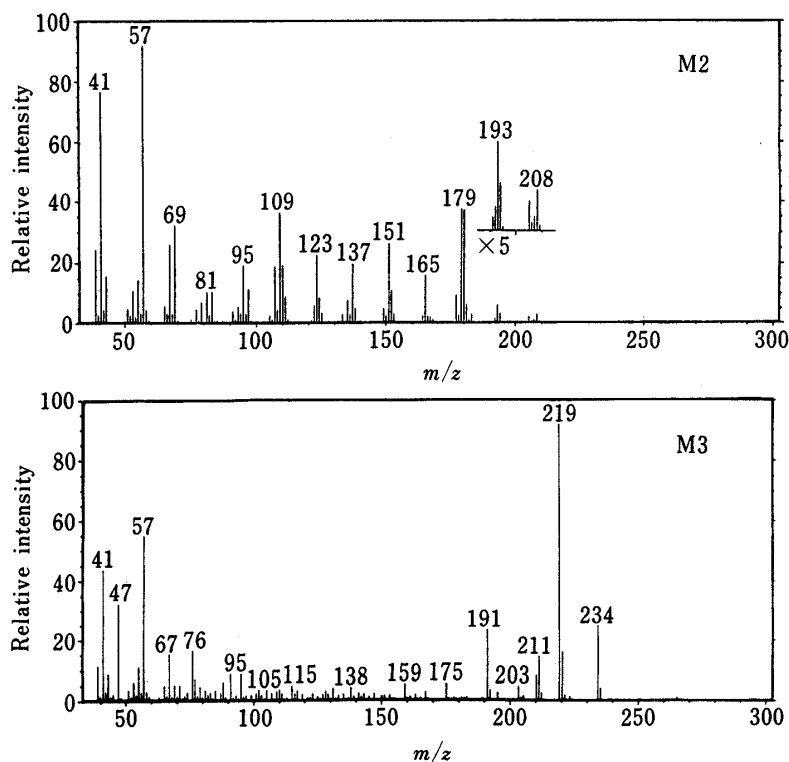


Fig. 9. Mass Spectra of M2 and M3 Methylester

were then converted to methyl esters of saturated fatty acids by hydrogenation and the retention times were compared with those of the methyl esters of authentic fatty acids. The retention times of these five peaks coincided with those of fatty acids with 10, 12, 14, 16 and 18 carbon atoms, respectively. Furthermore,  $\text{C}_{12:3}$  and  $\text{C}_{14:4}$  in peaks M2 and M3, respectively, were identified by GC-MS (Fig. 9).

Incubation of  $^{14}\text{C}$ -EPA-CoA with intact peroxisomes resulted in the formation of the chain-shortened products, as observed in the case of mitochondria (Fig. 10).

#### Effects of EPA-E and its Metabolites on Thrombus Formation in Rabbits

Thrombus formation was observed in six out of seven animals in the control group, whereas thrombi formed in only one out of seven animals in the EPA-E-treated group. The difference between these groups was statistically significant. The length of the thrombi (1.4 mm) in the EPA-E group was shorter than that ( $5.9 \pm 1.4$  mm) in the control group,

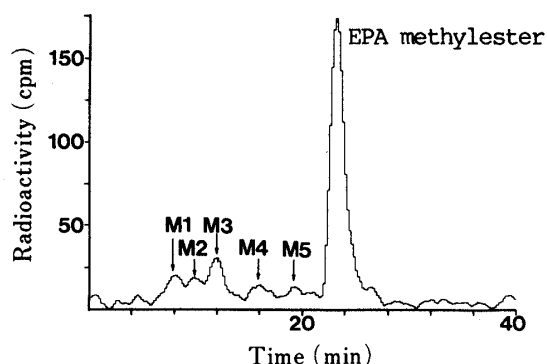


Fig. 10. Radio-High Performance Liquid Chromatogram of Methyl ester of Extract after Incubation of  $^{14}\text{C}$ -EPA-CoA with Rat Liver Peroxisomes

TABLE V. Effects of EPA-E, EPA, DPA and DHA on Thrombus Formation in the Stenosed Femoral Artery of Rabbit Induced by Ellagic Acid

Drug	Dose (mg/kg) <i>p.o.</i>	Incidence <sup>a)</sup>	Thrombus length (mm)
Control	—	6/7	$5.9 \pm 1.4$
EPA-E	300	1/7 <sup>b)</sup>	1.4
EPA	300	2/7	4.2
DPA	300	3/7	$6.4 \pm 0.8$
DHA	300	3/7	$6.7 \pm 0.3$

a) No. of rabbits with thrombus/No. of rabbits tested. Thrombus length value is evaluated from rabbits with thrombus. Significant difference by  $\chi^2$ -test from the control, b)  $p < 0.05$ .

though the difference was statistically not significant. The metabolites of EPA-E, such as EPA, DPA and DHA, moderately decreased the incidence of thrombus formation, but failed to decrease the length of thrombi (Table V).

### Discussion

In this work, we have analyzed the metabolites of EPA-E in the liver of rats given  $^{14}\text{C}$ -EPA-E and identified three metabolites, namely EPA formed by the deesterification of EPA-E, DPA formed by chain elongation of EPA and DHA formed by the desaturation of DPA.

These metabolites were incorporated into lipids rather specifically, mainly into PL, TG and cholesterol (Cho), and distributed in biological fluids, organs and tissues. Details of the distribution of the metabolites have been reported previously.<sup>13)</sup>

In this work, we examined the positions of TG and PL where the metabolites noted above were incorporated. In the case of TG from lymph, metabolites of EPA-E were mainly incorporated into the C-1 and/or C-3 positions because most of the radioactivity on TLC was detected in the FFA fraction of the products produced by treatment with lipase which specifically released acyl groups at the C-1 and C-3 positions of TG. TG from liver also incorporated radioactivity mainly into the C-1 and/or C-3 positions (probably one molecule of EPA-E metabolites because of the competition between EPA-E metabolites and naturally occurring fatty acids, which are undoubtedly more abundant than EPA-E metabolites). The radioactivity distributed to PL of the liver was mainly detected in PC and PE. Therefore the position of radioactivity in PL was determined for PC\* and PE\* from liver. The metabolites of EPA-E were incorporated only at the C-2 position of PC\* and PE\*, because most of the radioactivity on TLC was detected in the FFA fraction of the products produced by treatment with phospholipase A<sub>2</sub> which specifically released acyl groups at the C-2 position of PL. PL from lymph likewise incorporated the EPA-E metabolites at the C-2 position. These findings suggest that the metabolites of EPA-E may compete with arachidonic acid (AA) for the C-2 position of PL,<sup>14)</sup> including the PL of platelets and/or arteries, and may therefore interfere with the AA cascade. Free cholesterol released from peripheral tissues, including arteries, is known to be esterified by lecithin-cholesterol acyltransferase in plasma, receiving the acyl group from the C-2 position of PC. In fact, radioactivity was detected in cholesterol ester in the plasma of rats after the administration of  $^{14}\text{C}$ -EPA-E.<sup>13)</sup> It was reported<sup>15)</sup> that cholesterol ester with a polyunsaturated acyl group tended to be metabolized and/or excreted.

We have already reported<sup>13)</sup> that the metabolites of EPA-E are  $\beta$ -oxidized by mitochondria.



drial fraction. In this experiment, further *in vitro* studies were carried out to determine the organelle involved in the elongation and desaturation reactions of EPA and the organelle involved in degradation of the metabolites of EPA-E. The elongation reaction of EPA was found to occur in microsomes. Moreover, the desaturation reaction of DPA was also found to occur in microsomes. It has been reported by Suga *et al.*<sup>16)</sup> that peroxisomes produce DPA from EPA in the presence of acetyl-CoA as a cofactor for supply of the C-2 unit. In the present study, peroxisomes were found to produce DPA from EPA in the presence of not only acetyl-CoA but also malonyl-CoA as a cofactor. In the experiments on the degradation of  $^{14}\text{C}$ -EPA-CoA by mitochondria, the formation of  $^{14}\text{CO}_2$  was observed and other  $\beta$ -oxidation products with decreased chain length and double bond number were detected in radio-HPLC. Suga and Horie<sup>17)</sup> reported that peroxisomes were involved in the  $\beta$ -oxidation of long chain fatty acids such as EPA. In the present study, the participation of peroxisomes in the  $\beta$ -oxidation of EPA was confirmed by detecting the same  $\beta$ -oxidation products as those formed by mitochondria in radio-HPLC, except for  $^{14}\text{CO}_2$ .

Based on the manner of absorption, the chemical structure and the distribution of metabolites and *in vitro* metabolism, a metabolic pathway is proposed (Chart 1): EPA-E is first deesterified in small intestine and incorporated into TG and PL mainly as a compositional fatty acid, then distributed to various tissues through lymph and plasma, and finally  $\beta$ -oxidized by mitochondria and peroxisomes, mainly in the liver and also in each tissue. On the other hand, a certain portion of EPA is metabolized to DPA and DHA by elongation and desaturation reactions in microsomes and/or peroxisomes. It has been observed<sup>13)</sup> that  $^{14}\text{C}$ -DPA and  $^{14}\text{C}$ -DHA also undergo  $\beta$ -oxidation in mitochondrial fraction containing peroxisomes. DHA is a polyunsaturated fatty acid present in various tissues, especially in nerve tissues and synaptic membranes.<sup>18)</sup> Further, a retroconversion enzyme that converts DHA to EPA has been found in rat liver.<sup>19)</sup> These facts suggest that DPA and DHA may, either after conversion to EPA or directly, be also metabolized, through the  $\beta$ -oxidation process and tricarboxylic acid (TCA) cycle, to  $\text{CO}_2$  and water.

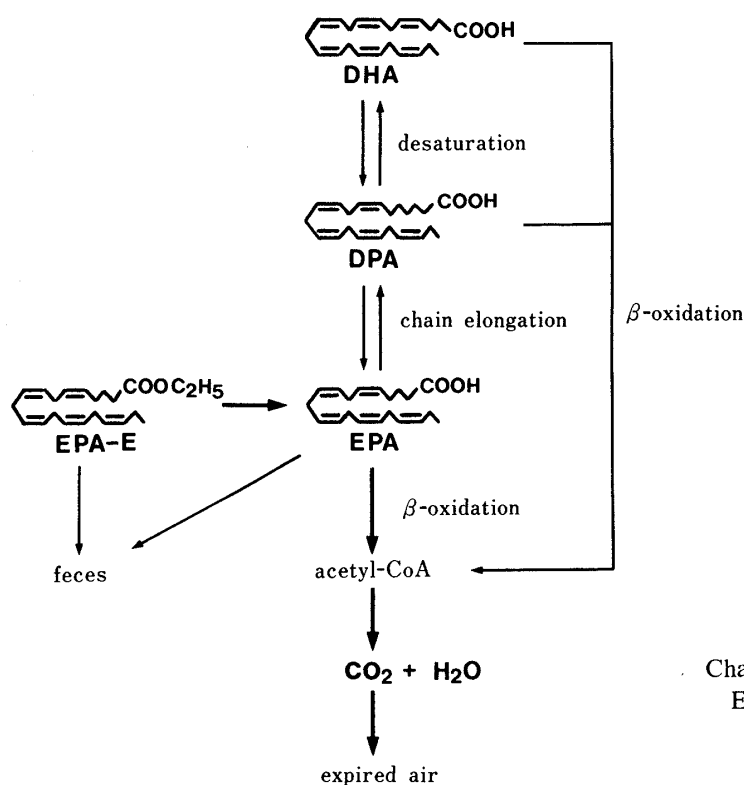


Chart 1. Possible Metabolic Pathway of EPA-E in Rats

We also examined the antithrombotic activities of EPA-E and its major metabolites, EPA, DPA and DHA, on ellagic acid-induced thrombus formation in the stenosed femoral artery of rabbits. We confirmed again the antithrombotic activity of EPA-E. EPA also decreased the incidence of thrombus formation, though its potency was somewhat weaker than that of EPA-E, and DPA and DHA also showed moderate activity to decrease the incidence of thrombus formation. Pharmacologically, antithrombotic activity of DPA has not been elucidated so far, but DHA has been reported<sup>20)</sup> to inhibit platelet aggregation and prolong the bleeding time. The effect of DHA on thrombus formation may be attributed to such antiplatelet activity of DHA.

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