Cardiac metabolism of ω-(*p*-iodo-phenyl)-pentadecanoic acid: a gas–liquid chromatographic–mass spectrometric analysis

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Abstract The ω-(*p*-iodo-phenyl)-pentadecanoic acid (I-PPA) has been used successfully for the investigation of the cardiac metabolic activity and for the imaging of the myocardium (Machulla, H. J., M. Marsmann, and K. Dutschka. 1980. *Eur. J. Nucl. Med.* **5:** 171–173). In the present study, the metabolic fate of I-PPA in the perfused rat heart was investigated. After application of I-PPA to the perfused rat heart, lipids were extracted, separated by thin-layer chromatography, and transesterified. The gas–liquid chromatographic-mass spectrometric (GLC–MS) analysis yielded the following results. *1*) Heart triglycerides contained 73% of the recovered I-PPA; only small amounts of unesterified I-PPA were found in the heart. This finding is in good agreement with the radioactivity distribution determined simultaneously. *2*) Three metabolites could be detected and characterized by GLC–MS: ω-(*p*-iodo-phenyl)-propionic acid, ω-(*p*-iodo-phenyl)-propenoic acid, and *p*-iodobenzoic acid. These short chain metabolites were found only in the perfusion medium demonstrating that they are not enriched but rapidly eliminated from the perfused rat heart.—**Schmitz, B., S. N. Reske, H. J. Machulla, H. Egge, and C. Winkler.** Cardiac metabolism of ω-(*p*-iodo-phenyl)-pentadecanoic acid: a gas–liquid chromatographic–mass spectrometric analysis. *J. Lipid Res.* 1984 **25:** 1102–1108.

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Iodinated phenyl-fatty acids have recently been developed as a tracer for the noninvasive assessment of cardiac lipid metabolism by means of external counting techniques (1–5). Remembering the early discovery of biologic oxidation of free fatty acids in β-position, first shown by Knoop in 1905 by means of terminal 'phenyl-labeled' free fatty acids, it was reasoned that an iodine atom, introduced in para-position of the terminal phenyl-substituent, would not significantly alter the biologic behavior of phenyl-fatty acids (1). Moreover, since the iodine atom is rather firmly bound to the phenyl-substituent, in vivo deiodination, which may pose major problems if iodinated aliphatic free fatty acids are used (2, 3), is prevented.

In several experimental models it was shown that ^{131}I or ^{123}I-labeled phenyl-pentadecanoic acid (I-PPA) is rapidly taken up and metabolized subsequently by the heart muscle (4–8). Major determinants for cardiac I-PPA uptake are regional myocardial blood flow (7) as well as cell viability and the metabolic state of the heart (8, 9). The clinical use of I-PPA in patients with acute myocardial ischemia, coronary artery disease, and cardiomyopathies is currently being investigated.

After initial I-PPA uptake in heart muscle radioactivity is rapidly incorporated into the main cardiac lipid fractions i.e., phospholipids, triglycerides, diglycerides, and the free fatty acid comigrating fraction (4). In double tracer studies labeling patterns of I-PPA or [1-^{14}C]palmitic acid-labeled cardiac lipids were found to be very similar (10, 11). The identity of radioactively labeled cardiac metabolites, however, could not be unequivocally identified. The purpose of this study was therefore *1*) to identify and to characterize potential metabolites of 15(*p*-I-phenyl)-pentadecanoic acid, produced by the heart muscle, by means of gas–liquid chromatography–mass spectrometry (GLC–MS) and *2*) to compare its fractional uptake in myocardial lipids to that encountered by radioactivity measurements after simultaneous administration of unlabeled I-PPA and ^{131}I-PPA.

MATERIALS AND METHODS

Tracers

Unlabeled I-PPA and ^{131}I-labeled PPA were prepared as described by Machulla, Marsmann, and Dutschka

Abbreviations: GLC–MS, gas–liquid chromatography–mass spectrometry; I-PPA, iodinated phenyl-pentadecanoic acid; TLC, thin-layer chromatography.

(12). Briefly, I-PPA was synthesized by electrophilic iodination of 15-phenyl-pentadecanoic acid in a solution of acetic acid and concentrated sulfuric acid in the presence of sodium nitrite (12). The ortho- and para-isomers of the iodinated phenyl-pentadecanoic acid were separated by high performance liquid chromatography (12). Approximately 50 mg of unlabeled I-PPA and 500 μCi of ^{131}I-labeled PPA (sp act > 900 Ci/mmol) were dissolved in 20 ml of 20% delipidated bovine serum albumin in Krebs-Henseleit buffer. The final solution was filtered through a Millipore filter (2.2 μm pore size, Nucleopore) and kept at 37°C.

Animal experiments

In total, six hearts of male Wistar rats (280–310 g body weight) were studied. The animals were supplied with food (Altromin, Fa.Trimborn, Bonn) and drinking water ad libitum before the study. After initiation of a 20–30 min pentobarbital anaesthesia (90–120 mg/kg, i.p.) and heparin pretreatment (10 mg/kg, i.p.), the trachea was cannulated with a polyethylene tube. The animals were respirated with room air by means of a Schuler respirator (Fa.Braun, Melsungen). The thorax was opened by a median incision, the pericardium was removed, and the aorta and pulmonary artery were cannulated. The superior and inferior vena cava and the lung veins were ligated according to the method described by Neely, Bowman, and Morgan (13). Subsequently the hearts were mounted on a Langendorff perfusion apparatus. The hearts were perfused according to the standard Langendorff technique without recirculation of the perfusion medium (13), i.e., with gassed (95% O_2, 5% CO_2) Krebs-Henseleit buffer (37°C) at a flow rate of 10 ml/min by means of a peristaltic pump (Fa.Braun, Melsungen). Tracer infusion was initiated after a 10-min equilibration period so that a stable ventricular performance (heart rate 170–180 beats per min) was achieved. Unlabeled I-PPA and ^{131}I-labeled PPA, complexed to the albumin solution, were infused via an aortic bypass at a constant flow rate of 1 ml/min with a precalibrated pump (Perfusor, Fa.Braun, Melsungen). Total perfusion time was 30 min. In previous experiments is was found that the heart rate during a 30-min perfusion and oxygen consumption (0.08–0.11 mmol per min per 1 g wet weight) was comparable to results reported from Langendorff hearts performing for more than 3 hr at stable ventricular pressure characteristics (13), indicating relatively uncompromised ventricular performance of myocardium in our experiments. The perfusate collected from the hearts was pooled and analyzed as described below. After termination of the perfusion period, the hearts were rapidly removed from the Langendorff apparatus, cleared of connective tissue, and cut into two pieces which were frozen and stored in liquid nitrogen.

Lipid extraction

Before extraction, hearts were powdered at liquid nitrogen temperature in order to minimize lipolysis (14) and extracted with $CHCl_3$–CH_3OH 1:1 (by vol) essentially according to Folch, Lees, and Sloane Stanley (15). The perfusion medium (300 ml/heart) was extracted three times with 600 ml of $CHCl_3$–CH_3OH 8:5.5 (by vol). Phase separation was achieved by centrifugation at 3000 *g*.

Analytical procedures

The quantitative determination of the lipids followed the method of Egge et al. (16). The preparative separation of the lipids was performed by thin-layer chromatography (TLC) on silica gel-60 plates (Fa.Merck, Darmstadt) using the following solvent system: *1*) $CHCl_3$–CH_3OH–H_2O 75:25:4 (by vol) to ¼ of the plate height; *2*) $CHCl_3$ to ⅔ of the plate height; and *3*) $CHCl_3$–n-hexane 1:3 (by vol) to the top of the plate. Methyl esters of fatty acids were prepared by transesterification of the separated lipids with 0.5 N potassium hydroxide in methanol at room temperature. Free fatty acids were esterified by the addition of etheral diazomethane.

GLC of fatty acid methyl esters was carried out on a packed column (2 m) with 10% SP 2330 as stationary phase (see also legend to Fig. 1). The GLC–MS analyses were performed on an LKB 9000 mass spectrometer using a S.C.O.T. column (50 m) with SE 30 as stationary phase and helium as carrier gas at a flow rate of 4 ml/min. Temperature program: 150 to 280°C at 1°C/min after an initial period of 15 min at 150°C. Mass spectrometric conditions are described elsewhere (17).

RESULTS

Cardiac lipids

The distribution of the major cardiac lipid classes is presented in **Table 1.** The data are in good agreement with those found in the literature (18). Free fatty acids could not be detected by densitometric measurement of the charred lipids which indicates that their amount is ≤1%. This was confirmed by weighing the extracted lipid fractions after their separation. The very small amount of unesterified fatty acids indicates that no lipolysis has taken place.

The fatty acid methyl esters obtained after transesterification of the separated lipid classes were analyzed by GLC. The exact determination of I-PPA even in small amounts was easily achieved since the retention

TABLE 1. Distribution of cardiac lipids

	Percent
Triglycerides	9.1 ± 1.8
Cholesterol	5.5 ± 1.9
Fatty acids	≤1.0 n.d.[a]
Cardiolipin	13.9 ± 2.9
Phosphatidylethanolamine	32.5 ± 2.5
Phosphatidylcholine	39.1 ± 2.1

[a] Not detected by densitometry.

time of the methyl ester of I-PPA differs significantly from those of all other fatty acid methyl esters normally present.

Fig. 1 shows the gas chromatogram of the methyl esters of the fatty acids from heart triglycerides that contain 0.4% I-PPA. The identity of the peak appearing after 135 min with I-PPA was confirmed by mass spectrometry. **Fig. 2** shows the mass spectrum of I-PPA which contains the molecular ion (m/e 458) and several characteristic fragments. Base peak m/e 299 is derived from the molecular ion by sequential elimination of iodine (m/e 331) and methanol. The second step is accompanied by a metastable ion at m/e 270.1. Fragment m/e 427 is formed by elimination of 31 amu (O − CH_3) from the molecular ion. In the lower mass region several fragments characteristic for the aromatic ring are found. M/e 217 corresponds to the iodine-containing tropylium ion; the tropylium ion itself ($C_7H_7^+$, m/e 91), a fragment present in the mass spectra of all aromatic hydrocarbons (19a), is subsequently produced by the loss of iodine from m/e 217. The peaks between 105 and 153 amu are interpreted as ions consisting of the phenyl group and/or shorter hydrocarbon chains. I-PPA was found in varying amounts in all heart lipids. The fatty acids found in the fraction "diglycerides + cholesterol" are certainly to be attributed to the diglycerides which are not completely separated from cholesterol with the solvent system used for TLC on silica gel-60 plates. The relative distribution as well as the total amount of incorporated I-PPA are in good

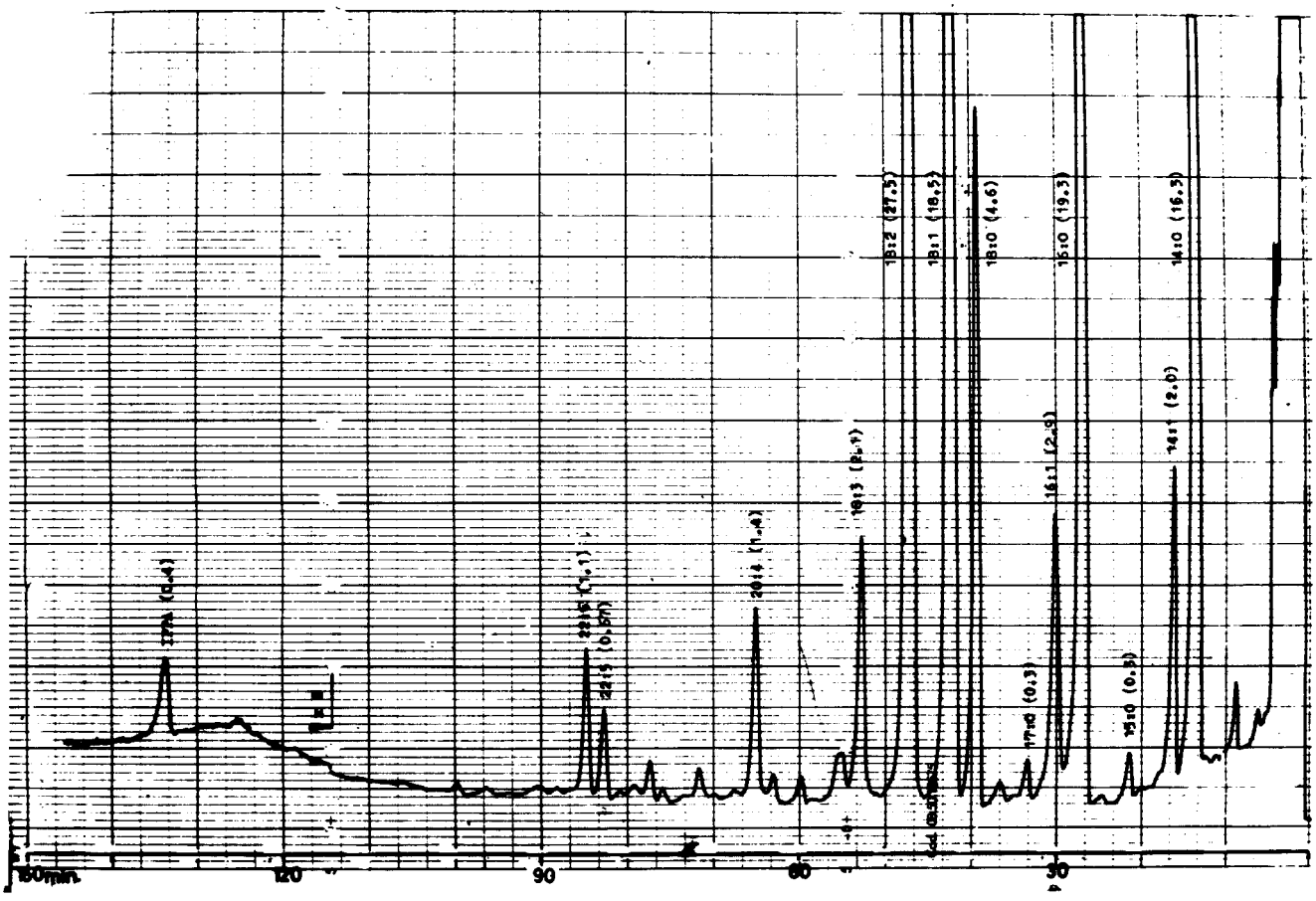

Fig. 1. Gas chromatogram of rat heart triglyceride fatty acid methyl esters. GLC conditions: packed column (2 m) with 10% SP 2330 as stationary phases; temperature program: 1°/min after 10 min at 160°C. Final temperature: 270°C. Fatty acids are given in % (values in brackets).

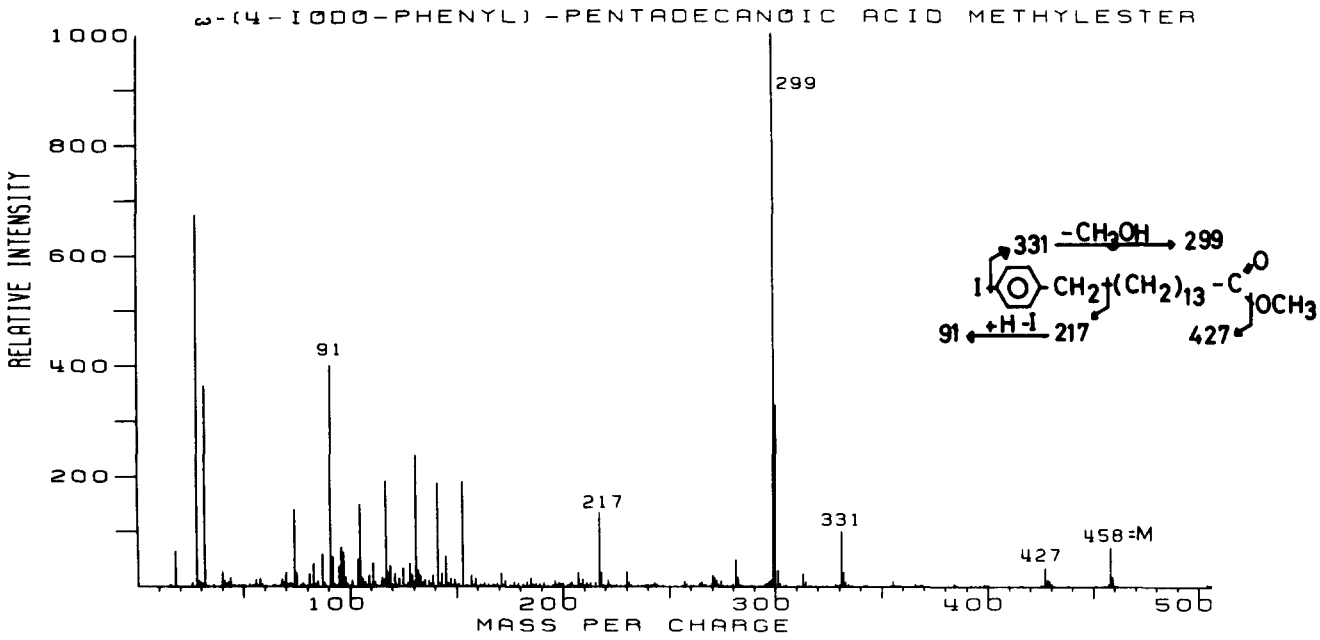

Fig. 2. Mass spectrum of the methyl ester of I-PPA.

agreement with radioactivity measurements of ^{131}I performed simultaneously.

A small amount, 1.5–2.5%, of the albumin-bound I-PPA added to the perfusion medium was taken up by the perfused rat hearts. Of this amount, more than 70% was incorporated into triglycerides, whereas phospholipids, free fatty acids, and the fraction "diglycerides + cholesterol" each contained 10% or less. The data are summarized in *Table 2.*

Lipids of the perfusion medium

In order to recover that portion of the I-PPA that was not incorporated into rat heart lipids, the total perfusion medium was extracted with chloroform–methanol 8:5.5 (by vol). TLC of the lipid extract revealed that in addition to the expected band of free fatty acids, triglycerides and phospholipids (and sugars from the medium itself) were also found. The amount of these lipids as well as the relative distribution differed widely from experiment to experiment, and possibly depended on the metabolic condition of the heart.

TABLE 2. Distribution of I-PPA in heart lipids

	GLC		
	1	2	^{131}I
	%		%
Triglycerides	71.8	74.8	77.6 ± 6.98
Cholesterol + diglycerides	9.2	1.6	5.8 ± 0.5
Fatty acids	10.1	11.7	7.6 ± 0.6
Phospholipids	8.9	11.9	0.1 ± 1.0

Comparison between gas–liquid chromatographic (GLC) and radioactivity (^{131}I) data; GLC data are taken from percent distribution of fatty acids related to the weight of each lipid class. Lipids of three hearts each were pooled for the fatty acid analyses. ^{131}I values are the means of six determinations.

After transesterification, fatty acid methyl esters were analyzed by GLC on SP 2330 as well as by GLC–MS on SE 30. In addition to I-PPA as the main component and several normally occurring fatty acids, three metabolites of I-PPA could be detected in small amounts: *p*-iodo-benzoic acid, ω-(*p*-iodo-phenyl)-propenoic acid, and ω-(*p*-iodo-phenyl)-propionic acid.

The mass spectra of these short chain metabolites are shown in **Fig. 3.** Three important ions are to be observed in the mass spectrum of the *p*-iodo-benzoic acid methyl ester (Fig. 3a): the molecular ion at m/e 262, and fragments m/e 231 ($M - O - CH_3$) and m/e 203, the iodo-phenyl-ion.

Fragment m/e 130 is the base peak in the mass spectrum of ω-(*p*-iodo-phenyl)-propenoic acid methyl ester (Fig. 3b). This fragment is probably produced by loss of 31 amu ($O - CH_3$, m/e 257) and iodine from the molecular ion which itself gives rise to a very intense peak at m/e 288. Fragment m/e 102 can be derived from the molecular ion by elimination of the ester group ($COOCH_3$) and iodine.

The ω-(*p*-iodo-phenyl)-propionic acid methylester (Fig. 3c) shows a mass spectrometric fragmentation behavior

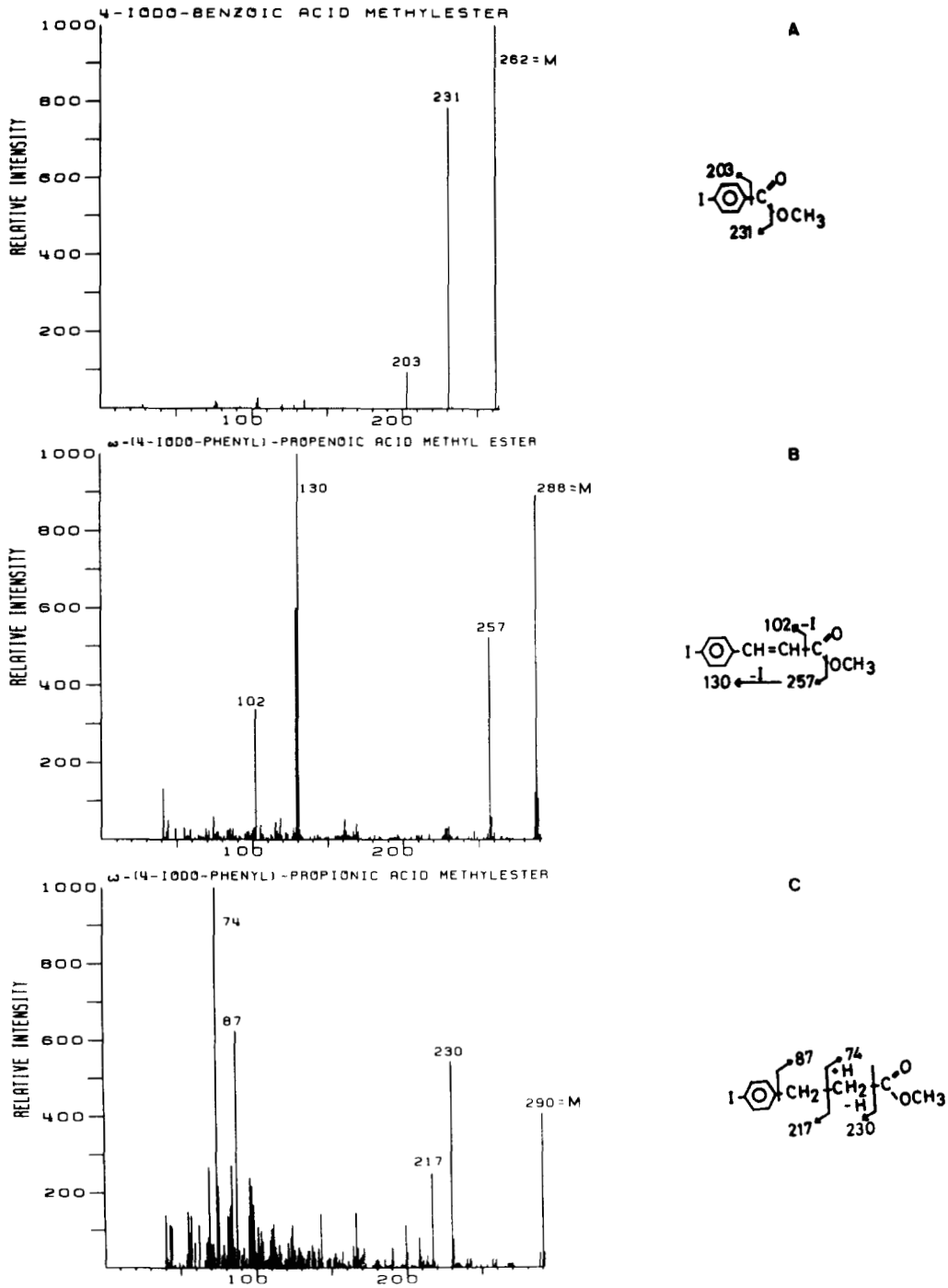

Fig. 3. Mass spectra of the methyl esters of the short chain metabolites of I-PPA. A) *p*-iodo-benzoic acid methylester; B) ω-(*p*-iodo-phenyl)-propenoic acid methylester; C) ω-(*p*-iodo-phenyl)-propionic acid methylester.

similar to that of normal fatty acids, because the McLafferty rearrangement (19b) can take place leading to fragments m/e 74 as base peak and m/e 87. The molecular ion (m/e 290) and two further ions of diagnostic value are present: m/e 230 which originates from the molecular ion after loss of 60 amu (the ester group + H) and m/e 217, the iodo-tropylium ion.

DISCUSSION

The purpose of this study was to show that the ^{123}I radioactivity of incorporated I-PPA as measured in earlier experiments (4) really coincides with this fatty acid, and to investigate the metabolism of I-PPA. The first point is established by the unequivocal mass spec-

trometric identification of I-PPA, as well as the agreement of the data obtained by the methods described in this study with the radioactivity measurements of the relative distribution of I-PPA in the lipids of the perfused rat heart.

In analogy to incorporation studies with [^{14}C]palmitate (20), I-PPA is recovered mainly in cardiac triglycerides. In conjunction with the rapid hydrolysis of at least one pool of I-PPA-labeled triglycerides, derived from the biphasic radioactivity elimination from triglycerides found in earlier experiments (4), this finding argues for the rapid turnover and, thus, for the importance of this lipid as a source of substrate for energy production.

From radioactivity measurements of the aqueous phase of the perfusate it was expected to demonstrate that I-PPA is catabolized to the water-soluble *p*-iodo-benzoic acid as is known for phenyl fatty acids shown by Knoop at the beginning of this century. The other two metabolites, ω-(*p*-iodo-phenyl)-propionic and -propenoic acid, found and identified in the perfusate also fit into the β-oxidation scheme.

In several investigations on the cardiac β-oxidation of fatty acids it was found that under special conditions as, for example, respiration inhibition or ischemia, various intermediates of the β-oxidation accumulate (21–24). These intermediates are mostly long chain saturated, unsaturated, or hydroxy fatty acids. Therefore, it is concluded that the short chain metabolites found in our study do not arise from incomplete β-oxidation as a consequence of metabolic inhibition of enzymes. Steric hindrance of the neighbouring iodophenyl group may slow down enzymatic reactions. It has been shown for the biosynthesis of ω-phenyl fatty acids with phenylacetyl-CoA as primer instead of acetyl-CoA (25) that the maximal velocity of the condensation between phenylacetyl-CoA and malonyl-CoA is much smaller than for the normal primer acetyl-CoA with malonyl-CoA. On the other hand, the β-oxidation of the short chain ω-iodo-phenyl fatty acids may be sterically hindered for the same reason.

The fact that the metabolites of I-PPA are detected only in the perfusate and not in heart lipids clearly demonstrates that the metabolites are able to pass the mitochondrial as well as the plasma membrane, so that under the experimental conditions they do not accumulate but are rapidly eliminated from the rat heart.

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