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The Metabolism of 3-Phenoxybenzoic Acid-containing Xenobiotic Triacylglycerols *In Vitro* by Pancreatic, Hormone-sensitive and Lipoprotein Lipases

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ABSTRACT. Two model substrates, *rac*-1-(3-phenoxy-[*ring*-^{14}C]benzoyl)-2,3-dipalmitoyl glycerol (1(3PBA)DPG) and *sn*-2-(3-phenoxy-[*ring*-^{14}C]benzoyl)-1,3-dipalmitoyl glycerol (2(3PBA)DPG), were compared with tri[1-^{14}C]palmitoylglycerol or tri[9,10(n)-^{3}H]oleoylglycerol as substrates for pancreatic lipase, lipoprotein lipase, and hormone-sensitive lipase. The loss of 3PBA from the *sn*-2 position was always low because of the positional specificity of the lipases. The loss of 3PBA from the *rac*-1 position was similarly low with hormone-sensitive lipase (about 7% of the loss of oleate), but higher with pancreatic lipase (about 35% that of oleate) and lipoprotein lipase (about 23% that of oleate). With one exception, more than 50% and up to 80% of the ^{14}C-3PBA was still in the form of a diacylglycerol after incubation with a lipase, whereas free acid or monoacylglycerol forms would have been expected. Lipoprotein lipase acting on 1-(^{14}C-3PBA)DPG produced nearly 70% of its product as nonesterified 3PBA and only 25% as the diacylglycerol. The results suggest that 3PBA-containing xenobiotic triacylglycerols, and the 3PBA-glycerol ester bond in particular, are poorer substrates for lipases than are their natural counterparts, with the result that high proportions of partially digested xenobiotic acylglycerols are produced. The three lipases performed differently with the xenobiotic substrates; this could have consequences for the relative rates of storage and clearance of the xenobiotic triacylglycerols from the body. BIOCHEM PHARMACOL **56**;12:1591–1598, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. 3-phenoxybenzoic acid; lipolysis; triacylglycerol; diacylglycerol; monoacylglycerol; metabolism

Over 40 different xenobiotic carboxylic acids have now been reported to form lipophilic conjugates, the majority being analogs of naturally occurring triacylglycerols—the so-called xenobiotic triacylglycerols [1–4]. Formation of small amounts of xenobiotic triacylglycerols, and their storage in adipose tissue, may be regarded, initially, as benign metabolism, but continued over a prolonged period, it suggests a mechanism of bioaccumulation. Any role for xenobiotic triacylglycerols in the storage of xenobiotic compounds depends upon the relative rates of synthesis and mobilization under the prevailing physiological conditions.

Although the mechanisms remain unclear, the biosynthesis of xenobiotic triacylglycerol has been demonstrated from a wide variety of xenobiotic acids [4], and in some cases there is good evidence for biosynthesis by the enzymes of the monoacylglycerol pathway [5, 6]. In contrast, little work has been reported on the breakdown of xenobiotic triacylglycerols by lipolytic enzymes. What work there has been suggests a degree of resistance to lipolysis of the ester bond between the glycerol and the xenobiotic acid [7–9]. Other workers have reported that xenobiotic triacylglycerols are relatively long-lived metabolites [10–16]. Recently, we have shown that triacylglycerol-conjugates of the non-steroidal anti-inflammatory drug fenbufen were both synthesized by and mobilized from cultured 3T3-L1 adipocytes. The mobilization responded to stimulation by the β-adrenergic agonist isoproterenol, but the products of lipolysis contained high proportions of the incompletely hydrolysed mono- and di-acylglycerol analogs [17].

A knowledge of the ability of individual lipase enzymes to use xenobiotic triacylglycerols as substrates is critical to understanding the metabolic fates of these conjugates. We report the results of studies on the action of three different lipase preparations upon two model xenobiotic triacylglycerols containing 3PBA§ at either the *sn*-2 or the *rac*-1 position (i.e. a mixture of the *sn*-1 and *sn*-3 positions). The lipases studied were relevant to understanding the processes of: 1) the uptake of dietary triacylglycerol from the gut (pancreatic lipase [triacylglycerol acylhydrolase; EC

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§ *Abbreviations*: apo-C_{II}, apolipoprotein-C_{II}; 1(3PBA)DPG, *rac*-1-(3-phenoxybenzoyl)-2,3-dipalmitoylglycerol; 2(3PBA)DPG, *sn*-2-(3-phenoxybenzoyl)-1,3-dipalmitoylglycerol; 3PBA, 3-phenoxybenzoic acid; and VLDL, very low density lipoprotein.

3.1.1.3]); 2) the uptake of circulating triacylglycerol from plasma lipoproteins by adipose and other tissues (lipoprotein lipase [triacylglycero-protein acylhydrolase; EC 3.1.1.34]); and 3) the mobilization of triacylglycerol from adipose tissue depots (hormone-sensitive lipase [triacylglycerol acylhydrolase; EC 3.1.1.3]). The latter two enzymes are undoubtedly involved in the metabolism of the xenobiotic triacylglycerols supplied to rats *in vivo* as very low-density lipoprotein reported in the accompanying paper [18]. 3PBA was chosen as the xenobiotic moiety because it is liberated during the metabolism of several synthetic pyrethroid insecticides, and its metabolism and incorporation into xenobiotic triacylglycerol is well documented [6, 8, 11, 12].

MATERIALS AND METHODS

Materials

Tri[1-^{14}C]palmitoylglycerol and tri[9,10(n)-^{3}H]oleoylglycerol were obtained from Amersham. Unlabelled tripalmitoylglycerol and trioleoylglycerol, bile salts (a mixture of sodium cholate and sodium deoxycholate), lipids for TLC standards, and the "micro-Lowry" kit were obtained from Sigma. TLC plates were Merck Type 60F_{254} silica gel plates. Other chemicals and biochemicals, solvents, and scintillation cocktails were as described earlier by Dodds *et al.* [19].

Substrates

rac-1-(3-Phenoxybenzoyl)-2,3-dipalmitoyl glycerol (1(3PBA)DPG) and *sn*-2-(3-phenoxybenzoyl)-1,3-dipalmitoyl glycerol (2(3PBA)DPG) were synthesized by reacting *rac*-2,3-dipalmitoyl glycerol or *sn*-1,3-dipalmitoyl glycerol, respectively, with 3-phenoxybenzoic acid (3PBA) in the presence of dicyclohexyl-carbodiimide in the molar ratio of 1:1:1 and catalytic quantities of dimethylaminopyridine. The reactants were dispersed in ethanol-free chloroform, stirred at room temperature for 5 hr, and refluxed for either 3 hr (1(3PBA)DPG) or 10 hr (2(3PBA)DPG). Unreacted acid was extracted in $NaHCO_3$ solution. The dried products were purified by TLC on silica gel developed with hexane/ethyl acetate (10:1, by volume) to remove a urea byproduct and ethyl-3PBA contamination. Further purification was achieved by column chromatography on silica eluted with 9% (v/v) diethyl ether in hexane. Purity was confirmed by TLC on silica gel developed with hexane/diethyl ether/acetic acid (40:60:1, by volume), and the identity of the products was confirmed by proton NMR using a 260 MHz NMR spectrometer (Varian, Walton on Thames, Surrey, U.K.).

Radioactive xenobiotic-triacylglycerols were made using the same method, except that 3-phenoxy-[*ring*-^{14}C]benzoic acid (^{14}C-3PBA, 10.8 Ci/mol) replaced the nonradioactive 3PBA; consequently, both the two synthesized xenobiotic triacylglycerols also had a specific radioactivity of 10.8 Ci/mol. In this case, purification was performed by TLC on silica gel developed with hexane/diethyl ether/acetic acid (40:60:1, by volume).

Preparation of Triacylglycerol Lipases

Pancreatic lipase (freeze-dried powder from porcine pancreas, Type VI-S) was obtained from Sigma and was dissolved in 5 mM $CaCl_2$ solution to give 10,000 U of lipase activity/mL.

An acetone/diethyl ether-dried powder preparation of lipoprotein lipase was prepared from rat epididymal adipose tissue as described previously [19, 20]. The enzyme preparation was stored at −20° as a solid until required.

Hormone-sensitive lipase was prepared from chicken adipose tissue by a method modified from that of Khoo and Steinberg [21]. A laying hen was anaesthetized with diethyl ether and adipose tissue from the abdomen and gizzard collected. The tissue was homogenized in 2 volumes of 0.25 M sucrose, containing 25 mM Tris-HCl and 1 mM EDTA, pH 7.4, using a blender (Atomix, MSE, Loughborough, Leics, U.K.). After centrifugation at 1,000 *g* for 10 min at 4°, the infranatant was filtered through glass wool to remove any remaining fat, and a particle free supernatant was prepared by centrifugation at 100,000 *g* for 60 min at 4°. The 100,000 *g* supernatant was adjusted to pH 5.2 with 0.2 M acetic acid, and the resulting precipitate collected by centrifugation at 1,000 *g* for 10 min at 4°. The precipitate was resuspended in 20% (w/v) glycerol containing 25 mM Tris-HCl and 1 mM EDTA, pH 7.4, frozen in aliquots and stored at −70° until required.

Measurement of Lipase Activity

PANCREATIC LIPASE. The activity of pancreatic lipase was determined by an adaptation of the method of Luddy *et al.* [22]. Triacylglycerol substrates were prepared as an emulsion. An appropriate volume of triacylglycerol solution to give a final concentration of 27 μM was dispensed into a glass vial and solvent removed in a stream of nitrogen. To this, 4 mL of 1 M Tris-HCl (pH 8.0), 0.4 mL of 0.2 M $CaCl_2$ and 1 mL of 0.05% (w/v) bile salts were added. The mixture was dispersed using an ultrasonic probe (Soniprep, MSE, Loughborough, Leics, U.K.) for between 30 and 60 sec. The standard assay consisted of a 0.5-mL sample of the triacylglycerol emulsion, which contained 0.738 M Tris-HCl (pH 8.0), 14.5 mM $CaCl_2$, 0.009% (w/v) bile salts and 27 μM triacylglycerol [^{3}H-trioleoylglycerol, 1.03 Ci/mol; 1(^{14}C-3PBA)DPG or 2(^{14}C-3PBA)DPG, both 10.8 Ci/mol]. The reactions were started by the addition of 5 μL of enzyme solution (2 U of lipase activity) and maintained at 40° with vigorous shaking for 30 min. The reaction was stopped by the addition of 1.88 mL of chloroform/methanol (1:2, v/v) and extracted by the method of Hajra *et al.* [23]. An aliquot (1 mL) of the chloroform phase was dried in a stream of nitrogen and redissolved in 100 μL of chloroform/methanol (1:1, v/v), 80 μL of which was analysed by TLC. A further sample of chloroform phase was taken for scintillation counting.

LIPOPROTEIN LIPASE. The method of Nilsson-Ehle and Schotz [24] as described by Dodds *et al.* [19] was used to measure the activity of the acetone-powder preparation. A total lipoprotein fraction prepared from pig serum [19] was used as the source of the apolipoprotein-C_{II} activator. The triacylglycerol used as substrate (emulsion in phosphatidylcholine and glycerol, prepared as before [19]) was present in a standard final concentration of 100 μM, the specific radioactivities being: ^{3}H-trioleoylglycerol (0.66 or 1.03 Ci/mol), ^{14}C-tripalmitoylglycerol (1.81 Ci/mol), 1(^{14}C-3PBA)DPG (0.86 Ci/mol), and 2(^{14}C-3PBA)DPG (0.80 Ci/mol). After 30 min, the incubations were stopped by the addition of 3.25 mL of chloroform/methanol/heptane (124:141:100, by volume), and nonesterified acids were extracted by the method of Belfrage and Vaughan [25]. Samples (1 mL) of the top phase containing the liberated acids were subjected to scintillation counting [19]. Samples (1 mL) of bottom phase were taken, dried in a stream of N_2, and redissolved in 100 μL of chloroform/methanol (1:1, v/v) prior to the analysis of any remaining partially-digested triacylglycerol by TLC.

HORMONE-SENSITIVE LIPASE. An emulsion of radiolabelled triacylglycerol (up to 2 mM trioleoylglycerol, or 0.5 mM tripalmitoylglycerol, 1(3PBA)DPG or 2(3PBA)DPG) was prepared in phosphatidylcholine and glycerol as for lipoprotein lipase. Before use, a working solution was dispersed in 100 mM sodium phosphate buffer (pH 7.0) using an ultrasonic probe. The final reaction mixture contained, in a volume of 0.2 mL, 0.2 mM radiolabelled triacylglycerol [specific radioactivities: 1(^{14}C-3PBA)DPG or 2(^{14}C-3PBA)DPG, 10.8 Ci/mol; ^{14}C-tripalmitoylglycerol, 1.81 Ci/mol; ^{3}H-trioleoylglycerol, 0.657 or 1.032 Ci/mol], 1.8 mg/mL of phosphatidylcholine, 50 mM sodium phosphate buffer, derived from both the substrate and additional buffer, 25 mg/mL of BSA and approximately 0.15 mg of enzyme protein. The reaction was started by the addition of the enzyme protein and maintained at 37° for 60 min in a shaking water bath. The reaction was stopped and nonesterified acids were extracted as described above.

Alterations to the standard conditions described for assaying the three lipases are signalled appropriately in the results section or in the legends of the tables.

TLC

Samples of lipid extracts were applied to 0.25-mm Merck silica gel 60F254 plates and developed twice in hexane/diethyl ether/acetic acid (40:60:1 by volume). Radioactive bands were located by autoradiography [8] or by radioscanning, using either a Berthold Dunnschicht-Scanner II (EG&G, Milton Keynes, U.K.) or a Raytest RITA-90 (LabLogic, Sheffield U.K.). Lipid standards were located by staining with iodine vapour, and 3PBA-containing bands were observed by their quenching of the fluorescent indicator in UV light at 254 nm. After scraping the silica from the plate, the radioactive content of individual bands was determined by liquid scintillation counting.

Determination of Radioactivity and Protein

Radioactivity was measured using a LKB 1211 liquid scintillation counter as described before for lipid extracts or silica scrapings from TLC plates [26] or for alkaline extracts containing fatty or xenobiotic acids [19]. Protein concentrations were determined by a microbiuret method [27] or, in the case of hormone-sensitive lipase, by Peterson's micro-Lowry method [28, 29].

RESULTS

Pancreatic Lipase

^{3}H-Trioleoylglycerol was used as the substrate in preliminary experiments, and the activity of pancreatic lipase was measured from the change in the concentrations of the ^{3}H-lipids identified by TLC. It was shown that oleic acid release was first order over at least the first 60 min of incubation. Similarly, the reaction was first order with respect to protein concentration up to 50 ng of enzyme protein per assay. Routine assays used 40 ng of pancreatic lipase protein per assay. Under these optimum conditions, the nonesterified oleic acid that was formed exceeded the dioleoylglycerol that was formed by 7:3 (mol/mol) and the monooleoylglycerol by 7:1. Maximum rates of acid release were obtained using 166 mM trioleoylglycerol but it was necessary to use lower concentrations (27 mM) routinely because of the limited availability of both the radioactive and the nonradioactive xenobiotic triacylglycerols.

Table 1 shows that release of radioactive, nonesterified acid from ^{3}H-trioleoylglycerol was about three-fold higher than from 1(^{14}C-3PBA)DPG and about five-fold higher than from 2(^{14}C-3PBA)DPG. Table 2 shows that lipolysis of both xenobiotic triacylglycerols yielded ^{14}C-diacylglycerol as the major radioactive product, exceeding the yield of nonesterified 3PBA. Monoacylglycerol was a minor product in both cases. The preferential formation of diacylglycerol was particularly apparent when 2(^{14}C-3PBA)DPG was the substrate. 2(^{14}C-3PBA)DPG also gave rise to a higher proportion of monoacylglycerol product and less nonesterified 3PBA than did 1(^{14}C-3PBA)DPG. In contrast, separate experiments under standard (rather than optimum) conditions showed that, while mono- and di-acylglycerols were both products of the action of pancreatic lipase on ^{3}H-trideoylglycerol, they were always exceeded by the oleic acid formed: after 30-min incubation, 57 mol % of the product was nonesterified oleic acid compared to 19 and 23% for the mono- and di-oleoylglycerols, respectively.

Lipoprotein Lipase

Lipoprotein lipase activity, as measured by the release of ^{3}H-oleic acid from the trioleoylglycerol substrate into the alkaline top phase of the extraction, was shown to be linear

TABLE 1. The activity of the three lipase preparations, assayed under the standard conditions described in the results section, with three different substrates

Substrate	Lipase activity (pmol of labelled acid released/min per mg of protein)		
	Pancreatic lipase	Lipoprotein lipase	Hormone-sensitive lipase
Trioleoylglycerol	225	1626 ± 257 (2)	1251 ± 508 (3)
1(3PBA)DPG	78	367 ± 270 (2)	90 ± 55 (5)
2(3PBA)DPG	42	123 ± 16 (2)	92 ± 67 (5)

The values used were obtained from graphs showing the effect of protein concentration, time of incubation, varying substrate concentration, and (for lipoprotein lipase) changing the concentration of activator. All the reported data were obtained under conditions where the rate of reaction was first order with respect to time of incubation and the concentration of enzyme protein.

Results shown are the mean ± standard deviation (hormone-sensitive lipase) or mean ± range (lipoprotein lipase) from the number of determinations shown in parentheses.

with respect to time of incubation for at least 60 min and with respect to the concentration of acetone powder protein up to at least 60 μg of protein per assay. (Excess casein was added as a carrier during the preparation of the acetone powder and the protein concentrations—and consequently the specific activities—reflect this.) Optimum activity was obtained in the presence of total lipoprotein equivalent to between 20 and 25 μL of serum, representing a stimulation of 1.64 ± 0.02-fold compared to incubations not containing a source of apo-C_{II}. In the presence of 1 M NaCl, the activity was reduced to 6% of its value in the absence of added NaCl. Stimulation by apo-C_{II} (supplied here as plasma lipoprotein) is regarded as definitive evidence of lipoprotein lipase activity [30], and inhibition by NaCl is another recognised property of the enzyme [31].

When measured as the release of nonesterified radioactive acid, the lipoprotein lipase preparation showed activity with all the triacylglycerol preparations, the highest activity being with trioleoylglycerol and the lowest with 2(3PBA)DPG. Table 1 shows that the activity with trioleoylglycerol as substrate exceeded that with 1(3PBA)DPG by a factor of 4.4 and with 2(3PBA)DPG by a factor of 13. When the concentration of lipoprotein activator was varied, activities with trioleoylglycerol, tripalmitoylglycerol, or 2(3PBA)DPG as substrates all showed stimulations of between 50 and 200% in the presence of total lipoprotein equivalent to 22 μL of serum, but the same activation was not apparent when 1(3PBA)DPG was the substrate.

Analysis of the products, again, revealed that ^{14}C-diacylglycerol was the major product when 2(^{14}C-3PBA)DPG was used, but when 1(^{14}C-3PBA)DPG was used the major radioactive product was the nonesterified 3PBA, although the diacylglycerol still constituted a quarter of the products (Table 2). Monoacylglycerol was formed in greater amounts when 2(3PBA)DPG was the substrate, especially after longer periods of incubation. As was the case with nonesterified acid release, the formation of diacylglycerol and monoacylglycerol from 2(3PBA)DPG, but not from 1(3PBA)DPG, was stimulated up to 2.5-fold by the inclusion of lipoprotein as activator. In contrast, separate experiments showed that dioleoylglycerol never represented more than 12% of the total radioactive product formed, when ^{3}H-trioleoylglycerol was used as the substrate.

Hormone-sensitive Lipase

Acylhydrolase activity of the preparation from chicken adipose tissue was initially assayed using ^{3}H-trioleoylglycerol emulsions as substrate and measuring the release of nonesterified oleic acid appearing in the alkaline top phase of a two-phase extraction system [25]. It was shown that the activity was first order with respect to time of incubation for

TABLE 2. The mole% compositions of radioactive products formed from the incubation of 1(^{14}C-3PBA)DPG or 2(^{14}C-3PBA)DPG with one of the three lipase preparations

Product of lipolysis	Pancreatic lipase		Lipoprotein lipase		Hormone-sensitive lipase	
	1(3PBA)DPG	2(3PBA)DPG	1(3PBA)DPG	2(3PBA)DPG	1(3PBA)DPG	2(3PBA)DPG
Unesterified acid	31.00 ± 0.32 (3)	10.07 ± 0.50 (3)	69.78 ± 0.77 (2)	21.15 ± 6.58 (3)	20.44 ± 4.04 (5)	16.74 ± 5.70 (5)
Monoacylglycerol	1.36 ± 0.15 (3)	7.11 ± 1.35 (3)	5.12 ± 0.82 (2)	14.73 ± 2.18 (3)	20.38 ± 4.09 (5)	29.79 ± 6.72 (5)
Diacylglycerol	67.3 ± 0.47 (3)	82.82 ± 0.98 (3)	25.10 ± 0.05 (2)	64.12 ± 6.94 (3)	59.17 ± 5.79 (5)	53.48 ± 4.46 (5)

All values were obtained from incubations carried out under standard conditions using pancreatic, between 30 and 60 min; lipoprotein between 40 and 80 μg protein; and hormone-sensitive, between 0.12 and 0.25 mg protein per assay. All the reported data were obtained under conditions where the rate of reaction was first order with respect to time of incubation and the concentration of enzyme protein and where the proportions of products formed was not affected by either of these variables.

Results shown are the mean ± standard error of the mean from the number of determinations shown in parentheses and derived from 3 (hormone-sensitive lipase) or from 2 (lipoprotein lipase) independent experiments, or from a single experiment (pancreatic lipase).

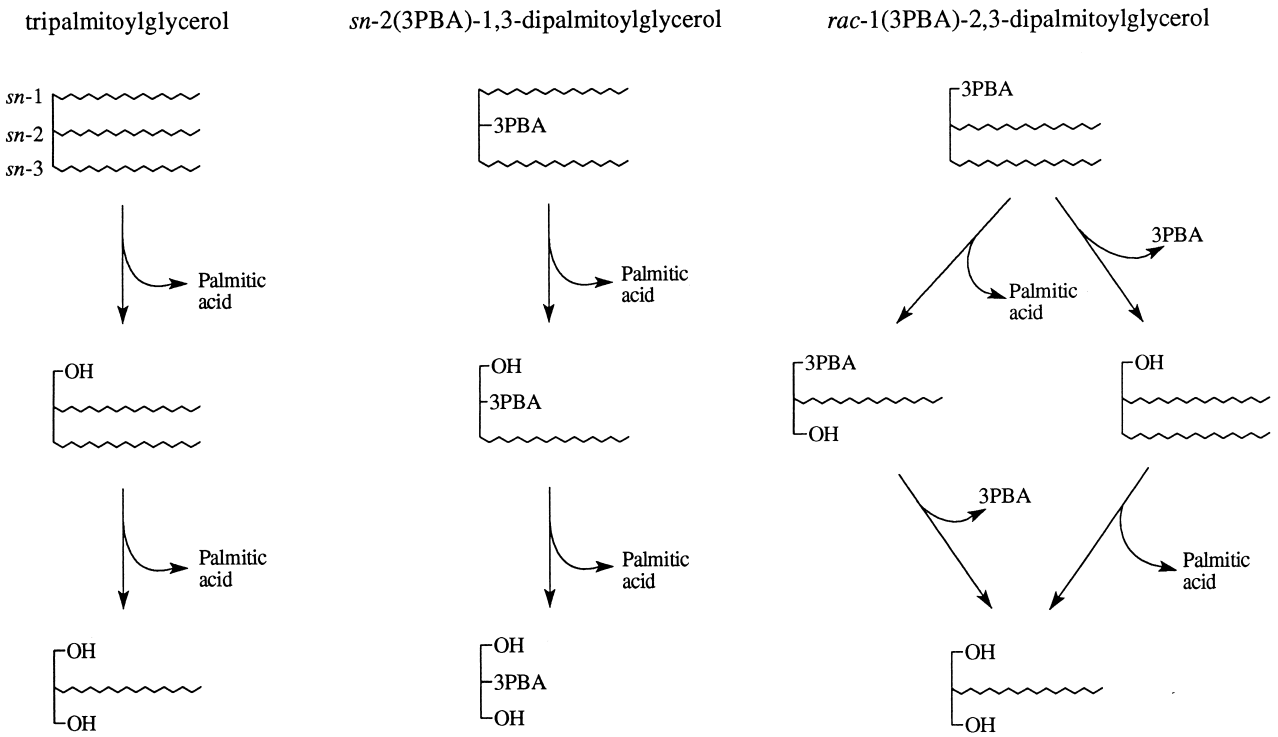

FIG. 1. The supposed order of hydrolysis by triacylglycerol lipases of the ester bonds in the natural and xenobiotic triacylglycerols substrates used. The course of lipolysis of trioleoylglycerol will be the same as the tripalmitoylglycerol shown. In the case of the *rac*-1(3PBA)-2,3-dipalmitoylglycerol, the 3PBA can be present in either the *sn*-1 or the *sn*-3 positions. Variations from this scheme are discussed in the text.

at least 90 min and to protein concentration up to about 0.2 mg of protein per assay. The activity increased with substrate concentration up to 0.2 mM, above which the activity continued to increase but more slowly.

Attempts to activate hormone-sensitive lipase activity by pre-incubating in the presence of cAMP-dependent protein kinase, ATP, and Mg^{2+} ions [21, 32] yielded inconsistent results, but activations were achieved on occasion. It was not possible to activate the lipase activity by including lipoprotein in the assay at the concentrations giving the maximum activity for lipoprotein lipase; furthermore, incubating in the presence of 50 mM citric acid/sodium dihydrogen phosphate buffer at pH 5.1, in place of Tris buffer, completely abolished acylhydrolase activity. Taken together, these results indicate the presence of a kinase-activatable, triacylglycerol lipase activity that could be neither lipoprotein lipase nor an acid lipase. It was concluded that the preparation contained genuine hormone-sensitive lipase, although contamination with other lipases, such as monoacylglycerol lipase (glycerol-ester acylhydrolase; EC 2.3.1.22), could not be discounted. The failure to observe consistent activation by protein kinase action suggests that the enzyme was isolated in an activated state.

After the initial characterisation studies, the acylhydrolase activity of the hormone-sensitive lipase preparation was compared using ^{3}H-trioleoylglycerol, 1(^{14}C-3PBA)DPG or 2(^{14}C-3PBA)DPG as substrates, all at a standard concentration of 0.2 mM. Table 1 shows that oleate release from trioleoylglycerol exceeded the release of 3PBA from both 1(3PBA)DPG and 2(3PBA)DPG by a factor of about 14. When the extract remaining in the heptane phase was analysed by TLC, it could be seen that, once again, a relatively low proportion of the esterified ^{14}C-3PBA was hydrolysed. In this case, the product distribution was similar when either of the xenobiotic triacylglycerols was used as substrate: over 50% of the product was in the form of xenobiotic diacylglycerol and roughly equal proportions of the remainder were present as the corresponding monoacylglycerol or as the nonesterified 3PBA (Table 2).

DISCUSSION

Degradation of triacylglycerols by triacylglycerol lipases is believed to involve the preferential hydrolysis of the two acyl chains esterified to the primary alcohols of glycerol, yielding first the *sn*-2,3- or *sn*-1,2-diacylglycerols then *sn*-2-monoacylglycerols [33]. Figure 1 illustrates how this scheme applies to the triacylglycerols used here. *In vivo*, a separate monoacylglycerol lipase is usually required to remove the final acyl chain [34–36]. These general characteristics have been reported for each of the lipases studied here [21, 37, 38]. Hydrolysis of the fatty acid from the *sn*-2 position, *in vitro*, may be brought about in one of three ways: 1) the enzyme may possess an intrinsic, albeit low, activity against the *sn*-2 position [39]; 2) the preparation (only partially purified) may be contaminated with a genuine monoacylglycerol lipase enzyme activity; or 3) non-enzymatic acyl-migration in diacylglycerols or mono-

acylglycerols may occur. Acyl-migration is observed in basic or acidic media [40], either during the incubation or the extraction phases, or in the presence of alcohols in the extraction medium [41], all of which are conditions that occurred in these experiments.

The results do indeed show that oleate was released from trioleoylglycerol faster than 3PBA was released from either of the xenobiotic triacylglycerols by each of the three lipase preparations. The low rate of 3PBA release from the *sn*-2 position probably resulted from isomerisation of mono- or di-acylglycerols or from contamination with monoacylglycerol lipase (lipoprotein lipase and hormone-sensitive lipase were both partially purified preparations). The release of 3PBA from the *rac*-1 position was also slow, although, in the case of pancreatic and lipoprotein lipases, considerably faster than from the *sn*-2 position. The discrimination by hormone-sensitive lipase against 3PBA is even more pronounced than by the other lipases. In experiments when ^{14}C-tripalmitoylglycerol was used as the substrate for hormone-sensitive lipase or lipoprotein lipase, the release of palmitate also exceeded the release of 3PBA, but by approximately 3-fold compared to 14-fold with trioleoylglycerol.

The conclusion that each of the three lipases discriminates, to some extent, against the glycerol-3PBA ester bond was confirmed when the radioactive products of the reactions were examined. Only when lipoprotein lipase was used with 1(^{14}C-3PBA)DPG was nonesterified 3PBA the major product: in every other case the corresponding xenobiotic diacylglycerol was the most abundant radioactive product. As would be anticipated, 3PBA was more readily hydrolyzed by each of the lipase preparations from the *rac*-1 position than from the *sn*-2 position and correspondingly more monoacylglycerol was formed when 3PBA was in the *sn*-2 position. That the glycerol-3PBA bond is relatively resistant to hydrolysis is, perhaps, less surprising than the finding of large proportions of xenobiotic diacylglycerol when 2(^{14}C-3PBA)DPG was the substrate. The inference is that the presence of the xenobiotic moiety in the *sn*-2 position interferes with the hydrolysis of the adjacent, non-xenobiotic, fatty acid.

Pancreatic lipase is secreted into the duodenum by the pancreas and acts in the presence of a colipase at the surface of the lipid droplets of the emulsion in bile [42]. The commercial preparation of pancreatic lipase used here was purified extensively but not to the point where the colipase was removed (information supplied by the Sigma Chemical Co.). Inasmuch as the substrate emulsion with bile salts and the state of the purified enzyme are reasonably similar to the conditions that occur *in vivo*, the low observed rate of 3PBA release compared to oleate release can be treated with confidence. Similar low rates of hydrolysis were observed when *sn*-1-acyl-2-3PBA glycerol [9] or ibuprofen- or fenbufen-containing triacylglycerol were used as substrates for pancreatic lipase, the latter two also exhibiting incomplete hydrolysis [8, 17]. In biochemical terms, the significance of the findings is that they confirm the concept that certain xenobiotic-glycerol ester bonds are relatively resistant to lipase attack. In toxicological terms, the significance is less certain. Dietary exposure to xenobiotic triacylglycerol has not been assessed, but is likely to be relatively small compared to the exposure to the nonesterified parent compound. If a dietary xenobiotic triacylglycerol is a good substrate for pancreatic lipase, then it will provide a source of acids and xenobiotic *sn*-2-monoacylglycerol for uptake by enterocytes. If it is not digested, then it will be excreted with the faeces. In this sense, poor digestion of dietary xenobiotic triacylglycerols in the gut would appear to protect the organism from additional systemic exposure to the compound.

Lipoprotein lipase is an enzyme found in active form anchored to heparan sulphate chains on the walls of capillaries in tissues expressing the activity. Its role is to digest the triacylglycerol content of the circulating lipoproteins, thus enabling their uptake by the tissue. To this end, the presence of apo-C_{II}, a normal component of mature chylomicrons and VLDL, is required as an activator [43] .

The use of an emulsion of triacylglycerol as substrate provides a convenient assay method. The addition of total lipoprotein fraction as the source of apo-C_{II} to assays of lipoprotein lipase supplies additional, unlabelled substrate with which the labelled substrate-emulsion must compete—this situation applies equally to natural and xenobiotic substrates. The acetone/diethyl ether dried preparation from rat epididymal adipose tissue is a commonly used source of lipoprotein lipase [20], but represents only a partial purification. The chance of contamination by other lipase activities (including monoacylglycerol lipase) cannot be ruled out and appears to be confirmed by the observation that the release of ^{14}C-3PBA from 1(^{14}C-3PBA)DPG was poorly stimulated by added apo-C_{II}. Both the incubations with lipoprotein lipase and the extraction of products were carried out at basic pH and both *rac*-1,2- and 1,3-diacylglycerol were detected in the products; as the latter probably arose by nonenzymatic isomerisation, they were pooled and counted simply as diacylglycerol product.

In vivo, xenobiotic substrates for lipoprotein lipase will be dietary-derived xenobiotic acids incorporated into the triacylglycerols of chylomicrons or hepatically synthesised VLDL. It would appear that 1-(3PBA)DPG makes a valid substrate for lipoprotein lipase, producing the majority of its xenobiotic product as the nonesterified acid; digestion and uptake are likely to be slower than with natural counterparts, but will take place. In comparison, the 2-isomer yields mostly the xenobiotic diacylglycerol as product and that at a slow rate. It is difficult to predict the fate of any xenobiotic diacylglycerol thus produced, but it seems likely that most will give rise to the corresponding xenobiotic monoacylglycerol *in vivo*, which can be taken up as such or after isomerisation and further lipolysis.

Working with a fat-free 800 *g* supernatant from rat adipose tissue, Fears *et al.* [7] were unable to detect lipoprotein lipase activity against synthetic triacylglycerols containing 4-benzyloxybenzoate. Similarly, Laurell [44] was

unable to demonstrate any action of human post-heparin lipoprotein lipase against the aliphatic triphytanoylglycerol. Our results are in agreement with the findings that 3PBA can be taken up from the circulation of rats *in vivo* and incorporated, albeit at a lower rate, into adipose tissue when they are presented as 3PBA-containing triacylglycerols in VLDL [18] .

Hormone-sensitive lipase is a multifunctional enzyme whose main role in adipose tissue is the hydrolysis of triacylglycerols [45]. The initial extract of hormone-sensitive lipase from adipose tissue is also active against monoacylglycerols, and the latter activity was not removed by our purification procedure [21, 46]. In our hands, the hormone-sensitive lipase preparation did discriminate strongly against the xenobiotic substrates yielding xenobiotic diacylglycerol as a major product. The results are consistent with those of Haselden *et al.* [18], where it is shown that 3PBA taken into adipose tissue from circulating VLDL is mobilized slowly compared to other tissues and suggests that there will be a tendency for the lipid-conjugate to accumulate. The results are also consistent with the earlier findings with cultured 3T3-L1 adipocytes, where ^{14}C-fenbufen, present as a triacylglycerol, was released from the cells in response to β-adrenergic stimulation of hormone-sensitive lipase mostly in the form of the xenobiotic diacylglycerol [17]. The toxicological consequences of such a release have yet to be fully evaluated [9, 47] .

In conclusion, the results presented here show that the three lipase preparations are less active when a triacylglycerol, in which one fatty acid is replaced with 3PBA, is used as substrate than when a triacylglycerol containing three long-chain fatty acids is used. Furthermore, the ester bond between 3PBA and glycerol is relatively resistant to hydrolysis, resulting in the formation of 3PBA-containing mono- and di-acylglycerols as major products. All the results were obtained with partially purified, but functionally distinct, enzymes assayed *in vitro*. The relative rates of hydrolysis *in vivo* will affect the tissue distribution, the rate of bioaccumulation, and the turnover rates of similar lipid conjugates. One attempt to extend the study of the metabolism of xenobiotic triacylglycerols *in vivo* is presented in the accompanying paper [18] .

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