Metabolism of fenbufen by cultured 3T3-L1 adipocytes: synthesis and metabolism of xenobiotic glycerolipids

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Abstract The storage of xenobiotic compounds as glycerolipids and their subsequent mobilization was studied using fenbufen and differentiated 3T3-L1 cells in culture. Fenbufen was taken up from the incubation medium and incorporated into triacylglycerol, diacylglycerol, and phospholipids. The triacylglycerol was susceptible to digestion by pancreatic lipase. The xenobiotic phospholipid contained three species, one of which behaved as fenbufenoyl phosphatidylcholine as judged by TLC, HPLC, choline analysis, and mass spectroscopy. After incubation with radioactive fenbufen for 18 h, the cells were transferred to a chase medium where radioactivity was lost from the cells and appeared in the medium. The rate was three times higher when 10 μM isoproterenol was present; insulin had no effect. Non-esterified fenbufen and analogues of mono- and di-acylglycerol were secreted. Monofenbufenoylglycerol was characterized by its ability to be used as a substrate by purified monoacylglycerol acyltransferase. When oleic acid was used in place of fenbufen, the majority of the radioactivity released in a chase experiment was the non-esterified acid (over 90%) and neither mono- nor di-acylglycerol was detected. These data indicate that 3T3-L1 adipocytes can synthesize fenbufen-containing lipids and release them into the medium on hormonal stimulation. The secretion of mono- and di-acylglycerols may have unforeseen pharmacological or toxicological implications.—**Dodds, P. F., S-C. Chou, A. Ranasinghe, and R. A. Coleman.** Metabolism of fenbufen by cultured 3T3-L1 adipocytes: synthesis and metabolism of xenobiotic glycerolipids. *J. Lipid Res.* 1995. **36:** 2493–2503.

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Xenobiotic glycerolipids are synthesized when a xenobiotic carboxylic acid, which may be a drug, an agrochemical, a food additive, a pollutant, or some other foreign compound, becomes esterified to the glycerol backbone of a lipid in place of a natural fatty acid. Over 35 different xenobiotic acids are now known to form such conjugates, the most common of which are analogues of triacylglycerols and diacylglycerols. The formation of xenobiotic phospholipids has not been well demonstrated except when the structure of the xenobiotic acid is very similar to that of a fatty acid (1–4).

Aside from the interest to lipid biochemists in what the synthesis of xenobiotic lipids tells us about the specificity of the acyltransferases involved, there is a natural concern about the possible toxic implications of their formation. Fears have been expressed that incorporation of xenobiotic phospholipids may disrupt membrane function (5), and that xenobiotic diacylglycerols may behave like the tumor-promoting phorbol diesters (6). Perhaps more importantly, incorporation into xenobiotic lipids represents a mechanism by which potentially toxic compounds may be retained in the body and accumulate over a period of prolonged, albeit low, exposure. The logical corollary of such storage is that the compound may be released later, as a result of dietary or hormonal change, in concentrations higher than those experienced during the initial exposure.

More is known about the formation of xenobiotic lipids than is known about their degradation. Both experiments in vivo (7–10) and those performed with individual lipases (6, 11–13) suggest that xenobiotic acylglycerols may be less readily mobilized than either natural acylglycerols or non-lipophilic xenobiotic conjugates.

In order to understand the metabolism of xenobiotic acids in adipose tissue, a primary storage site for xenobiotic glycerolipids, we studied the incorporation and mobilization of fenbufen [4-(4-biphenyl)-4-oxo-butanoic

Abbreviations: TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; FAB, fast atom bombardment; MS, mass spectroscopy; DMEM, Dulbecco's minimum essential medium.

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acid], a non-steroidal anti-inflammatory drug, by cultured 3T3-L1 cells. These 3T3-L1 fibroblasts can differentiate into adipocytes, synthesize and store triacylglycerol, and respond to hormones such as insulin and epinephrine (14–18). We present evidence that fenbufen can be incorporated into a variety of xenobiotic glycerolipids by 3T3-L1 cells, and can be mobilized in response to a lipolytic stimulus.

EXPERIMENTAL PROCEDURES

Materials

[ring-U-^{14}C]fenbufen was obtained from Amersham North America (Arlington Heights, IL) and dissolved in 95% (w/v) ethanol. The fenbufen was confirmed to be 98% radiochemically pure by thin-layer chromatography in chloroform–methanol–acetic acid 95:5:5 (v/v) on silica gel G. [1-^{14}C]oleic acid was obtained from ICN Biomedicals Inc. (Costa Mesa, CA). Fenbufen, methyl isobutylxanthine, dexamethasone, insulin, pancreatic lipase Type VI-S (triacylglycerol acylhydrolase; EC 3.1.1.3), and (±)-isoproterenol hydrochloride were obtained from Sigma Chemical Corporation, St. Louis, MO. 4-Biphenyl acetate was obtained from Aldrich Chemical Company Inc. (Milwaukee, WI). Lipid standards for TLC (oleic acid forms), and dipalmitoyl-, dioleoyl-, and dilinoleoyl-phosphatidylcholines for HPLC were obtained from Serdary Research Laboratories Inc., London, Ontario. All other chemicals were reagent grade. Thin-layer chromatography plates were from Analtech, Newark, DE, from Merck Ltd., Poole, U.K. (silica gel F_{254} plates), or from Whatman International Ltd., Maidstone, U.K. (Linear-K preparative plates). Tissue culture supplies were obtained from the Lineberger Cancer Center, UNC.

sn-1,3-Dipalmitoyl-2-fenbufenoyl-glycerol was made by Merlin Synthesis, Wye College, Wye, Ashford, U.K. by reacting *sn*-1,3-dipalmitoylglycerol with fenbufen in the presence of dicyclohexylcarbodiimide. The structure was confirmed by proton NMR. A sample of the product was converted to the corresponding *sn*-2-fenbufenoyl-glycerol by incubating with pancreatic lipase as described below. *sn*-1-Acyl-2-fenbufenoyl-glycerophosphocholine (fenbufenoyl-phosphatidylcholine) was prepared by reacting lysophosphatidylcholine (from egg; Lipid Products, Redhill, Surrey, U.K.) with fenbufen in the presence of *N,N'*-carbonyldiimidazole (19). The product was purified by TLC on a 1-mm thick "Linear-K" silica gel plate developed in chloroform–methanol–water 25:10:1 (v/v). The identity of the product was confirmed by fast atom bombardment (FAB)–mass spectroscopy (MS) carried out in the Chemistry Department, Kings College, University of London, U.K. The product showed a mass ion with a *m/z* equal to 236 more than the lysophosphatidylcholine precursor corresponding to the addition of one molecule of fenbufen and the loss of one molecule of water on esterification.

Cell culture

3T3-L1 cells, obtained from the American Type Tissue Culture Collection, were grown to confluence in 60-mm dishes in Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum and penicillin and streptomycin (standard culture medium) (15). Cells were differentiated at confluence by replacing the culture medium with the same medium supplemented with 0.25 μM dexamethasone, 0.5 mM methyl isobutylxanthine, and 10 μg/ml (equal to 1.7 μM) insulin. After 48 h the medium was again replaced with standard culture medium supplemented only with 10 μg/ml insulin. Medium was changed every 48 h and the cells were used for experiments 5 days after removal of methyl isobutylxanthine and dexamethasone when approximately 80% of the cells contained lipid droplets.

Individual 60-mm dishes of differentiated 3T3-L1 adipocytes were incubated at 37°C in 2 ml of standard culture medium to which 5 μl of ethanol containing [^{14}C]fenbufen had been added (yielding a concentration in the medium of 26.9 μM fenbufen, 13.2 μCi/μmol). Cells were then either washed and extracted or incubated with 'chase medium' as described in the legend to Fig. 5.

Extraction of lipid products

Floating cells and debris were removed from the medium by sedimenting at 1200 *g* for 5 min at room temperature in a bench centrifuge. Aliquots (100 μl) were counted. A larger sample (800 μl) was acidified with 10 μl 60% perchloric acid and extracted with 3 ml chloroform–methanol 1:2 (v/v). Chloroform (1 ml) and water (1 ml) were added. After vortexing, the phases were separated in a bench centrifuge at 1200 *g* for 5 min. Aliquots of each phase (100 μl of the upper phase and 50 μl of the lower phase) were counted.

To collect the cells, 1 ml ice-cold methanol was added to each dish. Cells were scraped with a rubber policeman and transferred to centrifuge tubes. The process was repeated with a further 1 ml methanol and then with 0.5 ml water. Chloroform (1 ml) was added to each pooled suspension and the tubes were vortexed. After at least 4 h at -20°C, the cellular debris was sedimented at 1200 *g* for 5 min in a bench centrifuge and the supernatant was collected. The pellet was re-extracted in 1.875 ml chloroform–methanol 1:2 (v/v) plus 0.5 ml water. The supernatants from the first and second extractions were pooled and 1.625 ml chloroform and 1.925 ml water were added. After mixing, the phases were separated in

a bench centrifuge at 1200 *g* for 5 min. Samples of the upper aqueous phase (100 μl) and lower chloroform phase (50 μl) were counted.

Thin-layer chromatography

Aliquots of the chloroform phases of cell (0.5 ml) and media extracts (1.0 ml) were dried using a SpeedVac concentrator (Savant, Hicksville, NY). Each residue was redissolved in 20 μl chloroform–methanol 1:2 (v/v) and applied to one lane of a silica gel G plate; each tube was washed twice with further 20-μl aliquots of the same solvent which were applied to the same lane of the TLC plate. Because fenbufen did not migrate adequately in systems designed to separate neutral glycerolipids, the plates were developed in Solvent System 1, chloroform–methanol–acetic acid 100:2.5:5 (v/v). In this system, fenbufen had an R_f of about 0.8; phospholipid standards migrated up to 1 cm from the origin but were well separated from monooleoyl- and monofenbufenoylglycerol standards. As with some other nonstandard solvent systems (20, 21) diacylglycerols migrated between triacylglycerol and fatty acid (see Fig. 1). After development, the plates were scanned by a BioScan 200 system (BioScan, Washington, DC) and the relative sizes of the radioactive peaks were determined after integration of the resulting trace. The accuracy of the integration method was confirmed initially by scraping silica from the TLC plate followed by liquid scintillation counting.

When it was necessary to retard fenbufen and possible acidic metabolites at the origin, the TLC plate was developed in chloroform–methanol–methylamine 100:2.5:0.5 (v/v) (Solvent System 2).

In order to separate phospholipid products, the TLC was developed either in acidic systems of chloroform–methanol–acetone–acetic acid–water 30:10:40:10:5 (v/v) (Solvent System 3), or in the more polar chloroform–methanol–acetic acid–water 60:40:9:3 (v/v) (Solvent System 4), or in basic systems of chloroform–methanol–ammonia 65:25:5 (v/v) or 50:40:5 (v/v) (Solvent Systems 5 and 6, respectively).

Identification of products

The band that was identified by co-chromatography with a known standard as fenbufenoyl-triacylglycerol (in which at least one of the fatty acids was replaced by fenbufen) was scraped from the TLC plate and extracted in chloroform–methanol 1:2 (v/v). Solvent was removed in a centrifugal concentrator and the lipid was sonicated in 0.75 M Tris, pH 7.4, 0.167 M $CaCl_2$, 0.167% (w/v) anhydrous sodium taurocholate. To 1.2 ml of the emulsion was added 1000 units of pancreatic lipase Type VI-S. Samples of the mixture were removed within 1 min after the enzyme was added and after 40 min incubation at room temperature. These samples were extracted three times with 2.5 ml diethyl ether. The extracts were combined, then dried, chromatographed in Solvent System 1, and scanned as described above.

The radiolabeled band identified by co-chromatography as monofenbufenoyl-glycerol was scraped from plates, extracted with chloroform–methanol 1:2 (v/v), and dried in a centrifugal concentrator. This extracted monofenbufenoyl-glycerol was incubated with 0.2 mM palmitoyl-CoA and monoacylglycerol acyltransferase (EC 2.3.1.22), partially purified from the livers of 11-day-old rats to the stage after chromatography on hydroxylapatite (22). After 0 or 60 min incubation at 23°C, samples of the reaction mixture were extracted into heptane (23). The extracts were dried, chromatographed on silica gel G plates in Solvent System 1, and scanned as described above.

In order to identify the radiolabeled compound that co-chromatographed with phosphatidylcholine, each of ten 100-mm dishes of differentiated 3T3-L1 adipocytes was incubated for 21 h with DMEM containing 10% fetal bovine serum, penicillin, streptomycin (15), and [^{14}C]fenbufen (0.50 μCi/μmol) added in 2.5 μl of ethanol per ml of medium to give a final concentration of 41.6 μM. The cells were extracted as described above but in double the volumes. The combined extracts were concentrated and applied to a TLC plate that was developed in Solvent System 3. Because scanning revealed that the plate was overloaded with extracted triacylglycerol, the lower 10 cm of silica was scraped from the plate and the lipids were re-extracted from the silica and applied to a preparative TLC plate (1 mm thick). The plate was developed in the same solvent and a radioactive band of silica, corresponding to a phosphatidylcholine standard, was scraped from the plate. The lipids were extracted and dried. (No additional phospholipid was present on the upper 10 cm of the original TLC plate.)

In order to purify the fenbufenoyl-lipids from the non-radioactive natural phospholipids that copurified on the silica gel, the putative fenbufenoyl-phosphatidylcholine was subjected to high performance liquid chromatography (HPLC) using a reverse phase column to separate components of the mixture on the basis of their fatty acid composition. Lipid was dissolved in methanol–acetonitrile–water 70:15:15 (v/v) and applied to a Beckman Ultrasphere ODS 5 μm 4.6 mm × 35 cm column. The column was eluted at 2 ml/min in methanol–acetonitrile–water 70:15:15 (v/v) for 5 min followed by a programmed linear gradient changing, over 15 min, to 100% methanol in which the elution was continued for a final 50 min. Fractions corresponding to peaks of radioactivity were collected and used for subsequent mass spectroscopic or choline analysis.

Mass spectroscopy

Liquid secondary mass spectra of putative fenbufenoyl-phosphatidylcholine were obtained using a VG70-250SEQ hybrid mass spectrometer equipped with a Cs^+ gun operating at a primary ion beam of 30 kV. The Cs^+ ions were allowed to bombard the sample dissolved in a matrix containing a mixture of dithiothreitol and dithioerythritol (3:1, w/w). Mass spectroscopy/mass spectroscopy (MS/MS) studies were performed by mass-selecting positive ions from the magnetic analyzer and allowing them to collide with neutral Ar atoms (pressure 2×10^{-5} torr) present in the first quadrupole at a collision energy of 30 eV followed by the mass analysis with the second quadrupole.

Other methods

Choline was measured by the method of Pomfret et al. (24). Data were analyzed by a paired Student's *t*-test.

RESULTS

Incorporation into neutral lipid

Five days after differentiation, 3T3-L1 adipocytes were incubated with [^{14}C]fenbufen for 18 h. During the incubation, 20.1 ± 5.7% (mean ± SD of 54 measurements in three independent experiments) of the fenbufen radioactivity was taken up from the medium. Most of the label was recovered in the chloroform phase of the washed and extracted cells: only 1.63 ± 0.35% (n = 54) remained in the aqueous phase. When the chloroform extract was chromatographed in Solvent System 1, typically 94% migrated between the fenbufen standard and the solvent front. The major portion co-chromatographed with a *sn*-1,3-dipalmitoyl-2-fenbufenoyl-glycerol standard. Radiolabeled spots running between that standard and fenbufen were tentatively identified as diacylglycerol analogues, but triacylglycerol analogues containing more than one fenbufen moiety could not be excluded. No particular precautions were taken to prevent isomerization of diacylglycerols during extraction and analysis (20); consequently, the radioactive spots corresponding to *sn*-1,2(2,3-)-diacylglycerol (having the lower R_f) and *sn*-1,3-diacylglycerol were combined as total diacylglycerol for most purposes. Less than 1% of the radioactivity co-chromatographed with fenbufen, but 5.56 ± 0.96% (9 observations) was routinely associated with more than one component in the first cm of the plate where phospholipid standards were also located (**Fig. 1**).

To confirm the composition of the putative fenbufenoyl-TG, the band was extracted from the silica, treated with pancreatic lipase, and resubjected to TLC. The fraction corresponding to the fenbufenoyl-TG standard decreased from 80% after incubation for 1 min to 32% after 40 min of incubation with pancreatic lipase. During this time, unesterified fenbufen rose from 13% to 40% of the radioactivity and the region correspond-

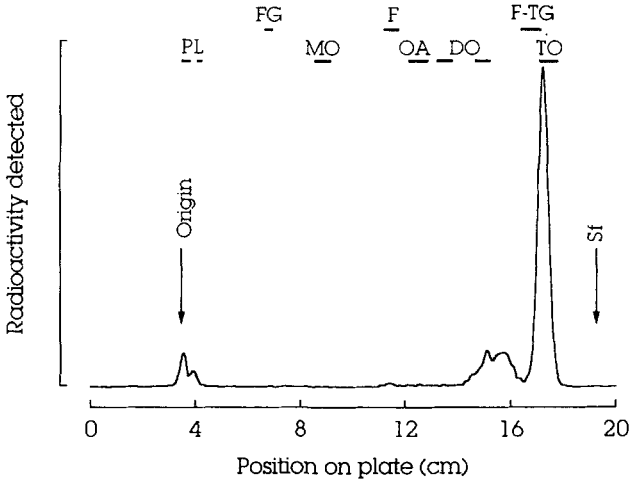

Fig. 1. TLC analysis of lipids from cells incubated with fenbufen. Cells were incubated for 18 h with [^{14}C]fenbufen, washed, extracted, and subjected to TLC in Solvent System 1 as described in Experimental Procedures. The TLC plate was scanned using the BioScan 200 and the resultant trace is shown. The position of genuine standards as seen by staining in I_2 are indicated by: PL, mixed phospholipid standards; MO, monooleoylglycerol; OA, oleic acid; DO, dioleoylglycerol; TO, trioleoylglycerol; FG, (mono)fenbufenoylglycerol; F, fenbufen; F-TG, *sn*-1,3-dipalmitoyl-2-fenbufenoyl-glycerol. The position of the origin and the solvent front (Sf) are indicated by arrows. The full vertical scale represents 5000 counts accumulated by the BioScan 200 over 20 min.

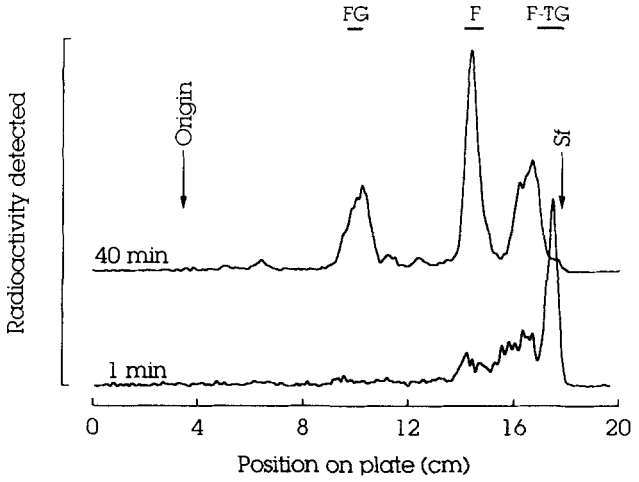

Fig. 2. Digestion of fenbufen-containing triacylglycerol with pancreatic lipase. The band corresponding to fenbufenoyl triacylglycerol was scraped from TLC plates and digested with pancreatic lipase for 1 min or 40 min as described in Experimental Procedures. The traces from the BioScan 200 of subsequent rechromatography in Solvent System 1 are shown. The full vertical scale represents 3000 counts accumulated by the BioScan 200 over 15 min. The genuine standards are as described under Fig. 1.

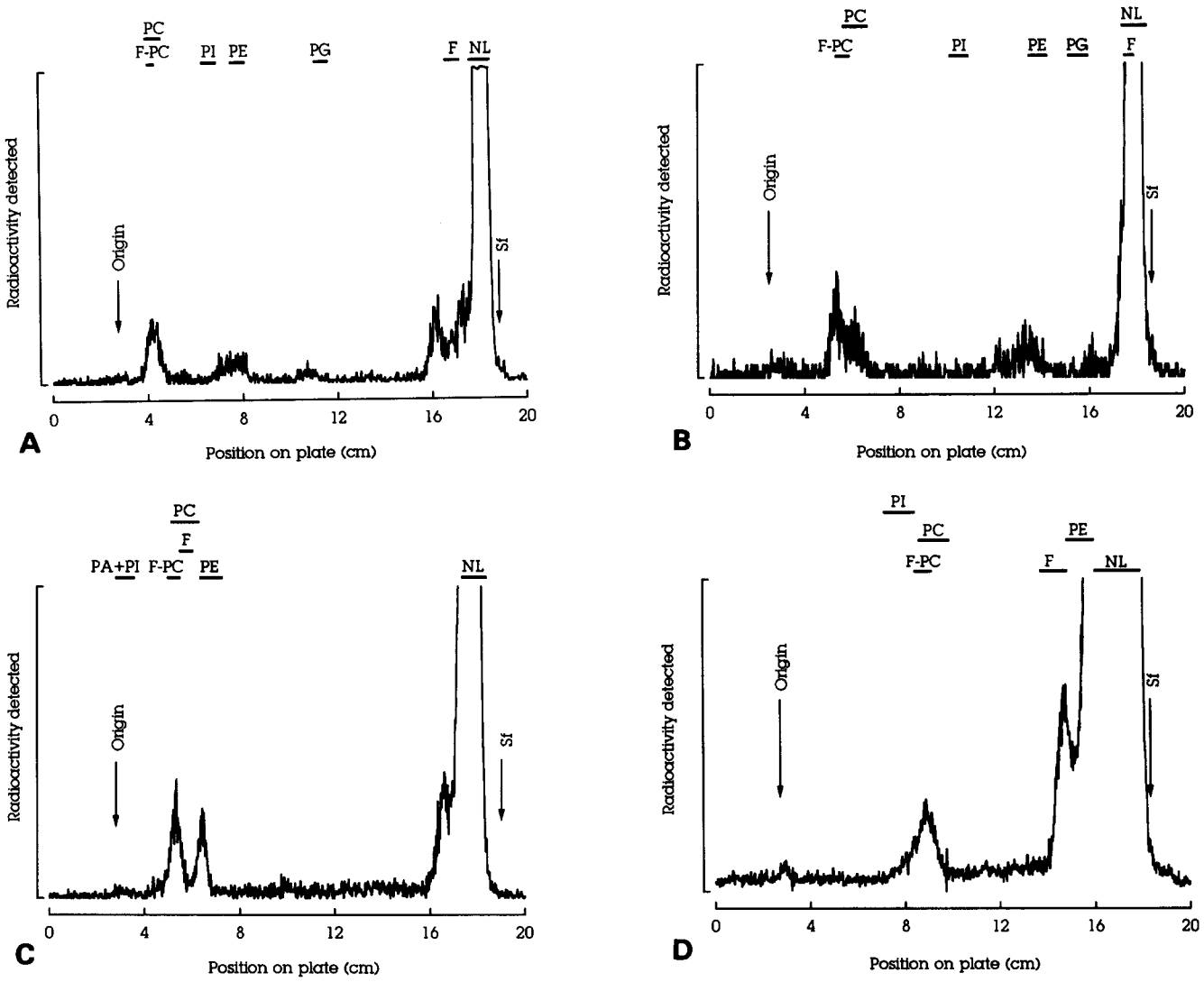

Fig. 3. TLC analysis of putative fenbufen-containing phospholipids. Lipids extracted from cells incubated with fenbufen were subjected to TLC in (A) Solvent System 3 (acidic), (B) Solvent System 4 (acidic, more polar), (C) Solvent System 5 (basic), or (D) Solvent System 6 (basic, more polar) to separate the phospholipids present. The traces from the BioScan are shown. The position of genuine standards as seen by staining in I_2 or by quenching of the fluorescent indicator at 254 nm are indicated by: NL, neutral lipids (mono-, di-, and tri-oleoylglycerol); PE, phosphatidylethanolamine; F-PC, synthetic fenbufenoyl-phosphatidylcholine; PC, phosphatidylcholine; PI, phosphatidylinositol; PG, phosphatidylglycerol. Other abbreviations are as Fig. 1. The full vertical scale represents 150 counts accumulated by the BioScan 200 over 30 min (A), 400 counts over 120 min (B), 150 counts over 30 min (C), or 400 counts over 120 min (D).

ing to the *sn*-2-monofenbufenoyl-glycerol standard increased from 4% to 23% (**Fig. 2**).

The possibility could not be ruled out that some of the ethanol added as solvent (about 55 μM in the incubations) reacted with fenbufen forming an ethyl ester that may have contributed to the product and have migrated with triacylglycerol on TLC; however, quantitatively and qualitatively similar results were obtained when acetonitrile was used instead as the solvent for fenbufen (data not shown).

Incorporation of fenbufen into phospholipid

In order to identify the radiolabeled products that migrated near the origin, samples of the chloroform phase were chromatographed in acidic (Solvent System 3 on Analtech plates and Systems 3 or 4 on Merck plates) or basic solvent systems (Solvent System 5 on Analtech plates and Systems 5 or 6 on Merck plates). In Solvent System 3, neutral lipids, including fenbufen, ran at the solvent front. The largest of three other bands migrated slightly behind a dioleoylphosphatidylcholine standard. On the Merck plates, in either System 3 or 4, this band, which again migrated slightly behind the dioleoylphosphatidylcholine standard, exactly co-chromatographed with the synthetic fenbufenoyl-phosphatidylcholine (**Fig. 3A** and **B** respectively). One of the other radiola-

beled bands was tentatively identified as fenbufenoyl-phosphatidylethanolamine. In Solvent Systems 5 and 6 on the Merck plates, (Fig. 3C and D respectively) the major non-neutral radiolabeled compound again migrated with the fenbufenoyl-phosphatidylcholine standard and slightly behind the dioleoylphosphatidylcholine standard. A less radioactive band migrated close to the phosphatidylethanolamine standard. The basic nature of these solvent systems greatly retards the migration of acidic phospholipids relative to phosphatidylcholine and phosphatidylethanolamine and reduces the likelihood of their being confused on the TLC plates. Extra standards used with the Analtech plates (not shown) revealed that no radioactivity comigrated with phosphatidic acid, phosphatidylserine, or sphingomyelin in Solvent System 5. Furthermore, the two radioactive peaks in Solvent System 5 (Fig. 3C), corresponding to standards for phosphatidylcholine and phosphatidylethanolamine, were separated further in the earlier run on the less active Analtech plates, showing clearly that the unesterified fenbufen standard, which migrated between the two peaks, was not associated with any radioactivity. In an additional TLC analysis, a Merck plate was developed first in Solvent System 4 and then in Solvent System 6 in the perpendicular direction to give a two-dimensional separation. There were three regions of radioactivity not associated with neutral lipid: the one that corresponded to the fenbufenoyl-phosphatidylcholine standards contained 3.3% of the total radioactivity and the one that behaved as phosphatidylethanolamine contained 1.6%.

In order to characterize further the product tentatively identified as fenbufenoyl-phosphatidylcholine, a larger amount of material was prepared and purified by TLC as described in Experimental Procedures. In an attempt to purify the fenbufenoyl product away from the (non-radiolabeled) natural phosphatidylcholines, we used reverse phase HPLC, which separates lipid species according to their fatty acid content (**Fig. 4**). Five radioactive peaks eluted between 14 and 23 min; natural phosphatidylcholine standards (dipalmitoyl-, dioleoyl-, and dilinoleoyl-) eluted after 25 min. The shorter elution time on the hydrophobic column is consistent with the presence of the more polar fenbufen in the structure. The presence of several peaks probably resulted from the presence of a variety of natural fatty acids in the remaining position. Two fractions were collected, one contained peaks with retention times between 14 and 18 min (peak 17) and the other between 18 and 22 min (peak 19). An additional radioactive peak eluted at about 2.5 min (called peak 2) close to the solvent front. This peak, which contained choline, remains enigmatic.

The amount of choline in peaks 2, 17, and 19 exceeded that predicted by the content of fenbufen-derived radioactivity (assuming one xenobiotic moiety per molecule of phosphatidylcholine) by a factor of 3.5 for peak 2, 12.5 for peak 17, and 15 for peak 19. Apparently, despite the HPLC purification step, the fractions still contained considerable quantities of naturally occurring choline-containing molecules.

Contamination of the putative fenbufenoyl-phosphatidylcholine with large amounts of natural phosphatidylcholine renders interpretation of mass spectra problematic. Two features further complicated the interpre-

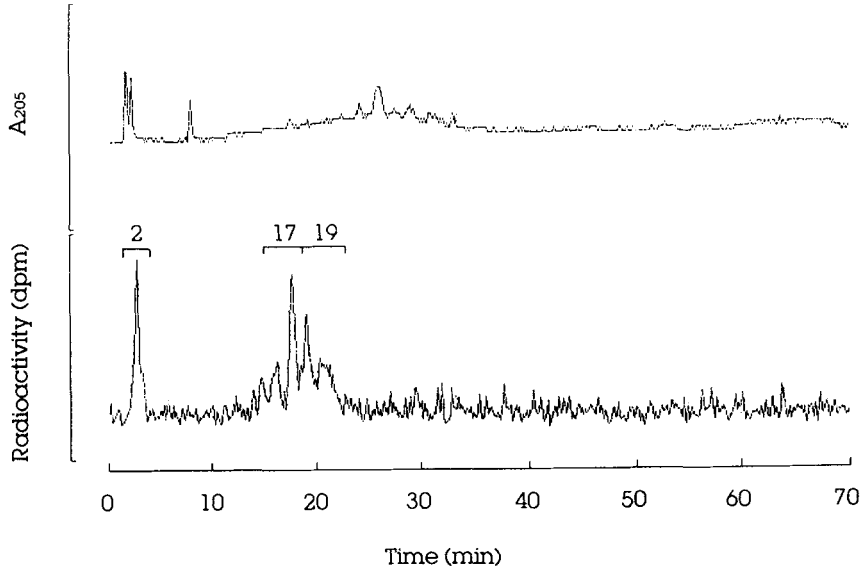

Fig. 4. Analysis of fenbufen-containing phosphatidylcholine by reverse-phase HPLC. The extracted cellular lipid that co-chromatographed with phosphatidylcholine was collected from a preparative TLC plate and analyzed by HPLC as described in Experimental Procedures. The upper trace shows the output from the UV monitor; the lower trace shows the output from the radio-detector with the full scale representing 6200 dpm.

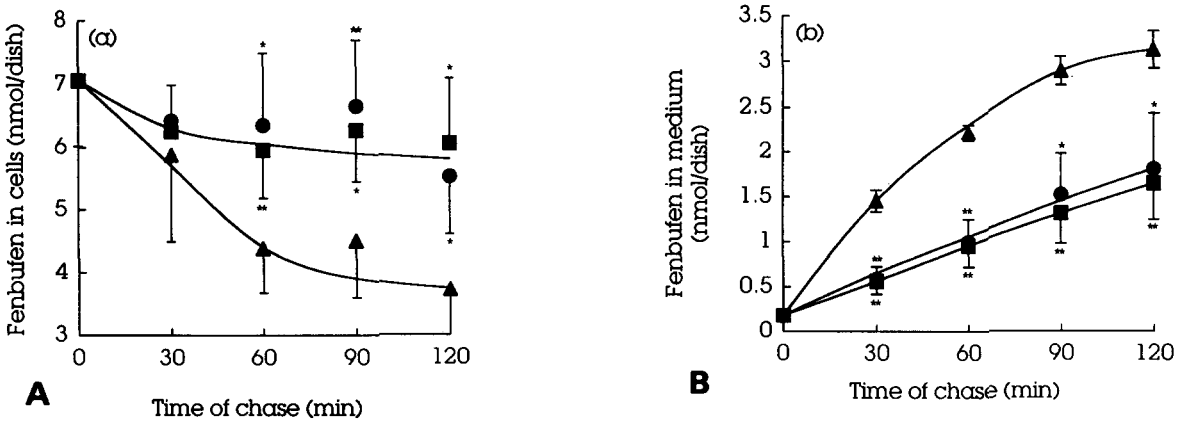

Fig. 5. Effect of insulin and isoproterenol on fenbufen release from 3T3-L1 cells. 3T3-L1 adipocytes were incubated with [^{14}C]fenbufen for 18 h. Medium was removed and cells were washed four times with 1 ml phosphate-buffered saline containing 1% bovine serum albumin. Cells were either extracted as described in Experimental Procedures or incubated for up to 2 h with 2 ml culture medium containing 50 μM non-radioactive fenbufen in 5 μl ethanol (the 'chase medium') plus either 10 μM (±)-isoproterenol (▲), 10 μg insulin/ml (■), or with no addition (●). At the indicated intervals the chase medium was collected and the cells were washed and extracted. Radioactivity associated with the lipid (chloroform) phase is shown for (A) cells and (B) media. Points represent the mean ± standard deviation for three independent experiments (n = 3 except where time = 0: n = 9). Error bars are not shown where they are smaller than the symbol. Control or insulin incubations with results significantly different from the isoproterenol incubations are indicated: $*P < 0.05$; $**P < 0.01$.

tation: *1)* the molecular weight of fenbufen is only two less than that of palmitic acid, and *2)* in addition to the common even-chain fatty acids, 3T3-L1 cells are rich in fatty acids with 15 or 17 carbons (25). Nevertheless, it is clear that most of the features predicted for fast atom bombardment (FAB) mass spectra of phosphatidylcholines were present (26). Both peak 17 and peak 19 showed quasi mass ions in the region of 740–770 and a major ion at 566. This is close to the position for diacylglycero-fragment ions, the major predicted fragments (26). Peak 17 also showed peaks with m/z = 496 and 524 that correspond to mono-palmitoyl- and mono-stearoyl-glycerophosphorylcholines, respectively. Further confirmation comes from the MS/MS analysis of peak 19 mass ions 746 and 748 which show strong peaks at m/z = 184 corresponding to phosphorylcholine. It should be noted that peaks 17 and 2 both also showed peaks with m/z = 184, although the mass ions were not analyzed by MS/MS in these cases because insufficient material was available. Direct evidence for phosphatidylcholines containing fenbufen is more difficult to come by, especially as these were shown by choline analysis to be minor components. The existence of a distinct peak at m/z = 494 suggests the formation of a mono-fenbufenoyl-glycerophosphocholine fragment, but the lack of purity means it is possible to find small peaks to match almost any predicted product. While these results are entirely consistent with the identification of the product as a fenbufen-containing phosphatidylcholine, the lack of absolute purity means that unequivocal evidence has not yet been obtained.

Hormone-stimulated lipolysis of fenbufenylated lipid

In order to examine the hormone-stimulated metabolism of the triacylglycerol analogue of fenbufen, differentiated 3T3-L1 adipocytes were incubated for 18 h with 27 μM [^{14}C]fenbufen. The cells accumulated 7.06 ± 1.05 nmol/dish from 9 dishes derived from three independent experiments. Seventy-two percent of the [^{14}C]fenbufen was incorporated into a triacylglycerol analogue; the remainder migrated as a putative diacylglycerol analogue (22%) or as phospholipid (6%). Non-esterified fenbufen and mono-fenbufenoyl-glycerol represented minor constituents (1%). These conditions provided cells that were loaded with sufficient fenbufen-containing triacylglycerol to enable studies of its further metabolism. Cells were then incubated in a chase medium containing 50 μM non-radioactive fenbufen to dilute any released radioactive fenbufen and reduce the likelihood of its reincorporation. (Measurement of radioactive fenbufen in the medium can therefore be regarded as total, and not as net, release). The amount of radiolabel associated with the cells decreased in a time-dependent manner (**Fig. 5A**) and there was a concomitant increase in the radioactivity released into the medium (Fig. 5B). During the first hour of the chase period, fenbufen was released at a rate that was close to first order. The rates of release into the medium or loss from the cells were 0.81 ± 0.22 and 1.12 ± 0.48 nmol/dish per h, respectively. The rates measured in the presence of insulin (0.77 ± 0.22 and 0.87 ± 1.05 nmol/dish per h appearing in the medium or lost from the cells, respectively) were not significantly different ($P > 0.05$). When isoproterenol was present, however, these rates increased to 2.04 ± 0.11 and 2.51 ± 0.24 nmol/ dish per h appearing in the medium or lost from the cells, respectively. The rates measured in the presence of isoproterenol were significantly higher than those meas-

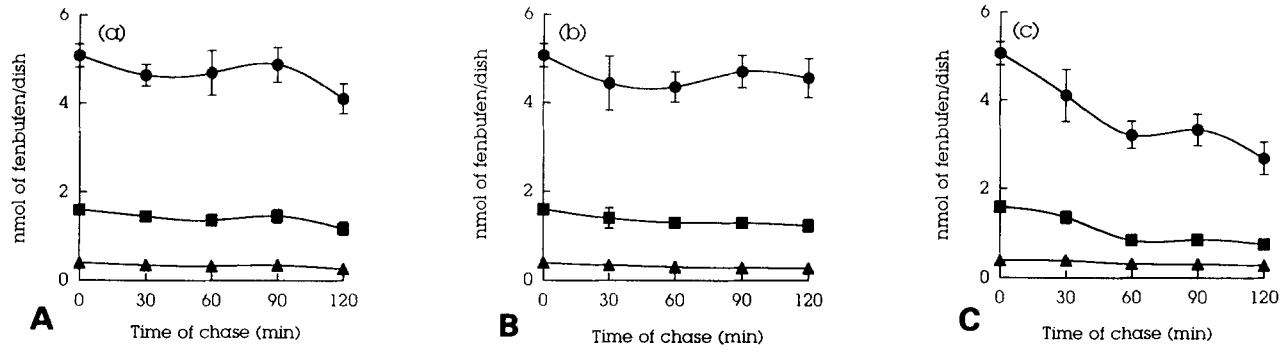

Fig. 6. Composition of fenbufenoyl-lipids extracted from 3T3-L1 cells. Cellular lipids from the experiment described under Fig. 5 were analyzed by TLC as described under Experimental Procedures for (A) control, (B) insulin, and (C) isoproterenol. The symbols represent the fenbufen-derived radioactivity associated with triacylglycerol (●), diacylglycerol (■), or phospholipid (▲). Points represent the mean ± standard error of the mean for three independent experiments (n = 3 except where time = 0: n = 9). Error bars are not shown where they are smaller than the symbol.

ured for the control medium ($P < 0.01$), control cells ($P < 0.05$), and insulin medium ($P < 0.01$). A more kinetically valid method of analysis is to fit an exponential expression to the above time courses and thereby to derive a decay constant and halflife ($t_{1/2}$) for the intracellular fenbufen, a measure that is independent of the original amount incorporated. The data in Fig. 5B give half-life values of 299 min for the control incubations changing to 146 min in the presence of isoproterenol and to 343 min in the presence of insulin.

Figure 6 and **Figure 7** show the composition of lipid products extracted from the cells and the media, respectively, during the course of the chase experiments. The percentage data, used to compile Fig. 6, show that the relative amounts of the different fenbufenoyl-lipids remaining in the cells were altered by neither the length of the chase period nor by the presence of isoproterenol or insulin. In the chase media, 95.1 ± 1.8% (n = 15 at times > 0) of the fenbufen-derived radioactivity was extracted into chloroform. The major radiolabeled products in the medium were nonesterified fenbufen, a diacylglycerol analogue, and a monoacylglycerol analogue (Fig. 7). The monoacylglycerol analogue was usually represented by a split peak presumably resulting from the isomerization by acyl-migration of either the monoacylglycerol itself or of its immediate precursor the diacylglycerol. These diacylglycerol and monoacylglycerol analogues did not result from the release of the contents of disrupted cells, as virtually no phospholipid or triacylglycerol was present in the media. Radiolabeled monoacylglycerol was released most rapidly during the first 30 min of the chase; it then fell as a proportion of the total label. This effect was most marked in the cells treated with isoproterenol. Labeled fenbufen and the diacylglycerol analogue were present in roughly equal quantities (between 38% and 42% of the total) at all times and under all conditions.

Normally when natural precursors are used, mono- and di-acylglycerols are not secreted by adipose cells. In order to check whether their formation from fenbufen could have been an artifact of the experimental conditions used, parallel experiments were conducted, label-

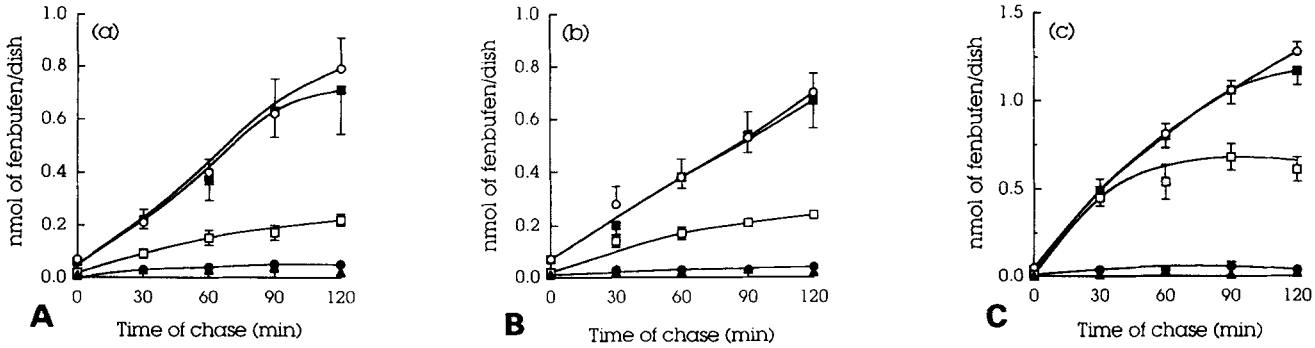

Fig. 7. Composition of fenbufenoyl-lipids extracted from 3T3-L1 incubation media. Media lipids from the experiment described under Fig. 5 were analyzed by TLC as described under Experimental Procedures for (A) control, (B) insulin, and (C) isoproterenol. The symbols represent the fenbufen-derived radioactivity associated with triacylglycerol (●), diacylglycerol (■), phospholipid (▲), non-esterified fenbufen (○), monoacylglycerol (□). Points represent the mean ± standard error of the mean for three independent experiments (n = 3 except where time = 0: n = 9). Error bars are not shown where they are smaller than the symbol.

ing 3T3-L1 adipocytes for 18 h with either [^{14}C]oleic acid or [^{14}C]fenbufen and then chasing them in the presence of 50 μM non-radioactive precursor as described above. Despite being present at a lower initial concentration, more oleic acid than fenbufen was incorporated into lipids with similar proportions forming triacylglycerol but a greater proportion (27% vs. 6%) forming phospholipid. The most important observation was that 92.4 ± 2.5% (9 observations) of the released oleic acid, measured under all conditions, was found unesterified in the medium (compared to only 44.9 ± 2.1% (9) of the fenbufen; significantly different, $P < 0.001$) and that no mono- or di-acylglycerol could be detected (compared to 14% and 39% respectively for the fenbufen).

It was necessary to confirm that the TLC bands identified as analogues of monoacylglycerol and diacylglycerol were not acidic metabolites of fenbufen, such as biphenyl acetic acid or a hydroxylated acid (27), that might have migrated to similar positions. Although acidic metabolites might migrate close to mono- or di-acylglycerols in an acidic solvent system for nonpolar lipids (Solvent System 1), they would be unlikely to move from the origin in a basic solvent system for nonpolar lipids (Solvent System 2). Accordingly, an extract of chase medium was analyzed by TLC in both Solvent Systems 1 (acidic) and 2 (basic). In Solvent System 1, fenbufen and biphenyl acetic acid standards both migrated to the same position on the plate, together with 33.6% of the radioactivity; in Solvent System 2, both acids and 33.3% of the radioactivity were retarded on the origin. As hydroxy acids are more polar than fenbufen, they would also have remained at the origin. If the mono- or di-acylglycerol bands had been contaminated with the acidic metabolites, then the percentage of radioactivity co-chromatographing with genuine fenbufen would have increased in the basic system. Consequently, the remaining 66% of radioactivity on the plate can not be attributed to acidic metabolites of fenbufen.

Further evidence that the band identified as monofenbufenoyl-glycerol was assigned correctly was obtained by incubating it with palmitoyl-CoA and partially purified hepatic monoacylglycerol acyltransferase. Very little of the radioactive precursor extracted into the heptane phase but, after incubation for 60 min, 97% of the heptane-extractable radioactivity chromatographed in Solvent System 1 ahead of the fenbufen standard and below the *sn*-1,3-dipalmitoyl-2-fenbufenoyl-glycerol standard in the area assigned to the diacylglycerol analogues. The *sn*-1,2-(2,3-)-diacylglycerol in the product exceeded the *sn*-1,3-diacylglycerol by 60%: this result could reflect the isomerization of the precursor, the specificity of the enzyme, and the isomerization of the product but does suggest that some of the precursor monoacylglycerol was in the *sn*-2 form, particularly since hepatic MGAT is highly specific for *sn*-2-monoacylglycerols (22). These results both *1)* confirm that the product identified as the monoacylglycerol analogue was a substrate for monoacylglycerol acyltransferase, and *2)* support the identification of the diacylglycerol analogue by TLC.

DISCUSSION

Our results show clearly that the nonsteroidal anti-inflammatory drug, fenbufen, is readily taken up from the incubation medium by differentiated 3T3-L1 adipocytes and incorporated into products analogous to natural lipids. 3T3-L1 adipocytes appear not to express the detoxication enzymes found in other tissues such as liver. Although the formation and subsequent incorporation of fenbufen metabolites, such as the chain-shortened biphenyl acetic acid, cannot be ruled out, it is unlikely that any of the documented hydroxy-derivatives (27–29) were formed because they would have been evident during the TLC analysis of the products secreted into the chase medium. The data did not support the formation of the unidentified hydrophilic conjugates (27) because less than 5% of the radioactivity in the chase media partitioned into the aqueous phase when extracted with chloroform–methanol. In contrast, biphenyl acetic acid, derived from fenbufen, was formed and incorporated into lipids in primary cultures of rat hepatocytes and 50% of the radioactivity in these hepatocytes extracted into the aqueous phase (T. Vrablik, P. F. Dodds, and R. A. Coleman, unpublished results). The lack of competing detoxication enzymes probably contributes to the relatively high rate of incorporation of fenbufen by 3T3-L1 cells. The presence of a 4-carbon aliphatic moiety at the carboxyl end of fenbufen may also make it a good substrate for fatty acid-CoA ligase or for the acyltransferases. The identity of the acyltransferase or acyltransferases responsible for the incorporation of fenbufen into glycerolipids cannot be inferred from the present experiments although it is likely that both monoacylglycerol acyltransferase and diacylglycerol acyltransferase contribute (30, 31). It will be of interest to learn in future experiments whether the natural fatty acids and fenbufen compete for ligase or acyltransferase active sites.

This is the first report to show that fenbufen is incorporated into neutral lipids and to provide good evidence that an acid with aromatic groups close to the carboxylic acid moiety can be incorporated into phospholipids. Previous studies have identified xenobiotic phospholipids that were formed from acids that either closely resembled a natural fatty acid or that were modified only at the ω-end of a long aliphatic chain (32–36). Evidence

has not been provided to support the claims that phospholipids can be formed from the hypoglycemic compounds tetradecylglycidic acid and 5-tetradecyloxy-2-furan carboxylic acid (37, 38). Preliminary reports from our laboratory have suggested that hepatocytes can synthesize phospholipids that contain 3-phenoxybenzoic acid or ibuprofen (39). The evidence presented here (behavior in a variety of different TLC systems using genuine synthetic fenbufenoyl-phosphatidylcholine as a standard, behavior on reverse-phase HPLC, choline analysis, and mass spectroscopy) strongly suggest that several molecular species of fenbufenoyl-phosphatidylcholine were formed. In addition, fenbufenoyl-phospholipids of two other classes were formed, one of which behaved similarly to phosphatidylethanolamine on chromatography. To be unambiguously identified, these products will require mass spectral analysis after they have been purified away from all natural counterparts.

The chase experiments indicate that radioactivity from fenbufen can be released from 3T3-L1 adipocytes and that isoproterenol, a β_2-adrenergic receptor agonist, stimulates release about threefold. Insulin, under the conditions used, did not inhibit the release of either [^{14}C]oleate or [^{14}C]fenbufen into the medium. The concentration of insulin used (1.7 μM) was well in excess of the 0.1 nM concentration that, in conditions of acute exposure, inhibits lipolysis by 50% (25). Insulin probably failed to affect the rate of lipolysis in the current experiments because the cells had been chronically exposed to the hormone (17, 18).

Hormone-sensitive lipase (EC 3.1.1.3), which is responsible for the initial steps of lipolysis in adipose tissue (40), appeared to be responsible for the increased release of fenbufen in response to isoproterenol. Hormone-sensitive lipase activity has not been measured with fenbufenoyl substrates, but we have shown that it hydrolyzes dipalmitoyl 3-phenoxybenzoyl glycerols at about one-third of the rate observed with tripalmitoylglycerol and one-tenth of the rate with trioleoylglycerol (41). Lipoprotein lipase (EC 3.1.1.34) and pancreatic lipase both also have lower activities with xenobiotic substrates than with the corresponding 'natural' substrates (1, 6, 11, 13, 41). It also appears that the ester bond between the xenobiotic acid and glycerol is selectively resistant to hydrolysis within a given triacylglycerol resulting in diacyl- and monoacyl-glycerols as the principal products of lipolysis (13, 41). Relative resistance to lipolysis may therefore be responsible for our results. Although the secretion rates of the fenbufen-labeled and the oleate-labeled lipids were comparable, only 32–43% of the fenbufen product in the medium was the non-esterified acid, and the remainder of the fenbufenoyl-product was released as mono- and di-acylglycerols (Fig. 7). In contrast, more than 90% of the label released by the oleate-labeled cells was oleic acid.

The appearance in the medium of monofenbufenoylglycerol and a fenbufenoyl-containing diacylglycerol raises some interesting questions. With natural triacylglycerols, the products secreted by adipose tissue are fatty acids and glycerol; apart from the secretion of monobutanoylglycerol (42), partial acylglycerols do not appear to be released, probably because they are good substrates for both hormone-sensitive lipase and monoacylglycerol lipase. Our work suggests that, when they are present in sufficient concentrations, acylglycerols can be transported out of the cell. If xenobiotic analogues of monoacyl- and diacyl-glycerols are also secreted in vivo, the fate of these products may have particular importance in view of the evidence that at least one xenobiotic analogue of *sn*-1,2-diacylglycerol stimulates protein kinase C in a manner similar to that of tumor-promoting phorbol diesters (6).

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