The participation of ethyl 4-benzyloxybenzoate (BRL 10894) and other aryl-substituted acids in glycerolipid metabolism

Robin Fears, Keith H. Baggaley, Roger Alexander, Brian Morgan, and Richard M. Hindley

Beecham Pharmaceuticals Research Division, Nutritional Research Centre, Walton Oaks, Tadworth, Surrey, KT20 7NT, U.K. and Chemotherapeutic Research Center,[1] Brockham Park, Betchworth, Surrey, RN3 7AJ, U.K.

Abstract Investigation into the mechanism of action of BRL 10894 (ethyl 4-benzyloxybenzoate), a compound possessing hypolipidemic activity in the rat, disclosed participation in glycerolipid metabolism. In the presence of BRL 10894, an abnormal metabolite was synthesized in vitro using liver slices or rings of small intestine with glycerol, palmitate, or monoolein as substrate, and using adipose tissue with pyruvate as substrate. Esters related chemically to BRL 10894 and other pharmacologically active acids (e.g., ibuprofen) also produced abnormal metabolites in vitro. With BRL 10894 in the diet, a similar metabolite was produced in vivo in rats and accumulated in adipose tissue. Chemical characterization of the material synthesized in vivo showed that the metabolite was a triglyceride in which one fatty acid moiety was substituted by the acid of BRL 10894. Additional proof of this structure was obtained by comparison with reference material synthesized in our laboratories. The study of the intimate involvement of exogenous acids in glycerolipid turnover is of value in the characterization of pharmacologically important acids and may be of use in achieving a greater understanding of certain aspects of lipid metabolism.

Supplementary key words glyceride biosynthesis

In a study of a series of compounds showing hypolipidemic activity in the rat, BRL 10894 (ethyl 4-benzyloxybenzoate, see **Fig. 1**) was selected for further investigation because of its ability to reduce the serum concentration of cholesterol and triglycerides without appreciable overt toxicity.

During the course of a detailed biochemical investigation into the mechanism of action of BRL 10894, it became apparent that the free acid of ethyl 4-benzyloxybenzoate (BRL 14280) behaved as a fatty acid in that it participated in glycerolipid metabolism.

Subsequent investigations examined the behavior of a number of other acids containing an aryl group and the present communication describes the intimate involvement of these synthetic acids in the normal processes of lipid metabolism.

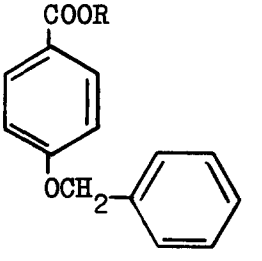

Fig. 1. Structure of BRL 10894 and BRL 14280. R = C_2H_5, BRL 10894; R = H, BRL 14280.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]Glycerol, trioleoyl[2(n)-^{3}H]glycerol, tri[1-^{14}C]-oleolylglycerol, [2-^{14}C]pyruvate, and [1-^{14}C]palmitate were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. [7-^{14}C]Benzyl chloride was obtained from New England Nuclear, Swindon, Wiltshire, U.K. [2-^{14}C]Pyruvate had a stated radiochemical purity greater than 96%; the other radiochemicals were stated to be greater than 98% pure. Pancreatic lipase, collagenase, and lipid standards for chromatography were obtained from Sigma (London) Chemical Co. Ltd, Surrey, U.K.

Physical measurements

Infrared spectra (KBr discs) were obtained with a Perkin-Elmer 257 grating infrared spectrophotometer.

Nuclear magnetic resonance spectroscopy was carried out with a Perkin-Elmer R24 spectrometer at 60 MHz, with $CDCl_3$ as solvent and TMS as internal standard.

Abbreviations: TLC, thin-layer chromatography; UV, ultraviolet; TMS, tetramethylsilane.

[1] Dr. Alexander.

High-pressure liquid chromatography was carried out with a 5 μm Spherisorb column (20 cm × 4 mm), using a Waters Associates pump and a Cecil UV detector. Elution was isocratic with a flow rate of 2 ml/min using hexane–dichloromethane 30:70 (v/v).

Mass spectra were obtained with an AE1 MS9 instrument, operating at 8 KV and 70 eV. Samples were introduced using the direct insertion probe at approximately 210°C; the source temperature was 240°C.

Thin-layer chromatography

All TLC was performed on layers of Silica Gel HF254 + 366 (Merck) in one of the following solvent systems: *i*) light petroleum (bp 40–60°C)–diethyl ether–glacial acetic acid 70:30:1 (v/v/v), *ii*) acetone–heptane 12:88 (v/v), or *iii*) chloroform–methanol–water 65:25:4 (v/v).

Animals

Male CFY rats (Carworth (Europe) Ltd., Alconbury, Hunts., U.K.) were used in all experiments and fed on a stock pelleted diet (Oxoid Breeding Diet) unless otherwise stated.

Glyceride synthesis

In vitro glyceride synthesis in rat liver slices was measured essentially by the method of Liberti and Jezyk (1). Liver slices, approximately 0.5 mm thick, were cut with a Stadie-Riggs microtome. The slices (approximately 250 mg) were pre-incubated at 37°C for 15 min with 5 ml of Krebs-Ringer Tris buffer, pH 7.4, and 100 μl of propylene glycol containing the appropriate addition. Substrate (200 μl) was then added and the flasks were incubated for 1 hr at 37°C with shaking at 100 oscillations/min with air as the gas phase. The amounts of the substrates added and their sp act are stated in the relevant tables. Glycerol, pyruvate, and monoolein were dissolved in water and sodium palmitate was solubilized with 10% fatty acid-free bovine serum albumin in Krebs-Ringer Tris buffer, pH 7.4. Monooleoyl[2(n)-^{3}H]glycerol was prepared by the action of pancreatic lipase on trioleoylglycerol (2); the products of the reaction were extracted with diethyl ether and separated by TLC using solvent system *i*.

Assays were terminated by transferring the flasks to an ice bath. Liver slices were collected by centrifugation, rinsed with ice-cold Krebs-Ringer Tris buffer, pH 7.4, and lipids were extracted by the method of Folch, Lees, and Sloane Stanley (3) as modified by Bligh and Dyer (4). The radiolabeled glyceride classes were separated by TLC using solvent system *i*. The areas corresponding to lipid standards, or to peaks of radioactivity as detected with a radiochromatogram scanner, were scraped off the plates directly into vials and scintillation fluid was added.

Preparations in vitro from other tissues (pieces of epididymal adipose tissue or rings of jejunem) were assayed in a similar manner. Glyceride synthesis from glycerol in rat liver slices, prepared from animals receiving BRL 10894 in the diet, was assayed in a similar manner, except that propylene glycol was not added to the incubation.

Tissue distribution studies

BRL 10894, ^{14}C-labeled in the oxymethylene bridge, was prepared by the reaction of [7-^{14}C]benzyl chloride in ethanol with the sodium salt of ethyl 4-hydroxybenzoate in ethanol at reflux temperature for 6 hr (5). The sp act of the product was 0.08 μCi/μmol.

Rats were given a single oral dose of BRL 10894 in 1% methyl cellulose, 200 mg(10 μCi)/kg body weight. Two animals were killed at various time intervals up to 48 hr after dosing and total radioactivity in the liver and adipose tissue samples was measured after combustion in an Intertechnique Oxymat tissue oxidizer. Where applicable, radioactivity in lipid soluble fractions was measured after extraction as described in the method for glyceride synthesis and TLC fractionation using solvent system *i*.

Lipoprotein lipase

Adipose tissue was pooled from the epididymal fat pads of five rats and was homogenized with five volumes of 0.025 M NH_4OH, pH 8.6, in a Potter-Elvehjem homogenizer at 4°C. After centrifugation at 800 *g* at 4°C for 10 min, an aliquot of the infranatant was taken as the enzyme source for the assay of lipoprotein lipase activity. Assay conditions were as stated by Greten, Levy, and Fredrickson (6) except that the substrate mixture included 0.2 ml/assay of serum from rats starved for 24 hr, and the products of the assay were separated in TLC solvent system *i*. Six assays were performed using triolein substrate and six assays with either of the synthetic triglycerides prepared in our laboratories (one, or all three, of the palmitate constituents replaced by BRL 14280). Results were compared with blank assays performed using a boiled enzyme preparation.

Hormone-sensitive lipase

The activity of hormone-sensitive lipase was measured in adipocytes prepared by the action of collagenase on epididymal fat pads pooled from four rats. Approximately 250 mg of adipose tissue cells was incubated in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 4% fatty acid-free bovine serum albumin.

TABLE 1. Effect of BRL 10894 and BRL 14280 on [1-^{14}C]glycerol incorporation into liver glycerides in vitro

Lipid Class	R_f Value[a]	Glycerol Incorporation[b]		
		Control	BRL 10894[c]	BRL 14280[c]
Phospholipid + Monoglyceride[d]	Origin–0.1	250	150	100
Diglyceride	0.21	63	36	30
Metabolite 'X'	0.49	1	90	80
Triglyceride	0.66	250	150	100
Total lipid		580	435	305

[a] Solvent system *i*.
[b] 1.5 μmol (0.5 μCi) of [1-^{14}C]glycerol added per assay (approximately 250 mg of liver slices) giving a final concentration of 3×10^{-4} M. Results are expressed as nmol glycerol incorporated/g liver per hr. Results are means of analyses of five animals (each analysis performed in triplicate).
[c] Added in propylene glycol to give final concentration of 5×10^{-4} M. Control assays received the same volume (100 μl) of propylene glycol alone.
[d] Not separated effectively in solvent system *i*.

One μmol of synthetic triglyceride substrate (one palmitate substituted) was added in 20 μl of ethanol–acetone 1:1 (v/v). Incubations with, and without, 5 μg of epinephrine were performed in triplicate for 1 hr at 37°C. At the end of the incubation period, free fatty acids were extracted and measured by a combination of the methods of Duncombe (7) and Itaya and Ui (8). For the measurement of BRL 14280 cleaved from the synthetic glyceride, lipid was extracted into chloroform and the lipid classes were separated by TLC using solvent system *i*. BRL 14280 was estimated by a semiquantitative method in which the size of the sample spot under UV light was compared with the sizes of spots of standard amounts of BRL 14280 run on the same plate.

Radioactivity counting procedures

The scintillation fluid consisted of 10 ml of 0.6% (w/v) 5-(4-biphenylyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD; Intertechnique Ltd., Brighton, Sussex, U.K.) in toluene. All samples were analyzed in an Intertechnique ABAC SL40 spectrometer and corrected for quenching by the external standard channels ratio method.

RESULTS

BRL 10894 and glyceride synthesis in vitro

The first experiments were primarily designed to investigate the effect of BRL 10894 on conventional glycerolipid synthesis from various substrates.

BRL 10894 or its acid, BRL 14280, was added to tissue preparations to give a final concentration of 5×10^{-4}M (see **Tables 1, 2 and 3**).

Glyceride synthesis from glycerol (liver and small intestine), 2-monoolein (liver), and perhaps pyruvate (adipose tissue) was inhibited; synthesis from palmitate in liver was not affected. We have shown[2] that BRL 10894 and its free acid inhibit fatty acid synthesis from acetyl CoA in rat liver and, in consequence, glyceride biosynthesis is limited unless exogenous fatty acid is supplied.

It was also apparent, however, that for each substrate, whether or not conventional glyceride synthesis was affected, there was appreciable incorporation of radioactivity into an unidentified metabolite 'X' when BRL 10894 or its acid was added to the assay. In each case, 'X' had the same mobility, running between the triglyceride band and the fatty acid band in solvent system *i*.

Visualization of the chromatograms under UV light showed fluorescence corresponding to the acid, BRL 14280, even when the ester had been added to the assay. A similar characteristic fluorescence was also associated with the unidentified metabolite 'X'. When the metabolite 'X' was eluted from the silica gel, saponified with alcoholic KOH, and the lipid extract was rechromatographed in solvent system *i*, neither the radioactivity nor the fluorescence associated with 'X' was present. However, fluorescent bands equivalent in mobility to free fatty acids and to the free acid, BRL 14280, could now be observed, suggesting that ester hydrolysis had occurred. It is likely therefore that, not only did the metabolite 'X' incorporate radio-

[2] Fears, R. B. Unpublished experiments.

TABLE 2. Effect of BRL 10894 on [1-^{14}C]palmitate incorporation and BRL 14280 on monooleoyl[2(n)-^{3}H]glycerol incorporation into liver glycerides in vitro

Lipid Class	Palmitate[a]		2-Monoolein[b]	
	Control	BRL 10894[c]	Control	BRL 14280[c]
Phospholipid + Monoglyceride[d]	330	360	130	60
Diglyceride	260	220	70	30
Metabolite 'X'	9	670	1	90
Triglyceride	1590	1530	180	80
Total lipid	2320	2750	400	265

[a] 4 μmol (0.2 μCi) of [1-^{14}C]palmitate added per assay (approximately 250 mg of liver slices) giving a final concentration of 8×10^{-4} M. Results are expressed as nmol substrate incorporated/g liver per hr. Results are means of three animals (each analysis performed in triplicate).

[b] 1 μmol (0.5 μCi) of monooleoyl[2(n)-^{3}H]glycerol added per assay (approximately 250 mg of liver slices) giving a final concentration of 2×10^{-4} M. Results are expressed as for palmitate. Results are means of three animals (each analysis performed in triplicate).

[c] Added in propylene glycol to give a final concentration of 5×10^{-4} M. Control assays received the same volume (100 μl) of propylene glycol alone.

[d] Not separated effectively in solvent system *i*.

activity from monoglyceride, glycerol, palmitate, or their precursor (pyruvate), but that it also contained some moiety of the acid BRL 14280. Consequently a tentative, preliminary identification of this metabolite was suggested as a substituted glyceride in which one or more fatty acid moieties was replaced by the acid BRL 14280. In the small intestine the situation is perhaps more complicated; it has been established (9) that, in that tissue, monoglyceride is used for triglyceride synthesis, whereas glycerol is mainly incor-

TABLE 3. Effect of BRL 14280 on in vitro incorporation of [2-^{14}C]pyruvate into adipose tissue glycerides, [1-^{14}C]glycerol and monooleoyl[2(n)-^{3}H]glycerol into jejunal glycerides

Lipid Class	Adipose Tissue		Jejunem			
	Pyruvate Incorporation[a]		Glycerol Incorporation[b]		Monoolein Incorporation[c]	
	Control	BRL 14280[d]	Control	BRL 14280[d]	Control	BRL 14280[d]
Phospholipid + Monoglyceride[e]			41	20	65	75
Diglyceride	1,300[f]	900[f]	20	8	17	24
Fatty acid						
Metabolite 'X'	2	390	1	15	1	16
Triglyceride	3,220	2,060	80	63	52	52
Total lipid	4,700	3,350	150	120	140	170

[a] 10 μmol (0.9 μCi) of [2-^{14}C]pyruvate added per assay (approximately 100 mg of adipose tissue pieces) giving a final concentration of 2×10^{-3} M. Results are expressed as nmol of substrate incorporated/g tissue per hr. Results are means of four animals (each analysis performed in triplicate).

[b] 1.5 μmol (0.5 μCi) of [1-^{14}C]glycerol added per assay (approximately 250 mg of rings of jejunem) giving a final concentration of 3×10^{-4} M. Results are expressed as for pyruvate. Results are means of four animals (each analysis performed in triplicate).

[c] 1 μmol (0.5 μCi) of monooleoyl[2(n)-^{3}H]glycerol added per assay (approximately 250 mg of rings of jejunem) giving a final concentration of 2×10^{-4} M. Results are expressed as for pyruvate. Results are means of four animals (each analysis performed in triplicate).

[d] Added in propylene glycol to give a final concentration of 5×10^{-4} M. Control assays received the same volume (100 μl) of propylene glycol alone.

[e] Not separated effectively in solvent system *i*.

[f] For pyruvate the results were not differentiated into lower glycerides and fatty acids.

porated into phospholipids and the respective diglyceride pools do not mix. The high triglyceride production from glycerol in the present experiments suggests that the internal organization of the cell may have been disrupted, and a similar explanation may account for the synthesis of the unknown metabolite 'X' from both substrates. TLC separation of the phospholipid fraction (isolated in system *i*) was performed using solvent system *iii* but disclosed no other metabolic abnormalities with small intestine or liver using either monoglyceride or glycerol as substrates.

BRL 10894 and hepatic glyceride synthesis ex vivo

Following the unexpected discovery of the participation of BRL 10894 in glycerolipid metabolism in vitro, it was decided to extend the investigation to studies in which BRL 10894 was fed to rats.

Groups of eight rats were fed for 7 days on a stock laboratory diet with or without a supplement (0.25% of diet) of BRL 10894. Glyceride synthesis from glycerol was measured in rat liver slices as before. Visualization of the chromatograms under UV light, or with iodine, disclosed a band of material in all of the rats treated with BRL 10894 that was not present in the controls. This band had an identical fluorescence and mobility to metabolite 'X' produced in vitro and was associated with a small but significant amount of radioactivity: 5.1 ± 0.9% of the total radioactive lipid compared with 1.6 ± 0.9% for the equivalent area in control samples ($P < 0.02$). Thus the same radiolabeled metabolite was produced whether BRL 10894 was included in the diet or added in vitro.

Glycerol incorporation into triglyceride, diglyceride, and phospholipid/monoglyceride was not reduced ex vivo, suggesting that fatty acid supply was not limiting under these conditions.

Metabolism of radiolabeled BRL 10894 in vivo

In an effort to understand more fully the metabolism of BRL 10894 in vivo, experiments were performed using radiolabeled material synthesized in our laboratories.

TABLE 4. Radioactivity in the liver after a single oral dose of radiolabeled BRL 10894

Time after Dosing[a]	Peak 1 $R_f = 0.13$[b]	Peak 2 $R_f = 0.33$[b]	Peak 3 $R_f = 0.53$[b]
hr		*dpm/g liver*[c]	
0.5	2,400	4,000	2,400
2	1,000	2,900	200
4	1,600	2,900	0
24	0	5,600	0

[a] Two rats per time period.
[b] R_f value in solvent system *i*.
[c] Peaks were identified by scanning the chromatogram of the lipid extract.

TABLE 5. Radioactivity in the liver and adipose tissue after a single oral dose of radiolabeled BRL 10894

Time after Dosing[a]	Radioactivity[b]		
	Liver	Epididymal Adipose Tissue	Perirenal Adipose Tissue
hr			
0.5	12,400	16,700	30,600
2	10,100	57,000	159,200
4	9,700	44,800	70,500
7	6,600	67,700	128,700
24	6,000	108,000	146,300
48	2,100	135,200	154,100

[a] Two rats per time period.
[b] Radioactivity expressed as dpm/g tissue.

Thirty minutes after rats were given an oral dose of ^{14}C-labeled BRL 10894, three peaks of radioactivity were observed on scanning the thin-layer chromatogram of the chloroform extract of liver fractionated in solvent system *i*. Peak 3 had a mobility similar to that of metabolite 'X' and peak 2 had a mobility equivalent to the acid BRL 14280. Peak 1 (running just above the origin) was unidentified. With increasing time after dosing, the radioactivity in peaks 1 and 3 disappeared (**Table 4**). When the lipid extract prepared from the liver 30 min after dosing was saponified with alcoholic KOH, peaks 1 and 3 disappeared while peak 2 remained; this suggested that peak 2 was indeed BRL 14280 whereas 1 and 3 were esters (glycerides).

In a second experiment, radioactivity in the liver at various times after the administration of labeled BRL 10894 was compared with the radioactivity in samples of adipose tissue (**Table 5**). At all times a greater proportion of the dose was found in the adipose tissue.

As the adipose tissue mass accounts for approximately 10% of the total body weight, and as the samples taken were known to be representative of the total fat content of the body,[2] it can be calculated that approximately 70% of the dose was present in adipose tissue 48 hr after dosing.

Fractionation of the chloroform extract of adipose tissue, pooled from samples taken from each of the rats, in solvent system *i* and *ii* showed only one peak of radioactivity, which was identical in mobility and fluorescence to metabolite 'X' previously described.

Further quantification and chemical characterization of metabolite 'X'

Although the principal part of a single dose of BRL 10894 appears to be accounted for by metabolite 'X' in adipose tissue, it was considered of importance to de-

termine the contribution made by this fraction when BRL 10894 was administered over a longer period of time. It was also necessary to obtain a larger amount of material for chemical characterization.

Therefore, BRL 10894 was administered to eight rats for 8 days as an oral dose, 5000 mg/kg in 1% methyl cellulose. At the end of the experimental period, the chloroform-soluble lipid was extracted from pooled samples of epididymal adipose tissue, weighed, and fractionated by TLC using solvent system *i*. A band equivalent in mobility and fluorescence to metabolite 'X' was eluted with chloroform, purified in solvent system *ii*, and weighed. Approximately 30% of the lipid in adipose tissue, 220 mg/g adipose tissue, was found to be metabolite 'X'. Eight control rats that had received an equal volume of 1% methyl cellulose alone contained no such metabolite in their adipose tissue.

The structure of the metabolite 'X' sample obtained from this dietary experiment was investigated by various physical methods.

The infrared spectrum of the metabolite exhibited strong absorption bands at 2740–3000 cm^{-1} (CH stretching and deformation), 1720–1740 cm^{-1} (C=O stretching), 1610 cm^{-1} (aromatic ring breathing mode), 1250–1279 cm^{-1} (C–O stretching), 1170 and 1125 cm^{-1} (aromatic mono and 1,4-disubstitution), and 850, 770, 740, 685 cm^{-1} (aromatic CH out-of-plane deformation). The nuclear magnetic resonance spectrum exhibited characteristic bands associated with a fatty acid ester at 0.1 (m), 1.24 (singlet on methylene envelope) and 2.32 (m) ppm, as well as a glyceryl function at 4.32 (m), 4.44 (m) and 5.41 (m) ppm and a 4-benzyloxybenzoyl moiety at 5.10 (s), 6.89 (d, J9Hz), 7.98 (d, J9Hz) and 7.37 (s) ppm. The integral indicated the ratio of glyceryl to 4-benzyloxybenzoyl moieties to be 1:1 and that the sample contained approximately 90 aliphatic protons. This information, in conjunction with the biochemical evidence, indicated that the metabolite constituted a triglyceride containing at least one 4-benzyloxybenzoyl moiety.

The mass spectrum of the metabolite provided further evidence for this assignment. Three molecular ions were observed in the mass spectrum at *m/e* 804, 778, and 750, in the ratio 1:5:2, indicating the metabolite to have three constituents. High resolution mass determination on these ions gave their respective molecular compositions as $C_{51}H_{80}O_7$ (1.6 ppm), $C_{49}H_{78}O_7$ (1.5 ppm) and $C_{47}H_{74}O_7$ (1.9 ppm). Ions at *m/e* 265 and 239 were assigned to the fragments $C_{17}H_{33}CO^+$ (oleyl) and $C_{15}H_{31}CO^+$ (palmityl). High resolution determination of the ion at *m/e* 211 showed it to consist of a doublet (9:1) of molecular formula $C_{14}H_{11}O_2$ (1.6 ppm), assigned the 4-benzyloxybenzoyl moiety, and $C_{14}H_{27}O$ (2.5 ppm), assigned the myristyl moiety. The base peak at *m/e* 91 was assigned to $C_7H_7^+$ from the benzylic fragment of *1*. Fragment ions at *m/e* 313, 354, and 367 were assigned to $[C_{15}H_{31}CO + 74]^+$, $[C_{15}H_{31}CO + 115]^+$, and $[C_{15}H_{31}CO + 128]^+$ and ions at *m/e* 551 and 550 to $[M - C_{13}H_{11}OCO_2]^+$ and $[M - C_{13}H_{11}O \cdot CO_2H]^+$ from the principal constituent *m/e* 778, all characteristic of triglycerides (10). The corresponding losses of $C_{13}H_{11}OCO_2^+$ and $C_{13}H_{11}OCO_2H^+$ from the minor constituents were also apparent by ions at *m/e* 576^+ and 577^+ from M^+ 804 and 523 and 522 from M^+ 750.

The evidence obtained from the chemical characterization studies shows, in fact, that the metabolite 'X' is a mixture of three triglycerides. Each triglyceride component of this mixture appears to have contained a palmitate moiety and a 4-benzyloxybenzoyl moiety (from BRL 10894). The third position in the triglyceride was occupied by oleate (mol wt of triglycerides 804), myristate (750) or an additional palmitate (778). The results for the integral from nuclear magnetic resonance spectroscopy indicate, however, that we have not completely characterized all the components of the isolated material since there appeared to be more aliphatic protons present than could be fully accounted for by the mass spectroscopy data. As yet we have no information on the positional specificity of any of the substituents. A synthetic triglyceride containing two palmitates and a 4-benzyloxybenzoyl moiety was prepared in our laboratories by the treatment of 1,2-dipalmitin with 4-benzyloxybenzoyl chloride (11) in dry chloroform in the presence of pyridine (12).

The thin-layer and high-pressure liquid chromatograms of the synthetic material and of the metabolite 'X' were identical. The mass spectrum of the former exhibited a molecular ion at *m/e* 778 and the fragmentation pattern of the synthetic material was consistent with that of the metabolite 'X'. No information as to the position of the 4-benzyloxybenzoyl moiety on the glycerol moiety in the metabolites could be adduced from the mass spectrum since the peak at $[M - C_{14}H_{11}O \cdot CO_2CH_2]^+$ was absent.

Lipolysis studies

It may be concluded that the result obtained from the structural analyses is consistent with the results obtained using the labeled substrates and so there can be little remaining doubt that metabolite 'X' is a mixture of triglycerides in which one fatty acid is replaced by the acid moiety of BRL 10894. In subsequent investigations, we examined in more detail the involvement of the substituted glyceride in various enzyme systems. Synthetic triglycerides, where one or all three palmitate moieties were replaced by BRL 14280, were assayed as substrates for the enzymes lipoprotein li-

pase and hormone-sensitive lipase. Conventional activity of our lipoprotein lipase preparation, using tri[1-^{14}C]oleoylglycerol as substrate, was 2.1 μmol of free fatty acid released/hr per g of adipose tissue. No significant lipolytic activity could be detected when the synthetic glycerides were used as substrates. In the assay of hormone-sensitive lipase, 5.3 μmol of free fatty acid was released/hr per g of adipose tissue together with 0.044 μmol of BRL 14280 from the synthetic glyceride under basal conditions. In the presence of 5 μg of epinephrine, the rates were 11.2 and 0.040 μmol/hr per g, respectively. Thus, addition of epinephrine to the assay did not enhance the rate of hydrolysis and it is possible that the glyceride was cleaved by an 'esterase' rather than by hormone-sensitive lipase. Such esterases have not been fully characterized but they are known to have properties and functions differing markedly from hormone-sensitive lipase and other long chain glyceride hydrolases (13). Further biochemical investigations are continuing.

Other acids

Following the observation that BRL 10894 participated in glycerolipid metabolism, a number of other acids containing aryl groups, or their ethyl esters, were investigated. The compounds examined comprised four groups: ethyl esters possessing hypolipidemic properties and chemically related to BRL 10894; commercially available antiinflammatory compounds (flurbiprofen, ketoprofen, ibuprofen, fenoprofen); other pharmacologically important acids (clofibric, *p*-aminosalicylic, nicotinic, and acetyl salicylic acids); and naturally occurring plant acids (chlorogenic, *p*-coumaric, ferulic, and vanillic acids). All experiments were performed with liver slices in vitro and each compound was added to give a final concentration of 5×10^{-4}M. The results are shown in **Tables 6** (glycerol substrate) **and 7** (palmitate substrate).

TABLE 6. Effect of other acids or their precursor on [1-^{14}C]glycerol incorporation into an abnormal hepatic metabolite in vitro

Acid or Precursor[a]	Metabolite Production[b]
Ethyl *p*-(2-methoxybenzyloxy)phenyl acetate (BRL 14227)	240
Ethyl *p*-(4-fluorobenzyloxy)phenyl acetate (BRL 14635)	230
Clofibrate	1
Clofibric acid	0
p-Aminosalicylic acid	0
Nicotinic acid	0
Acetyl salicylic acid	0
Chlorogenic acid	6
p-Coumaric acid	2
Ferulic acid	4
Vanillic acid	3
2-(2-Fluoro-4-biphenylyl)-propionic acid (Flurbiprofen)	2
2-(3-Benzoylphenyl)-propionic acid (Ketoprofen)	48
2-(4-Isobutylphenyl)-propionic acid (Ibuprofen)	110
2-(3-Phenoxyphenyl)propionic acid (Fenoprofen)	140

[a] Added in propylene glycol to give a final concentration of 5×10^{-4} M.

[b] 1.5 μmol (0.5 μCi) of [1-^{14}C]glycerol added per assay (approximately 250 mg of liver slices) giving a final concentration of 3×10^{-4} M. Results are expressed as the percentage of the incorporation found using BRL 10894 in the same assay. Results are means of analyses of at least two animals (each analysis performed in triplicate).

TABLE 7. Effect of ketoprofen, fenoprofen, and ibuprofen on [1-^{14}C]palmitate incorporation into an abnormal hepatic metabolite in vitro

Acid[a]	Metabolite Production[b]
Ketoprofen	76
Fenoprofen	201
Ibuprofen	70

[a] Added in propylene glycol to give a final concentration of 5×10^{-4} M.

[b] 4 μmol (0.1 μCi) of [1-^{14}C]palmitate added per assay (approximately 250 mg of liver slices) giving a final concentration of 8×10^{-4} M. Results are expressed as the percentage of the incorporation found using BRL 10894 in the same assay. Results are means of analyses of two animals (each analysis performed in triplicate).

Production of an abnormal metabolite was identified by radioscanning the chromatogram of the lipid extract of the products of the assay. This method does not detect any metabolite having a mobility similar to a conventional glycerolipid. In each case, the radioactivity attributed to an unidentified metabolite was found to be associated with fluorescence under UV light. The amount of each abnormal metabolite synthesized was compared with the amount synthesized with BRL 10894 under the same experimental conditions.

With glycerol as substrate, BRL 14227, BRL 14635, ketoprofen, ibuprofen, and fenoprofen each produced an abnormal metabolite but no other acid tested behaved in this way. With palmitate as substrate, fenoprofen, ketoprofen, and ibuprofen again produced an abnormal metabolite, in each case of mobility similar to that synthesized with glycerol. When the abnormal metabolites produced from glycerol with fenoprofen, ibuprofen, or ketoprofen were eluted, saponified with alcoholic KOH, and rechromatographed in solvent system *i*, no radioactivity could be detected, but fluorescence of the parent acid and of free fatty acids could be observed in each case. It is likely, therefore, that fenoprofen, ketoprofen, and ibuprofen produce substituted glycerides in vitro in a manner similar to BRL

10894, although it remains to be established whether they have similar effects in vivo.

When BRL 14227 or BRL 14635 were included as a supplement (0.25%) in the diet of eight rats for 7 days, characteristic UV-fluorescent material was found in epididymal adipose tissue. For both compounds, the metabolite that accumulated in vivo was of similar chromatographic mobility to that synthesized in vitro and it seems likely, therefore, that their behavior in vivo is similar to that of BRL 10894.

DISCUSSION

The rationale for undertaking the research described here was to investigate in detail the biochemical basis for the mechanism of action of compound BRL 10894, known to exhibit hypolipidemic activity in laboratory animals. For example, in a typical experiment, when BRL 10894 was included as a supplement (0.25%) in the diet of eight rats, serum cholesterol was reduced by 17% and serum triglyceride was reduced by 61%. There was no effect on the concentration of liver lipids (5).

It seems likely however, that the discovery of the intimate involvement of an exogenous agent in glycerolipid metabolism may have a wider application. Our observation of such behavior appears to be novel, though a number of synthetic compounds, including carboxylic hypolipidemic agents, are known to affect conventional glycerolipid synthesis (14).

Although the majority of naturally occurring fatty acids are aliphatic, a number of unusual cyclic fatty acids have been identified in naturally occurring triglycerides. These include cyclopropenoid acids (malvalic and sterculic), cyclopentene acids (chaulmoogric, hynocarpic, and gorlic) and furanoid acid, in plants (15). Similarly, furanoid fatty acids have been identified in the liver and testes of fish such as the pike (16) and sterculic acid is found in the triglycerides of the rainbow trout fed with methyl sterculate (17). Esters of various aromatic acids such as benzoic acids and also aryl-substituted aliphatic acids such as hydroxycinnamic acid, are known in plants; for example, chlorogenic acid has been identified in coffee and tobacco (18). There is no evidence for the participation in glycerolipid turnover of naturally occurring acids containing aryl groups in animals but a recent identification has been reported (19) of ω-cyclohexyl fatty acids in acidophilic, thermophilic bacteria. These unusual fatty acids were esterified in glyceride type complex lipids and accounted for between 74 and 93% of the total fatty acids.

Thus, although there appear to be no reported accounts of the involvement of exogenous aryl-substituted acids in glycerolipid metabolism, there are natural precedents and it seemed that the behavior of BRL 10894 may not be exceptional. In fact, our results show that the incorporation of an aryl-substituted fatty acid into glycerides is not confined to BRL 10894 but is also shown by chemically related compounds possessing hypolipidemic activity and by other pharmacologically important acids. There is no evidence, however, that the intimate participation in glycerolipid metabolism is synonymous with hypolipidemic activity.

From the limited number of acids investigated it is not possible to derive any clear structure–activity relationships. All acids active in producing an abnormal metabolite could be categorized as 'activated' aryl or aryl-substituted acids.

Participation of an unnatural acid in the formation of glycerides presumably necessitates prior activation to the acyl-CoA derivative. It is of relevance to note that benzoyl-CoA and phenacetyl-CoA can be produced in vivo (20) and medium chain fatty acid:CoA lipase is active with a variety of aliphatic and aromatic carboxylic acids (21). In the present case de-esterification of the ethyl ester is unlikely to be rate-limiting for the metabolism of BRL 10894 as many mammalian tissue carboxylesterases are highly efficient catalysts of ester hydrolysis (22).

Besides their inherent interest, studies such as the one reported here may be of use in comprehending normal lipid metabolism and its control. Hancock, Greenwald, and Sable (23) recently reported the synthesis of pseudoglycerides (tris-homoacyl derivatives of cyclopentane 1,2,3-triols) with the hope that such analogues may provide a better understanding of some of the physical and biological properties of natural glycerol-containing lipids. Similarly, Chakrabarti and Khorana have described (24) synthetic fatty acids and phospholipids containing photosensitive groups, such as 4-azido-2-nitrophenyl, for use in studying hydrophobic interactions between proteins and phospholipids in biological membranes.

More recently Stoffel and Michaelis (25) have produced ω-anthracene-labeled fatty acids in an attempt to obtain fluorescent probes suitable for incorporation into the lipid components such as glycero-, phospho-, and sphingolipids of biological membranes. The use of synthetic fatty acids to obtain information about normal lipid metabolism was, in fact, started by Knoop (26) who derived the principles of fatty acid β-oxidation with phenyl-substituted even- and odd-numbered fatty acids.

Possible future studies with BRL 10894 could include, for example, the investigation of the positional specificity of naturally occurring fatty acids in the arti-

ficial glycerides and whether such glycerides behave in a manner different from the conventional glycerides in depot fat.

The possibility that the unusual glyceride might participate in lipoprotein metabolism is also of interest, especially perhaps from the point of view of understanding the metabolism of other atypical fatty acids such as phytanic acid in Refsum's disease (27).

From the present results it may be concluded that a number of exogenous acids can participate in glycerolipid turnover. The identification of other similarly acting agents, therapeutic or otherwise, is of undeniable importance.

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