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DISPOSITION OF CLOFIBRATE IN THE RAT

ACUTE AND CHRONIC ADMINISTRATION*†

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Abstract—The disposition of 1-[^{14}C]clofibrate (0.4 mmole/kg) was studied in rats after acute (single dose) and chronic (b.i.d., for 14 days) administration. With a single dose (orally or by intraperitoneal injection) of clofibrate, most (~90 per cent) of the ^{14}C-dose appeared in the urine within 24 hr and the recovery of ^{14}C from the urine and feces was nearly quantitative within 72 hr. Little fecal excretion of ^{14}C (< 5 per cent) occurred after a single or chronic clofibrate administration. Clofibrate was readily absorbed and eliminated, as evidenced by a rapid increase in plasma ^{14}C level within 90 min and a calculated biological half-life of 4.1 hr. The pharmacokinetic profile of ^{14}C-elimination in rats was unaffected by pretreatment with cholestyramine. Clofibric acid [2-(4-chlorophenoxy)-2-methylpropionic acid] was identified as the major metabolite in plasma (~97 per cent) whereas the glucuronide of clofibric acid was the main urinary and biliary metabolite (~96 per cent). Clofibric acid, as the free acid and glucuronide form, accounted for 99 per cent of the total ^{14}C-dose in rats, and unchanged clofibrate was not detected in any of the biological samples. Two unidentified, minor urinary metabolites were also detected. In cannulated bile duct studies, it was found that [^{14}C]clofibrate, as clofibric acid, was rapidly and efficiently excreted in the bile. The biliary excretion rates of ^{14}C and of the glucuronide of clofibric acid were also not altered by phenobarbital pretreatment. Chronic treatment with [^{14}C]clofibrate did not alter the qualitative or quantitative nature of biotransformation *in vivo*. An increased rate of urinary ^{14}C-elimination was observed following chronic 1-[^{14}C]clofibrate treatment, with concomitant reductions in blood and heart ^{14}C-content and an elevation in ^{14}C-content of epididymal fat tissue. Subcellular fractionation of liver, from rats given [^{14}C]clofibrate chronically, indicated an increased distribution of ^{14}C into mitochondria and peroxisomes. Tissue ^{14}C-levels, achieved in these *in vivo* studies, were an order of magnitude lower than those required for the pharmacological activities of clofibrate and clofibric acid *in vitro*.

Clofibrate, the ethyl ester of 2-(4-chlorophenoxy)-2-methylpropionic acid (hereafter designated as clofibric acid), is widely used as an antihyperlipidemic agent [1, 2]. Pharmacokinetic data for clofibrate have appeared in the literature [3–5], however, very little work has been done on the biotransformation and the tissue distribution of clofibrate [6, 7]. This is due, in part, to the initial report by Thorp [6] showing that clofibrate was rapidly hydrolyzed to clofibric acid by tissue esterases, glucuronidated, and quantitatively excreted in the urine. Based upon these findings and those of Cayen *et al.* [7], clofibric acid was proposed to be the pharmacologically active form of clofibrate in man, rat and dog.

To discern the mode of antilipidemic or toxicological actions of clofibrate, the effects of clofibrate and clofibric acid on selected tissues and enzyme systems have been evaluated in experimental studies [8–12]. These pharmacological studies involved either a direct addition of clofibrate to the biological medium or measurement of various enzyme activities after pretreatment of animals with clofibrate for a predetermined period. It remains to be established whether this drug accumulates to an effective level at its proposed pharmacological site(s) of action. In the present study, we examined the biotransformation, elimination, and tissue distribution of clofibrate in rats, using a dosage regimen of clofibrate that exhibited significant antilipidemic activity [13]. The serum–protein binding and tissue accumulation of clofibrate were also monitored after acute and chronic administration. While our studies were in progress, Cayen *et al.* [7] reported on the disposition of clofibrate and clofibric acid in the rat and dog.

MATERIALS AND METHODS

Materials. Clofibrate was supplied by Ayerst Laboratories (New York NY). Radiolabeled 1-[^{14}C]clofibrate (sp. act. 45.3 μCi/mmole) was synthesized in our laboratories according to the procedure of Ferdinandi [14], using [^{14}C]-chloroform as the reactant (see Fig. 1). Radiochemical purity (> 98 per cent) was assessed by thin-layer chromatography (t.l.c.) in three solvent systems (see below). Glucose-6-phosphate dehydrogenase (grade II), β-glucuronidase (bovine liver, type B-1), and cytochrome *c* (horse heart, type III) were purchased

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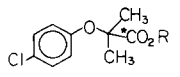

*location of 1-^{14}C atom

Clofibrate	R = CH_2CH_3
Clofibric Acid	R = H
Clofibric Acid-Glucuronide	R = 

Fig. 1. Structures of clofibrate, clofibric acid, and the glucuronide of clofibric acid. The asterisk indicates the presence of the ^{14}C-atom of 1-[^{14}C]clofibrate.

from the Sigma Chemical Co. (St. Louis, MO). Ethylmorphine (Dionin) and sodium phenobarbital were obtained from Merck & Co., Inc. (Rahway, NJ). Cholestyramine was received from Mead Johnson & Co. (Evansville, IN). Soluene-100, Permafluor V, Carbo-Sorb, Combusto-Pads, and Combusto-Cones were purchased from the Packard Instrument Co., Inc. (Downers Grove, IL), while Thrift-Solve was obtained from Kew Scientific, Inc., (Columbus, OH). Anasil t.l.c. plates (silica gel G and GF, 5 × 20 cm, 250 μm thickness) were purchased from Analabs, Inc. (North Haven, CT). All other materials were reagent grade materials, from commercial sources, and were used without further purification.

Animals. Harlan male Sprague–Dawley rats (Cumberland, IN) weighing approximately 180 g at the beginning of the experiment were used throughout. Animals had free access to water and Purina lab chow.

Measurment of radioactivity. Samples containing ^{14}C were counted in a Beckman model LS-355 liquid scintillation spectrometer (Fullerton, CA). The extent of quenching was corrected by external and internal standardization techniques. Plasma, urine, bile and silica gel scrapings were counted in 10 ml of Thrift-Solve with an 86–94 per cent counting efficiency. Tissue, fecal and whole blood samples were combusted in a Packard sample oxidizer (model 306, Downers Grove, IL), which automatically collected $^{14}CO_2$ in a Permafluor–CarboSorb solution. The counting efficiency of ^{14}C in these samples ranged from 72 to 85 per cent.

Thin-layer chromatography. Metabolites of clofibrate were separated on silica gel by ascending t.l.c. chromatography using three solvent systems: I, benzene–glacial acetic acid–methanol (76:4:20), II, 2-propanol–ammonia (95:5), and III, hexane–formic acid (90 per cent)–ethanol (57:3:40). After allowing the plates to develop 15 cm, they were air dried and visualized in u.v. light (254 nm); the radioactive peaks were isolated with a t.l.c. zonal scraper (model TLE-001, Analabs, Inc.). The R_f values of clofibrate and clofibric acid in the solvent systems were I, 0.73 and 0.57; II, 0.69 and 0.30 and III, 0.91 and 0.88 respectively (N = 4).

Radioactivity in whole blood. For the 6-hr study, three rats were anesthetized with urethane (1.2 g/kg, i.p.) followed by intubation of 0.4 mmole/kg of clofibrate (3.5 μCi/mmole) suspended in 0.25 per cent methylcellulose. The right carotid artery was cannulated with PE-II tubing connected to a heparinized syringe. Samples of blood (0.2 ml) were drawn every 30 min and dispensed onto Combusto-Pads and allowed to dry overnight. For the 14-day study, three rats were given 0.4 mmole/kg of clofibrate (3.5 μCi/mmole) every 12 hr by intubation. The animals were lightly anesthetized with ether, and blood was drawn (0.4 ml) from the orbital plexus with heparinized capillary tubes 12 hr after the last dose on days 1, 7, and 14.

Isolation of metabolites in plasma, urine and bile. Animals used in the 6-hr blood level study were exsanguinated upon termination of the experiment. ^{14}C in the plasma samples was extracted according to the procedure of Karmen and Haut [15]. Ether extracts were concentrated under nitrogen and then an aliquot was subjected to thin-layer chromatography using solvent systems I and II.

Aliquots of urine (2–3 ml) were acidified with 2 N HCl to pH 2, and extracted twice with ethyl acetate (6 ml). Recovery of ^{14}C into the organic phase exceeded 96 per cent. Aliquots of the organic phase were then placed in 25-ml Erlenmeyer flasks and evaporated. For enzymatic hydrolysis, 4 ml of 0.1 M sodium acetate buffer, pH 5, containing 100,000 E.U. of β-glucuronidase was added to the Erlenmeyer flasks and incubated for 24 hr at 37°. After 24 hr, 0.5 ml of 30 per cent $HClO_4$ was added, and the pH was adjusted to 6.5 with 1 M K_2CO_3. The solution was centrifuged for 10 min, and the supernatant portion was then acidified with 2 NHCl and extracted twice with ethyl acetate (6 ml). The organic phase was evaporated to near dryness and metabolites were separated using solvent systems I and II. Chemical treatment of samples was also done with 0.2 N HCl at 40° for 60 min and with 0.2 N NaOH at 37° for 20 hr. Final incubation mixtures were treated as described for β-glucuronidase.

Biliary metabolites were assayed at the fourth collection period when ^{14}C-levels were most concentrated. Aliquots were taken from the bile of both nontreated and phenobarbital-treated animals (40 mg/kg, b.i.d., for 4 days) and were treated in a fashion identical to that described for urine samples.

Plasma binding of ^{14}C. Plasma samples were collected from individual animals upon the termination of the 12 hr (acute) and 14-day (chronic) treatment studies. Duplicate plasma samples (1 ml) were placed in multicavity dialysis cells (Chemical Rubber Co., Cleveland, OH) and dialyzed with shaking against 1 ml of 0.16 M phosphate buffer, pH 7.4, for 24 hr at 37°. Each half-cell contained a small glass bead to aid in mixing. At the end of 24 hr, 0.3-ml samples were removed from each half-cell, placed in scintillation counting vials containing 1.5 ml Soluene-100, and heated to 50° for 30 min. Samples were cooled and radioactivity was measured after addition of 10 ml of Thrift-Solve.

Urinary and fecal radioactivity. Urinary and fecal elimination rates were studied in animals receiving [^{14}C]clofibrate (0.4 mmole/kg) either orally or i.p. Rates of elimination were also studied in a group of animals receiving 75 mg cholestyramine per day

orally for 4 days prior to and continuing 3 days after an i.p. injection of [^{14}C]clofibrate. Urine and feces were collected from animals every 12 hr for the first day and every 24 hr thereafter. The animals were housed in Delmar (Perkin-Elmer, Maywood, IL), all-glass metabolism cages, which permitted the separation of urine and fecal material in a collection chamber packed in dry ice. Carbon dioxide was trapped by aspirating all expired air through a column containing 100 ml of 2.5 N NaOH. Duplicate samples of urine (0.5 ml) were added directly to Thrift-Solve (15 ml) and counted. Fecal material was weighed, and an equal amount (g) of 1 per cent Tween 80 was added to the sample prior to homogenization in a glass mortar. Triplicate samples were weighed in Combusto-Cones containing a small amount of cellulose powder to aid the combustion process, and the amount of $^{14}CO_2$ present was determined as before. The possibility of $^{14}CO_2$ being cleaved from clofibrate was monitored by taking 1.0-ml samples from the column (2.5 N NaOH) at various times and dissolving them directly in 10 ml of Thrift-Solve.

Liver microsomal metabolism of clofibrate. Liver microsomes were isolated from nontreated and phenobarbital-treated rats according to the procedure described by Mellon *et al.* [16]. Treated rats received 80 mg/kg sodium phenobarbital, once daily, for 4 days while the control group received a requisite volume of saline. The extent of hepatic microsomal induction due to phenobarbital treatment was assessed by measurement of ethylmophine *N*-demethylase activity as described previously [16]. Reaction mixtures for clofibrate consisted of 5 mg of microsomal protein, 30 μmoles of 0.1 M HEPES* buffer, pH 7.4, and an NADPH-generating system (containing 15 μmoles $MgCl_2$, 1 mg $NADP^+$, 5.4 mg glucose-6-phosphate, and 2 units of glucose-6-phosphate dehydrogenase) in a final volume of 3.0 ml. Reactions were initiated with 10 μmoles [^{14}C]clofibrate (6.22 μCi), delivered in 25 μl ethanol, and terminated after 10, 30 and 60 min by adjusting the pH to 2 with 2 N HCl. ^{14}C was extracted twice using ethyl acetate (6 ml); the ethyl acetate extracts were concentrated under nitrogen, and the ^{14}C-metabolites were separated on silica gel by t.l.c. using solvent systems I and II.

Isolation and characterization of urinary and hepatic microsomal metabolites of clofibrate. The condensed ethyl acetate extracts of ^{14}C from urine and hepatic microsomal preparations were streaked across 20 × 20 cm silica gel t.l.c. plates (Anasil, silica gel G, 250 μm), air dried, and metabolites separated in solvent system I. The single radioactive band corresponding to the R_f of clofibric acid was removed, and the silica gel scrapings were eluted successively with 10 ml of ether methanol. The eluant was concentrated to dryness under nitrogen, and the ^{14}C-metabolite was crystallized in methanol and the precipitate isolated by centrifugation. A 90 MHz Bruker nuclear magnetic spectrometer (Bruker Instruments, F.R.G.) was used to characterize these metabolites. The n.m.r. spectrum of each isolated metabolite was identical to that of clofibric acid in the aromatic and methyl proton regions. Proton resonances were observed as a typical AA'BB' pattern in the range of 6.87–7.23 δ and a methyl proton resonance was observed at 1.58 δ, which were consistent with the intact 1,4-disubstituted ring and isobutyric acid side chain of clofibric acid respectively.

Biliary excretion of clofibrate. Rats were anesthetized with urethane, 1.2 g/kg, i.p., and bile ducts were cannulated with PE-10 tubing through an abdominal incision. Body temperatures were monitored with a rectal probe and maintained at 38° with a heating lamp. Clofibrate was injected (0.4 mmole/kg; 1.2 μCi) i.p., in propylene glycol and ethanol (9:1, v/v). The injection volume was 0.5 ml. Successive 15-min bile samples were taken for the first hour, at 30-min intervals for the second hour, and then at 45 min intervals until the last hour. Samples were collected directly in preweighed scintillation vials throughout the 6-hr time period and were dissolved in 10 ml of Thrift-Solve for measurement of radioactivity.

Liver cell fractionation. Subcellular fractions of liver were obtained from the 12-hr and 14-day [^{14}C]clofibrate-treated animals, using the differential centrifugation and discontinuous density gradient techniques outlined by Borowitz [17].

Livers were quickly excised, perfused with saline, blotted dry, and then homogenized in 4 vol. of ice-cold 0.32 M sucrose solution. Subcellular fractions of cell debris, mitochondria and microsomes were prepared by differential centrifugation of each liver homogenate (35 ml) at 480 *g* for 10 min, 10,000 *g* for 30 min, and 105,000 *g* for 75 min respectively. Cytosol was recovered as the 105,000 *g* supernatant fraction. Aliquots (1 ml) of the resuspended mitochondrial fraction (10 ml) were layered onto a discontinuous sucrose gradient and centrifuged at 120,000 *g* for 48 min. Fractions recovered were designated as F_1 (3.5 ml, 0.32 M sucrose), F_2 (2.5 ml, 1.25 M sucrose), F_3 (2.5 ml, 1.50 M sucrose), F_4 (2.5 ml, 1.75 M sucrose), and F_5 (2.5 ml, 2.0 M sucrose). Three gradient tubes were prepared for each liver such that combination of their respective fractions (F_1–F_5) yielded a total volume of 10 ml. Each of these fractions (F_1–F_5) was analyzed for cytochrome *c* reductase and catalase activities by the methods of Borowitz [17] and Luck [18], respectively. The protein content of each fraction was determined by the method of Lowry *et al.* [19] using crystalline bovine albumin as a standard. From each fraction, 1.5-ml aliquots were added to Combusto-Cones, and the amount of ^{14}C present was determined after sample combustion. Prior to each fractionation step, 1.5-ml aliquots were also assayed for ^{14}C to monitor recoveries. The total percent recovery of ^{14}C from all fractionation steps except for the sucrose gradient was 106 ± 9 (mean ± S.E.M.; N = 3). As an internal standard, 156 μg [^{14}C]clofibric acid (7593 dpm) was added to livers from nontreated animals prior to homogenization. These homogenates were fractionated as before, and the ^{14}C and protein contents were monitored in each fraction.

Tissue radioactivity. All tissue samples except those from adrenals, small intestine, and epididymal

* HEPES =4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

fat pads were homogenized in a 10-ml volume of distilled water with a Polytron model PT OD tissue homogenizer (Brinkmann Instruments, Westbury, NY). Intestinal tissue was homogenized in 20 ml of water after elimination of all fecal material by rinsing in water. Aliquots (1.5 ml) of the homogenates were placed in Combusto-Cones, allowed to dry, and subjected to combustion. Adrenal glands from each animal were combined, dried, and combusted directly, whereas epididymal fat pads were divided into 200-mg sections for combustion.

Carcass radioactivity. After removal of the various tissues, the residual carcass for each rat was weighed and homogenized in a Waring blender containing 300 ml of water. Six aliquots (1–2 ml) of the homogenate were placed in Combusto-Cones, air dried, and combusted.

Serum cholesterol and triglyceride assays. Serum cholesterol and triglyceride levels were measured by the method of Parekh and Jung [20] and Soloni [21] respectively.

Statistical analysis. Student's *t*-test was employed to make comparisons between means.

RESULTS

^{14}C-Absorption and plasma concentration. ^{14}C was observed in the plasma within 30 min after oral administration of clofibrate (0.4 mmole/kg) to rats (see insert in Fig. 2). The maximum plasma ^{14}C level was attained 90 min after administration, indicating that clofibrate was readily absorbed. At 6 hr, the plasma level had declined from a peak level of 40.9 μg/ml to 28.6 μg/ml. The half-life for the disappearance of ^{14}C from plasma was estimated to be 8.3 hr. In the group of rats treated with clofibrate (0.4 mmole/kg, b.i.d.) for 14 days (chronic), the plasma levels of ^{14}C declined significantly ($P < 0.05$) (by 27 per cent on day 7 and by 48 per cent on day 14) after chronic clofibrate administration.

^{14}C-Metabolites and plasma protein binding, in vivo. Drug metabolites in plasma were separated by t.l.c. using solvent system I; authentic standards of clofibric acid and clofibrate migrated to $R_f = 0.62$ and $R_f = 0.74$ respectively. In 6-hr plasma samples, two radioactive peaks appeared on t.l.c. plates after development. The major peak represented clofibric acid (96.7 per cent of the total ^{14}C); the remaining ^{14}C (3.2 per cent) was located at $R_f = 0.31$. The latter metabolite was determined to be the glucuronide conjugate of clofibric acid, for it had an R_f identical to that of the glucuronide characterized in urine

Table 1. Plasma protein binding of ^{14}C following acute or chronic clofibrate administration in the rat

Variable	Drug treatment*	
	Acute	Chronic
Microgram present† (μg/ml)	30.9 ± 2.1	16.2 ± 1.2‡
Microgram bound† (μg/ml)	19.2 ± 1.1	11.4 ± 0.6‡
Percent ^{14}C bound	62.1 ± 0.6	70.4 ± 3.4

* Rats were given 0.4 mmole/kg 1-[^{14}C]clofibrate as a single dose (acute treatment) or twice daily for 14 days (chronic treatment). Blood was drawn 12 hr after the last dose, and plasma drug binding was measured by equilibrium dialysis as described in Materials and Methods.

† Data are expressed as μg of clofibric acid equivalents per ml of plasma. Each value is mean ± S.E.; N = 3.

‡ Value is significantly different (at the $P<0.05$ level) from acute drug treatment.

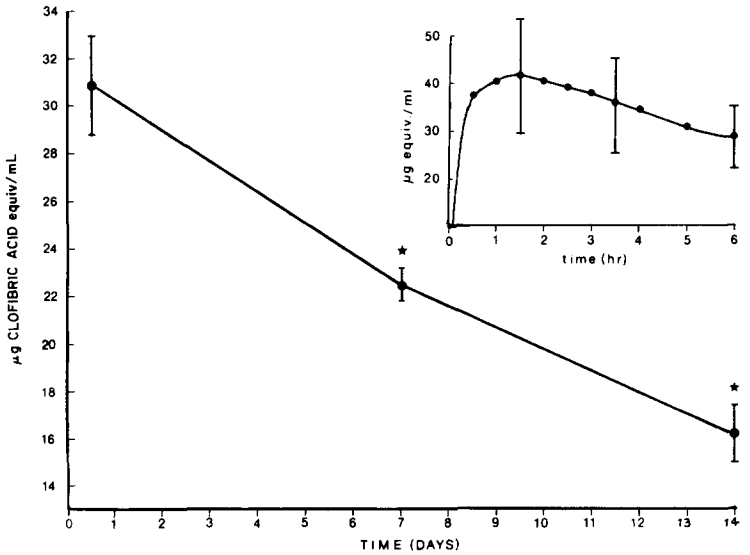

Fig. 2. Time course of mean plasma ^{14}C-levels in rats (N = 3) given clofibrate (0.4 mmole/kg) acutely (single dose) or chronically (b.i.d., for 14 days). Data are expressed as μg clofibric acid equivalents per ml of plasma and values are means ± S.D. An asterisk indicates a significant difference ($P < 0.05$) between mean plasma levels (7-day and 14-day vs initial 12-hr values). Insert: Plasma ^{14}C after oral administration of clofibrate (0.4 mmole/kg).

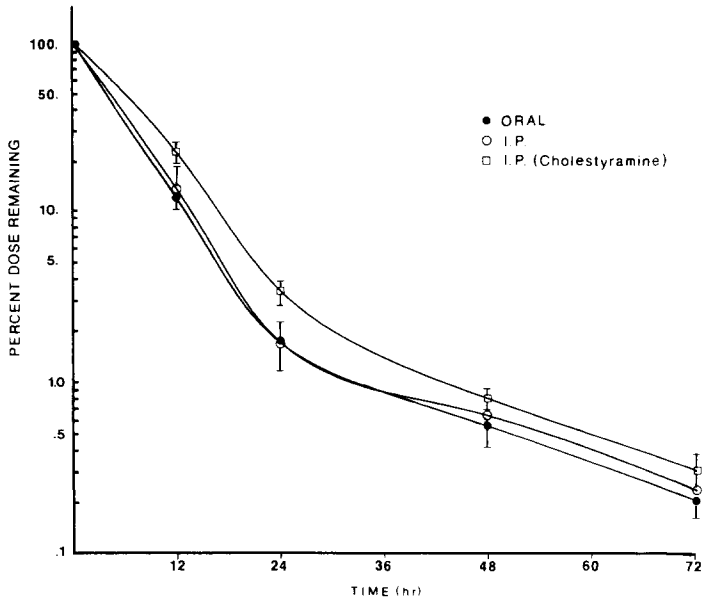

Fig. 3. Time course of ^{14}C-elimination in rats (N = 3) given a single oral or i.p. dose of clofibrate (0.4 mmole/kg); effect of cholestyramine pretreatment. Values are the mean percent of total ^{14}C remaining ± S.D. Key: (●—●) oral administration; (○—○) i.p. administration; and (□—□) i.p. administration to cholestyramine-treated rats.

samples (see below). In no case did a t.l.c. radiochromatogram of 6-hr extracts of plasma samples reveal any ^{14}C corresponding to the R_f of clofibrate.

Plasma samples were obtained from rats given [^{14}C]clofibrate (0.4 mmole/kg orally) as a single oral dose (acute) or twice daily for 14 days (chronic). The study was designed to determine not only the amounts of free and bound drug but also any quantitative difference that might occur during multiple dosing with clofibrate. As indicated in Table 1, total plasma drug concentrations and bound drug were significantly reduced by 52 per cent and 41 per cent in rats given a single dose or after chronic clofibrate treatment, respectively. The small difference between the two treatment groups, in the percentage of ^{14}C-drug bound to plasma proteins, is most likely related to the differences in the total plasma drug concentration.

^{14}C-Elimination of clofibrate. As presented in Fig. 3, the disappearance rate of ^{14}C was monitored for 72 hr in rats given a single dose of clofibrate (i.p. or orally) or in a group of rats pretreated with cholestyramine. It can be seen that the elimination rate of [^{14}C]clofibrate from the body was unaffected by the route of administration or by pretreatment with cholestyramine. The biological half-lives (mean ± S.D.; N = 3) derived from the ^{14}C-elimination curves were 4.1 ± 0.6 hr for the oral and i.p. routes and 4.9 ± 0.4 hr for the i.p. route treated with cholestyramine.

As shown in Table 2, > 96 per cent of an administered ^{14}C-dose was eliminated within 24 hr, and 90–96 per cent of the ^{14}C was excreted in the urine. Small amounts of the ^{14}C-doses were excreted in the feces; the least amount of ^{14}C excreted into feces was in the group receiving clofibrate orally (Table 2). In addition, very little residual carcass ^{14}C (< 0.3 per cent of the total dose) was found in the animals

Table 2. Effects of the routes of administration of cholestyramine on the urinary and fecal excretion of 1-[^{14}C]clofibrate in the rat

Route of administration	Per cent of total ^{14}C dose eliminated*			
	24 hr		72 hr	
	Urine	Fecal	Urine	Fecal
Oral	96.4 ± 1.6	1.9 ± 1.2	97.7 ± 1.2	2.1 ± 1.2
Intraperitoneal	92.8 ± 5.1	5.5 ± 5.1	94.0 ± 4.9	5.8 ± 5.0
Intraperitoneal cholestyramine†	90.1 ± 5.2	6.6 ± 5.8	92.7 ± 5.7	7.2 ± 5.7

* Rats were given clofibrate (0.4 mmole/kg) as a single dose, and urine or fecal samples were assayed for ^{14}C after 24 and 72 hr. Data are the means ± S.E. of N = 3 in each treatment group.

† Rats were given cholestyramine (75 mg, orally) once daily for 10 days. Clofibrate was given as a single dose (0.4 mmole/kg) on day 7.

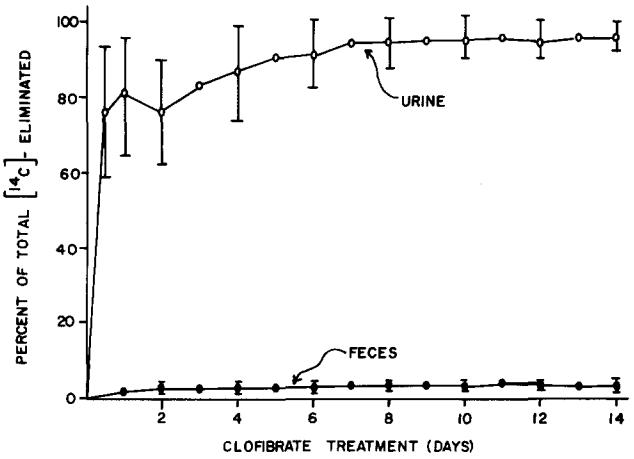

Fig. 4. Cumulative elimination of ^{14}C in rats (N = 3) during chronic clofibrate administration (0.4 mmole/kg, orally, b.i.d. for 14 days). Values are means ± S.D. Data are represented as the per cent of total ^{14}C eliminated into urine (○—○) or feces (●—●).

72 hr after they had received single doses of [^{14}C]-clofibrate either orally, i.p., or i.p. in combination with cholestyramine, and no ^{14}C was detected in the expired air of these animals within 72 hr.

Urinary and fecal excretion of ^{14}C were monitored every 24 hr in the group of animals treated chronically with [^{14}C]clofibrate (twice daily for 14 days) (Fig. 4). The total ^{14}C eliminated in the feces was small and relatively constant throughout the 14 days (3.7 ± 0.4 per cent, mean ± S.D., N = 3, at day 14). The major portion of ^{14}C (~95 per cent) was excreted in the urine. The percentage of total ^{14}C excreted in the urine within each 24-hr period increased only slightly throughout 14 days of clofibrate treatment (from a mean level of 80 per cent at day 1 to 95 per cent at day 14) (see Fig. 4).

Urinary metabolites. Thin-layer chromatographic separation of ^{14}C-metabolites extracted from urine (0–12 hr) produced two major radioactive peaks located at $R_f = 0.33$ and $R_f = 0.62$ (Table 3). None of the extracts of urine showed a radioactive peak with an R_f value of clofibrate ($R_f = 0.74$). The sum of these two peaks represented 99 per cent of the radioactivity on the plate and the remaining 1 per cent or less was located in two other radioactive peaks of $R_f = 0.42$ and $R_f = 0.48$. Extracts of urine collected during 12 hr were subjected to various chemical treatments (Table 3). Treatment of the extracts with β-glucuronidase prior to t.l.c. separation increased the ^{14}C-peak which corresponded to clofibric acid ($R_f = 0.62$). Treatment of the urine extracts with 0.2 N NaOH completely shifted the peak at $R_f = 0.33$ to the R_f of clofibric acid, whereas no change in t.l.c. behavior was noted after treatment with 0.2 N HCl. Moreover, a reduction in the smaller ^{14}C-peaks of R_f 0.42 and 0.48 was noted only after alkali treatment. The n.m.r. spectrum of the metabolite of $R_f = 0.62$ isolated after treatment with β-glucuronidase or 0.2 N NaOH was identical to that of clofibric acid.

Table 3. Metabolites of 1[^{14}C]clofibrate in 0–12 hr urine*

	Metabolites‡ (% of total ^{14}C) R_f				
Treatment	0.33	0.42	0.48	0.62	0.74
Control	88.5	0.5	0.3	10.7	0.0
β-Glucuronidase	10.8	0.3	0.4	88.5	0.0
Alkali (0.2 N NaOH)	0.0	0.2	0.0	99.8	0.0
Acid (0.2 N HCl)	87.7	0.5	0.5	11.3	0.0

* Urine samples (0–12 hr) were collected from rats given a single oral dose of clofibrate (0.4 mmole/kg) and ^{14}C-metabolites were isolated and chromatographed as described in Materials and Methods.

† Aliquots of ^{14}C-metabolites isolated from urine were subjected to treatment with β-glucuronidase (700 I.U. for 30 min at 37°), alkali (incubated for 20 hr at 37°), or acid (incubated for 10 min at 25°).

‡ Metabolites are: $R_f = 0.33$, glucuronide of clofibric acid; $R_f = 0.42$ and 0.48, undefined metabolites; $R_f = 0.62$, clofibric acid; and $R_f = 0.74$, clofibrate. Data are given as a per cent of the ^{14}C applied to the chromatogram.

The percentages of total ^{14}C represented by clofibric acid (free acid and glucuronide) in urine samples are presented in Table 4. The data are also representative of the urinary metabolites found in groups of rats given clofibrate alone (oral or i.p.) or in rats treated with cholestyramine. In rats receiving a single injection of clofibrate, there was no significant change in the profile of metabolites produced during the 24–48 hr period compared with that of the 0–12 hr period. Similarly, in rats receiving multiple injections (chronic treatment) no significant differences were found between the profiles of urinary metabolites after the 14-day treatment period, and the amounts of metabolites were essentially identical to those found during the initial 0–12 hr period. Additionally, only minute quantities of the ^{14}C-metabolites (< 0.5 per cent) of $R_f = 0.42$ and 0.48 were detected in the urine samples from rats given acute or chronic clofibrate treatment.

Hepatic microsomal metabolism of clofibrate. To examine the possibility that clofibrate was a substrate for Phase I metabolism, [^{14}C]clofibrate was incubated with hepatic microsomes prepared from control and phenobarbital-pretreated rats (Table 5). Microsomes prepared from phenobarbital-treated rats exhibited

Table 4. Urinary metabolites of [^{14}C]clofibrate after acute and chronic administration in rat*

	Per cent of total ^{14}C present			
	Acute		Chronic	
Metabolite†	0–12 hr	24–48 hr	0–12 hr	14 day
Glucuronide of clofibric acid	90.3 ± 1.8	87.7 ± 1.3	82.2 ± 7.5	75.7 ± 4.5
Clofibric acid	9.1 ± 1.7	12.3 ± 1.2	17.0 ± 7.4	23.5 ± 4.4

* Rats were given 0.4 mmole/kg 1-[^{14}C]clofibrate as a single dose (acute) or twice daily for 14 days (chronic), and urine samples were collected at the times indicated.

† ^{14}C-metabolites present in urine samples were extracted into ethyl acetate, chromatographed on silica gel, and quantitated as described in Materials and Methods. Each value is the mean per cent of total ^{14}C ± S.D. of N = 3.

a 2.7-fold increase in ethylmorphine *N*-demethylase activity when compared to control microsomes, whereas little difference was noted in the metabolism of clofibrate. Thin-layer chromatographic separation of the ^{14}C-metabolites of clofibrate in control and treated microsomes (1 hr) in solvent systems I and II yielded a single major ^{14}C-peak with an R_f value equal to that of clofibric acid. This metabolite was isolated and the n.m.r. spectrum of this metabolite was identical to that of clofibric acid. Most of the clofibrate was converted to clofibric within 15 min in both microsomal preparations, and less than 0.1 per cent remained unmetabolized after 1 hr.

Biliary metabolites and excretion. The excretion of [^{14}C]clofibrate in bile was studied in phenobarbital-treated and control rats (Fig. 5). The peak concentrations of ^{14}C in the bile of control and treated rats were reached 60 and 45 min, respectively, after [^{14}C]clofibrate administration. As shown in the insert of Fig. 5, the rates of ^{14}C-accumulation (expressed as cumulative percent dose excreted) in the control and phenobarbital-treated rats were indistinguishable. In both groups, about 48 per cent of the total ^{14}C administered was recovered within the 6 hr period. When the data were then plotted as percent of ^{14}C dose per ml bile collected, the specific activity of ^{14}C per ml bile was reduced by phenobarbital pretreatment versus control animals (Fig. 5). This observation can be related to the increased biliary flow rate observed in phenobarbital-treated rats. Initial flow rates for phenobarbital-treated and control animals were 1.36 ml/hr and 0.76 ml/hr, respectively; the biliary flow after 6 hr decreased to 90 per cent of the initial rate for the treated animals and to 80 per cent of the initial rate for the control animals.

^{14}C-Metabolites in the bile of phenobarbital or control rats were chromatographed using solvent system I and only two radioactive peaks of $R_f = 0.33$ and $R_f = 0.62$ were detected. These R_f values were identical to the glucuronide conjugate and the free acid form of clofibric acid respectively. In the bile samples obtained from phenobarbital and control rats, approximately 96 and 4 per cent of the total ^{14}C was associated with the glucuronide and free forms of clofibric acid respectively. These results demonstrate that pretreatment with phenobarbital did not modify the biliary excretion rate or metabolite profile and that the excretion of ^{14}C-metabolites of clofibrate was independent of the biliary flow rate.

Hypolipidemic activity of clofibrate and tissue distribution of clofibrate in rats. After 14 days of clo-

Table 5. Time course of the metabolism of ethylmorphine and 1-[^{14}C]clofibrate in rat hepatic microsomes: Effect of phenobarbital pretreatment*

	Micromoles present		
Substrate-product	15	30	60
Control microsomes			
Clofibric acid	8.86	9.57	9.90
1-[^{14}C]Clofibrate	1.09	0.43	0.09
Ethylmorphine (HCHO)	0.33		
Phenobarbital-treated microsomes			
Clofibric acid	8.99	9.86	9.92
1[^{14}C]Clofibrate	0.90	0.37	0.07
Ethylmorphine (HCHO)	0.91†		

* Rats were given phenobarbital (40 mg/kg) or saline, twice daily for 4 days. Microsomes were isolated 12 hr after the last dose. The amounts of clofibrate and ethylmorphine added were 10 μmoles (6.22 μCi) and 5 μmoles, respectively.

† Value is significantly different at the $P < 0.05$ level from the control microsomes.

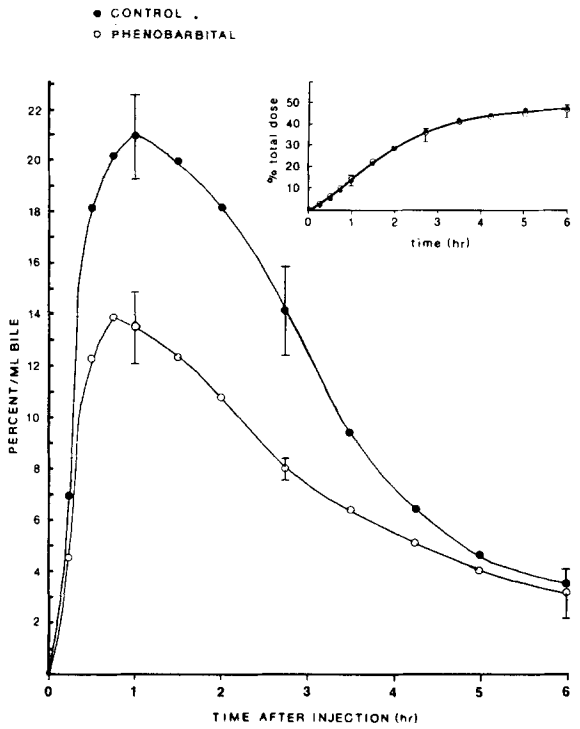

Fig. 5. Time course of biliary excretion of ^{14}C in bile-duct cannulated rats (N = 3) given a single dose of clofibrate (0.4 mmole/kg, i.p.); effect of phenobarbital pretreatment. Values are means ± S.E. Data are expressed as the per cent of total ^{14}C excreted/ml of bile or as the cumulative per cent of total ^{14}C dose excreted (figure insert) versus time in control (●—●) and phenobarbital-treated (○—○) rats.

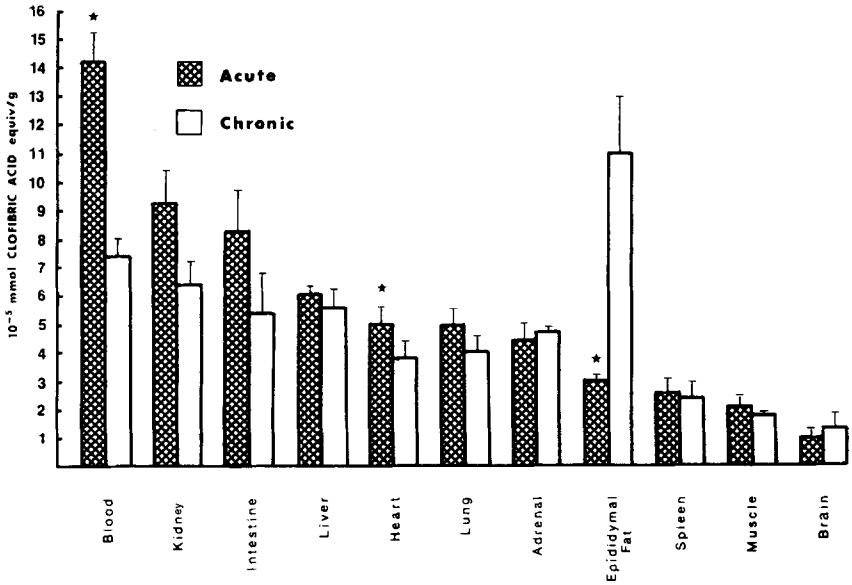

Fig. 6. Tissue levels of ^{14}C in rats (N = 3) given clofibrate (0.4 mmole/kg orally) acutely (single dose) or chronically (b.i.d. for 14 days). Rats were killed 12 hr after the last dose and the tissues were analyzed for radioactivity as described in Materials and Methods. Data are expressed as the mean mM clofibric acid equivalents per g of tissue ± S.E. as indicated by the vertical bars. An asterisk (*) indicates a significant difference ($P < 0.05$) between data of acute versus chronic clofibrate administration.

Table 6. Antilipemic effect of pretreatment with clofibrate on serum triglyceride and cholesterol levels in rat*

Variable measured†	Concentration (mg/100 ml ± S.D.) Control	Clofibrate
Serum cholesterol		
Day 0	72.6 ± 4.8	82.2 ± 11.8
Day 7	68.7 ± 4.2	40.9 ± 7.0‡,§
Day 14	71.8 ± 3.0	42.5 ± 7.2‡,§
Serum triglyceride		
Day 14	80.9 ± 19	40.9 ± 15.9‖

* Animals (N = 3) received clofibrate (0.4 mmole/kg), or vehicle, b.i.d., orally for 14 days.

† Serum cholesterol and triglycerides were assayed at the times indicated by procedures outlined in Materials and Methods.

‡ $P < 0.05$ (clofibrate group vs control group).

§ $P < 0.05$ (treatment group vs day 0 values).

‖ $P < 0.20$ (clofibrate vs control).

fibrate treatment (0.4 mmole/kg, b.i.d.), serum cholesterol and triglyceride levels were reduced to 58.4 and 50.6 per cent of the control values, respectively (Table 6). Serum cholesterol was reduced significantly at both the 7 and 14-day treatment periods.

Levels of ^{14}C were determined in several organs and tissues 12 hr after the last dose in those animals receiving a single dose of clofibrate (acute) and those receiving clofibrate twice daily for 14 days (chronic) (Fig. 6). Molar clofibric acid equivalents in tissues of acute and chronically treated animals ranged from 0.1 to 1.4×10^{-4}M. With the exception of blood, heart and epididymal fat, the radioactivity in the various tissues of acute and chronic treated rats did not differ statistically ($P > 0.05$). However, most tissues accumulated less ^{14}C after chronic clofibrate administration. The residual amounts in the carcasses of these animals also showed this relationship. After 14 days of treatment (28 doses) the ^{14}C-levels in epididymal fat were 3.7-fold greater than those in rats given a single injection and 1.5–6.1-fold greater than in the remaining tissues, including blood.

Liver subcellular fractions prepared from nondrug, acute, and chronically treated rats were analyzed for ^{14}C (Table 7). Values of ^{14}C-distribution in the subcellular fractions did not differ significantly ($P < 0.05$) between nondrug and acute treatment groups. Significant differences in ^{14}C-levels, however, did exist between fractions isolated from acutely and chronically treated groups. The ^{14}C-contents of fractions F_3, F_4 and F_5 were significantly higher in rats given chronic clofibrate treatment. Moreover, chronic clofibrate treatment also produced changes in the protein content of the fractions indicated as F_4 and cytosol (compare acute versus chronic treatment). Only the specific activity (dpm bound/mg protein) of F_3 in the chronic group was increased when compared to that fraction in the acute or nondrug treatment groups.

It was also found that 69 per cent of the succinate cytochrome *c* reductase activity was in fraction F_3, with 6, 3, 21 and 1 per cent found in fractions F_1, F_2, F_4 and F_5, respectively. Forty-nine per cent of the total catalase activity was in fraction F_4, the remainder of the activity being distributed in fractions F_1 (6 per cent), F_2 (13 per cent), F_3 (15 per cent) and F_5 (17 per cent).

DISCUSSION

Our combined data on the assay of ^{14}C in urine, plasma, bile and fecal samples indicate that orally or parenterally administered clofibrate is rapidly absorbed and almost completely eliminated within

Table 7. *In vivo* subcellular ^{14}C distribution and protein content in livers of acute and chronic clofibrate-treated rats*

	Percent of total liver ^{14}C†			Protein content (mg/g liver)	
	Nondrug‡	Acute	Chronic	Acute	Chronic
Cell debris	25.6 ± 5.9	26.4 ± 3.0	22.9 ± 17.4	79.3 ± 6,2	74.8 ± 6.9
Microsomes	15.9 ± 2.8	14.7 ± 2.2	13.3 ± 2.0	38.7 ± 2.6	27.9 ± 7.8
Cytosol	43.4 ± 2.8	42.4 ± 3.1	30.1 ± 8.4	85.3 ± 2.9	65.3 ± 8.8§
F_1	4.5 ± 3.6	4.4 ± 0.9	4.5 ± 0.4	5.1 ± 0.7	3.3 ± 2.2
F_2	4.0 ± 1.4	4.0 ± 0.7	5.9 ± 2.4	11.3 ± 0.9	10.9 ± 3.3
F_3	2.5 ± 0.2	3.5 ± 1.3	7.5 ± 1.3§,‖	29.0 ± 7.1	25.8 ± 7.3
F_4	3.0 ± 1.0	2.6 ± 0.8	8.2 ± 2.0§	7.3 ± 2.8	16.4 ± 2.9§
F_5	2.0 ± 1.4	2.2 ± 0.5	7.6 ± 4.4§	2.8 ± 2.8	2.9 ± 1.9

* Rats (N = 3) were given 1-[^{14}C]clofibrate (0.4 mmole/kg, orally) as a single dose (acute) or twice daily for 14 days (chronic) and killed 12 hr after the last dose. Subcellular fractions were isolated as described in Materials and Methods. Data are expressed as the mean ± S.D. of N = 3 in each treatment group.

† Mean ^{14}C contents of livers from nondrug, acute, and chronic treatment groups were 2.75, 2.5 and 2.88 nCi, respectively.

‡ Liver homogenates (N = 3) of nondrug-treated rats were spiked with 1-[^{14}C]clofibric acid and fractionated. No significant differences ($P > 0.05$) were noted between control and acute treatment groups.

§ Significant difference ($P < 0.05$) between acute and chronic treatment groups.

‖ Specific activity of this fraction (dpm/mg protein) was significantly ($P < 0.05$) increased, compared to the acute and nondrug treatment groups.

24 hr (Figs. 2 and 3). Peak plasma and bile levels of ^{14}C in rats were reached 1–2 hr after dosing which is slightly earlier than the range of 2–8 hr reported for clofibrate levels in human plasma [4, 22]. We also observed that clofibrate was eliminated primarily in the urine as clofibric acid (free acid and glucuronide); our estimated plasma biological half-life of ^{14}C in the rat (8.3 hr) is about one-third to one-half of that calculated for clofibrate in man (by Thorp [6] and others [4, 22, 23]) or in dog [7]. The higher elimination rate in the rat versus man is probably due to the differences in serum protein binding, as was recently noted by Cayen *et al.* [7]. Values for the binding of clofibrate, as clofibric acid, to bovine albumin and human plasma have been reported to be 96 per cent [7, 24] whereas in the present study only 62–70 per cent was bound to rat plasma proteins (Table 1) after acute or chronic administration. Since a greater fraction of the total plasma drug concentration in the rat is in the unbound form, relatively more drug is available for biotransformation and excretion, therefore giving a higher clearance rate in this species.

Very little ^{14}C (< 5 per cent of the dose) was found in the feces of rats given a single dose (acute) or after chronic clofibrate treatment (Fig. 4); thus, fecal excretion appears to play a minimal role in the elimination of clofibrate in this species. Whereas earlier investigations were unable to detect the presence of clofibrate or metabolites in bile [6, 25], Sedaghat and Ahrens [23] and Pertsemlidis *et al.* [26] recovered measurable quantities of the conjugated and free forms of clofibrate in human bile. In addition, the dog eliminates 25 per cent of a single oral clofibrate dose into the feces within 3 days [7]. In our bile cannulation studies, it was found that more than 48 per cent of a single dose of [^{14}C]clofibrate was eliminated into rat bile within 6 hr (Fig. 5), and the only biliary metabolites identified were the free and glucuronide forms of clofibric acid. The high and low concentrations of the glucuronide of clofibric acid in bile and plasma, respectively, coupled with the low recovery of ^{14}C in feces of rats suggest that in this species, as in man [23, 26], enterohepatic recirculation of clofibric acid would appear to play a major role in the maintenance of plasma drug levels. Accordingly, we expected coadministration of cholestyramine (an anionic resin) with clofibrate to modify the pharmacokinetics of this drug in rat, but no significant alterations in the biological half-life and fecal excretion rate of clofibrate metabolites were found (Fig. 3 and Table 2). Similarly, Sedaghat and Ahrens [23] also reported that the administration of cholestyramine to patients maintained on clofibrate therapy did not modify the pharmacokinetic profile.

Studies were also initiated to examine the influence of phenobarbital pretreatment on the biliary excretion of [^{14}C]clofibrate. Treatment of rats with phenobarbital did not increase the total output of biliary metabolites of clofibrate (Fig. 5) or change the glucuronide to clofibric acid ratio. This observation was surprising since phenobarbital is a known hepatic inducer of hepatic microsomal UDPGA-glucuronosyl transferase [27] and increases bile flow [28] in this species. The absence of an effect of phenobarbital is explicable if the rate-limiting step is biliary transport of the glucuronide or hepatic uptake of the drug, or if the liver is not the major site of the glucuronidation of clofibric acid. In a personal communication to Sedaghat *et al.* [29], Thorp noted that, when clofibrate was incubated with rat liver and kidney slices, the glucuronide metabolite was produced only by the kidney preparation. If this is the case, phenobarbital treatment would most likely have little, or no, effect on the quantity or the nature of biliary or urinary metabolites of clofibrate in rat *in vivo*. Inasmuch as large quantities of the ^{14}C in rat bile (~96 per cent) was identified as glucuronide, and very little glucuronide metabolite was present in plasma (2.3 per cent), it would appear that significant quantities of clofibrate may, in fact, be formed by liver in this species. In this regard, an increase in the biliary elimination of the glucuronide of clofibric acid after chronic administration could explain, in part, the gradual increase in the amounts of urinary metabolites (24-hr intervals) found during the first 10 days of chronic clofibrate treatment (Fig. 4). An increased biotransformation and/or elimination of clofibrate is also indirectly supported by the decreased plasma and tissue concentrations of ^{14}C found in animals after 14 days of clofibrate treatment.

In man, Houin *et al.* [4] reported the existence of other metabolites of clofibrate that were sensitive to acid treatment but not to treatment with β-glucuronidase. In dog, an alkali-labile, urinary metabolite of clofibrate has also been found. The chemical structure of this metabolite is unknown [7]. We were also able to detect one or two minor (< 1 per cent) urinary metabolites of clofibrate (Table 3). In an attempt to generate compounds that may correspond to these undefined urinary metabolites, the biotransformation of clofibrate was studied in hepatic microsomes obtained from control and phenobarbital-pretreated rats. Our *in vitro* studies of [^{14}C]clofibrate metabolism in hepatic microsomes indicate that clofibrate is converted to clofibric acid (Table 5) and does not undergo measurable conversion to other metabolites catalyzed by Phase I drug-metabolizing enzymes in liver microsomes. Therefore, the chemical nature of these minor urinary metabolites of clofibrate remains to be characterized. Identification of these metabolites is of interest due to the reported potential risk of malignant tumors in man [30] and in male rats [31] following long-term clofibrate treatment.

In our study, as reported by other investigators [7, 23, 32], we were unable to detect any unmetabolized clofibrate in the various biological samples. We were able to detect clofibric acid and only small amounts of the glucuronide (2.3 per cent of total ^{14}C) clofibric acid in rat plasma, an observation verified in dog and rat by Cayen *et al.* [7]. This was expected, for clofibrate is known to be rapidly hydrolyzed by serum and tissue esterases both *in vivo* and *in vitro* [6]. Clofibric acid has a pK_a of 4.46 and is also an excellent substrate for glucuronidation. An efficient and rapid urinary and biliary clearance of the glucuronide of clofibric acid was seen after administration of this drug in rat. It is evident, therefore, that the major metabolites of clofibrate in the rat are the free acid and glucuronide of clofibric acid,

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as originally suggested by Thorp [6] and verified by Cayen *et al.* [7].

Thorp [6] reported that the apparent volume of distribution of clofibric acid was equivalent to 10–15 per cent of the body weight, which suggested that the distribution of clofibrate, as of clofibric acid, was limited to plasma and extracellular fluids. On the basis of their pharmacokinetic data in man [3, 23], other investigators have also concluded that clofibrate does not enter or accumulate in most tissues the body. Cayen *et al.* [7] recently reported that clofibric acid was distributed to various tissues and was localized in the intracellular compartment of heart, kidney, fat and muscle in rat. By contrast, the accumulation of clofibric acid in the lungs and spleen was in the extracellular compartment [7]. Our whole tissue and subcellular distribution studies show that only a small amount of the total clofibrate dose enters the tissues studied and, in most cases, does not accumulate after several doses. The ^{14}C-content decreased in blood and heart and increased in fat after chronic clofibrate treatment (Fig. 6). The accumulation of clofibrate in fat may be related to the known antilipidemic action of this drug. The reduction in serum lipids may be due to the antilipolytic effect of clofibrate on FFA mobilization and to the decreased hepatic utilization of FFA for lipoprotein synthesis [33]. It has been found that high concentrations ($> 10^{-3}$ M) of clofibric acid significantly inhibit catecholamine- and ACTH-induced increases in cAMP and FFA mobilization in isolated rat adipocytes *in vitro* [13, 33, 34]. Chronic treatment with clofibrate would not be expected to obtund hormone-induced lipolysis, *in vivo*, since the tissue concentrations of ^{14}C (10^{-4} M) were about 10-fold lower than those reported necessary for the inhibition of lipolysis, *in vitro*. These findings do not rule out any indirect antilipolytic effect that might develop upon the accumulation of clofibrate in fat tissues during chronic administration. Greene *et al.* [35] reported a reduction in the activity of adenylate cyclase in adipose tissue after clofibrate administration, which may be responsible for the possible *in vivo* actions of this agent.

Investigators have attempted to correlate changes in hepatic mitochondrial [11, 12] or peroxisomal [31, 36–38] function with the antilipemic and/or tumorigenic effects of clofibrate; however, no studies have appeared on subcellular organelle distribution. In the present study, the percentage of total ^{14}C in the liver, after chronic clofibrate administration, was reduced in the nuclear and cytosolic fractions and was increased in the mitochondrial and peroxisomal fractions. In the latter fractions, only the ^{14}C specific activity of ^{14}C that was bound to the hepatic mitochondrial fraction was increased after chronic clofibrate administration (Table 7). At this time, the significance of ^{14}C-sequestration by liver mitochondria remains unclear. However, the antilipidemic effects of clofibrate have been associated with changes in specific activity of the side chain oxidation of cholesterol [11] and in the modification of energy production or utilization in this subcellular organelle [39–41].

Svoboda and Azarnoff [31] recently proposed that the tumorigenic properties of clofibrate and related hypolipidemic agents may be associated with the ability to produce peroxisomal proliferation. In contrast, Lazarow [38] has reported that clofibrate and structurally diverse hypolipidemic drugs increased the peroxisomal system of palmitoyl-CoA oxidation, and suggested that liver peroxisomes may play a role in lowering serum lipid concentrations during clofibrate treatment. The elevation of ^{14}C-levels in liver peroxisomes after chronic clofibrate treatment in rats (Table 7) is suggestive of a direct interaction of clofibrate or metabolites in this organelle. Irrespective of the nature and role of ^{14}C-binding of clofibrate in liver peroxisomes, however, it is of interest to note that the increased accumulation of ^{14}C in peroxisomes after chronic clofibrate administration paralleled the changes in protein content of this fraction (Table 7). Clearly, additional studies are required to evaluate the importance of this finding and its potential relationship to malignant transformations [31] or antilipidemic properties [38].

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