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THE IMMEDIATE AND LONG TERM EFFECTS OF CLOFIBRATE ON THE METABOLISM OF THE PERFUSED RAT LIVER

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Abstract—A study was made of the immediate effects of CPIB (chlorophenoxy-isobutyrate) and of the effects of clofibrate (ethyl-CPIB) pretreatment on the metabolism of the perfused liver. Both treatments caused an increased hepatic uptake of lactate and free fatty acids. Pretreatment with clofibrate resulted in a decrease in perfusate glucose, an increase in ketogenesis and a decreased output of very low density lipoprotein triacylglycerol. A more oxidized redox state of both the cytosol and the mitochondria was indicated by decreased ratios of perfusate [lactate]/[pyruvate] and [3-hydroxybutyrate]/[acetoacetate] respectively. Increased hepatic O_2 consumption was associated with the increased liver weight of rats treated with the drug for 1 week. The fate of free fatty acids was followed by infusing [1–^{14}C]oleate. The increased oxidation of oleate to both CO_2 and ketone bodies in livers from animals pretreated with clofibrate was accompanied by a corresponding decreased incorporation of ^{14}C into very low density lipoprotein triacylglycerol. Lipogenesis was depressed upon addition of CPIB to the perfusate, but was increased after pretreatment with clofibrate. No changes in cholesterol synthesis were detected. A hypothesis to account for the hypolipidaemic and other effects of clofibrate pretreatment is advanced. This is based on a primary enhancement of fatty acid oxidation accompanied by a reciprocal decrease in hepatic triacylglycerol secretion. It is suggested that increased peroxisomal oxidation of fatty acids may be a cause of the decreased redox potential. A consequent activation of pyruvate dehydrogenase would explain both the changes in carbohydrate metabolism and the increase in lipogenesis.

Clofibrate [atromid-S, ethyl-2-(4-chlorophenoxy)-2-methylpropionate] is used clinically as a hypolipidaemic drug, but numerous reports, some conflicting, ascribe to it multiple actions on lipid metabolism and a variety of other metabolic and histological effects. It appears to act both by decreasing the production of serum lipoproteins and by enhancing their removal from the plasma, but as yet, there is no unifying hypothesis to account for its many properties.

Once absorbed, the drug is transported in the blood as the anion, CPIB*, which is mainly protein bound [1]. It has been suggested that it acts by displacing other organic ligands, e.g. free fatty acids, acidic coenzymes, hormones, etc. from plasma albumin into tissues, where they could affect carbohydrate and lipid metabolism [1]. Direct effects of CPIB have been reported on lipid metabolism in isolated hepatocytes [2] and subcellular liver fractions [3], and on purified enzyme preparations [4]. However, some investigators have failed to find any direct effects *in vitro* but do find metabolic differences in tissues and tissue fractions from animals pretreated with clofibrate, indicating adaptive changes or actions arising from the metabolism of clofibrate.

The validity of the reported direct actions of the drug have been questioned due to the uncertainty over whether CPIB actually enters hepatocytes *in vivo*. However, our recent studies, utilizing the perfused liver, indicate a substantial hepatic capacity to remove and metabolize radioactive CPIB [5]. In the present investigation, preliminary studies were carried out to monitor the serum CPIB concentration in rats given a standard dose of clofibrate in the diet for 7 days and to determine the magnitude of the hypolipidaemic effect of this treatment. Using this information, the main study was carried out utilizing rat liver perfusions. The direct actions of CPIB, when it was present in the perfusate at the concentration found *in vivo*, were compared with the adaptive effects produced in the liver as a response to 24 hr and 7 days of consuming the standard dose of clofibrate in the diet.

In virtually all previous investigations of the action of clofibrate, only one or two metabolic parameters have been studied at any one time. Our present approach, utilizing the perfused liver, has allowed us to follow not only all the major pathways of hepatic lipid metabolism, but also some aspects of carbohydrate metabolism, in one physiological preparation. It has therefore been possible to study both interrelated and independent actions of the drug, simultaneously.

MATERIALS AND METHODS

Animals. Male Wistar rats, purchased from A. Tuck & Sons, Rayleigh, Essex, U.K., were kept under constant daylength (18 hr) and temperature (25°), and fed a pellet diet (Diet 86, E. Dixon, Ware, Herts, U.K.) for a minimum of 3 weeks prior to use. Rats used as liver donors for control perfusions continued on this diet until they attained 340–360 g, the weight chosen for all liver donors.

Clofibrate was dissolved in ether (2.5 g in 20 ml) and sprayed over 1 kg of the pellet diet, to give the standard dose of the drug (0.25%, w/w of the diet). Some rats,

* Abbreviation: CPIB, chlorophenoxy isobutyrate [2-(4-chlorophenoxy)-2-methylpropionate]

weighing 325–335 g, were transferred on to this diet for 1 week prior to perfusion. Others, weighing 340–360 g, were fed on it during the 24 hr preceding perfusion.

Whole animal experiments. Twelve rats (315–350 g) were used; six were fed on the clofibrate-treated diet and six were maintained on the stock diet (controls). During a period of 7 days, the food and water consumption, and weight gain of each rat were recorded. At the end of this period, each animal was anaesthetized (at 9.30–10.00 a.m.) with an i.p. injection of sodium pentobarbital (60 mg/kg of body weight, Abbott Laboratories Ltd., Queenborough, Kent, U.K.) dissolved in 0.9% NaCl. Blood was withdrawn from the abdominal aorta and the concentrations of serum FFA*, triacylglycerol, cholesterol and CPIB determined. A portion of each liver was taken for estimation of the glycogen content and the remainder was used for analysis of the triacylglycerol and cholesterol content.

Liver perfusions. Rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital. Livers were surgically prepared between 10.00 a.m. and 11.00 a.m. and perfused for 3 hr, the bile duct having been cannulated. The perfusate was 148 ml of whole defibrinated rat blood which had been dialysed for 24 hr against Krebs–Henseleit bicarbonate buffer [6], containing glucose (250 mg/100 ml), amino acids (50 mg/100 ml—Stuart Company, Pasadena, CA, U.S.A.) and half the specified concentration of Ca. The perfusion apparatus and techniques were essentially as described by Mayes and Felts [7], except that the liver was retained *in situ* and a silicone–rubber gas exchanger was used. The liver temperature was maintained at 37°, the pO_2 of the inflowing blood was stabilized at 12–13 kPa (90–100 mmHg) and its rate of flow was adjusted to 12 ml/min.

Additions to perfusate. 190.5 mg of oleic acid (Sigma Chemical Co. Ltd., Norbiton, London, U.K.), labelled with 1 μCi of [1-^{14}C]oleic acid (specific radioactivity 50 mCi/mmol) was dissolved in 1.2 ml of 1 M KOH and 1.8 ml of H_2O, and mixed at 40° with a solution of 0.3 g of crystalline bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.) in 10.0 ml of 0.9% NaCl. A rapid 3 min priming infusion of this albumin–FFA complex was introduced into the perfusate, followed by a slower constant infusion. This achieved and maintained a serum FFA concentration of approx 0.7 μmol/ml for the 3 hr perfusion period. 5 mCi of ^3H_2O in 1.0 ml of 0.9% NaCl was added, with rapid mixing, into the perfusate at 1.5 min after the start of the FFA infusion.

Sampling. Blood was withdrawn from the system at times 0 min, 60 min, 120 min and 180 min after the start of the infusion, and chilled in ice. The serum volume at these times was calculated from the perfusate volume and knowledge of the haematocrit determined with MSE microhaematocrit tubes. After the final blood sample had been taken, the liver was flushed with 40 ml of ice-cold 0.9% NaCl and approx 0.75 g was taken for estimation of the glycogen content. The remainder of the liver was used for lipid analysis.

Analytical techniques. The pO_2 of both the blood flowing into the liver via the portal vein cannula and the blood leaving the liver via the cannulated hepatic vein, was monitored continuously using O_2 electrodes (Radiometer, Copenhagen, Denmark) connected to a recording potentiometer (Speedomax W, Leeds and Northrop Ltd., Tyseley, Birmingham, U.K.). The electrodes were housed in thermostatic perspex holders through which blood was circulated.

Glucose in blood and glucose liberated from liver glycogen after hydrolysis [8], were measured with a glucose oxidase method [Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K.]. L(+)Lactate and pyruvate were determined with lactate dehydrogenase [9], and D(−)-3-hydroxybutyrate and acetoacetate with 3-hydroxybutyrate dehydrogenase [10].

Serum was prepared from blood samples by centrifugation at 4° for 25 min at 2500 r.p.m. Serum FFA was determined by a modified Dole procedure [11] and corrections were made for the interference due to the presence of CPIB. This was approximately 26.8% of the total fatty acid estimated. The serum CPIB content was assayed spectrophotometrically† at 226 nm, after acidification and extraction with 2:2:4 trimethylpentane:ethanol (95:5, v/v). Portions (1 ml) of serum were layered under 0.9% NaCl (d = 1.006) in cellulose nitrate tubes (Beckman Ltd., Fife, Scotland, U.K.) and centrifuged at 30,000 r.p.m. for 18 hr at 4° (MSE Superspeed 65—rotor SP2582). VLD lipoproteins‡ (d < 1.006) in the supernatants were separated from d > 1.006 lipoproteins, in the infranatants, by a tube slicer (Beckman Ltd.). The lipids in each fraction were harvested with 3 washings of minimal volumes of 0.9% NaCl and extracted with 20 volumes of chloroform:methanol (2:1, v/v). The sample of liver for lipid analysis was homogenised in a Waring Blendor with methanol (50 ml). Chloroform (100 ml) was added and the mixture allowed to stand overnight at room temperature and then filtered through Whatman No. 1 paper. All chloroform:methanol extracts were shaken with two fifths of their volume of 0.03 M HCl and the chloroform layer taken for lipid analysis.

Triacylglycerol in serum and liver was determined by the method of Carlson [12] and cholesterol by the method of Rudel *et al.* [13].

Determination of radioactivity. The lipid classes in the chloroform extracts were separated by t.l.c. using silica gel G (E. Merck AG; obtained from Anderman & Co., London, U.K.), and development in a hexane:ether:glacial acetic acid mixture (80:19:1, by vol). The position of the lipids was located with I_2 vapour. After the I_2 had evaporated, each band of silica gel containing lipid was transferred to a counting vial except for the phospholipids which underwent prior extraction with ethanol:chloroform:H_2O:acetic acid (100:30:20:2, by vol) before transference to counting vials.

Fatty acid and cholesterol synthesis were measured by following the incorporation of ^3H from ^3H_2O into hepatic and perfusate total fatty acid (esterified + FFA) and total cholesterol (free cholesterol + ester cholesterol) according to Windmueller and Spaeth [14]. The specific radioactivity of ^3H_2O was calculated from the H_2O content of 1.0 ml samples of perfusate and from

* Abbreviation: FFA, non-esterified long chain fatty acids.

† J. M. Thorp, personal communication.

‡ Abbreviation: VLD lipoproteins, very low density lipoproteins (d < 1.006).

the radioactivity present in 0.1 ml portions of deproteinized perfusate. Samples were saponified with ethanolic KOH and extracted with light petroleum (b.p. 40–60°) to remove the cholesterol. The residue was acidified and the fatty acids extracted into light petroleum [15]. The two extracts were evaporated in counting vials and the radioactivity measured after addition of scintillant.

Respiratory gases containing $^{14}CO_2$ were passed through 2 M NaOH, and the CO_2 absorbed was displaced by acid into hyamine hydroxide (Packard Instrument Ltd., Reading, U.K.) and counted [8]. ^{14}C in plasma bicarbonate was displaced into hyamine hydroxide in a similar manner. ^{14}C in carbon 1 of acetoacetate and 3-hydroxybutyrate was determined by decarboxylation and trapping of $^{14}CO_2$ in hyamine hydroxide using a modification of the procedure of Mayes and Felts [16]. ^{14}C present in carbon 3 of ketone bodies could not be determined directly because of the presence of ^3H_2O. It was estimated, using the linear relationship between the rate of ketone body production and the distribution of ^{14}C between carbon 1 and carbon 3 *.

The scintillant used for counting radioactivity comprised 12.0 ml of toluene containing 2,5-diphenyloxazole (0.5%) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl) benzene (0.025%). The volume in each vial was increased to 18.0 ml by the addition of toluene to the lipid samples and ethanol to all other samples. Radioactivity was counted in a Tri Carb liquid scintillation spectrometer (Model 3003, Packard Instrument Ltd). Quenching was estimated by the external standard method, and all radioactivity was corrected for efficiency by a computer programme method [17].

Reagents. All chemicals and solvents were of analytical grade and were obtained from British Drug Houses Limited, Poole, Dorset, U.K., May & Baker Limited, Dagenham, Essex, U.K. or Hopkins & Williams, Chadwell Heath, Essex, U.K. Enzymes were supplied by the Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. and radiochemicals by The Radiochemical Centre, Amersham, Bucks, U.K. Clofibrate and sodium CPIB were generously provided by Mr. J. M. Thorp of Imperial Chemical Industries Limited, Macclesfield, Cheshire.

Experimental design. Five groups of perfusions were carried out in which the individual perfusions were allocated evenly between the groups with respect to time. The control group (Group I) consisted of normal liver donors and perfusate. In Group II, sodium-CPIB was dissolved in 0.9% NaCl and added to the perfusate to give a concentration of 0.75 mM, the concentration found in the serum of rats fed on the standard dose of the drug [18]. In Group III, livers from rats fed 24 hr on the treated diet were perfused, but without addition of the drug to the perfusate. Livers from rats fed 1 week on the treated diet were perfused both without (Group IV) and with (Group V) CPIB present in the perfusate. All results are reported as the mean ± S.E.M. of replicate experiments. The significance of differences between means was ascertained by Student's '*t*' test. All results are corrected for withdrawal of samples during perfusion.

RESULTS

A summary of the effects of administering clofibrate in the diet of rats for 7 days is shown in Table 1. There was a noticeable hypolipidaemic effect. The plasma concentrations of both triacylglycerol and cholesterol were decreased as compared with the concentrations in corresponding controls. Accompanying the pronounced hepatomegaly, the liver content of both triacylglycerol and cholesterol was increased. There were tendencies towards a decrease in serum FFA concentration and a retardation in growth rate. No detectable change in liver glycogen concentration occurred. The serum CPIB concentration was 0.49 ± 0.01 mM in clofibrate treated animals.

* P. A. Mayes and J. M. Felts, unpublished observations.

Table 1. Summary of the effects of administering clofibrate in the diet of rats for 7 days

Parameter	Control value	Clofibrate treated	P
Serum CPIB concentration (μmol/ml)	—	0.49 ± 0.01	
Increase in body weight (g)	27.0 ± 2.3	20.8 ± 2.4	0.05–0.1
Liver weight (g)	12.9 ± 0.1	17.9 ± 0.4	< 0.001
Liver glycogen (% of wet wt)	2.28 ± 0.33	1.86 ± 0.22	> 0.1
Serum FFA (μmol/ml)	0.49 ± 0.01	0.32 ± 0.05	0.05–0.1
Serum VLD-lipoprotein triacylglycerol fatty acid (μmol/ml)	4.19 ± 0.37	2.45 ± 0.07	0.001–0.01
Liver triacylglycerol content (μmol)	142.6 ± 10.0	181.9 ± 12.2	0.01–0.05
Liver triacylglycerol concentration (μmol/g)	11.1 ± 0.9	10.2 ± 0.8	> 0.1
Serum VLD-lipoprotein cholesterol (μmol/ml)	0.25 ± 0.03	0.15 ± 0.04	> 0.1
Serum d > 1.006-lipoprotein cholesterol (μmol/ml)	1.35 ± 0.15	0.82 ± 0.15	0.01–0.05
Total serum cholesterol (μmol/ml)	1.60 ± 0.16	0.97 ± 0.18	0.01–0.05
Liver cholesterol content (μmol)	63.8 ± 3.2	73.8 ± 3.5	0.05–0.1
Liver cholesterol concentration (μmol/g)	4.95 ± 0.21	4.14 ± 0.23	0.01–0.05

Six male Wistar rats (315–350 g) were fed on the stock diet containing 0.25% clofibrate for 1 week, and six controls were fed on the stock diet only. All rats were caged singly, and had free access to drinking water. After 7 days, each animal was anaesthetized with an intraperitoneal injection of sodium pentobarbital and samples of blood and liver were taken and analysed by the methods described in the text. The results are expressed as the mean ± S.E.M. and differences were tested for significance using Student's '*t*' test. There were no significant differences in food or water consumption between the two groups.

Table 2. Immediate and long term effects of clofibrate on the metabolism of the perfused rat liver during FFA (^{14}C-oleate) infusion—Summary of results

Parameter	Group II—CPIB in perfusate (5)	Group III—Clofibrate in the diet for 24 hr (5)	Group IV—Clofibrate in the diet for 1 week (6)	Group V—Clofibrate in the diet for 1 week plus CPIB in perfusate (5)
Liver weight-increase	N.S.	N.S.	***	***
O_2 consumption per liver-increase	N.S.	N.S.	**	*
O_2 consumption per g of liver-decrease	N.S.	N.S.	*	N.S.
Bile production	N.S.	N.S.	N.S.	N.S.
Perfusate blood [glucose]-decrease	N.S.	**	†	*
Liver [glycogen]	N.S.	N.S.	N.S.	N.S.
Perfusate blood [pyruvate]	N.S.	N.S.	N.S.	N.S.
Perfusate blood [lactate]-decrease	*	**	***	***
Perfusate blood [lactate]/[pyruvate]-decrease	N.S.	N.S.	***	**
Perfusate blood [acetoacetate]-increase	N.S.	†	***	***
Perfusate blood [3-hydroxybutyrate]-increase	N.S.	*	†	*
Perfusate blood [3-hydroxybutyrate]/[acetoacetate]-decrease	N.S.	N.S.	*	*
Perfusate serum [FFA]-decrease	***	*	***	***
Perfusate VLD-lipoprotein cholesterol	N.S.	N.S.	N.S.	N.S.
Perfusate serum d > 1.006-lipoprotein cholesterol	N.S.	N.S.	N.S.	N.S.
Liver [cholesterol] or content	N.S.	N.S.	N.S.	N.S.
VLD-lipoprotein triacylglycerol production-decrease	N.S.	*	***	**
Total liver triacylglycerol-increase	N.S.	*	**	†
^{14}C oxidation to CO_2 + ketone bodies-increase	N.S.	**	**	**
^{14}C oxidation to CO_2-increase	N.S.	**	**	†
^{14}C oxidation to ketone bodies-increase	N.S.	†	†	*
Total ^{14}C esterification-decrease	N.S.	**	**	N.S.
^{14}C incorporation into liver lipids	N.S.	N.S.	N.S.	N.S.
^{14}C incorporation into VLD lipoproteins-decrease	*	*	***	*
^{3}H incorporation into fatty acids	↓*	N.S.	↑*	↑*
^{3}H incorporation into cholesterol	N.S.	N.S.	N.S.	N.S.

Livers were perfused with whole defibrinated rat blood for 3 hr in the presence of $^{3}H_2O$, under the conditions described in the text. The number of experiments in each group is given in parentheses. Significance of the effects was tested against the control Group I by Student's '*t*' test, and is indicated as follows: N.S., $P > 0.1$; †, $0.05 < P < 0.1$; *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$; ↑, increased; ↓, decreased

The major effects of immediate, short and long term clofibrate treatment on the metabolism of the perfused rat liver are summarized in Table 2.

Liver weight. Clofibrate feeding for 1 week (Groups IV and V) increased the mean liver weight from 11.3 ± 0.2 g to 14.7 ± 0.4 g. However, clofibrate feeding for 24 hr only gave a value of 11.6 ± 0.6 which was not significantly different from the control Group I.

Oxygen consumption. The total O_2 consumption of livers that had not increased in weight as a result of clofibrate treatment (Groups II and III) was similar to that of livers from control rats (Table 3). Total O_2 consumption for the heavier clofibrate pretreated livers in Group IV and Group V was significantly greater than in controls (Group I), but when allowance was made for differences in liver weight, the rate of O_2 consumption per g of tissue was slightly less than in controls. The mean pO_2 of the effluent blood in the hepatic vein cannula was 32.9 mm Hg for the larger livers in Groups IV and V compared with a control value of 38.7, indicating increased O_2 extraction. When the rate of flow of the perfusate in some heavier (clofibrate-pretreated) livers (Group IVa) was increased so that the pO_2 of the effluent blood was at the control value, the total O_2 consumption was much increased, but the rate per g became similar to the value found in controls.

Bile. The mean bile production over the 3 hr experimental period was 2.4 ± 0.2 ml in control Group I, and this did not differ significantly from that of the four experimental groups (Fig. 1). Initially, bile production was in excess of 1 ml per hr but this rate became less towards the end of the 3 hr period.

Carbohydrate metabolism

Perfusate glucose and liver glycogen. In control (Group I) perfusions, the initial perfusate glucose concentration of 151.6 ± 9.5 mg/100 ml was maintained relatively constant, reaching 194.4 ± 20.3 at the end of the 3 hr period (Fig. 2). In the four experimental groups, the initial glucose concentration did not differ significantly from the control value. However, in all the groups of perfusions of livers derived from rats fed on clofibrate (Groups III–V), the concentrations recorded at 120 and 180 min were lower than at the corresponding times in the controls. This effect was significant in Groups III and V.

Table 3. Immediate and long term effects of clofibrate on the oxygen consumption of the perfused rat liver

		μmol O_2/liver per min	μmol O_2/g of liver per min
Group I—	Control (8)	58.7 ± 2.1	5.2 ± 0.2
Group II—	CPIB in perfusate (6)	58.9 ± 3.8	5.1 ± 0.5
Group III—	Clofibrate in the diet for 24 hr (5)	61.8 ± 3.8	5.4 ± 0.3
Group IV—	Clofibrate in the diet for 1 week (6)	69.4 ± 2.7**	4.8 ± 0.1*
Group V—	Clofibrate in the diet for 1 week plus CPIB in perfusate (7)	67.4 ± 2.1*	4.8 ± 0.3
Group IVa—	Clofibrate in the diet for 1 week with increased perfusate flow rate (3)	92.0 ± 9.9**	5.6 ± 0.7

Livers were perfused for 3 hr under the conditions described in the text. FFA (oleate) was infused continuously. The pO_2 of both the blood entering the liver and the blood leaving the liver was monitored (see text), the corresponding O_2 content of the blood was found from the O_2-haemoglobin dissociation curve for the rat and the O_2 consumption of the liver was calculated. In group IVa the rate of flow of perfusate was increased until the pO_2 of the blood leaving the liver attained the control value, at which it was maintained throughout the perfusion. Results are expressed as the mean ± S.E.M., and the number of perfusions in each group is given in parentheses. Significance of the means was tested against the control Group I by Student's '*t*' test. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$.

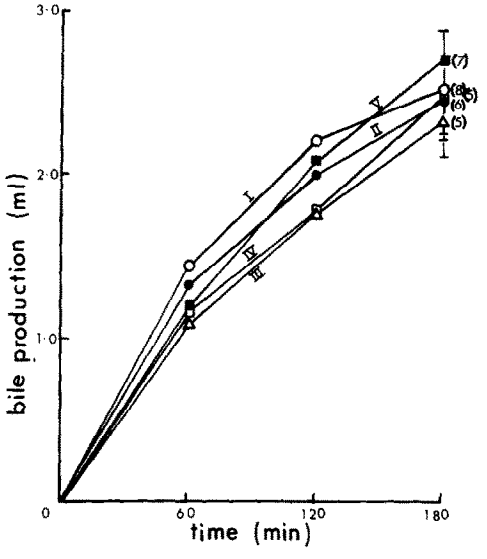

Fig. 1. Bile production during 3-hr liver perfusions: ○, control (Group I); ●, with CPIB added to the perfusate (Group II); △, pretreated for 24 hr with clofibrate in the diet (Group III); □, pretreated for 1 week with clofibrate in the diet (Group IV); ■, pretreated for 1 week with clofibrate in the diet and with CPIB added to the perfusate (Group V). Mean values are shown and the bars indicate the S.E.M., the number of perfusions in each group being given in parentheses.

The mean value for the liver glycogen concentration in Group I, at the termination of the perfusion, was 1.1 ± 0.2% of the wet weight, and did not differ significantly from the value found in the four experimental groups.

Pyruvate and lactate. In all experimental groups the perfusate pyruvate concentration was within the control range, which increased from an initial 0.4 ± 0.0 μmol/ml to 0.8 ± 0.1 at the end of the 3 hr period. In control perfusions, the perfusate lactate concentration increased from approximately 4 μmol/ml to 6 μmol/ml (Fig. 3). However, the initial (zero time) lactate concentration was significantly decreased in all perfusions of livers that had been pretreated with clofibrate in the diet (Groups III–V), and this decrease was evident throughout the perfusions. By the end of the 3 hr period, the perfusate lactate concentration of Group II, where the sole experimental change had been the addition of CPIB to the perfusate, was also significantly lower than that of controls. This decrease in lactate concentration was not sufficient to significantly alter the perfusate [lactate]/[pyruvate] ratios in Group II (Fig. 4). In Group III, although the initial ratio was significantly less than in controls ($P < 0.01$), the ratio at 60, 120 and 180 min was not. In the perfusions of livers from animals that had been on the clofibrate-treated diet for 1 week (Groups IV and V), the [lactate]/[pyruvate] ratio was significantly less initially and throughout the 3 hr period.

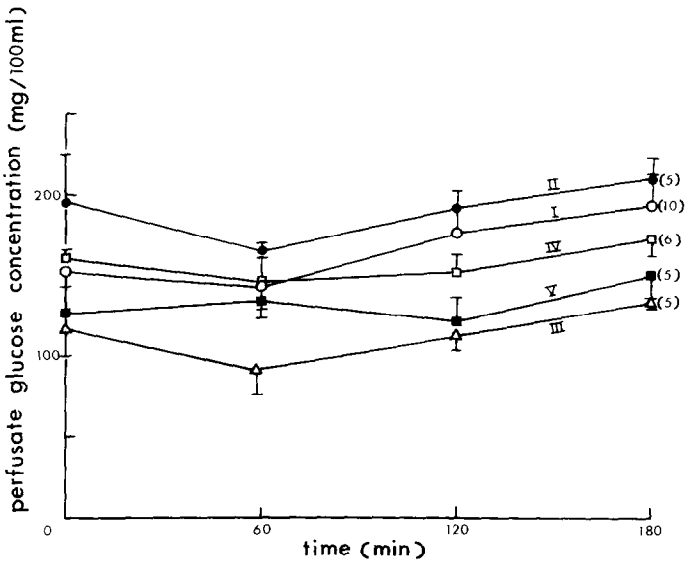

Fig. 2. Perfusate glucose concentrations during 3-hr liver perfusions: ○, control (Group I); ●, with CPIB added to the perfusate (Group II); △, pretreated for 24 hr with clofibrate in the diet (Group III); □, pretreated for 1 week with clofibrate in the diet (Group IV); ■, pretreated for 1 week with clofibrate in the diet and with CPIB added to the perfusate (Group V). Mean values are shown, and the bars indicate the S.E.M., the numbers of perfusions in each group being given in parentheses.

Lipid metabolism

Acetoacetate and 3-hydroxybutyrate. The terminal perfusate acetoacetate concentration in control perfusions (Group I) was 0.6 ± 0.1 μmol/ml (Table 4). Similar values were obtained in Group II. In perfusions of livers from animals given clofibrate for 24 hr (Group III), the initial acetoacetate concentration was significantly greater than in controls ($P < 0.05$): however, the values during the remainder of the experiment were not. Group IV and V showed an approximate doubling of the acetoacetate concentration throughout the perfusions when compared with controls. At the end of the 3 hr period, the perfusate 3-hydroxybutyrate concentration in Groups III and V exceeded that in Group I and the value in Group IV showed a similar trend. When CPIB was added to the perfusate of untreated livers (Group II) the 3-hydroxybutyrate concentration did not differ from the control value. Expressing these results as total production of ketone bodies (acetoacetate + 3-hydroxybutyrate), showed that ketogenesis in Groups III–V was significantly greater than in the control group (Table 4).

The terminal [3-hydroxybutyrate]/[acetoacetate] ratio in the perfusate was 1.8 in control perfusions (Table 4) and similar values were found in Groups II and III.

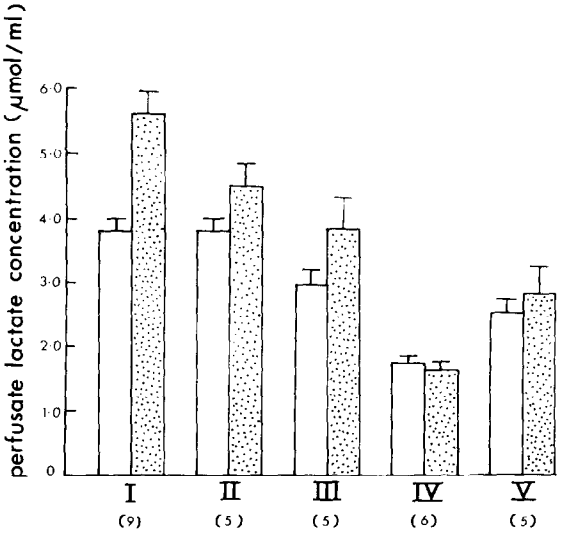

Fig. 3. Initial (□) and final (▨) concentrations of lactate in the perfusate for 3-hr liver perfusions. I, control; II, with CPIB added to the perfusate; III, pretreated for 24 hr with clofibrate in the diet; IV, pretreated for 1 week with clofibrate in the diet; V, pretreated for 1 week with clofibrate in the diet and with CPIB added to the perfusate. Mean values are shown and bars indicate the S.E.M., the numbers of perfusions in each group being given in parentheses.

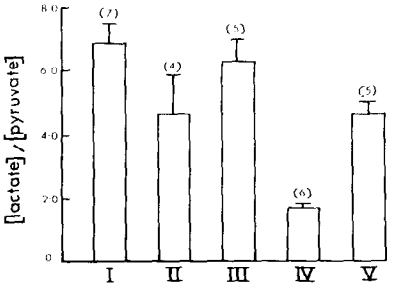

Fig. 4. Ratio of perfusate [lactate]/[pyruvate] after 3 hr of liver perfusion. I, control; II, with CPIB added to the perfusate; III, pretreated for 24 hr with clofibrate in the diet; IV, pretreated for 1 week with clofibrate in the diet; V, pretreated for 1 week with clofibrate in the diet and with CPIB added to the perfusate. Mean values are shown and bars indicate the S.E.M., the number of perfusions in each group being given in parentheses.

Table 4. Immediate and long term effects of clofibrate on perfusate ketone body concentration of perfused livers

		Perfusate acetoacetate (μmol/ml)	Perfusate 3-hydroxybutyrate (μmol/ml)	Total ketone body production (μmol)	Perfusate [3-hydroxybutyrate]/[acetoacetate] ratio
Group I—	Control (9)	0.6 ± 0.1	1.0 ± 0.1	203.2 ± 17.0	1.8 ± 0.2
Group II—	CPIB in perfusate (5)	0.5 ± 0.1†	0.9 ± 0.1	157.2 ± 15.3†	1.9 ± 0.1
Group III—	Clofibrate in the diet for 24 hr (5)	0.9 ± 0.2†	1.6 ± 0.2*	310.4 ± 10.9*	2.3 ± 1.0
Group IV—	Clofibrate in the diet for 1 week (6)	1.1 ± 0.1***	1.3 ± 0.1†	280.2 ± 9.1**	1.3 ± 0.1†
Group V—	Clofibrate in the diet for 1 week plus CPIB in perfusate (5)	1.3 ± 0.1***	1.6 ± 0.2*	376.5 ± 46.6**	1.2 ± 0.1*

Livers were perfused under the conditions described in the text and FFA (oleate) was infused continuously. Results are expressed as the mean ± S.E.M., after 3 hr of perfusion, and the number of experiments in each group is given in parentheses. Significance of the effects was tested against the control Group I by '*t*' test. †, $0.05 < P < 0.1$; *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$.

In the perfusions of livers from rats treated with clofibrate for 1 week (Groups IV and V) the ratio was significantly less at 60 min ($P < 0.05$) and in Group V it was also significantly less at 180 min ($P < 0.05$). In these two groups a decreased ratio was found at all other time intervals, including 0 min, which approached statistical significance ($0.1 > P > 0.05$).

Free fatty acids. In the control Group I (Fig. 5), the initial perfusate serum FFA concentration was 0.4 μmol/ml. The infusion of the albumin–oleate complex caused this to rise rapidly and stabilize at approximately 0.7 μmol/ml. In the perfusions of Groups III–V, whose liver donors had been fed clofibrate, the zero time perfusate serum FFA concentration was lower ($P < 0.01$), than in controls and this difference was maintained during the perfusion. The serum FFA concentrations in the perfusions from Group II were also significantly lower after 1 hr had elapsed from the addition of CPIB ($P < 0.05$). Thus in all four experimental groups, a significantly lower concentration of perfusate serum FFA was maintained following either previous or actual contact of the liver with the drug.

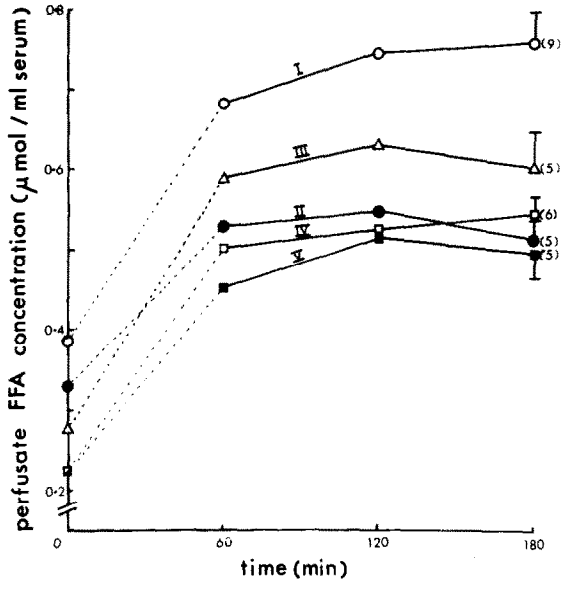

Fig. 5. Concentration of serum FFA during 3-hr liver perfusions: ○, control (Group I); ●, with CPIB added to the perfusate (Group II); △, pretreated for 24 hr with clofibrate in the diet (Group III); □, pretreated for 1 week with clofibrate in the diet (Group IV); ■, pretreated for 1 week with clofibrate in the diet and with CPIB added to the perfusate (Group V). The livers were infused continuously with oleate complexed to serum albumin. Mean values are shown and the bars indicate the S.E.M., the number of observations being given in parentheses.

Cholesterol. The cholesterol concentration in the $d < 1.006$ lipoprotein fraction of perfusate serum in the control group increased from an initial value of 0.43 ± 0.04 μmol/ml to 0.50 ± 0.04 at the end of 3 hr. The cholesterol concentration in the $d > 1.006$ lipoprotein fraction remained constant at a mean value of 1.39 ± 0.06 μmol/ml throughout and the total liver content of cholesterol at the conclusion of the experiment was 67.5 ± 5.2 μmol (5.99 ± 0.45 μmol per g of liver). None of the experimental groups differed significantly from the control group with respect to these parameters.

Lipoprotein triacylglycerol secretion. The output of triacylglycerol in VLD lipoproteins (Fig. 6) was virtually linear throughout the experimental period for all groups of perfusions. Although the mean output was less, livers in Group II showed no significant difference in this respect from those in the control Group I, which secreted a total of 338.2 ± 29.0 μmol of triacylglycerol fatty acid during the 3 hr period. However, the total secretion by livers from animals fed clofibrate for 24 hr (Group III) or for a week (Groups IV and V) was significantly less than that of controls ($P < 0.02$). When the rate of secretion was expressed as μmol of triacylglycerol fatty acid/h per g of liver, the mean rate for Group 1 was 10.0 ± 0.09. The rates were significantly less in perfusions of livers from animals fed on clofibrate, namely, 5.7 ± 1.1 ($P < 0.05$) in Group III, 4.6 ± 0.4 ($P < 0.001$) in Group IV and 5.9 ± 0.9 ($P < 0.01$) in Group V.

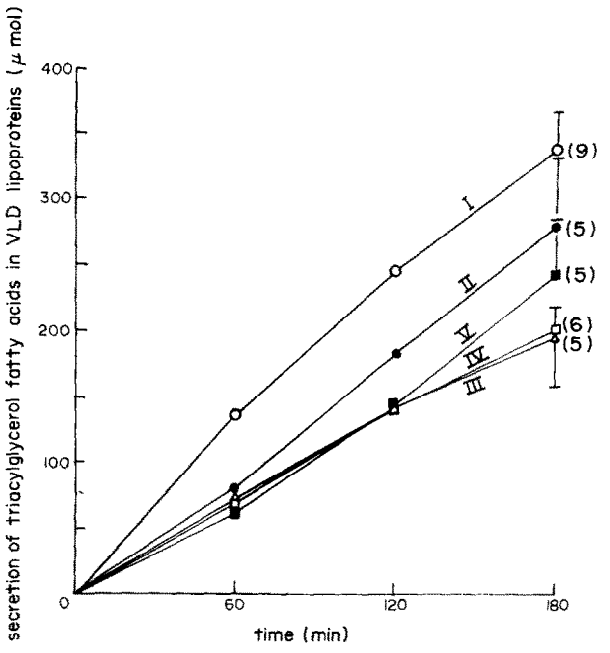

Fig. 6. Secretion of triacylglycerol fatty acids in VLD lipoproteins during 3 hr liver perfusions. ○, control (Group I); ●, with CPIB added to the perfusate (Group II); △, pretreated for 24 hr with clofibrate in the diet (Group III); ▽, pretreated for 1 week with clofibrate in the diet (Group IV); ■, pretreated for 1 week with clofibrate in the diet and with CPIB added to the perfusate (Group V). The livers were infused continuously with oleate complexed to serum albumin. Mean values are shown, and the bars indicate the S.E.M., the number of observations being given in parentheses.

Liver triacylglycerol. The total liver triacylglycerol content was increased from the control value of 177.9 ± 18.8 μmol to 248.2 ± 24.3 in Group III ($P < 0.05$) and to 282.6 ± 26.6 in Group IV ($P < 0.01$). A similar trend was observed in Group V ($0.1 > P > 0.05$). These changes were not apparent in any group when the results were expressed as μmol triacylglycerol per g of liver.

Fate of ^{14}C-labelled free fatty acid

Oxidation of [1-^{14}C]oleate FFA. Total oxidation of FFA, measured as the sum of ^{14}C incorporated into CO_2, including that remaining in perfusate bicarbonate at 180 min, plus total ketone bodies, was least in the control perfusions (Group I) and did not increase significantly when CPIB was added to the perfusate (Table 5). However, there was a significant increase in oxidation in all perfusions of livers from animals fed clofibrate, which was represented by an enhanced incorporation of ^{14}C into both CO_2 and total ketone bodies.

The course of oxidation of the labelled fatty acid to $^{14}CO_2$, shown in Fig. 7, indicated that it was linear from approximately 30–60 min to the termination of perfusion. The initial delay in equilibration of $^{14}CO_2$, due to the kinetics of the system, includes equilibration of ^{14}C in the citric acid cycle, in intracellular and extracellular bicarbonate pools, and, finally, of $^{14}CO_2$ in the gas exchanger.

Incorporation of [1-^{14}C]oleate FFA into liver lipids and serum lipoproteins. Total esterification of FFA, represented by the sum of ^{14}C incorporated into lipids in the liver plus serum lipoproteins, was greatest in the control group, and was significantly decreased in Groups III and IV (Table 6). Although it was 8% less in Group V, the variability of these particular results prevented the attainment of statistical significance.

The ^{14}C incorporated into total liver lipids was 39.5% in the control group and did not differ significantly from this in any other group. The relative incorporation of the label into the lipid classes within the liver (Table 7) was also similar in all groups, most of the radioactivity being present in triacylglycerol and phospholipid.

Table 5. Immediate and long term effects of clofibrate on the oxidation of [1-^{14}C]oleate by perfused livers

		% of [1-^{14}C]oleate infused incorporated into:		
		CO_2 (*a*)	Total ketone bodies (*b*)	Total oxidized products (*a* + *b*)
Group I—	Controls (10)	3.4 ± 0.5	2.2 ± 0.4	5.5 ± 0.8
Group II—	CPIB in perfusate (5)	3.3 ± 0.3	2.7 ± 0.4	5.9 ± 0.7
Group III—	Clofibrate in the diet for 24 hr (5)	5.9 ± 0.6**	3.3 ± 0.3†	9.2 ± 0.7**
Group IV—	Clofibrate in the diet for 1 week (6)	6.6 ± 0.8**	3.8 ± 0.6†	11.1 ± 1.5**
Group V—	Clofibrate in the diet for 1 week plus CPIB in perfusate (5)	5.6 ± 1.0†	4.2 ± 0.5*	9.8 ± 1.1**

Livers were perfused for 3 hr and [1-^{14}C]oleate was infused continuously under the conditions described in the text. Results are expressed as the mean ± S.E.M. and their significance was tested against the control Group I by '*t*' test. The number of perfusions in each group is given in parentheses. †, $0.05 < P < 0.1$; *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$.

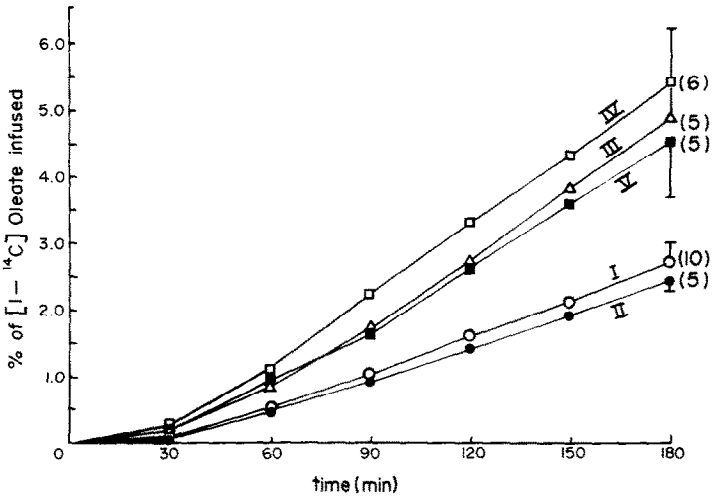

Fig. 7. Oxidation of [1-^{14}C]oleate FFA to $^{14}CO_2$ during 3 hr liver perfusions. ○, control (Group I); ●, with CPIB added to the perfusate (Group II); △, pretreated for 24 hr with clofibrate in the diet (Group III); □, pretreated for 1 week with clofibrate in the diet (Group IV); ■, pretreated for 1 week with clofibrate in the diet and with CPIB added to the perfusate (Group V). The livers were infused continuously with [1-^{14}C]oleate complexed to serum albumin. Mean values are shown, and the bars indicate the S.E.M., the number of observations being given in parentheses.

Incorporation of [1-^{14}C]oleate into the VLD-lipoprotein lipid fraction was significantly decreased, not only by the livers from animals fed clofibrate (Groups III–V), but also by livers (Group II) exposed solely to CPIB during the perfusion (Table 6). The greater proportion of the ^{14}C incorporated (90.4 per cent in control perfusions) was found in the triacylglycerol fraction. Changes due to the drug were apparent only in this fraction. The [1-^{14}C]oleate incorporated into the other lipid classes in VLD-lipoproteins in the experimental groups did not differ significantly from the control values shown in Table 7. The rate of incorporation of ^{14}C into VLD-lipoprotein triacylglycerol increased during the first hour and was linear for the remainder of the perfusion. This time course was similar to that previously reported [7].

12.5 ± 1.3% of the ^{14}C infused remained in the lipoproteins of density > 1.006 at the termination of control perfusions. However, 11.3 ± 1.2% remained in Group II and significantly less ($P < 0.05$) remained in Groups III (9.1 ± 0.6%), IV (9.0 ± 0.2%) and V (8.5 ± 0.4%). Virtually all the ^{14}C was present in the

Table 6. Immediate and long term effects of clofibrate on the incorporation of [1-^{14}C]oleate into esterified products by perfused livers

		% of [1-^{14}C]oleate infused incorporated into:		
		Liver lipids	VLD-lipoprotein lipids	Total
Group I—	Controls (10)	39.5 ± 2.3	30.0 ± 2.0	73.6 ± 3.4
Group II—	CPIB in perfusate (5)	43.1 ± 1.5	22.4 ± 2.8*	69.9 ± 2.1
Group III—	Clofibrate in the diet for 24 hr (5)	35.6 ± 2.3	22.9 ± 1.5*	60.8 ± 1.6**
Group IV—	Clofibrate in the diet for 1 week (5)	42.2 ± 3.0	17.8 ± 1.7***	62.1 ± 2.6*
Group V—	Clofibrate in the diet for 1 week plus CPIB in perfusate (5)	41.3 ± 3.3	21.5 ± 2.8*	65.4 ± 5.5

FFA (oleate) was continuously infused during 3 hr perfusions of livers under the conditions described in the text. Included in the column headed 'total' is the incorporation into esterified lipids in d > 1.006 lipoproteins, which did not differ significantly between the Groups. Results are expressed as the mean ± S.E.M., and were tested for significance against the control Group I by '*t*' test. The number of experiments in each group is given in parentheses. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$.

Table 7. Incorporation of [1-^{14}C]oleate into lipid classes of rat liver and of VLD lipoproteins during perfusion of untreated (Group I) livers

	% of [1-^{14}C]oleate infused in:	
	Liver lipid	VLD-lipoprotein lipid
Triacylglycerol	26.1 ± 1.8	27.2 ± 2.2
Phospholipid	12.2 ± 1.0	1.8 ± 0.8
Monoacylglycerol	0.4 ± 0.8	0.2 ± 0.1
Diacylglycerol + cholesterol	0.2 ± 0.5	0.1 ± 0.1
Cholesteryl esters	0.2 ± 0.0	0.6 ± 0.3
Non-esterified fatty acids	0.3 ± 0.1	0.7 ± 0.3

Perfused livers from fed rats were infused continuously for 3 hr with [1-^{14}C]oleate under the conditions described in the text. Lipid extracts were separated by t.l.c. into lipid classes, and their radioactivity measured. Values shown are the mean ± S.E.M. of 10 perfusions.

FFA fraction derived from the infusion. The small amount of radioactivity present in other lipid fractions did not differ between the perfusion groups.

Lipogenesis and cholesterolgenesis

Total hepatic fatty acid synthesis (calculated from the incorporation of ^{3}H from $^{3}H_2O$) was 104.6 ± 13.1 μmol in control perfusions (Table 8) and was increased significantly in those livers derived from rats administered clofibrate for 1 week (Groups IV and V). This increase was still apparent when the results were calculated on a per g basis, demonstrating that the effect of the drug was not due entirely to the increased mass of the liver. In addition, a decreased rate of synthesis per g of liver was revealed in Group II, where CPIB was added to the perfusate.

When the distribution ratio of ^{3}H between hepatic triacylglycerols and phospholipids in the experimental groups was compared with the control value of 1.51 ± 0.06, it was found to decrease significantly in those groups given clofibrate in the diet for 1 week, viz: Group IV, 1.04 ± 0.08 ($P < 0.001$); Group V, 1.08 ± 0.20 ($P < 0.05$). No differences from the control value of 2.25 ± 0.22 were found for the ratio of distribution of ^{14}C-oleate between hepatic triacylglycerols and phospholipids.

There were no differences in percentage distribution of ^{3}H in VLD-lipoprotein lipids between any of the groups. Most of the label was in triacylglycerol (92.6 ± 1.8% in controls) and phospholipids (2.9 ± 1.2% in controls).

The rate of cholesterol synthesis in livers from control rats was 0.50 ± 0.21 μmol/h per g. This rate did not differ significantly in any of the experimental groups.

DISCUSSION

Studies in whole animals

Previous studies on clofibrate-treated rats have shown a consistent hypocholesterolaemic action of the

Table 8. Immediate and long term effects of clofibrate on fatty acid synthesis of perfused livers

		fatty acid synthesized	
		total (μmol)	rate (μmol/hr per g of liver)
Group I—	Control (7)	104.6 ± 13.1	3.1 ± 0.4
Group II—	CPIB in perfusate (4)	77.1 ± 1.0†	2.1 ± 0.1*
Group III—	Clofibrate ir the diet for 24 hr (5)	91.1 ± 9.4	2.6 ± 0.3
Group IV—	Clofibrate in the diet for 1 week (5)	173.6 ± 10.7**	3.9 ± 0.2†
Group V—	Clofibrate in the diet for 1 week + CPIB in perfusate (5)	199.7 ± 25.4**	5.0 ± 0.5*

Livers were perfused for 3 hr, with a constant infusion of [1-^{14}C]oleate and after addition of $^{3}H_2O$. Hepatic fatty acid synthesis was calculated from the incorporation of ^{3}H, assuming that the average molecule of fatty acid synthesized has 31.7 carbon-bound hydrogen atoms, of which 13.3 are derived from $^{3}H_2O$ [14]. The results are expressed as the mean ± S.E.M. (the numbers of experiments in each group being given in parentheses) and were tested for significance against the control Group I using Student's '*t*' test. †, $0.05 < P < 0.01$; *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$.

drug, but the response of the plasma triacylglycerol has been variable and dependent on the period of treatment, type of diet, age, sex, strain, etc. In the present work, when male rats were fed 0.25% clofibrate in the diet for 1 week, there was a definite hypolipidaemic effect, with decreased concentrations of both cholesterol and triacylglycerol. The pronounced hepatomegaly reported has also been found by other investigators [19], as have the tendencies towards a decrease in serum FFA concentration [20] and a retardation in growth rate [21]. Although a decrease in liver glycogen has been reported to occur after 2 weeks of clofibrate treatment [19, 22], we did not detect any alteration after 1 week of treatment. Since the dose of clofibrate employed in these initial studies in whole animals produced typical reactions to the drug, animals treated similarly were used as liver donors in the subsequent perfusion studies.

The plasma CPIB concentration, established by administering clofibrate in the diet of rats in the present study, was similar (0.49 mM) to that observed in patients receiving therapeutic doses of clofibrate [23]. In our liver perfusions, CPIB was added to give an initial perfusate concentration of 0.75 mM. The rate of removal of CPIB from the perfusate by the liver [5] was such that the perfusate CPIB concentration during most of the 3 hr experimental period was within the range found in the whole animal.

Perfusion studies

O_2 *consumption.* Matched clofibrate treated and control patients [23] have a similar splanchic O_2 consumption and RQ. Studies of CPIB action on O_2 consumption in isolated mitochondria have utilized very high drug concentrations, whose detergent action uncouples the respiratory chain [24]. Mitochondria isolated from clofibrate treated rats have shown a decrease [25], increase [26], or no alteration [27] in respiration. Perfusions of clofibrate treated livers [26] have demonstrated an increase in O_2 consumption, but to a rate which was still much less than that of our control perfusions.

Comparison of results from perfusions of livers from untreated rats with perfusions of livers from rats pretreated with clofibrate, as in the present work, is complicated by the increase in liver weight due to the drug. We have therefore considered the effects described both in terms of the total liver and per g of liver. Despite the reported increase in liver mitochondrial protein occurring within 2–5 hr after a single injection of clofibrate [28], the livers from animals pretreated with clofibrate for 24 hr (Group III) showed no detectable increase in weight or alteration in O_2 consumption. Although the liver weight and total O_2 consumption were increased after 1 week of pretreatment (Group IV and V), the fact that the flow rate of the perfusate through the liver was kept constant, restricted the O_2 consumption per g of liver to below the control value. When the flow rate was increased to give an O_2 consumption per g similar to that of controls (Group IVa, Table 3), apart from further decreases in perfusate glucose and lactate concentrations, all other parameters investigated, including the fate of the added radioactivity, gave similar results to those in Groups IV and V (Table 2). This showed that the effects of 1 week of clofibrate treatment on the metabolism of the perfused liver were not altered by any insufficiency of O_2 during perfusion.

It has been suggested [1] that hepatic actions of clofibrate are due to displacement of thyroxine from plasma into the liver. Present results showed that, provided the blood flow was increased to compensate for the increased mass of clofibrate treated livers, the O_2 consumption per g remained unchanged. In this respect clofibrate treatment is unlike the effect of thyroid hormone [29], which increases not only the total O_2 consumption but also the O_2 consumption per g of tissue.

Bile production. The initial rate of bile production in these perfusions compares favourably with previous results from 90 min perfusions [30]. The decrease in rate during the 3 hr of perfusion is probably due to the absence of an enterohepatic circulation which, in the intact animal, would return bile salts to the liver.

Carbohydrate metabolism. Since blood is used as perfusate, two significant metabolizing tissues are present in the system, liver and erythrocytes. Under our conditions, erythrocytes utilize glucose and form lactate at a constant rate, which remains unchanged in the presence of 0.5 mM CPIB*. Consequently, a stable concentration of these metabolites in the perfusate is indicative of constant hepatic release of glucose and utilization of lactate and changes in perfusate concentrations may be ascribed to the liver.

The slight increase in perfusate glucose concentration in the control group could have been due to progressive depletion of insulin by the end of 3 hr [31]. Despite reports of an association between clofibrate treatment and a decreased serum insulin concentration *in vivo* [32, 33], present perfusions indicate an apparent 'insulin like' effect of clofibrate in lowering perfusate glucose concentration, especially after 24 hr administration. This effect demonstrated that the drug decreased hepatic glucose output. The glycogen concentration after the present perfusions was similar in all livers, despite reports of decreased liver glycogen after longer periods of clofibrate feeding *in vivo* [19, 22]. Thus there were no detectable effects of clofibrate feeding, or of added CPIB, on glycogenolysis or glycogenesis.

The perfusate lactate concentration was decreased from the control value in all experimental perfusions, indicating increased lactate uptake by the liver. As the glucose output by the liver decreased and the glycogen content did not change significantly, the increased lactate uptake was most likely due to enhanced oxidation via pyruvate rather than to increased gluconeogenesis. This was one of the direct effects of CPIB added to the perfusate and was also observed in perfusions of livers from rats fed clofibrate in the diet for only 24 hr, when there was no detectable increase in liver mass. Other investigators have suggested an inhibition in gluconeogenesis between 3-phosphoglycerate and fructose 1,6-diphosphate [22], but Gans and Cater [34] did not detect any effect of clofibrate on gluconeogenesis from ^{14}C alanine in dog liver. The decreased lactate concentration was sufficiently pronounced in the perfusions of livers from rats fed on clofibrate (Groups IV and V) to cause a significant decrease in the [lactate]/[pyruvate] ratio, indicative of a more oxidized redox state in the cytosol. A decrease in blood and liver lactate during

* M. E. Laker and P. A. Mayes, unpublished observations.

prolonged clofibrate treatment, and correspondingly more oxidized redox states have been reported recently [22, 35, 36].

Free fatty acid uptake. Our results show an immediate decrease in perfusate FFA concentration in all experimental groups, demonstrating an initial increased hepatic FFA uptake. Hitherto, it has been considered that clofibrate does not affect hepatic extraction of FFA [21, 22, 37] but a recent report of clofibrate pretreatment enhancing the uptake of palmitate–FFA by the isolated rat liver [38] is in agreement with present results. The immediate enhancement of FFA uptake by livers from untreated rats due to contact with CPIB in the perfusate (Group II), indicates that the well-documented lowering of serum FFA concentration in animals given clofibrate is not due solely to an extrahepatic antilipolytic action of the drug [1, 7] but must also be ascribed to an effect by the liver. However, perfusions in Group III demonstrated that, once the rat had been treated with clofibrate for 24 hr, the increased uptake of FFA did not require the presence of CPIB in the perfusate. The persistence of this effect in the absence of CPIB in the perfusate, shows that the proposed displacement of fatty acids from plasma albumin binding sites by CPIB [1, 39] is not the only explanation of the effect. It may be due to the reported direct stimulation of the synthesis of hepatic cytoplasmic Z (fatty acid binding) protein by clofibrate treatment [40, 41]. It has been suggested [42] that increased extrahepatic utilization of FFA, resulting from clofibrate treatment, decreases the flux of FFA to the liver, accounting for the changes in liver metabolism. However, it should be appreciated that once the perfusate FFA concentration had stabilized after the first few minutes of FFA infusion, the flux of FFA into all the livers in our experiments was the same, due to the constant rate of infusion of the oleate–FFA. Thus, none of the effects of the drug found in the present perfusions can be ascribed to major alterations in flux of FFA into the liver.

Fatty acid oxidation. The increase in total ketone body production indicated that clofibrate treatment resulted in greater oxidation of fatty acids, which was detectable within 24 hr of treatment. This increase in oxidation was confirmed by the increased incorporation of ^{14}C from [1-^{14}C]oleate into CO_2 and into ketone bodies by livers from rats treated with clofibrate for 1 week. There have been other reports of increased fatty acid oxidation resulting from clofibrate pretreatment, for example, octanoate by perfused livers [26], octanoate and palmitate by hepatic mitochondria [43] and palmitoyl CoA by liver homogenates [44]. However, there have also been conflicting reports of decreased hepatic mitochondrial rates of fatty acid oxidation and ketone body formation following subcutaneous clofibrate administration [25]. Our results show that production of 3-hydroxybutyrate was similar in all liver perfusions, and that the increase in total ketone body production, which followed clofibrate treatment, was almost entirely accounted for by an increased production of acetoacetate, leading generally to a decreased [3-hydroxybutyrate]/[acetoacetate] ratio, indicative of a more oxidized redox state in mitochondria. In agreement, Miyazawa *et al.* [22] found an increase in blood acetoacetate but no change in 3-hydroxybutyrate of rats fed for 2 weeks on a clofibrate treated diet [22]. It is noted that we were unable to detect any significant increase in FFA oxidation in untreated livers in which CPIB was added to the perfusate.

Fatty acid esterification. The inverse relationship between fatty acid oxidation on the one hand and fatty acid esterification and VLD lipoprotein formation on the other, found previously [45], was confirmed in the present investigation. The increased ^{14}C incorporation from [1-^{14}C]oleate into CO_2 and ketone bodies by livers from rats pretreated with clofibrate was counterbalanced by a corresponding decreased incorporation into esterified products, accounted for almost entirely by the decreased incorporation into VLD–lipoprotein triacylglycerol. There was also a decreased incorporation of ^{14}C into VLD lipoproteins by livers exposed solely to CPIB in the perfusate but the total output of triacylglycerol into VLD lipoproteins was not significantly decreased under this particular treatment. Our evidence for an inhibition of hepatic fatty acid esterification by clofibrate is at variance with work on liver slices [46, 47] which showed increased incorporation of precursors into triacylglycerol, and with studies on hepatocytes [48, 49] describing a stimulation of esterification of glycerol and palmitate, respectively. However, Fulton and Hsia [50] claimed that, *in vitro*, the synthesis of all classes of lipid was depressed, and perfusion experiments [51, 52, 37] also showed a decreased hepatic secretion of triacylglycerol after clofibrate treatment. Within 6 hr of a single intraperitoneal injection of CPIB, perfused livers exhibited a reduced capacity to incorporate palmitate into perfusate triacylglycerol [52].

It has been suggested [53] that the greater need for protein synthesis associated with the considerable increase in liver size during the first 2 weeks of clofibrate feeding would compete with lipoprotein apoprotein formation and lead to an accumulation of triacylglycerol in the liver. Despite decreased VLD-lipoprotein output in the present perfusions, the results showed no accumulation of esterified oleate within the liver during any perfusions, and hence did not support the hypothesis that clofibrate acts by blocking the final stages of assembly and release of preformed lipids as lipoproteins. Our results also show that, whereas the total liver triacylglycerol content was increased by administration of clofibrate in the diet for 7 days the concentration did not differ from that found in the smaller control livers.

Lipogenesis. Hepatic total fatty acid synthesis, as measured by the incorporation of tritium from ^3H_2O into lipids, was decreased during 3 hr of exposure to CPIB in the perfusate. Studies on hepatocytes [49] have shown an immediate inhibition by CPIB of acetate incorporation into fatty acids and work on enzyme preparations [54] and hepatocytes [55] has indicated an immediate inhibition of the activity of acetyl-CoA carboxylase, which could explain the immediate effect of CPIB in the perfused liver, since this enzyme catalyzes the first commited step in the pathway of fatty acid synthesis. There have also been reports of a direct inhibition of microsomal fatty acid synthesis *in vitro* [56, 57]. In the present experiments, after 24 hr pretreatment with clofibrate, hepatic fatty acids were synthesized at the control rate but after a week of clofibrate treatment there was an increased rate of fatty acid synthesis, which was also significantly higher than that of controls when calculated on a per g basis. It was of interest that the presence of CPIB did not inhibit the

increased fatty acid synthesis found after a week of clofibrate treatment. Miyazawa *et al.* [22] have also reported an increase in lipogenesis; with these authors we conclude that there can be no long term inhibition of the carboxylase reaction in the rat.

In all the perfusion groups, it was found that a greater proportion of the newly formed ^{3}H-labelled fatty acids was incorporated into phospholipids than into triacylglycerol, compared with ^{14}C-oleate FFA. This may be due to the initial formation of saturated fatty acids by lipogenesis and previous work * has indicated that there is a greater tendency for the more saturated fatty acids to become incorporated into phospholipids rather than triacylglycerol.

Lipogenesis vs *ketogenesis.* Stadie [58] proposed that a decreased utilization of acetyl CoA in fatty acid synthesis was a direct cause of ketogenesis. Lately, this concept of a direct inverse relationship between these two parameters has been revived [59]. However, present results show that, under the influence of clofibrate in the diet for 7 days, there is a concomitant increase in both ketone body production and in fatty acid synthesis, demonstrating that there can be no inverse causal relationship between lipogenesis and ketogenesis.

Cholesterol synthesis. There have been many reports of the existence of a block in the pathway of hepatic cholesterol synthesis resulting from clofibrate treatment [60–64]. However, Kim *et al.* [65] found no effect in swine, and d'Costa *et al.* [66] found no effect in liver slices. We did not find any effects of clofibrate on the absolute rate of hepatic cholesterol synthesis, as measured in the intact organ with $^{3}H_2O$.

Mechanism of the hypolipidemic effect of clofibrate. Peroxisomal proliferation results from clofibrate treatment in the rat, mouse, dog, hamster and human, but not in the guinea pig, chicken, rabbit or squirrel monkey [67]. This occurs within 18–24 hr in the rat [68]. In man, an enlarged palpable liver has been observed [69] and there is some increase in the number of hepatic peroxisomes [67], although an increase in mitochondria has not been reported, as in the rat [28]. Lazarow and de Duve [44] have shown that the proliferation of peroxisomes is accompanied by their increasing ability to oxidize palmitoyl-CoA. If this effect on the peroxisomes and the accompanying increase in mitochondrial protein is taken to be a primary effect of the drug, our results in livers from rats pretreated with clofibrate can be explained. Thus, the increased capacity to oxidize fatty acyl-CoA would account for the increased oxidation of FFA to CO_2 and ketone bodies found in our perfusions. Mayes and Felts [45] have demonstrated that hepatic oxidation of FFA is reciprocally related to esterification and VLD-lipoprotein secretion. Therefore, the decreased production of VLD-lipoprotein triacylglycerol by clofibrate-treated livers would follow as a consequence of the diversion of some of the FFA influx into the oxidative pathways. The drug would reduce hypertriacylglycerolaemia *in vivo*, at least in part, as a result of the decreased output of VLD-lipoprotein triacylglycerol from the liver. Because cholesterol is an integral part of the VLD-lipoprotein structure, decreased output of VLD lipoproteins resulting from the decreased esterification of FFA by the liver would also account for the hypocholesterolaemic effect of clofibrate *in vivo*, without the necessity to postulate any effect of clofibrate on cholesterol synthesis. An increased removal of intracellular fatty acids by the augmented oxidative pathways of the clofibrate treated liver might also cause more FFA to enter liver cells and hence decrease the serum FFA concentration, as observed both *in vivo* and in the perfused liver.

The increase in ketogenesis in perfusions of livers from clofibrate treated animals was due mainly to augmented production of acetoacetate rather than 3-hydroxybutyrate. This may be related to the increased capacity of peroxisomes to oxidize fatty acids. Acetoacetate might be formed directly by peroxisomes, whose oxidation pathway would favour acetoacetate production, since 3-hydroxybutyrate dehydrogenase, which is required to convert acetoacetate into 3-hydroxybutyrate, is located exclusively in mitochondria [70]. However, it is more likely that acetyl-CoA-carnitine acyltransferase, whose activity in mitochondria increases 10 fold as a result of clofibrate treatment [71], enables accelerated transport of acetyl-CoA from peroxisomes into mitochondria, where it is converted to acetoacetate via the normal pathway for ketogenesis. When acetyl-CoA is formed during β-oxidation of long chain fatty acids in mitochondria, the NADH produced is immediately available for the conversion of acetoacetate to 3-hydroxybutyrate. However, intramitochondrial NADH would not be formed when acetyl-CoA is derived from extramitochondrial sources such as peroxisomes. Thus ketogenesis would tend to terminate in acetoacetate rather than in 3-hydroxybutyrate, which would explain our results. For similar reasons proportionally less ATP would be generated within the mitochondria from fatty acid oxidation.

Generally, increased oxidation of fatty acids leads to decreased mitochondrial $[NAD^+]/[NADH]$ ratios and increased [ATP]/[ADP] ratios which, in turn, cause inactivation of pyruvate dehydrogenase [72, 73]. It is of interest that when increased fatty acid oxidation is due to clofibrate, it leads to opposite effects on these parameters. The decreased [3-hydroxybutyrate]/[acetoacetate] ratio found in these perfusions is indicative of a more oxidized intramitochondrial redox state. This would be reflected by an increased mitochondrial $[NAD^+]/[NADH]$ ratio and a decreased [ATP]/[ADP] ratio [70]. Both the shift in redox state and in adenine nucleotide ratios would be expected to convert pyruvate dehydrogenase from the inactive to active form. Activation of this enzyme would not only increase pyruvate utilization but would also explain the other effects of clofibrate on carbohydrate metabolism in the present perfusions, such as enhanced lactate uptake and decreased glucose output. It would also account for the reported long term decrease in hepatic glycogen *in vivo* [19, 22]. We have shown previously that synthesis of long chain fatty acids in the perfused liver parallels the activity of pyruvate dehydrogenase [74–76]. An increased activity of this enzyme would account, therefore, for the increased synthesis of long chain fatty acids found in the present investigations.

A primary effect of clofibrate in decreasing the intramitochondrial redox potential, such as might be caused by uncoupling of oxidation and phosphorylation, cannot be excluded. However, this seems unlikely in view of the lack of increase in O_2 consumption in our perfusions when the rate was expressed on a per g basis.

* P. A. Mayes, Unpublished work.

It is difficult to reconcile the immediate effects of CPIB when added to the perfusate (Group II) with the proposed hypothesis to account for the longer term effects of clofibrate administration. For example, lactate uptake by the liver increased in Group II without a detectable increase in oxidation of fatty acids. It is probable that the immediate and direct effects of the drug are different from the longer term adaptive effects. It is noteworthy that some of the adaptive effects described for perfusions of livers from animals treated with clofibrate for 1 week were not as marked, or were more variable, when CPIB was also present in the perfusate. It would appear that at least some of the immediate effects of CPIB may counteract its long term adaptive effects.

A preliminary account of this work has appeared [77].

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