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FURTHER STUDIES CONCERNING THE EFFECTS OF CLOFIBRATE ON RESPIRATION AND OXIDATIVE PHOSPHORYLATION OF RAT LIVER MITOCHONDRIA

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Abstract—Clofibrate, administered *in vitro*, inhibited rat liver mitochondrial respiration at two sites within the respiratory chain. One site was between the interaction of NADH with NADH dehydrogenase and the point at which electrons from succinate oxidation enter the electron transport chain; another, less sensitive site, was between the interaction of succinate with succinate dehydrogenase and cytochrome c. In addition to these specific sites, clofibrate inhibited respiration by causing a depletion of pyridine nucleotides that was accompanied or followed by large-amplitude, non-energy-linked swelling. Clofibrate uncoupled oxidative phosphorylation at coupling sites II and III but not at site I. The concentrations required to cause loss of pyridine nucleotides were lower than those required to inhibit at the specific sites. *p*-Chlorophenoxyisobutyrate (CPIB) also inhibited succinate and β-hydroxybutyrate-linked respiration, and uncoupled oxidative phosphorylation, but at much higher concentrations (50 per cent inhibition of β-hydroxybutyrate oxidation at about 3·7 μmoles/mg of protein) than were required of clofibrate (50 per cent inhibition of β-hydroxybutyrate oxidation at about 0·17 μmole/mg of protein). Clofibrate administration to rats (100 and 300 mg/kg p.o. daily for 1 week) lowered serum lipid levels and increased the liver size, the amount of mitochondrial protein/g of liver, and the oxygen consumption of liver slices. However, mitochondria, isolated from livers of the treated rats, respired normally. A single administration of clofibrate (100 or 300 mg/kg, p.o.) did not affect liver slice respiration.

CLOFIBRATE (Atromid-S) is used clinically as a hypolipidemic agent; its primary effect appears to be on the triglycerides of the low density serum lipoprotein fraction.[1–4] Toxicity has not been associated with clofibrate therapy and there appears to be no microscopic pathology,[5,6] although rat liver size[7,8] and the number of peroxisomes[9–11] and mitochondria[12,13] within the liver are increased. The activities of certain enzymes associated with mitochondria (e.g. α-glycerophosphate dehydrogenase)[9,14] and peroxisomes (e.g. catalase)[9,14] are also increased. The enhanced α-glycerophosphate dehydrogenase activity appears to be caused by a hyperthyroid condition within the liver, resulting perhaps from selective accumulation of thyroxine or potentiation of the activity of thyroxine already present in the liver.[15,16]

Recently, several reports have shown that clofibrate and chlorophenoxyisobutyrate (CPIB), when added *in vitro*, can inhibit oxidative phosphorylation of liver mitochondria.[17–21] Mackerer *et al.*[21] suggested the presence of at least two sites at which clofibrate can inhibit mitochondrial respiration. The present report describes the results of studies that were designed to obtain more information concerning the mechanisms by which clofibrate inhibits oxidative phosphorylation. In these experiments, clofibrate was administered *in vivo* to rats, and *in vitro* to rat liver mitochondria. It was found that, in addition to the two sites of inhibition already described, clofibrate, *in vitro*, caused a depletion of mitochondrial protein and NAD which was

accompanied or followed by large-amplitude, non-energy-linked swelling. Oral administration of clofibrate (100 and 300 mg/kg/day) produced hypolipidemic effects and increased the rate of oxygen consumption by liver slices but did not affect oxidative phosphorylation of isolated mitochondria.

METHODS

Animals and drug administration. Male rats (Charles River CD–CR strain; body weight, 150–200 g) were used for all experiments. These rats were separately housed and fed a commercial pelleted diet [Rockland Mouse/Rat Diet (Complete)], *ad lib.*, until killed by decapitation.

For the studies involving administration of clofibrate *in vivo*, rats received a single daily oral dose of 100 or 300 mg/kg dissolved in polyethylene glycol 400. Control rats received only the vehicle. On the day of sacrifice, rats were killed 1 hr after drug administration.

Oxygen consumption of mitochondria. Intact mitochondria were isolated by differential centrifugation and suspended in 0·25 M sucrose.[22] Damaged mitochondria, which were permeable to added NAD and NADH, were prepared by exposing suspensions of intact mitochondria to one or two cycles of freezing and thawing.

Oxygen consumption was measured polarographically at 30° in a Gilson Oxygraph (Gilson Medical Electronics Inc., Middleton, Wis.) equipped with a Clark oxygen electrode. The basic incubation medium was: 65 mM Tris buffer (pH 7·4), 75 mM KCl, 5 mM $MgCl_2$, 12 mM phosphate buffer (pH 7·4), 1 mM EDTA and 10 mM substrate. Further details are supplied in the text. The volume of the reaction mixture was always 1·9 ml. Oxygen consumption and ADP:O ratio were determined by the methods of Chance and Williams.[23,24] State 3 was initiated by adding either 400 nmoles, when P:O ratios were determined, or 2 μmoles ADP.

Oxygen consumption of tissues. Oxygen consumption of several rat tissues was determined by a modification of the procedure of Huston and Martin.[25,26] Slices of fat pad, liver, kidney and rectus abdominus muscle were made with a Stadie–Riggs type of tissue slicer.[27] Both tissue holder and blade were slightly moistened with 0·9% NaCl. After the blade was passed through the tissue, the resulting slice was removed with forceps and placed on a 2-cm square gauze pad which was premoistened with 0·9% NaCl. Pieces of intestine, with the serosa and mucosa intact, were taken from a segment of jejunum 4–7 in. from the pylorus. These pieces were cut longitudinally and spread, with the mucosal sides upward, on moistened gauze pads.

Each pad was pushed into a 15-ml, single sac, Warburg flask without center well that contained: (a) 0·4 ml of 5% KOH (in the sac) for absorbing CO_2, (b) two drops of 0·9% NaCl (in the main chamber) to prevent dehydration of the tissues, and (c) a piece of 30-mesh, stainless steel, wire (1·4 × 3·0 cm) with the two short sides bent down 3 mm from the ends (in the main chamber) to prevent the pad from contacting the bottom of the flask.

The flasks were connected to a Gilson Respirometer apparatus at 37·5° and gassed for 5 min with 100% O_2 which had been passed through a filter (Molecular Sieve, type 4A) and a hydrator. After a 15-min equilibration period, measurements of O_2 consumption were made at 5-min intervals up to 1 hr. After incubation, tissues were removed from the pads and weighed. It is important to note that in this procedure tissues were not incubated in aqueous media but were in direct contact with oxygen.

Mitochondrial swelling. The effects of clofibrate on swelling were followed by recording absorbance changes at 520 nm wavelength in 4-ml Silica cells of 1-cm light pathlength at 27°.[28,29] Mitochondria, equal to 2 mg protein, were added to 3 ml medium (0·17 M sucrose–0·003 M Tris-HCl, pH 7·3) and the resulting mixture was allowed to equilibrate for 2 min. Initial absorbance was between 1·2 and 1·3. After equilibration, various substrates and inhibitors including clofibrate were added and effects were observed for 25–30 min. In several experiments, 6 mM ATP was added after 15 min.

Analyses. Blood levels of glucose,[30] triglyceride,[31] cholesterol[32] and free fatty acids[33] were determined by the cited procedures. Mitochondrial levels of NAD and NADH were determined by the spectrophotometric methods of Klingenberg.[34,35] Mitochondrial protein was determined by the biuret procedure of Gornall *et al.*[36]

Clofibrate solution. Clofibrate, which is rather insoluble in water and buffered solutions, was dissolved in alcohol. Stock solutions of clofibrate in ethanol or propylene glycol and polyethylene glycol 400 were chosen for the studies *in vitro* and *in vivo* respectively. In most of the experiments *in vitro*, the vehicle alone (1%, v/v) did not produce any effects.

Chemicals. Clofibrate (*p*-ethyl-chlorophenoxyisobutyrate) was obtained from Ayerst Laboratories, N.Y. Organic reagents were from Sigma Chemical Co., St. Louis, and inorganic salts from Mallinckrodt Chemical Co., St. Louis. Fatty acid-free, bovine serum albumin was prepared from Pentex, Fraction V Powder (Miles Laboratories, Inc., Kankakee) by the method of Chen[37] as modified by Hanson and Ballard.[38]

RESULTS

Effects of clofibrate on respiration and ADP:O ratio. The effects of increasing concentration of clofibrate on state 3 respiration and on coupling activity are summarized in Fig. 1. The contribution of each coupling site to the ADP:O ratio was calculated by subtraction of the ADP:O ratio that was obtained with each substrate according to the method of Katyare *et al.*[39] When ascorbate + TMPD and succinate were the substrates, rotenone was added to prevent the oxidation of endogenous NAD-linked substrate.

As shown in Fig. 1a, clofibrate inhibited β-hydroxybutyrate oxidation at the lower concentrations and inhibited succinate oxidation at the higher concentrations. Site specificity for the uncoupling action of clofibrate was clearly evident (Fig. 1b). Site I was completely resistant to uncoupling at all concentrations tested. Both sites II and III were uncoupled in a dose-dependent manner with site II slightly more resistant to this effect. At 0·2 μmole clofibrate/mg of protein, site II showed a 43 per cent loss and site III a 72 per cent loss of coupling activity.

Effect of washing on clofibrate inhibition of state 3 oxidation. Clofibrate, at 0·075, 0·1 and 0·2 μmole/mg of protein, was added to 5-ml aliquots of mitochondrial suspension containing 19 mg protein/ml. These were incubated at 0° for 10 min and then centrifuged at 15,000 *g* for 5 min. The mitochondrial pellet was washed once with 5 ml of 0·25 M sucrose and resuspended. State 3 oxidations of β-hydroxybutyrate by the washed and unwashed mitochondria were determined and compared. It was found that preincubation with clofibrate produced inhibitory effects, similar to those

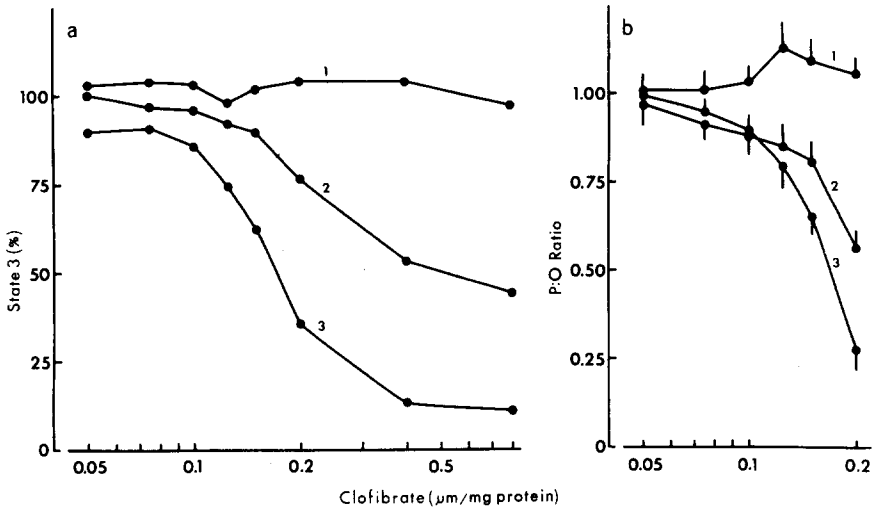

FIG. 1. Effects of clofibrate on state 3 respiration and on ADP:O ratio of rat liver mitochondria. Propylene glycol (1%, v/v) was the vehicle. (a) State 3 respiration: (1) ascorbate + TMPD, (2) succinate and (3) β-hydroxybutyrate were the substrates. Each data point represents the mean of ten replicate experiments. (b) The calculated ADP:O ratio at each phosphorylation site (see text for explanation of calculations). Each data point represents the mean ± S.E.M. of ten replicate experiments. (1) site I, (2) site II, (3) site III.

shown in Fig. 1a, which were not reversed by washing. Additions of 5, 10 and 20 mg albumin (fatty acid-free) to the inhibited, washed, mitochondria also failed to reverse the inhibition.

The degree of inhibition of respiration by clofibrate was dependent on the ratio of clofibrate:mitochondrial protein in the system (Fig. 2) and there was a stoichiometric relationship between mitochondrial protein and clofibrate concentrations. The inhibitory effects were not altered by varying the sequence of clofibrate and protein additions. These findings suggest that clofibrate was bound to the mitochondria and, therefore, removed from the medium.

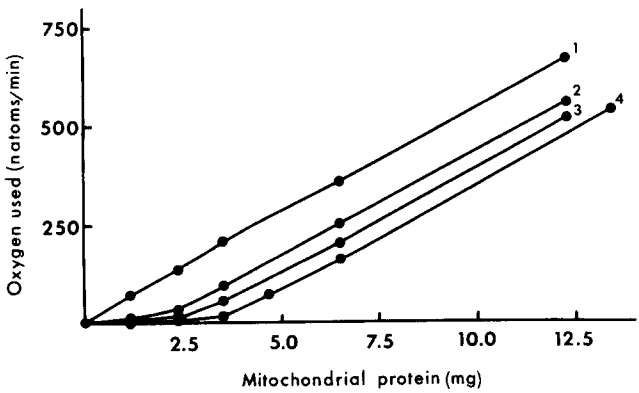

FIG. 2. Inhibition of state 3 respiration by clofibrate as a function of mitochondrial protein concentration. β-Hydroxybutyrate was the substrate and propylene glycol (1%, v/v) was the vehicle. Clofibrate additions: (1) none, (2) 0·69 μmole, (3) 1·03 μmoles, (4) 1·37 μmoles.

Effect of clofibrate on Ca^{2+}-stimulated respiration. The uptake of Ca^{2+} by mitochondria is an energy-requiring process which proceeds, in the presence of substrate and inorganic phosphate, with the consumption of oxygen.[40,41] This phenomenon can be studied in the Oxygraph in much the same way that ADP-stimulated oxygen consumption is studied. For our experiments, the basic incubation medium as described in Methods (but lacking EDTA) was used and Ca^{2+}, instead of ADP, was added to stimulate respiration.

When 550 nmoles Ca^{2+} was added to intact mitochondria, oxidizing β-hydroxybutyrate in state 4 (Figs. 1 and 3), there was a rapid and pronounced stimulation of oxygen consumption followed by an inhibition. This inhibition could be reversed by NAD (Figs. 1 and 3) but not by further additions of Ca^{2+} (data not shown). Figure 3 (Curves 2–5) shows the effects of increasing concentrations of clofibrate on Ca^{2+} and NAD-mediated responses. Clofibrate prolonged the duration of the respiratory stimulation elicited by Ca^{2+} but inhibited the rate of this respiration, and these effects were proportional to the clofibrate:protein ratio. At the lower clofibrate concentrations, NAD completely reversed the Ca^{2+}-induced respiratory depression and that caused by clofibrate; but, at the higher concentrations, reversal was only partial.

Clofibrate-induced inhibition of β-hydroxybutyrate oxidation and reversal by NAD. As shown previously[18,21] and in Figs. 1 and 2, clofibrate depresses NAD-linked mitochondrial respiration, perhaps through a mechanism involving the contribution of at least two inhibitory sites within the respiratory chain. However, it is probable that these sites do not entirely account for the clofibrate inhibition of β-hydroxybutyrate oxidation because, as shown in Fig. 4, this effect was also reversed by adding

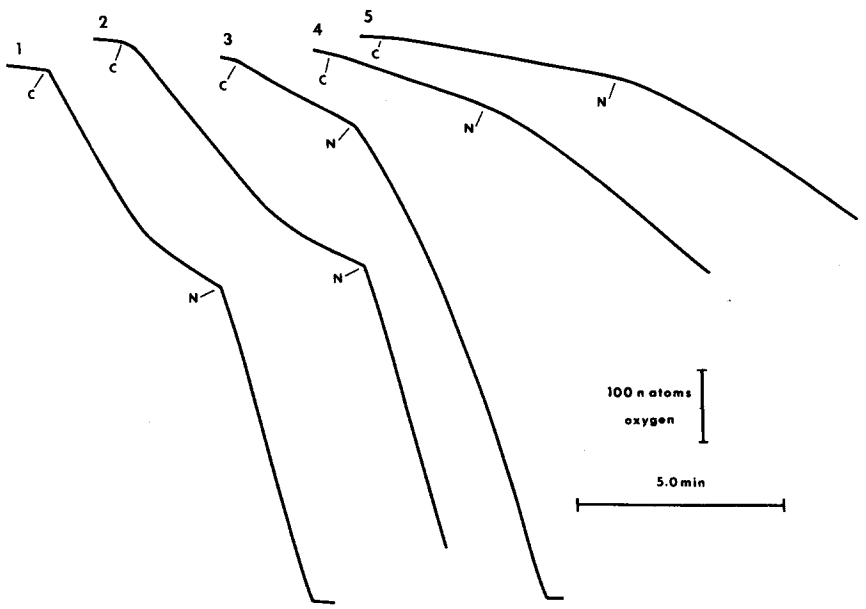

FIG. 3. Effects of Ca^{2+} and clofibrate on the respiration of mitochondria with β-hydroxybutyrate as the substrate. Ethanol (1%, v/v) was the vehicle. Clofibrate concentrations: (1) no clofibrate, (2) 0·15 μmole/mg of protein, (3) 0·20 μmole/mg of protein, (4) 0·30 μmole/mg of protein, (5) 0·40 μmole/mg of protein. Mitochondrial protein concentration was 2 mg/ml. C. Ca^{2+} (290 μ M): N, NAD (1 mM).

NAD. When succinate was used as the substrate, inhibition by clofibrate was not reversed by NAD.

Clofibrate inhibition of respiration in frozen–thawed mitochondria. Since NAD partially reversed the clofibrate inhibition of β-hydroxybutyrate oxidation, further experimentation was required to support the suggestion that a specific site of clofibrate inhibition exists between the interaction of NADH and NADH dehydrogenase and cytochrome b.[21]

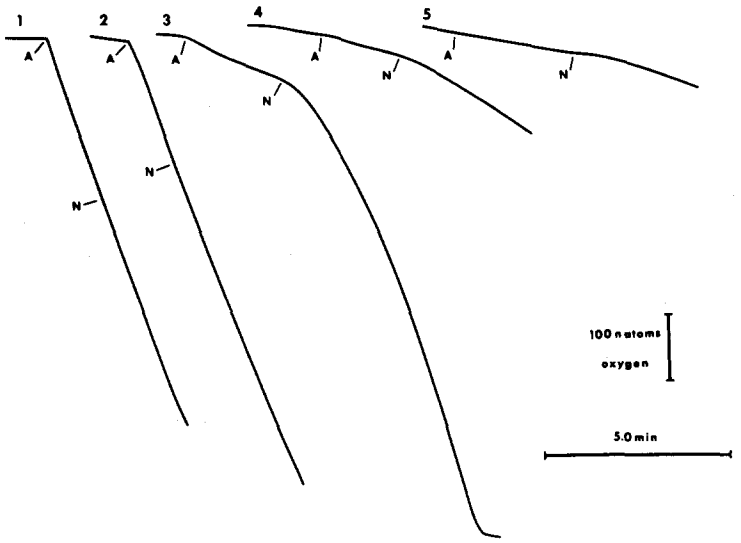

FIG. 4. Effects of clofibrate and NAD on state 3 respiration with β-hydroxybutyrate as the substrate. Ethanol (1%, v/v) was the vehicle. Clofibrate concentrations: (1) no clofibrate, (2) 0·1 μmole/mg of protein, (3) 0·20 μmole/mg of protein, (4) 0·30 μmole/mg of protein, (5) 0·40 μmole/mg of protein. Mitochondrial protein concentration was 2 mg/ml. A, ADP (1 mM); N, NAD (1 mM).

Frozen–thawed mitochondria, prepared as in Methods, were used to determine rates of respiration linked to oxidation of succinate and β-hydroxybutyrate. In the absence of added NAD, respiration with succinate was considerably faster than that with β-hydroxybutyrate, but slower than that of intact mitochondria. The rate of β-hydroxybutyrate oxidation was proportional to the concentration of NAD (Fig. 5a) and, therefore, it was possible to adjust the rate of this oxidation to be equal to that of succinate. As shown in Fig. 5b, when the initial rates of respiration were equal, progressive increases in the clofibrate concentration produced a greater inhibition of β-hydroxybutyrate oxidation than of succinate oxidation. In a separate series of experiments performed spectrophotometrically at 340 nm, it was found that clofibrate did not inhibit the reduction of NAD via β-hydroxybutyrate dehydrogenase when respiration was inhibited by rotenone.

Effect of clofibrate on mitochondrial protein and nucleotide content. The content of pyridine nucleotides and of protein was determined under conditions similar to those used for the measurements of respiration. Rat liver mitochondria (3 ml of suspension containing 16·9 mg protein/ml) were incubated for 5 min at 30° in 26·4 ml medium (see Methods) which, in addition to the standard components, contained 10 mM β-hydroxybutyrate and 1% propylene glycol or propylene glycol plus clofibrate. After

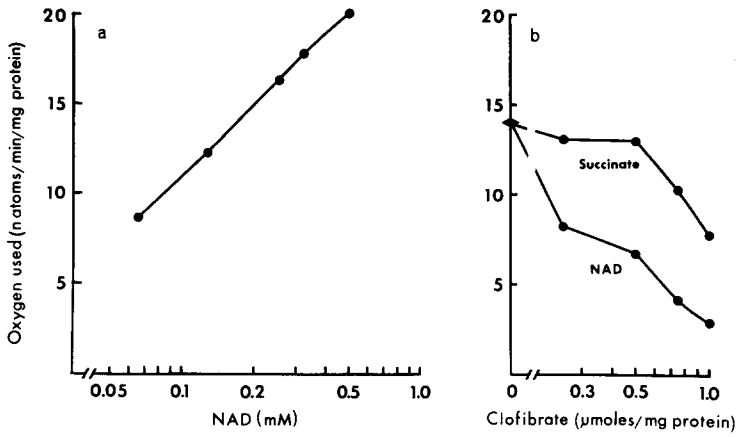

FIG. 5. Respiration of mitochondria which were exposed to two cycles of freezing and thawing. Mitochondrial protein concentration was 5·9 mg/ml. (a) β-Hydroxybutyrate oxidation as a function of the NAD concentration; (b) β-hydroxybutyrate and succinate oxidations, in the presence of 0·18 mM NAD, as a function of the clofibrate concentration.

5 min, the 29·4-ml samples were poured into tubes and centrifuged at 15,000 *g* at 4° for 5 min. The supernatant was poured off and discarded, and the mitochondrial pellet was saved for determinations of protein,[36] NAD[34] and NADH.[35] It was found (Fig. 6) that progressive increases in the clofibrate concentration from 0·15 to 0·6 μmole/mg of protein caused progressively larger depletions of oxidized and reduced NAD and of protein.

Clofibrate-induced mitochondrial swelling. It has been shown that a variety of agents, when added *in vitro* to mitochondrial suspensions, cause absorbance to decrease, and these decreases have been correlated with mitochondrial swelling.[42–44] Clofibrate was tested as a potential swelling agent in several experiments and the results are summarized in Fig. 7. Clofibrate produced large-amplitude decreases in absorbance when added at concentrations between 0·05 and 0·4 μmole/mg. Swelling

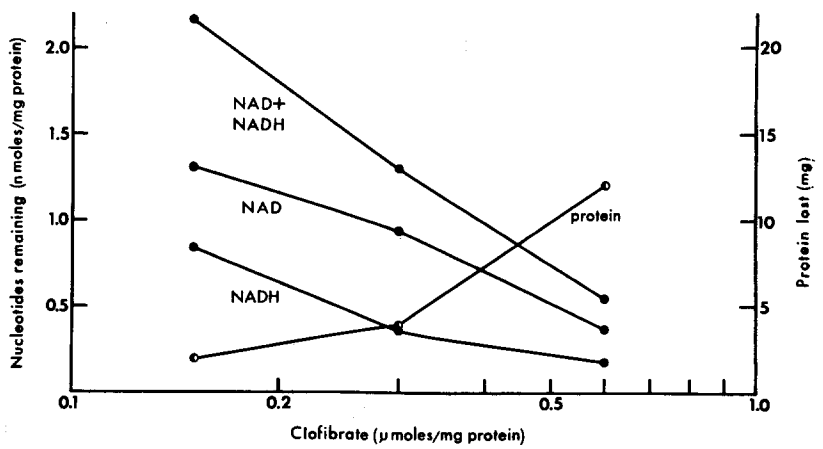

FIG. 6. Effects of clofibrate on mitochondrial protein and pyridine nucleotides. Propylene glycol (1%, v/v) was the vehicle. The reaction mixture (29·4 ml) contained 50·7 mg mitochondrial protein.

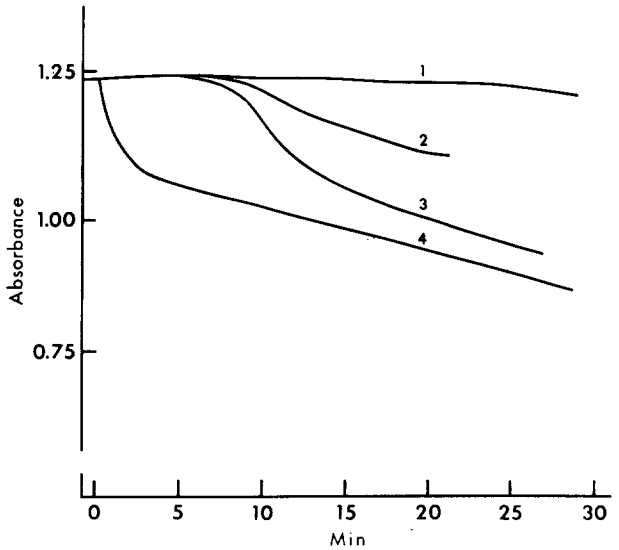

FIG. 7. Effects of clofibrate on absorbance of mitochondria at 520 nm wavelength. Mitochondrial protein concentration was 0·67 mg/ml. Propylene glycol (1%, v/v) was the vehicle. Clofibrate concentrations: (1) no clofibrate, (2) 0·05 μmole/mg of protein, (3) 0·1 μmole/mg of protein, (4) 0·4 μmole/mg of protein.

occurred in the presence and absence of 15 μM DNP, 10 mM β-hydroxybutyrate or succinate, 3·3 nmoles rotenone/mg of protein, 3·3 μg antimycin A/mg of protein, and 6 mM ADP or ATP. Addition of 6 mM ATP, 15 min after clofibrate, did not produce a reversal of swelling.

Effects of the vehicle on clofibrate-mediated responses. Clofibrate produced the same qualitative effects on the various mitochondrial parameters when either ethanol or propylene glycol was used as the vehicle. However, quantitatively, clofibrate was more active when added as an ethanolic solution. An example of this effect is presented in Fig. 8 where it can be seen that inhibition of state 3 respiration with both

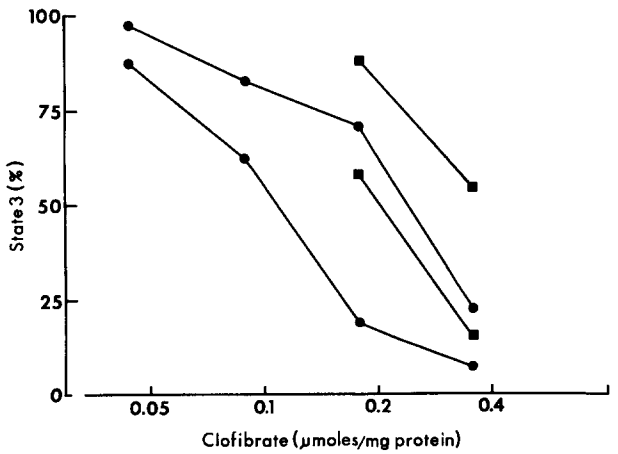

FIG. 8. Effects of the vehicle on inhibition of state 3 respiration by clofibrate. Closed circles, ethanol vehicle (1%, v/v); upper tracing, succinate oxidation; lower tracing, β-hydroxybutyrate oxidation. Squares, propylene glycol vehicle (1%, v/v); upper tracing, succinate oxidation; lower tracing, β-hydroxybutyrate oxidation.

β-hydroxybutyrate and succinate as substrates required twice as much clofibrate when propylene glycol was used as the vehicle.

Effects of clofibrate and p-*chlorophenoxyisobutyrate* (*CPIB*) *on state* 3 *respiration.* Clofibrate is rapidly converted to CPIB, *in vivo*, and does not accumulate to an appreciable degree in tissues.[5] Therefore, it was of interest to compare the effects of clofibrate and CPIB on mitochondrial respiration and oxidative phosphorylation. State 3 responses, with both β-hydroxybutyrate and succinate as substrates, are shown in Fig. 9. We found that CPIB inhibited respiration and uncoupled oxidation from phosphorylation in the concentration range of 0·56–6·3 μmoles/mg of protein (i.e. 1·25–10 mM). These concentrations were more than 10-fold those required of clofibrate.

Kurup *et al.*[13] previously reported that CPIB does not inhibit respiration or oxidative phosphorylation. However, Panini[19] has also reported inhibition and uncoupling by CPIB. The inhibitory effects of CPIB and clofibrate are probably not produced via the same mechanism because the CPIB inhibition can be reversed by adding DNP.[19] DNP does not reverse the clofibrate-induced inhibition of respiration.[21]

Effects of clofibrate on blood lipids and glucose, growth and liver weight. Clofibrate was administered orally, as described in Methods, at 100 and 300 mg/kg/day for 1 week. As shown in Table 1, the 100 mg/kg dose significantly lowered the serum triglyceride level, but did not alter the levels of cholesterol, free fatty acid and glucose. At 300 mg/kg, clofibrate also lowered the levels of cholesterol and free fatty acid.

As shown in Table 2, clofibrate produced liver enlargement, and increased the liver weight:body weight ratio at 300 mg/kg; however, these effects were not observed at 100 mg/kg. Clofibrate did not affect the rate of weight gain at either 100 or 300 mg/kg.

Effects of clofibrate and p-*chlorophenoxyisobutyrate* (*CPIB*) *on state* 3 *respiration.* Livers of the rats used for the experiments described in Table 1 were quickly removed and weighed, and 5-g pieces were utilized for the isolation of mitochondria.

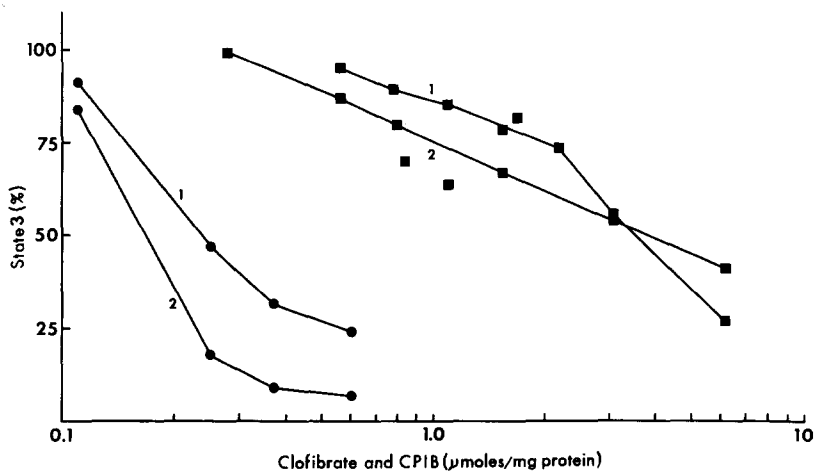

FIG. 9. Effects of clofibrate (closed circles) and CPIB (squares) on state 3 oxidation of (1) succinate and (2) β-hydroxybutyrate. Ethanol (1%, v/v) was the vehicle.

TABLE 1. EFFECT OF CLOFIBRATE ON BLOOD SERUM LEVELS OF TRIGLYCERIDE, CHOLESTEROL, FREE FATTY ACID AND GLUCOSE*

Rat treatment	Triglyceride† (mg/100 ml)	Cholesterol† (mg/100 ml)	Free fatty acid†,‡ (μequiv/l.)	Glucose† (mg/100 ml)
PEG 400	93 ± 8·7[a]	54 ± 2·3	422 ± 31·6	150 ± 2·6
+ Clofibrate (100 mg/kg)	54 ± 5·7[a]	46 ± 3·2	392 ± 40·2	146 ± 2·8
PEG 400	108 ± 11·2[b]	58 ± 2·2[a]	273 ± 24·2[a]	138 ± 3·2
+ Clofibrate (300 mg/kg)	68 ± 4·4[b]	50 ± 2·6[a]	182 ± 24·2[a]	144 ± 2·6

* Clofibrate was dissolved in polyethylene glycol 400 (PEG 400); each rat received 1 ml/kg of the appropriate solution daily via oral intubation. On the day of sacrifice, rats were killed 1 hr after dosing.

† All values represent the mean ± S.E.M. of eight rats. Values in each column with the same superscript are significantly different ($P < 0.05$).

‡ The values were not corrected for possible contribution of CPIB to the fatty acids as determined by the procedure of Dalton and Kowalski.[33]

Respiratory rates in states 3 and 4, respiratory control ratios and ADP:O ratios were determined for the mitochondria from the four groups of rats.

It was found that clofibrate at 100 and 300 mg/kg produced significant increases in the amount of protein recovered in the mitochondrial fraction (Fig. 10). However, when results were expressed per mg of protein, there were no differences in the several respiratory parameters between mitochondria from control and treated rats (Table 3).

Respiration of tissues from clofibrate-treated rats. The effects of clofibrate on the oxygen consumption of liver, kidney, epididymal fat pad, rectus abdominus muscle and intestine were determined as described in Methods. The results of these experiments are shown in Table 4. The oxygen uptake of liver slices was slightly, but significantly, elevated by the 7-day oral pretreatment with both the 100 and 300 mg/kg daily doses of clofibrate. Rectus abdominus muscle respiration appeared to be slightly depressed by the 300 mg/kg dose, but this effect was not statistically significant. Respiration of fat pad, kidney and intestine was not altered by clofibrate administration.

TABLE 2. EFFECT OF CLOFIBRATE ON WEIGHT GAIN, LIVER WEIGHT AND LIVER WEIGHT:BODY WEIGHT RATIO*

Rat treatment	Wt gain (g)	Liver wt (g)	Liver wt:Body wt
PEG 400	47 ± 3·3	10·9 ± 0·30	0·047 ± 0·001
+ Clofibrate (100 mg/kg)	46 ± 2·8	10·4 ± 0·38	0·043 ± 0·001
PEG 400	43 ± 2·4	9·2 ± 0·55[a]	0·042 ± 0·002[a]
+ Clofibrate (300 mg/kg)	39 ± 2·9	13·2 ± 0·40[a]	0·060 ± 0·001[a]

* The rats were those used for the experiments described in the legend of Table 1. The weight gains are for the 7 days of drug administration. All values represent the mean ± S.E.M. of eight rats. Values in each column with the same superscript are significantly different.

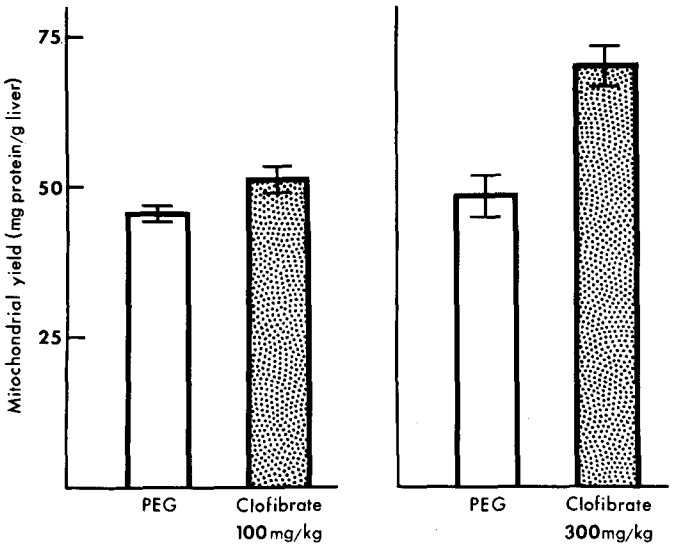

FIG. 10. Effects of oral administration of clofibrate to rats (100 and 300 mg/kg/day, for 1 week) on the amount of mitochondrial protein isolated from the liver.

Oxygen consumption of liver slices was also determined for rats which received a single oral dose of clofibrate (not diluted with vehicle) at 100 and 300 mg/kg. The rats were killed at 0·25, 0·5, 1, 2, 6 and 16 hr after receiving drug. In this experiment, we did not detect any effect of clofibrate on oxygen consumption.

DISCUSSION

Studies in vitro. Previous experimentation in our laboratory[21] has indicated that there are at least two distinct sites at which clofibrate can inhibit mitochondrial respiration. One site was postulated to exist between the interaction of NADH with NADH dehydrogenase and the point at which electrons from succinate oxidation enter the respiratory chain, and a second, less sensitive site, between the interaction

TABLE 3. EFFECT OF CLOFIBRATE *in vivo* ON MITOCHONDRIAL RESPIRATION AND OXIDATIVE PHOSPHORYLATION*

Rat treatment	Substrate (10 mM)	State 3 (n-atoms oxygen/min/mg protein)	State 4	R.C. ratio†	ADP:O
PEG 400	β-Hydroxybutyrate	57·4 ± 4·58	9·46 ± 0·67	6·06 ± 0·23	2·92 ± 0·063
	Succinate	163 ± 11·9	25·2 ± 1·37	6·44 ± 0·18	1·89 ± 0·026
+ Clofibrate	β-Hydroxybutyrate	60·2 ± 2·53	9·78 ± 0·413	6·18 ± 0·199	2·87 ± 0·049
(100 mg/kg)	Succinate	155 ± 9·48	26·1 ± 1·27	5·96 ± 0·215	1·88 ± 0·026
PEG 400	β-Hydroxybutyrate	53·0 ± 2·60	9·08 ± 0·381	5·88 ± 0·029	2·92 ± 0·077
	Succinate	152 ± 5·42	21·8 ± 0·769	7·01 ± 0·146	2·15 ± 0·023
+ Clofibrate	β-Hydroxybutyrate	50·6 ± 2·22	8·52 ± 0·332	5·98 ± 0·31	3·00 ± 0·056
(300 mg/kg)	Succinate	146 ± 2·67	19·8 ± 0·596	7·40 ± 0·149	2·12 ± 0·047

* Experimental details are given in the text. Mitochondria were isolated from livers of the rats used for the experiments described in the legend of Table 1. Values represent the mean ± S.E.M. of eight rats.
† Respiratory control ratio.

TABLE 4. RESPIRATION OF SEVERAL RAT TISSUES AFTER ADMINISTRATION OF CLOFIBRATE AT 100 AND 300 mg/kg*

Rat treatment	Liver	Intestine	Kidney	Rectus abdominus muscle	Epididymal fat pad
		(ml O_2 consumed/hr/g wet wt)			
PEG 400	3·11 ± 0·090[a]	1·71 ± 0·067			0·70 ± 0·053
+ Clofibrate (100 mg/kg)	3·46 ± 0·097[a]	1·83 ± 0·077			0·75 ± 0·090
PEG 400	3·01 ± 0·17[b]	1·36 ± 0·10	4·32 ± 0·22	1·54 ± 0·13	
+ Clofibrate (300 mg/kg)	3·53 ± 0·12[b]	1·30 ± 0·066	3·97 ± 0·19	1·22 ± 0·074	

* The rats used for these experiments were those described in the legend of Table 1. All values represent the mean ± S.E.M. of eight rats. Values in each column with the same superscript are significantly different ($P < 0·05$).

of succinate with succinate dehydrogenase and cytochrome c. These conclusions were based primarily on results similar to those presented in Fig. 1a, which were drived from experiments utilizing intact rat liver mitochondria. An additional experiment included in the original report, utilizing sonicated mitochondria, showed that respiration which was coupled to the oxidation of succinate and NADH was also inhibited by clofibrate. However, since the sonicated mitochondria maintained a faster oxidation of NADH than of succinate, we could not suggest the presence of a specific site of inhibition proximal to cytochrome b. In other experiments, we found that frozen–thawed mitochondria behaved similarly to the sonicated mitochondria. In the experiments summarized in Fig. 5, the rate of β-hydroxybutyrate oxidation by frozen–thawed mitochondria was adjusted to equal that of succinate oxidation by varying the concentration of NAD. It was observed that β-hydroxybutyrate oxidation was inhibited at lower concentrations of clofibrate than were required to inhibit succinate oxidation and, therefore, we can suggest the existence of an inhibitory site proximal to cytochrome b.

Clofibrate has been shown to be an uncoupler of oxidative phosphorylation when added to liver mitochondria *in vitro*.[21] However, site specificity for this effect has not been previously noted. As shown in Fig. 1b, clofibrate produced uncoupling only at sites II and III but not at site I. Specific effects at sites II and III have been reported for aliphatic biguanides[45] and for amyl azide,[46] but the classical uncoupler, DNP, appears to act only at sites I and III.[39] The mechanism by which clofibrate uncouples oxidative phosphorylation is entirely unknown; however, since mitochondria were found to swell after administration of clofibrate (Fig. 7), it is possible that structural alterations within the membrane system cleaved sites II and III, but not site I, from the respiratory chain.

The accumulation of Ca^{2+} by mitochondria is an energy-dependent process which is coupled to energy utilization at the level of the respiratory chain.[40,41,47,48] When Ca^{2+} is added to intact mitochondria which are oxidizing NAD-linked substrates in state 4, there is an abrupt increase of oxygen consumption, which represents an energy-dependent uptake of Ca^{2+} followed by a decrease. This secondary respiratory depression is caused by a depletion of mitochondrial pyridine nucleotides and can be reversed by adding NAD[41] (Fig. 3). As shown in Fig. 3, the inhibitory effects of

low concentrations of clofibrate on Ca^{2+}-stimulated respiration of intact liver mitochondria were also reversed by adding NAD. This finding suggested that clofibrate could inhibit respiration by causing a loss of nucleotides. It is of interest that this type of inhibition occurred at concentrations which were lower than those required to inhibit at the previously established sites. Subsequent experiments (Fig. 4) revealed that inhibition of state 3 respiration at the lower clofibrate concentrations, with β-hydroxybutyrate as substrate, could also be reversed by adding NAD. Finally, direct measurements proved that clofibrate caused a loss of pyridine nucleotides and protein (Fig. 6).

It is not known how the loss of both the pyridine nucleotides and protein was brought about, but it was almost certain that clofibrate altered the integrity of the mitochondrial membranes. This was clearly evident from the results of Fig. 7 which show that clofibrate caused non-energy-linked, irreversible swelling. The relationship betwen swelling produced by clofibrate and the inhibition of respiration, which was reversed by adding NAD, is not clear, since swelling *per se* does not necessarily lead to depletion of pyridine nucleotides. As shown by Vinogradov *et al.*,[41] swelling induced by valinomycin and K^+ did not inhibit the NAD-linked oxidation of glutamate and malate.

Studies in vivo. In agreement with previous reports (as cited), we found that clofibrate (100 and 300 mg/kg/day, p.o., for 1 week) lowered the blood levels of triglyceride,[3] cholesterol[3] and free fatty acids;[49] increased liver size[7,8] and oxygen consumption per g of liver;[15,50] and increased the yield of mitochondria per g of liver.[13] Also, we found that respiration and oxidative phosphorylation of liver mitochondria were not inhibited. However, unlike the findings of Kurup *et al.*,[13] we did not observe an increase in the ADP:O or respiratory control ratio after clofibrate treatment, nor did we have any indication that mitochondria from the treated rats were more refractory to damage during the isolation procedure.

Our results permit us to conclude that the hypolipidemic effects of clofibrate are not mediated through a direct effect of clofibrate or CPIB on oxidative phosphorylation; if this were not the case, we would have observed a decrease in the rate of liver slice respiration rather than in increase. The data on liver slices (Table 4) were most important in allowing us to arrive at this conclusion. Previous experiments,[15,50] which also showed increased liver slice respiration, were performed in buffered aqueous media and, since CPIB, the free-acid metabolite of clofibrate, is soluble in water, it was possible that inhibition could have been washed out during incubation. Our technique, modified from that of Huston and Martin,[26] obviated this possibility because an aqueous medium was not used. Effects produced by clofibrate (in contrast to CPIB) would not have been washed out during mitochondrial isolation procedures or during incubation of slices in aqueous medium because these effects appeared to be irreversible. It is possible that clofibrate concentrations in the liver were not high enough to produce the alterations that we observed *in vitro*.

The capacity for drug metabolism, which generally occurs in the liver, is usually lowest upon the first administration of a drug and is progressively increased, to a maximum, by repetitive administration. It was expected that the most pronounced and direct effects of clofibrate and CPIB would occur shortly after administration of the first dose. However, we found that single oral doses of clofibrate, at 100 and 300 mg/kg, did not produce detectable effects on liver slice respiration over the first

16 hr. It is most likely that we did not use doses which were high enough to produce an effect. Kaneko *et al.*[51] have reported that daily administration of clofibrate (1 g/kg, p.o.) for 7 days causes a decrease in liver succinic oxidase activity which persists for 12 hr after the initial dose but does not recur thereafter. This decrease was accompanied by swelling, elongation and deformation of the mitochondria.

Ruegamer *et al.*[16] have reported that the livers of CPIB-treated animals become hyperthyroid with respect to metabolic changes, such as increased oxygen consumption, while the animals remain euthyroid. These findings are in accord with the results of our rat tissue metabolism studies (Table 4), which showed oxygen consumption to be increased in liver but not in kidney, skeletal muscle, intestine and fat pad.

It is not clear what role an increased content of liver mitochondria might play in stimulating liver respiration. Kurup *et al.*[13,52] have shown that clofibrate increases the content of mitochondria in both liver and kidney, but we have found an increased rate of respiration only in the liver. Furthermore, in an experiment in which rat hepatic mitochondrial content was not increased by clofibrate treatment, Kähönen *et al.*[50] have observed increased liver slice respiration. It is probable that thyroxine did not cause the rise in mitochondrial content, since clofibrate administration,[13] but not thyroxine administration,[53] has been shown to increase the ratio of mitochondrial protein: total protein in the liver.

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