Protection of rats by clofibrate against the hypoglycaemic and toxic effects of hypoglycin and pent-4-enoate

An ultrastructural and biochemical study

François VAN HOOFF, Louis HUE,* Joseph VAMECQ and H. Stanley A. SHERRATT†
Laboratoire de Chimie Physiologique, Université de Louvain and International Institute of Cellular and Molecular Pathology, UCL 75.39, avenue Hippocrate 75, B-1200 Brussels, Belgium

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1. An ultrastructural and biochemical study of the toxic and hypoglycaemic effects of hypoglycin and pent-4-enoate was made on the livers of normal and clofibrate-fed rats. 2. Injection of hypoglycin to rats doubles (from 22% to 44%) the volume fraction of mitochondria and decreases (from 1.05% to 0.26%) the volume fraction of peroxisomes in hepatocytes. The fast-acting toxin pent-4-enoate causes few ultrastructural changes except for the accumulation of lipids. 3. In male adult rats fed with 0.5% clofibrate in their diet for 1–2 months, the volume fraction occupied by peroxisomes and mitochondria in hepatocytes rose to 6.26% and 29.5% respectively. Clofibrate feeding apparently protected the animals against the toxic, hypoglycaemic and hypothermic effects of hypoglycin and of pent-4-enoate, and completely prevented the ultrastructural damage caused by hypoglycin. 4. After hypoglycin administration, hepatic mitochondrial butyryl-CoA dehydrogenase activity was inhibited by more than 90% and, surprisingly, the activity of the peroxisomal enzymes studied was largely preserved. 5. When hypoglycin was given to rats fed on a clofibrate-containing diet, the oxidation of decanoylcarnitine, which was incomplete after hypoglycin treatment alone, remained incomplete with uncoupled mitochondria, but became apparently complete with coupled mitochondria. In the latter condition, there was a slowing of the rate during the last quarter of the pulse of oxygen uptake. Further, butyryl-CoA dehydrogenase activity was much less affected by hypoglycin in clofibrate-fed animals. 6. Pent-4-enoate does not inhibit β -oxidation in coupled mitochondria from clofibrate-treated rats.

Hypoglycin (2-amino-3-methylenecyclopropyl-propionic acid) is the toxic hypoglycaemic principle of the Jamaican ackee fruit, *Blighia sapida* (see Sherratt, 1969). Its active metabolite, methylenecyclopropylacetyl-CoA, inhibits several, but not all, acyl-CoA dehydrogenases (Billington *et al.*, 1978a; Wenz *et al.*, 1981). Inhibition of butyryl-CoA dehydrogenase (EC 1.3.99.2) decreases both the rate and the extent of the β -oxidation of long-

chain fatty acids, which are only oxidized as far as butyrate. In rats, large concentrations of isovalerate and 2-methylbutyrate are found in plasma after inhibition of isovaleryl-CoA dehydrogenase (EC 1.3.99.10) and 2-methylbutyryl-CoA dehydrogenase (Tanaka, 1972). The hypoglycin-induced hypoglycaemia results from the impairment of gluconeogenesis at the level of pyruvate carboxylase, which is inhibited by the intramitochondrial accumulation of acyl-CoA esters (Sherratt, 1981). Another hypoglycaemic compound, pent-4-enoate, often considered as an analogue of methylenecyclopropylacetate, inhibits β -oxidation at a different site (see Sherratt, 1981; Sherratt *et al.*, 1985). One of its metabolites, 3-oxopent-4-enoyl-CoA, inhibits 3-oxoacyl-CoA thiolase (EC 2.3.1.9) (Schulz, 1983). Gluconeogenesis is also inhibited

* Present address: Hormone and Metabolic Research Unit, Université de Louvain and International Institute of Cellular and Molecular Pathology, UCL 75.29, avenue Hippocrate 75, B-1200 Brussels, Belgium.

† Permanent address: Department of Pharmacological Sciences, Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 2HH, U.K.

by pent-4-enoate, which, however, does not cause a branched-chain organicacidaemia (Billington *et al.*, 1978b). The onset of hypoglycaemia caused by hypoglycin may take several hours and lasts for up to a further 20 h, whereas that caused by pent-4-enoate is more rapid (1–2 h) and glycaemia remains low for only 2–3 h (Billington *et al.*, 1978b).

The administration of hypoglycaemic doses of hypoglycin caused ultrastructural changes in rat liver: gross swelling of mitochondria, loss of dense matrix granules and presence of autophagic vacuoles containing mitochondria (Brooks & Audretsch, 1970, 1971a,b, 1975). In parallel with a study of the effects of hypoglycin on carbohydrate metabolism (L. Hue & H. S. A. Sherratt, unpublished work), the livers from rats given hypoglycin were examined by electron microscopy. It was noticed that, in addition to the changes previously seen by Brooks & Audretsch (1975), the number of peroxisomes in hepatocytes was decreased by approx. 70%. It was therefore decided to give hypoglycin and pent-4-enoate to rats that had been fed with the hypolipidaemic drug clofibrate [Atromid S; ethyl 2-(4-chlorophenoxy)isobutyrate], since it is known that clofibrate causes in hepatocytes a large increase in both the volume fraction of peroxisomes and the activity of peroxisomal β -oxidation of fatty acids (Lazarow & de Duve, 1976; Lazarow, 1978; Mannaerts *et al.*, 1978). It was found that clofibrate gave very efficient protection against the toxic, hypoglycaemic and hypothermic effects of hypoglycin and pent-4-enoate. Parts of this work have been presented at various scientific meetings (Van Hoof *et al.*, 1979, 1980a,b).

Materials and methods

Materials

Hypoglycin was prepared from ackee seeds as described by Billington & Sherratt (1981). Pent-4-enoate was from Fluka, Buchs, Switzerland. Butyryl-CoA and decanoyl(–)-carnitine were prepared and characterized as described by Holland *et al.* (1973) and by Holland & Sherratt (1973) respectively. Clofibrate and nafenopin were gifts from Imperial Chemical Industries Ltd. and Ciba-Geigy respectively. Other biochemicals were obtained from Boehringer Corp. or Sigma Chemical Co.

Animals

Male albino Wistar rats (approx. 200 g at the start of the experiments) were used. They were fed on a solid diet made by mixing 1 kg of powdered laboratory-animal chow with 500 ml of a warm solution of 10% (w/v) gelatin in water, with or

without 5 ml of clofibrate. Rats were maintained with a 12 h-light/12 h-darkness cycle.

Design of the experiments

All animals were starved at least for 24 h before being killed, and were injected intraperitoneally with hypoglycin (80 mg/kg body wt.) after 9 h of fasting, or with pent-4-enoate (300 mg/kg body wt.) or with 0.15 M-NaCl, after 23 h of fasting. After anaesthesia (Nembutal, 60 mg/kg intraperitoneally) the rectal temperatures were measured, the abdomen was opened, blood samples were taken for measurement of blood glucose concentrations, and samples of liver were directly taken for electron microscopy, for the preparation of mitochondrial fractions, or were freeze-clamped for further biochemical studies.

Electron microscopy and morphometry

Liver samples were either fixed directly in 2% (v/v) OsO₄ or prefixed in 2% (v/v) glutaraldehyde. After dehydration, they were embedded either in Epon or in the mixture described by Spurr (1969). Ultrathin sections, stained with uranyl acetate and lead citrate, were examined with a Philips EM-301 electron microscope. The morphometric analysis of micrographs taken at a constant magnification of $\times 18000$ was performed by the method and with the computer program designed by Baudhuin (1974). Additional description of this technique has been reported (Aubert-Tulkens *et al.*, 1979).

Preparation of mitochondrial fractions

The livers were homogenized in 5 vol. of 0.25 M-sucrose/2 mM-Hepes/0.1 mM-EDTA, at pH 7.2. The homogenate was centrifuged at 0–4°C for 3 min at 1000 g, and the supernatant was then centrifuged at 17300 g for 5 min to sediment the mitochondrial fraction (mitochondria, lysosomes, peroxisomes). This fraction was washed once, and finally suspended in the homogenization medium to give 40 mg of protein/ml. Mitochondrial fractions were kept at 0°C and used within 4 h.

Measurement of mitochondrial β -oxidation

Oxygen uptake by mitochondrial fractions was measured polarographically at 30°C and pH 7.2 in a final volume of 2.3 ml containing 1–5 mg of protein, 120 mM-KCl/5 mM-Hepes/5 mM-MgCl₂/2.5 mM-potassium phosphate/1 mM-EDTA and 5 mM-malonnate to prevent oxidation of acetyl-CoA. Decanoylcarnitine (20 μ M) was used as a substrate. To obtain coupling conditions 0.5 mM-ADP was also added, and for uncoupling conditions, 20 μ M-2,4-dinitrophenol and 10 mM-arsenate were included (Holland & Sherratt, 1973). During acylcarnitine oxidation, endogenous respiration is apparently suppressed (Lopez-Cardozo & van den

Bergh, 1972; Osmundsen & Sherratt, 1975). Under these conditions, fatty acyl groups are quantitatively oxidized to acetoacetate or to acetoacetate plus butyrate, and the oxygen uptake is a direct measure of β -oxidation (Sherratt & Osmundsen, 1976). The reaction may be written:

and when butyryl-CoA dehydrogenase is inactivated by hypoglycin metabolites:

The oxygen uptake by all mitochondrial fractions was completely inhibited by 1 mM-NaCN.

Extraction and assay of acyl-CoA dehydrogenase

Freeze-clamped livers or pelleted mitochondrial fractions were homogenized at 0°C in 4 vol. of 1.0M-KCl/40mM-potassium phosphate (pH 7.4)/0.5% (v/v) Triton X-100, and the resulting extracts were centrifuged at 150000g_{av}. for 60 min. The supernatant contained the acyl-CoA dehydrogenases, and some dispersed lipids, which interfered with the assays. These lipids were extracted by four washes with an equal volume of peroxide-free ether. The apparent acyl-CoA dehydrogenase activity (EC 1.3.99.-) was assayed with butyryl-CoA as a substrate, as described by Holland *et al.* (1973).

Assay of peroxisomal enzymes

Peroxisomal enzymes were assayed in freeze-clamped liver samples. Catalase activity (EC 1.11.1.6) was assayed by the titanium sulphate procedure (Baudhuin *et al.*, 1964). Urate oxidase (EC 1.7.3.3) and other H₂O₂-generating oxidases were assayed in the presence of 80mM-glycylglycine buffer, pH 8.3, by following fluorimetrically the production of H₂O₂ as described by Vamecq & Van Hoof (1984). The substrates were 200mM-D-proline, 0.15mM-palmitoyl-CoA and 5mM-glycolate for D-amino acid oxidase (EC 1.4.3.3), palmitoyl-CoA oxidase (EC 1.1.3.-) and glycolate oxidase (EC 1.1.3.1) respectively.

Standard methods

Glucose was measured by the glucose oxidase method (Huggett & Nixon, 1957), and protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Effects of clofibrate feeding on the toxicity of hypoglycin and of pent-4-enoate

In all animals fed on a normal diet, the administration of hypoglycin caused severe pros-

tration, hypoglycaemia (blood [glucose] = 1-3 mM) and hypothermia (rectal temperature ≤ 30°C). The dose given was in the region of the LD₅₀ for the strain of rats. The dose of pent-4-enoate injected was generally fatal within 60min in fasted rats.

Animals given 0.5% clofibrate in their diet appeared normal, except for a smaller gain of weight (15% less than controls after 2 weeks). The protective effect of clofibrate (feeding for 3 weeks or more) was striking: animals injected with hypoglycin or pent-4-enoate appeared virtually normal, and the hypoglycaemic and hypothermic effects of these toxins were completely prevented (blood [glucose] ≥ 5mM, rectal temperature > 35°C). The development of resistance to pent-4-enoate is shown in Table 1. It suggests that individual animals became resistant at different times, and that either they died or they survived without apparently showing any symptom. When after 18 days clofibrate feeding was discontinued, it took about 2 weeks before sensitivity to pent-4-enoate was restored in all animals, again with an apparent all-or-nothing response (Table 1). In a preliminary experiment, feeding another hypolipidaemic drug, nafenopin {2-methyl-2-[4-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionic acid}, which also causes peroxisomal proliferation (Stäubli *et al.*, 1977), protected rats against the toxic and morphological effects of hypoglycin (results not shown).

Ultrastructure and morphometry (Table 2, Figs. 1 and 2)

Effects of clofibrate. Clofibrate feeding for 1 or 2 months induced the following changes in parenchymal cells. There was a 6-fold increase in the volume fraction occupied by peroxisomes. Both the number (0.110 to 0.272 per μm³ of hepatocyte)

Table 1. Development and persistence of the resistance to pent-4-enoate toxicity in animals fed with clofibrate
The first rat was fasted for 24h after having been given the clofibrate-containing diet for 1 day, and was then given 0.2M-sodium pent-4-enoate (300 mg/kg body wt.) intraperitoneally. Control rats were also fasted for 24h and matched for age and weight. The administration of clofibrate was discontinued after 18 days and rats were fed on a control diet.

No. of days after start of the experiment	No. of deaths/no. of animals given pent-4-enoate	
	Control diet	Clofibrate-fed
2-5	1/1	4/5
6-10	2/2	3/10
11-18	1/1	1/6
28-29		2/4
32-35		3/3

Fig. 1. Morphometric analysis of the effect of clofibrate, hypoglycin, pent-4-enoate or combined treatments on the peroxisomal population in hepatocytes

The upper part of each histogram shows the distribution of the section profiles, as observed on the micrographs, with the minimal corrections made by the calculation procedure to account for the thickness of the section (black, negative correction), and for the lack or recognition of the smallest profiles, which are polar or tangential sections through peroxisomes (white, positive correction). The distribution of the actual sizes of the peroxisomes appears in the lower part of each histogram as a percentage of the population of these organelles.

and the average volume (0.094 to $0.207 \mu\text{m}^3$) of peroxisomes were increased. Further, there was an increase in the volume fraction of mitochondria (+33%), and lipid droplets practically disappeared

from hepatocytes, but not from the other hepatic cells (results not shown).

Effects of hypoglycin. The most striking effects caused by administration of hypoglycin to rats

Fig. 2. Ultrastructure of hepatocytes in (a) untreated fasted rat, (b) fasted rat given hypoglycin 17 h before death, (c) clofibrate-fed rat, (d) a clofibrate-fed rat given hypoglycin

In (b), only two peroxisomes are visible (arrows), one within an autophagic vacuole. In (d), the paler aspect of the peroxisomal matrix is due to a different embedding (Epon). The four micrographs are at the same magnification ($\times 8650$).

were swelling of mitochondria, decrease in the number of peroxisomes and an increased number and size of fat droplets. Peroxisomes were scarce, but they usually appeared normal.

In clofibrate-fed animals which resisted the effects of hypoglycin, the liver ultrastructure remained unchanged, except for rare images of stacking of mitochondrial cristae. This protective effect was, however, only consistently obtained when the treatment with clofibrate was longer than 1 month. In an experiment in which two clofibrate-fed animals had only increased the volume fraction of their liver peroxisomes to 2.40% and 2.64% respectively (results not shown in Table 2), administration of hypoglycin, although failing to elicit any clinical symptom, increased the volume fraction occupied by fat droplets in hepatocytes from 0.21 to 5.27%, without change in the volume fraction of the mitochondria.

Effects of pent-4-enoate. Few obvious effects of pent-4-enoate on liver ultrastructure were seen except for a dramatic increase in the number, size and osmiophily of fat droplets in hepatocytes. As noted above, previous clofibrate feeding for a few weeks completely protected the animals but, in this case, with a 5-fold increase in the average volume of peroxisomes ($0.470 \mu\text{m}^3$).

Biochemical results

Butyryl-CoA dehydrogenase. The almost complete inhibition (90%) of butyryl-CoA dehydrogenase activity in the livers of normal rats given hypoglycin (Billington *et al.*, 1978a,b) was confirmed. There was a 2.4-fold increase in the specific activity of liver butyryl-CoA dehydrogenase as a result of clofibrate feeding. When hypoglycin was given to clofibrate-fed rats, butyryl-CoA dehydrogenase activity was lowered by only 50–70% and went back to control values

(1.54 ± 0.26 as compared with 1.40 units/g of liver in controls).

Effects of clofibrate feeding on the inhibition of mitochondrial β -oxidation by hypoglycin. The expected incomplete oxidation of decanoylcarnitine (75% of the theoretical value) by coupled or uncoupled liver mitochondria from untreated rats after administration of hypoglycin (Osmundsen & Sherratt, 1975) was confirmed. By contrast, coupled mitochondrial fractions from the livers of clofibrate-fed rats given hypoglycin displayed an unusual pattern of decanoyl-carnitine oxidation with biphasic kinetics (Figs. 3b and 5a). Indeed, decanoylcarnitine was apparently completely oxidized to acetoacetate, but with a progressive slowing of the rate towards the end of the pulse of oxygen uptake, which started after about 75% of the theoretical oxygen uptake had occurred (Fig. 3b); when uncoupled, the same mitochondrial preparations incompletely oxidized decanoylcarnitine (Fig. 4b).

Effects of clofibrate feeding on the inhibition of mitochondrial β -oxidation by pent-4-enoate. Uncoupled mitochondria were not used, since formation of pent-4-enoyl-CoA is necessary for the inhibition of β -oxidation (Holland & Sherratt, 1973). The inhibition (up to 85% in our experiments) by 1 mM-pent-4-enoate of the decanoylcarnitine oxidation in coupled mitochondrial fractions from untreated rat livers (Holland & Sherratt, 1973) was minimal or absent in mitochondrial fractions from clofibrate-fed rats (Fig. 6). Metabolites of pent-4-enoate do not inhibit butyryl-CoA dehydrogenase (results not shown; Billington *et al.*, 1978a). They affect only the rate, but not the stoichiometry, of oxygen consumption when fatty acids are being oxidized (Billington *et al.*, 1978a,b). Clofibrate feeding did not increase the very small oxygen uptake which follows the addition of pent-4-enoate

Table 2. *Quantitative ultrastructural analysis of hepatocytes from starved rats after various treatments*
Results are expressed as percentages of total cell volume. Extreme values from individual rats are given in brackets.
Numbers of animals (n) are given in parentheses.

	Peroxisomes	Mitochondria	Lipid droplets
Controls (n = 4)	1.05 [0.72–1.33]	22.2 [19.5–25.0]	0.92 [0.15–1.70]
Clofibrate (1–2 months) (n = 4)	6.26 [6.07–6.77]	29.5 [27.3–31.6]	<0.03 [0–0.05]
Hypoglycin (17 h) (n = 2)	0.26 [0.24, 0.285]	44.0 [40.1, 47.8]	4.40 [3.28, 5.51]
Pent-4-enoate (40 min) (n = 2)	1.08 [1.05, 1.10]	21.8 [21.3, 22.3]	4.68 [2.93, 6.42]
Clofibrate (2 months)+ hypoglycin (17 h) (n = 3)	6.25 [4.21–7.97]	29.3 [27.6–31.0]	<0.03 [0–0.05]
Clofibrate (2 months)+ pent-4-enoate (40 min) (n = 3)	6.72 [4.52, 8.02]	26.7 [24.5–30.9]	0.03 [0–0.08]

Fig. 3. *Stoichiometry of the oxidation of decanoylcarnitine by coupled liver mitochondria*

To obtain coupling conditions, 0.5 mM-ADP was added to the medium, or 20 μ M-2,4-dinitrophenol and 10 mM-arsenate for uncoupled mitochondria. Other experimental details are given in the Materials and methods section.

Fig. 4. *Stoichiometry of the oxidation of decanoylcarnitine by uncoupled liver mitochondria*
For details, see legend to Fig. 3.

to mitochondrial fractions, as reported by Holland & Sherratt (1973); see Fig. 6.

Peroxisomal enzymes. The activities of peroxisomal enzymes were little affected by hypoglycin: only D-amino acid oxidase was consistently less active (24–37% of controls); palmitoyl-CoA and urate oxidase activities were lower in most animals, but sometimes remained normal (42–106% of controls); glycolate oxidase and catalase were unaffected. On the other hand, feeding animals for 3 weeks with clofibrate induced larger changes in

the activity of some liver peroxisomal enzymes. D-Amino acid oxidase activity became lower (9–40% of controls); glycolate oxidase and urate oxidase were unaffected, and palmitoyl-CoA oxidase was stimulated between 3.5- and 11.4-fold; catalase activity was increased about 2-fold. Finally, when both clofibrate and hypoglycin treatment were combined, D-amino acid oxidase activity was lowered further (down to 2–30% of that in untreated animals). The increased activities of palmitoyl-CoA oxidase (3.7–9-fold) and of catalase

Fig. 5. Oxidation of decanoylcarnitine by mitochondrial fractions from the livers of clofibrate-fed rats given hypoglycin. For details, see legend to Fig. 3.

Fig. 6. Inhibition of decanoylcarnitine oxidation by pent-4-enoate in coupled mitochondria from rat liver. For details, see legend to Fig. 3.

(about 2-fold) caused by clofibrate were not affected by the further administration of hypoglycin to these animals.

Discussion

Feeding clofibrate induces several changes in the liver of rats. In addition to the induction of peroxisomes (for a review, see Reddy & Lalwani, 1983) and of peroxisomal β -oxidation (Lazarow, 1978), it increases the total capacity for mitochondrial β -oxidation (Mannaerts *et al.*, 1978), particu-

larly the oxidation of medium-chain and short-chain acyl-carnitine esters (Osmundsen *et al.*, 1980). The total liver content of CoA and carnitine is increased 3-fold (Mannaerts *et al.*, 1978) and the concentration of the cytosolic fatty-acid-binding Z-protein is doubled (Renaud *et al.*, 1978). These appear to be co-ordinated changes, which allow the liver to metabolize very-long-chain fatty acids more effectively (Osmundsen *et al.*, 1980).

We show here that hypoglycin toxicity is accompanied by a large diminution in the number of peroxisomes in hepatocytes. Clofibrate protects

animals against this toxin as well as against pent-4-enoate, but this protection is neither complete (organic aciduria is only partially prevented; H. S. A. Sherratt & R. R. Veitch, unpublished work) nor appears from our data to result primarily from the proliferation of peroxisomes.

Ultrastructural changes

The ultrastructural changes observed in the liver after hypoglycin treatment confirm the previous reports by Brooks & Audretsch (1970, 1971*a,b*, 1975), although we cannot explain the apparent swelling of the mitochondrial matrix. The decrease in the number of peroxisomes was not reported by these authors. As the half-life of peroxisomes is about 1.5–2 days (de Duve & Baudhuin, 1966), a 70% decrease in the peroxisomal population during 16–18 h cannot be accounted for by simple blockage of their biogenesis. Specific autophagy of peroxisomes might have occurred. This explanation has been evoked in two conditions, in a patient with a major liver metabolic imbalance (Landrieu *et al.*, 1982) and in the course of a continuous clofibrate treatment in mice (Vamecq & Van Hoof, 1984). In our experiments, only one animal out of seven displayed an apparent excess of peroxisomes in autophagic vacuoles. The simple explanation of autophagic destruction of peroxisomes would, however, not explain the persistence of the activity of most peroxisomal enzymes in the livers of rats treated with hypoglycin. Therefore, alternative explanations such as, for example, leaking or lysis of peroxisomal membranes must be considered.

The rapid toxicity of pent-4-enoate explains why little significant abnormality was detected in the hepatocytes, except for the prominent accumulation of very osmiophilic fat droplets, suggesting the presence of large amounts of unsaturated lipid. The same amount of lipid accumulated in rat liver within 40 min after pent-4-enoate and within 17 h after hypoglycin treatment (Table 2). Occasionally, a limited stacking of the cristae was observed in mitochondria from rats treated with pent-4-enoate. Similar stacking has been observed in hepatic mitochondria of rats given small doses of hypoglycin (10 mg/kg body wt.) for 10 days (Brooks & Audretsch, 1975) and in riboflavin-deficient rats (Sugiska *et al.*, 1969). The mitochondrial acyl-CoA dehydrogenases that are inactivated by hypoglycin metabolites are flavoproteins.

The administration of clofibrate induced the expected 6-fold increase in the peroxisomal population in hepatocytes, which was not altered by treatment with either hypoglycaemic toxin. It must be stressed, however, that morphometric analysis also disclosed an increase in the volume fraction of mitochondria which is of similar absolute magnitude (22.2 to 29.5%; Table 2). This increase also was

largely unaffected by the subsequent administration of either toxin.

Biochemical changes

Effects of hypoglycin. Incomplete oxidation of acyl groups after hypoglycin poisoning is thought to result from the inhibition of butyryl-CoA dehydrogenase by methylenecyclopropylacetyl-CoA (Osmundsen & Sherratt, 1975), which forms irreversible adducts with the FAD prosthetic group (Wenz *et al.*, 1981). Butyryl-CoA accumulates in the matrix and is thought to be hydrolysed by the high- K_m short-chain acyl-CoA hydrolase, allowing recycling of CoA and liberation of free butyrate. The total CoA content of normal rat liver mitochondrial fractions (about 2 nmol/mg of protein; Holland & Sherratt, 1973) is much less (at least 4–12 times) than the amounts of decanoylcarnitine shown to be oxidized in our experiments. This is also less than the maximum possible CoA content in mitochondria from clofibrate-fed rats (see Mannaerts *et al.*, 1978). β -Oxidation would have ceased unless butyryl-CoA formed was deacylated, allowing CoA to recycle (see Osmundsen & Sherratt, 1975), yet the extent of the second phase of oxygen uptake was always proportional to the amount of substrate added.

A remarkable finding was that coupled, but not uncoupled, liver mitochondria from hypoglycin-treated rats fed with clofibrate oxidized decanoylcarnitine to the same extent (probably to acetoacetate), but with a biphasic time course (Fig. 5). The kinetics of decanoylcarnitine oxidation suggest that, in addition to the classical butyryl-CoA dehydrogenase present, clofibrate feeding induces another butyryl-CoA dehydrogenase in the mitochondrial matrix which is not inactivated by methylenecyclopropylacetyl-CoA and which is apparently only active when the mitochondria are energized. Such an enzyme may represent the remaining butyryl-CoA dehydrogenase activity in the livers of clofibrate-fed rats given hypoglycin. Its activity is also assumed to be insufficient to metabolize all the butyryl-CoA as quickly as it is formed during the first phase of oxygen uptake, but its contribution would also account for the faster rate during this phase of oxygen uptake compared with uncoupled mitochondria (Fig. 5). One explanation considered for the second slower phase of oxygen uptake with coupled mitochondria is that free butyrate formed during the first rapid phase would be slowly reconverted into butyryl-CoA in the matrix by butyryl-CoA synthetase (EC 6.2.1.2), accounting for the slow oxidation of accumulated butyrate after the first phase was completed. However, this explanation is not tenable, as there was no additional oxygen uptake on adding either 20 μ M- (the amount that would be

formed from 20 μ M-decanoylcarnitine) or even 1.0 mM-butyrate to coupled mitochondrial fractions from clofibrate-fed rats or from clofibrate-fed rats given hypoglycin.

The mechanism of the partial protective effect of clofibrate feeding against inhibition of β -oxidation found *in vitro* remains unknown, but if this also occurs *in vivo* it could explain the protection against hypoglycin toxicity. Further evidence that clofibrate does not prevent all the changes caused by hypoglycin is that the massive and complex organicaciduria found in poisoned rats (Tanaka, 1972) is decreased, but not prevented, by clofibrate feeding (R. K. Veitch & H. S. A. Sherratt, unpublished work).

Hypoglycin had almost no effect on the activities of the liver peroxisomal enzymes that we measured. It also did not affect significantly the response to clofibrate of these enzymes. It must be stressed, however, that not all biochemical functions of the peroxisomes were investigated.

Effects of pent-4-enoate. Pent-4-enoate must first be converted into pent-4-enoyl-CoA in the mitochondrial matrix (Holland & Sherratt, 1973). With low concentrations of pent-4-enoate and physiological concentrations of CO_2 , pent-4-enoyl-CoA is metabolized by the pathway pent-4-enoyl-CoA $\rightarrow$ penta-2,4-dienoyl-CoA $\rightarrow$ pent-2-enoyl-CoA $\rightarrow$ 3-hydroxypentanoyl-CoA $\rightarrow$ 3-oxopentanoyl-CoA $\rightarrow$ CoA $\rightarrow$ propionyl-CoA + acetyl-CoA (Hiltunen *et al.*, 1978).

Pent-2-enoyl-CoA is formed in coupled mitochondria by a reduction by NADPH of penta-2,4-dienoyl-CoA catalysed by enoyl-CoA reductase (Borrebaek *et al.*, 1980). When this pathway is overloaded by high concentrations of pent-4-enoate, hydration of penta-2,4-dienoyl-CoA competes with reduction, to give 3-hydroxypent-4-enoyl-CoA, which is oxidized to 3-oxopent-4-enoyl-CoA, which inactivates 3-oxoacyl-CoA thiolase (EC 2.3.1.16) (Schulz, 1983). Borrebaek *et al.* (1980) showed that clofibrate feeding increases the specific activity of the NADPH-linked enoyl-CoA reductase 4-fold in rats, preventing the metabolism of penta-2,4-dienoyl-CoA to inhibitory products, since NADPH is formed in energized mitochondria by the energy-linked transhydrogenase (Borrebaek *et al.*, 1980). A further difference between poisoning by pent-4-enoate and by hypoglycin may be that 4-enoyl-CoA reductase is inhibited by hypoglycin metabolites (see Sherratt & Veitch, 1984).

Conclusions

The mechanisms of action of the hypoglycaemic toxins hypoglycin and pent-4-enoate have been extensively investigated (for references, see Sherratt & Osmundsen, 1976; Billington *et al.*, 1978a,b;

Billington, 1979; Sherratt, 1981; Sherratt *et al.*, 1985). However, their effects on intermediary metabolism are still only partly understood (L. Hue & H. S. A. Sherratt, unpublished work). The use of these toxins as tools revealed quite unexpected changes in the enzyme systems for oxidizing fatty acids in mitochondria after clofibrate feeding. Chronic feeding of very-long-chain fatty acids also causes some increase in peroxisomal β -oxidation, but less than that produced by clofibrate and its analogues (Osmundsen *et al.*, 1980). Some enzyme changes may constitute an adaptive mechanism to protect animals against the toxic effects of unusual fatty acids which may be ingested chronically in their diet, and the changes caused by clofibrate may be an exaggeration of this natural response. It is remarkable that clofibrate protects rats against the hypoglycaemic and toxic effects of two toxins which inhibit mitochondrial β -oxidation in different ways. Further, the mechanism of protection against each toxin appears to be different.

The possible application of some of these findings to human pathology is speculative. Fibrate drugs increase the capacity of liver mitochondrial β -oxidation in both rodents and primates (Capuzzi *et al.*, 1983), but the extent of induction of peroxisomal β -oxidation in non-rodent species is controversial (Kindl & Lazarow, 1982; Reddy & Lalwani, 1983). Induction of increased mitochondrial β -oxidation activity might be useful in the treatment of some inborn errors of metabolism where there may be impaired mitochondrial β -oxidation. This would come in addition to the well-known use of these drugs in hypercholesterolaemia and hypertriglyceridaemia.

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