Effect of Gemfibrozil on Centrifugal Behavior of Rat Peroxisomes and Activities of Peroxisomal Enzymes Involved in Lipid Metabolism

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The effect of gemfibrozil, an analogue of clofibric acid, on the centrifugal behavior of peroxisomes and activity of peroxisomal enzymes involved in lipid metabolism was studied. Rats were fed chow containing 0.2% gemfibrozil for 2 weeks. Nycodenz gradient centrifugation of the light mitochondrial fraction revealed that peroxisomes of gemfibrozil-treated rats were concentrated in fractions of higher density compared with control rats. The activity of fatty acyl-CoA oxidase, crotonase, β-hydroxybutyryl-CoA dehydrogenase, and thiolase (individual enzymes of the peroxisomal fatty acid β-oxidation system) were enhanced 9.6, 2.3, 3.4 and 9.1 times respectively compared with controls, by treatment. The hydroxymethylglutaryl-CoA (HMG-CoA) reductase (rate-limiting enzyme of cholesterol synthesis) activity of peroxisomes and microsomes was greatly increased by *in vivo* treatment with gemfibrozil, but was decreased by addition of the agent to the assay mixture of the enzyme. Gemfibrozil directly inhibited the reductase activity and did so at a lower concentration than clofibric acid. Peroxisomal reductase was more resistant to damage by the agent than the microsomal enzyme. The HMG-CoA reductase activity of peroxisomes and microsomes of hyperlipidemic rats was also increased by *in vivo* treatment with gemfibrozil, whereas the serum cholesterol level was hardly changed.

These results indicate that the effect of gemfibrozil differs from that of clofibric acid, the main difference being the effect on HMG-CoA reductase. Gemfibrozil increased reductase activity *in vivo*, unlike clofibric acid, but inhibited the enzyme *in vitro* to a greater extent than clofibric acid.

Key words peroxisome; gemfibrozil; HMG-CoA reductase; clofibric acid; cholesterol

Clofibrate is a hypolipidemic agent. Although the mechanism of its hypolipidemic effect is as yet unknown, the reduction in serum cholesterol is believed to be caused by inhibition of hydroxymethylglutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol synthesis.[1,2] The effect of clofibrate on peroxisomes is well known, since it is a standard peroxisome proliferator.[3-6]

Gemfibrozil is also a hypolipidemic agent with a similar structure to clofibrate. However, its effect on peroxisomes has only been partially elucidated.[7-10] Gemfibrozil, like clofibrate, is a peroxisome proliferator and induces catalase.[7,9] However, unlike clofibrate, it has not been shown to inhibit HMG-CoA reductase activity *in vivo*.

We have been investigating the role of the peroxisomal fatty acid β-oxidation system.[11-13] In our last report, we studied the effect of gemfibrozil on lipid synthesis from acetyl-CoA derived from the peroxisomal fatty acid β-oxidation system. It was shown that gemfibrozil clearly activates HMG-CoA reductase as well as the peroxisomal fatty acid β-oxidation system of peroxisomes and microsomes *in vivo*, thereby increasing both cholesterol and bile acid biosynthesis from acetyl-CoA derived from peroxisomes.[14]

In the present report, we further investigated the effect of gemfibrozil on peroxisomes: the density of peroxisomes and the activity of the individual enzymes of the peroxisomal fatty acid β-oxidation system. Furthermore, we investigated the direct effect of gemfibrozil on HMG-CoA reductase of peroxisomes and microsomes. The results show that the density of peroxisomes of gemfibrozil-treated rats was greater than that of control rats. The activity of all the individual enzymes in the peroxisomal fatty acid β-oxidation system was enhanced. When clofibrate is added to the diet or assay mixture, HMG-CoA reductase activity is reported to be reduced.[2,12] However, gemfibrozil treatment increased such activity *in vivo*, although direct addition of gemfibrozil to the assay mixture resulted in a reduction in reductase activity.

MATERIALS AND METHODS

Materials Gemfibrozil was kindly donated by Warner–Lambert (U.S.A.). Nycodenz, palmitoyl-CoA, crotonyl-CoA, acetoacetyl-CoA, HMG-CoA, CoA, FAD, NAD, NADH, NADP, cytochrome c, *o*-nitrophenyl-acetate, glucose-6-phosphate, glycose-6-phosphate dehydrogenase, mevalonate and clofibric acid were purchased from Sigma (U.S.A.). Triton WR-1339 was obtained from Ruger Chemicals (U.S.A.). [3-^{14}C]HMG-CoA (2.1 Gbq/mmol, 57.6 mCi/mmol), [5-^{3}H]mevalonolactone (1221 Gbq/mmol, 33.0 Ci/mmol) and Aquazol 2 were purchased from New England Nuclear (U.S.A.). All other reagents were of analytical grade from Wako Pure Chemicals (Japan).

Treatment of the Rats Male Wistar rats (200—300 g) were fed *ad lib.* standard diet CE-2 (Clea Japan, Japan) and kept under a 12-h light-dark cycle. The treated rats were fed chow containing 0.2% gemfibrozil (w/w) for 2 weeks. The rats were then starved overnight and, 4 h into their light cycle (a.m. 10:00), blood was collected under anesthesia through the abdominal aorta. The rats were sacrificed and the livers excised after perfusion with cold saline. Hyperlipidemia was induced by intra-peritoneal injection of Triton WR-1339 (80 mg/100 g body weight) 2 d before sacrifice.

Cell Fractionation Livers were homogenized in 0.25 M sucrose, using a Potter–Elvehjem Teflon homogenizer. Ten

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percent (w/v) liver homogenate was fractionated according to the method of de Duve *et al.*[15] Nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P) and supernatant (S) fractions were obtained by centrifugation at $600 \times g$ for 10 min, $3300 \times g$ for 10 min, $12500 \times g$ for 20 min, and $105000 \times g$ for 60 min, respectively. Centrifugation at $105000 \times g$ was carried out in a Hitachi ultracentrifuge (model SCP70H) with an RP65 rotor. Each fraction was washed once at the same centrifugal force, and suspended in 0.25 M sucrose.

Nycodenz Density Gradient Centrifugation Livers were homogenized in 0.25 M sucrose, containing 1 mM EDTA, 0.1% ethanol, and 5 mM HEPES (pH 7.4) (SVEH). The light mitochondrial fraction was prepared from liver homogenate. A linear Nycodenz density gradient (30 ml) from 1.15 to 1.22 was prepared on a cushion of 2 ml Nycodenz solution ($d=1.25$) in a 40 ml centrifuge tube. About 4 ml of the light mitochondrial fraction was then layered on top of the gradient. The tubes were centrifuged at 25000 rpm overnight in a Hitachi model SCP70H ultracentrifuge with a Hitachi RPS27-109 swing rotor. After centrifugation, the tubes were divided into 19 fractions each of 2 ml, from the top of the gradient with an ISCO gradient fractionator (U.S.A.). Each fraction was diluted with SVEH, and centrifuged at $20000 \times g$ for 30 min. The precipitates were suspended in 0.25 M sucrose, and the activity of the enzymes in each fraction was determined.

Determination of Cholesterol and Triglyceride in Serum The total serum cholesterol was assayed by the method of Zlatkis and Zak.[16] Triglyceride was estimated using a Triglyceride-Test Wako assay kit (number 271-1430), as supplied by Wako Pure Chemicals (Japan).[17]

Enzyme and Protein Assays Catalase, D-amino acid oxidase and urate oxidase are marker enzymes of peroxisomes. Catalase activity was determined according to the method of Leighton *et al.*[18] with slight modification.[19] D-Amino acid oxidase and urate oxidase were assayed as described previously.[20]

The activity of the peroxisomal fatty acyl-CoA oxidizing system was determined by the method of Lazarow and de Duve[3] with slight modification.[21] One unit of activity was defined as the amount of enzyme that reduced 1 nmol NAD per min.

The fatty acyl-CoA oxidase activity was estimated by the method of Osumi and Hashimoto.[22] The activity of crotonase, 3-hydroxybutyryl-CoA dehydrogenase and thiolase was assayed by the method of Lazarow.[23]

Esterase is a marker enzyme of microsomes. Its activity was determined using *o*-nitrophenyl acetate as a substrate according to Beaufay *et al.*[24]

The acid phosphatase activity was determined as a marker of lysosomes, using the method described previously.[19]

The cytochrome c oxidase activity was assayed as a marker of mitochondria, using the method of Wharton and Tzagoloff[25] with slight modification.[26]

The activity of HMG-CoA reductase was determined by the method of Keller *et al.* using [3-^{14}C]HMG-CoA and [5-^{3}H]mevalonolactone as substrate and internal standard, respectively.[27] The enzyme sample was diluted in 50 mM potassium phosphate buffer, pH 7.4, containing 30 mM EDTA, 200 mM NaCl and 10 mM dithiothreitol. Approximately 50 μg protein was used.

Protein was determined by the Lowry method, using bovine serum albumin as a standard.[28] Since Nycodenz inteferes with the determination of protein, trichloroacetic acid co-precipitation of the protein with deoxycholate[29] was carried out before the Lowry protein assay.

RESULTS

Effect of Gemfibrozil on Rat Serum Lipids We studied the effect of gemfibrozil on the serum lipid concentrations of normal and hyperlipidemic rats. The triglyceride concentration in normal rats was reduced to about 28% of the control by gemfibrozil, but cholesterol was hardly altered. The cholesterol level of hyperlipidemic rats increased to about 10 times that of normal rats, while triglyceride increased to about 80 times. Gemfibrozil did not affect the cholesterol level of hyperlipidemic rats. The triglyceride level of hyperlipidemic rats was reduced to about 58% of the control by gemfibrozil treatment, but did not decrease to the level of normal rats (Table 1).

Effect of Gemfibrozil on Enzyme Activity Patterns in Subcellular Fractions of Liver Homogenate Figure 1 shows the result of the subcellular fractionation of liver homogenate. The specific activity of cytochrome c oxidase of control rats was highest in the mitochondrial fraction. Peroxisomal enzymes (catalase, D-amino acid oxidase, urate oxidase and the enzymes of the peroxisomal fatty acyl-CoA β-oxidation system) and acid phosphatase were localized in the light mitochondrial fraction while esterase was localized in the microsomal fraction. Gemfibrozil had little effect on the enzyme activity patterns in the subcellular fractions. Among the peroxisomal enzymes, the specific activity of the fatty acyl-CoA β-oxidation system was markedly increased by gemfibrozil treatment, similar to our previous results.[14]

Figure 2 shows the distribution of enzyme activity after Nycodenz density gradient centrifugation of the light mitochondrial fraction. The esterase (microsomal marker) and acid phosphatase (lysosomal marker) activity of control rats was distributed in the lower density fractions 3 and 4. The cytochrome c oxidase activity (mitochondrial marker) was mainly localized in fractions 4 and 5. Gemfibrozil had little effect on the localization of the

Table 1. Effect of Gemfibrozil on Rat Serum Lipids

Lipids	Control (C) (mg/ml)	Gemfibrozil (T) (mg/ml)	T/C ratio
Normal rats			
Cholesterol	111±27	128±36	1.15±0.32
Triglyceride	79.8±19.9	22.0±5.3	0.28±0.07*
Hyperlipidemic rats			
Cholesterol	1130±170	1020±110	0.90±0.10
Triglyceride	6240±720	3640±1440	0.58±0.23*

Rats were fed laboratory chow, with or without 0.2% gemfibrozil, for 2 weeks. Blood was collected from the rats and cholesterol and triglyceride levels were determined as described in the text. Hyperlipidemia was induced by intra-peritoneal injection of Triton-WR 1339 2 d before the blood collection. Data are mean values±S.D. of 5 animals. * Indicates significant difference ($p<0.001$).

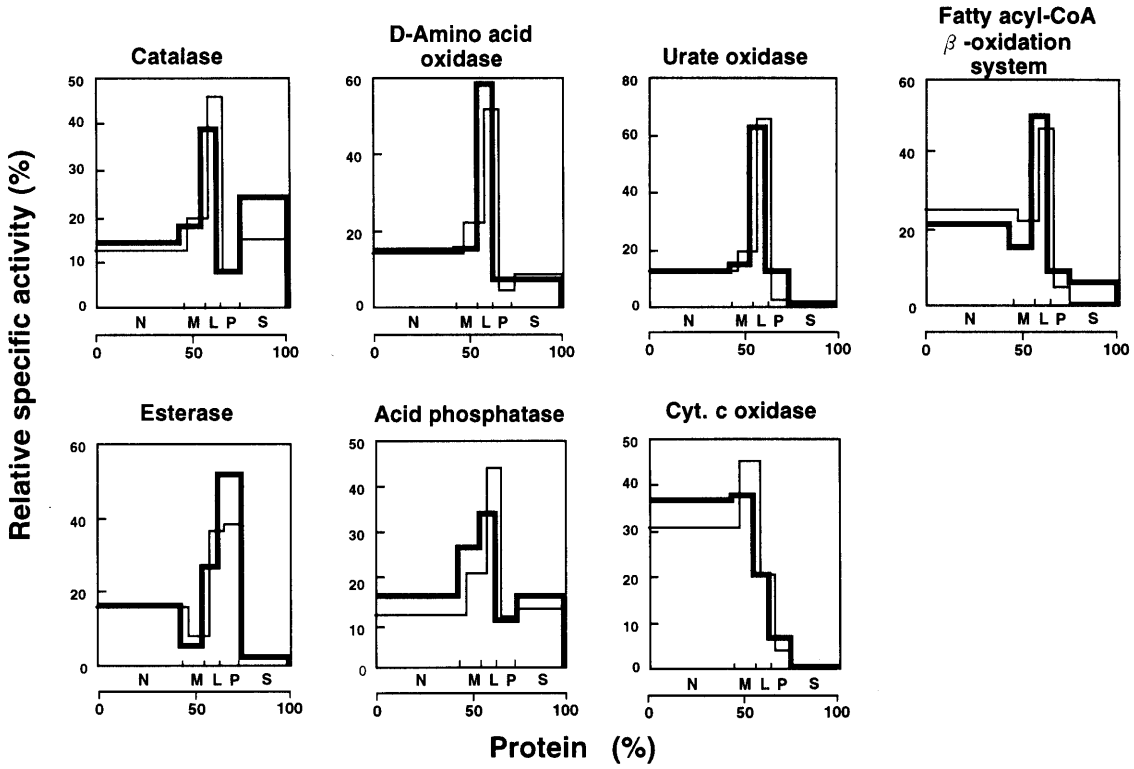

Fig. 1. Pattern of Enzyme Activity in the Subcellular Fraction of Liver Homogenate

Rats were fed chow, with (▬) or without (——) 0.2% gemfibrozil, for 2 weeks. Homogenate was prepared from rat liver, and fractionated according to the method of de Duve *et al.*[15] The ordinate represents the relative specific activity of the peroxisomal enzymes: catalase, D-amino acid oxidase, urate oxidase, fatty acyl-CoA β-oxidation system, and the relative specific activity of the marker enzymes: esterase (microsomal marker), acid phosphatase (lysosomal marker), cytochrome c oxidase (mitochondrial marker). The abscissa represents the protein content relative to the total. The specific activity of catalase, D-amino acid oxidase, urate oxidase and fatty acyl-CoA β-oxidation system of the light mitochondrial fraction of the treated rats was 251 (control rats: 177) U/mg protein, 19.3 (13.7) mU/mg protein, 22.3 (16.4) mU/mg protein and 15.6 (4.8) U/mg protein, respectively. The specific activity of esterase (microsomal fraction), acid phosphatase (light mitochondrial fraction) and cytochrome c oxidase (mitochondrial fraction) of the treated rats was 4.59 (control rats: 2.61) U/mg protein, 83 (137) mU/mg protein and 14.0 (25.8) U/mg protein, respectively. N, nuclear fraction; M, mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, cytosol fraction.

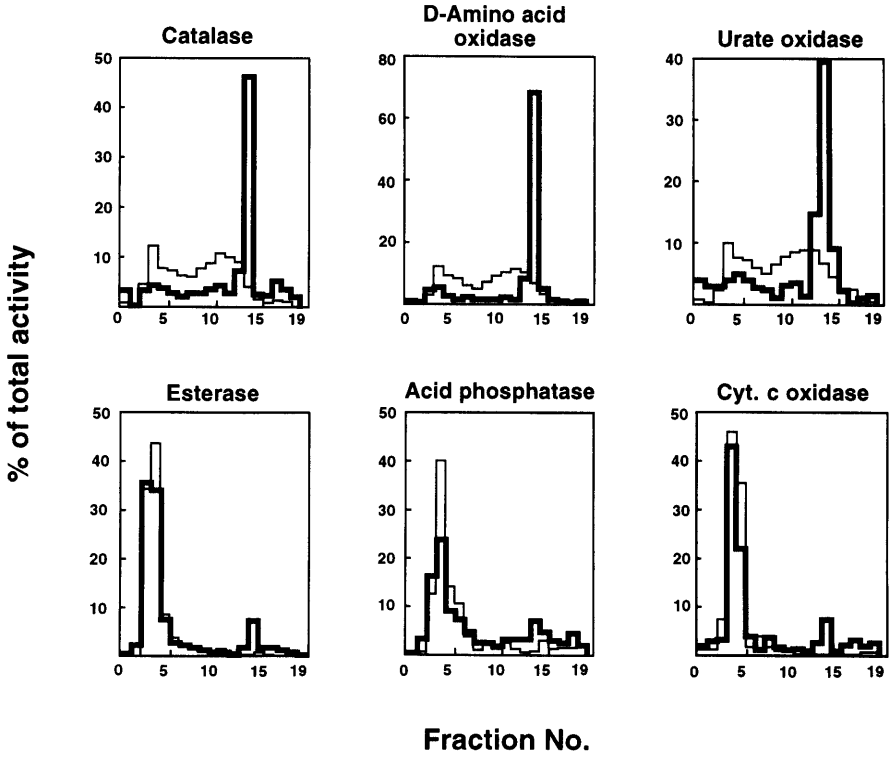

Fig. 2. Patterns of Enzyme Activity in Nycodenz Density Gradient Centrifugation of the Light Mitochondrial Fraction of Rat Liver

Rats were fed a diet, with (▬) or without (——) 0.2% gemfibrozil, for 2 weeks. The light mitochondrial fraction was prepared from rat liver, and subjected to Nycodenz density gradient centrifugation. The ordinate represents the enzyme activity of the fraction relative to the total activity of all fractions. The abscissa represents the fraction numbers from the top of the tube.

enzyme. In contrast, the peroxisomal enzymes (catalase, D-amino acid oxidase and urate oxidase) of control rats were widely distributed between lower density fraction 4 and higher density fraction 15, while the enzymes of gemfibrozil-treated rats were concentrated in high density fraction 14. From these results, it appeared that high density peroxisomes were produced by gemfibrozil treatment.

Effect of Gemfibrozil on Individual Enzymes of the Peroxisomal Fatty Acid β-Oxidation System We have reported that the activity of the peroxisomal fatty acid β-oxidation system is increased to about 5 times that of controls by gemfibrozil treatment.[14] In this experiment, it is also clear that the specific activity of the peroxisomal fatty acid β-oxidation system was increased to a greater extent than that of the other peroxisomal enzymes (Fig. 1). We studied the effect of gemfibrozil on the individual enzymes of the peroxisomal fatty acid β-oxidation system, *i.e.*, fatty acyl-CoA oxidase, crotonase, β-hydroxybutyryl-CoA dehydrogenase and thiolase (Table 2). The specific activity of all enzymes of gemfibrozil-treated rats was increased, compared with control rats. Among these enzymes, acyl-CoA oxidase and thiolase increased markedly to about 8.6 and 9.1 times that of controls, respectively.

Effect of Gemfibrozil on HMG-CoA Reductase of Peroxisomes and Microsomes Clofibrate, a typical peroxisomal proliferator is known to inhibit HMG-CoA reductase (key regulatory enzyme in the biosynthesis of cholesterol). We have reported that gemfibrozil activates the HMG-CoA reductase of peroxisomes and microsomes *in vivo* and, consequently, that the biosynthesis of cholesterol and bile acids is enhanced.[14] In this report, we studied the effect of *in vivo* treatment with gemfibrozil on the reductase of normal and hyperlipidemic rats (Table 3).

Gemfibrozil stimulates the HMG-CoA reductase activity of both peroxisomes and microsomes of normal rats. Among control rats, the activity of the HMG-CoA reductase of peroxisomes was lower than that of microsomes, but was increased by gemfibrozil treatment reaching the level of microsomal activity of control rats. The HMG-CoA reductase activity of peroxisomes and microsomes of hyperlipidemic rats was enhanced to approximately 3.3 and 3.7 times, respectively, that of the normal rats. Gemfibrozil also increased the reductase activity of both the peroxisomes and microsomes of hyperlipidemic rats. The gemfibrozil/control (T/C) ratios of peroxisomes and microsomes were similar, though they differed from those of normal rats.

In order to discover if the increase in HMG-CoA reductase activity is caused by the direct effect of gemfibrozil on the enzyme, we added the agent to the assay mixture. We also studied the effect of clofibric acid, which is a known metabolite of clofibrate and pharmacologically active,[30] and then compared its effect with that of gemfibrozil.

Figure 3 shows the effect of gemfibrozil and clofibric acid on the microsomal HMG-CoA reductase activity of control rats. The addition of 0.3 mM gemfibrozil to the mixture had little effect on activity. However, 1.5 mM gemfibrozil reduced the activity to about 27.6% of the control (no gemfibrozil was added to the mixture).

Table 2. Effect of Gemfibrozil on the Specific Activity of Individual Enzymes of the Peroxisomal Fatty Acyl-CoA β-Oxidation System

	Control (C)	Gemfibrozil (T)	T/C ratio
Fatty acyl-CoA oxidase[a]	7.44±2.39	63.8±4.0	8.58±0.54**
Crotonase[b]	1.31±0.55	2.99±1.36	2.28±1.04*
β-Hydroxybutyryl-CoA dehydrogenase[a]	78.5±25.3	270±112	3.44±1.43*
Thiolase[c]	72.6±36.9	660±135	9.09±1.86**

After treatment with 0.2% gemfibrozil for 2 weeks, rats were killed and livers were excised. Peroxisomes were prepared from the rat livers and the activity of individual enzymes of the fatty acyl-CoA β-oxidation system was assayed. Data are mean values±S.D. of 5 animals. ** and * represent significant changes (** $p<0.001$; * $p<0.05$). *a*) mU/mg protein. *b*) U/μg protein. *c*) U/mg protein.

Table 3. Influence of Gemfibrozil on HMG-CoA Reductase Activity of Peroxisomes and Microsomes

	HMG-CoA reductase (pmol/min/mg protein)		
	Control (C)	Gemfibrozil (T)	T/C ratio
Normal rats			
Peroxisomes	9.47±3.84	70.9±24.8	7.5±2.6*
Microsomes	65.2±16.1	1490±260	22.9±4.0*
Hyperlipidemic rats			
Peroxisomes	31.7±5.3	195±95	6.2±3.0*
Microsomes	238±44	1890±870	7.9±3.7*

Rats were fed chow containing 0.2% gemfibrozil for 2 weeks. Hyperlipidemia was induced by intra-peritoneal injection of Triton WR-1339 2 d before sacrifice. Peroxisomes and microsomes were prepared from rat livers, and HMG-CoA reductase activity was assayed. Data are means±S.D. of 5 animals. * Indicates significant difference ($p<0.005$).

Although the activation effect of gemfibrozil on HMG-CoA reductase observed *in vivo* was not detected, an inhibitory effect was. In the presence of 5 mM clofibric acid, the reductase activity changed little, while at 20 mM, it fell to 40.9%. Thus, gemfibrozil suppressed the activity of reductase at a much lower concentration than clofibric acid.

We carried out the same experiment using microsomes from rats treated with gemfibrozil. The addition of 0.3 mM and 1.5 mM gemfibrozil reduced HMG-CoA reductase activity to about 92.7 and 13.0%, respectively, of controls (Fig. 4). The activity of gemfibrozil-pretreated rats was also inhibited by direct addition of gemfibrozil. Consequently, the activation effect of gemfibrozil shown *in vivo* was not observed in gemfibrozil-treated rats. The direct inhibitory effect of gemfibrozil on the enzyme of gemfibrozil-pretreated rats seems to be greater than that of control rats. In the presence of 5 mM clofibric acid, about 92.9% of the enzyme activity was present, while this was reduced to 34.3% at a concentration of 20 mM. Similar to control rats, gemfibrozil directly inhibited reductase activity at lower concentrations than clofibric acid.

Figure 5 shows the result of an experiment performed using peroxisomes, instead of microsomes, from gemfibrozil-pretreated rats. Gemfibrozil at 0.3 mM had little effect on HMG-CoA reductase activity, while at 1.5 mM, it reduced it to 72.9%. Thus, gemfibrozil had a direct

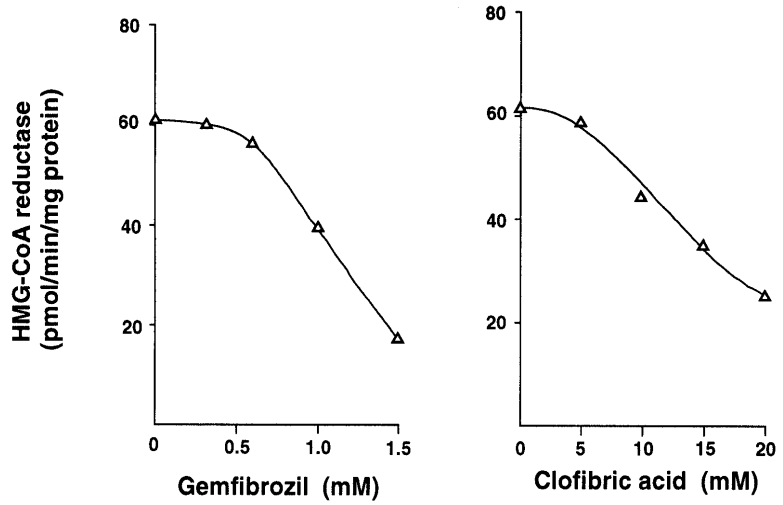

Fig. 3. Direct Effect of Gemfibrozil and Clofibric Acid on the Activity of Microsomal HMG-CoA Reductase of Control Rats

The microsomal preparation (see Table 3) was used. Assay of HMG-CoA reductase was carried out as described in the text, using about 50 μg microsomal protein. Final concentration of gemfibrozil and clofibric acid (sodium salt) was as indicated.

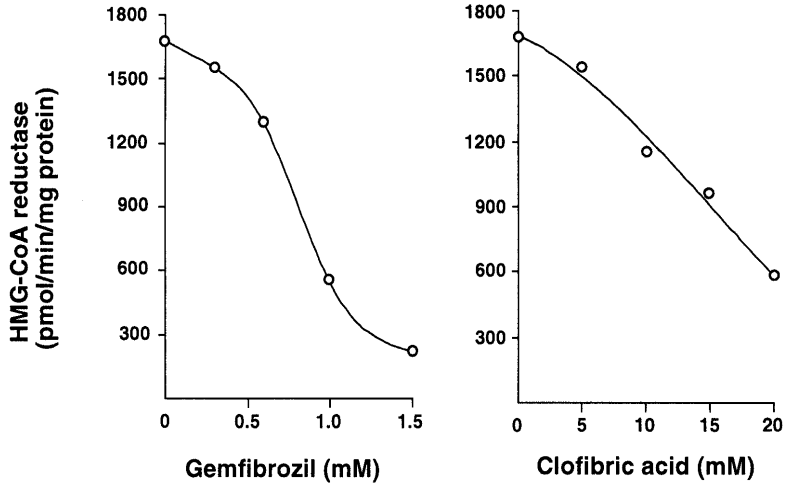

Fig. 4. Direct Effect of Gemfibrozil and Clofibric Acid on the Activity of Microsomal HMG-CoA Reductase of Gemfibrozil-Treated Rats

Microsomal preparations of gemfibrozil-treated rats (see Table 3) were used. HMG-CoA reductase activity was assayed with gemfibrozil or clofibric acid.

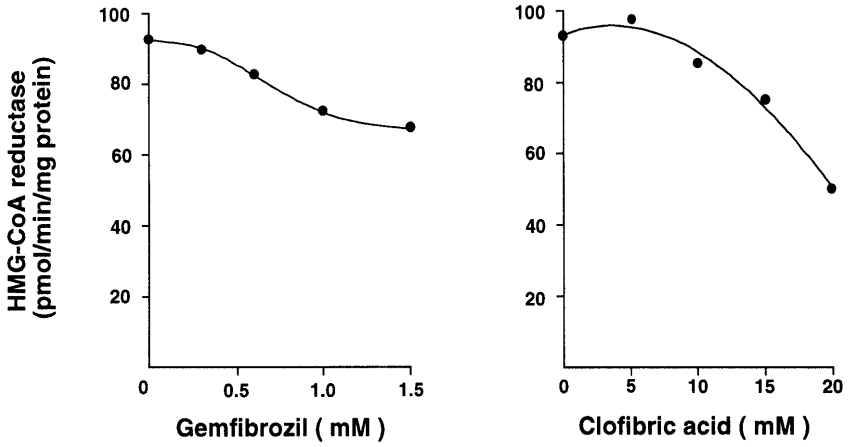

Fig. 5. Direct Effect of Gemfibrozil and Clofibric Acid on the Activity of Peroxisomal HMG-CoA Reductase of Gemfibrozil-Treated Rats

Peroxisomal preparations of gemfibrozil-treated rats (see Table 3) were used. HMG-CoA reductase activity was assayed with gemfibrozil or clofibric acid.

inhibitory effect on the peroxisomal enzymes, but a smaller effect relative to the microsomal enzymes (Figs. 3 and 4). Clofibric acid at 5 mM did not affect the enzyme, but at 20 mM its activity was reduced to 53.4%. As with the microsomes, gemfibrozil inhibited the enzyme activity of peroxisomes at lower concentrations than clofibric acid. The HMG-CoA reductase activity of control rats was too weak to allow the effect of the agents to be studied.

From these results, it was found that gemfibrozil directly inhibits the HMG-CoA reductase activity of both microsomes and peroxisomes. Consequently, it was suggested that the activation effect of gemfibrozil on HMG-CoA reductase shown *in vivo* is not caused by the direct effect of gemfibrozil on the enzyme. Furthermore, it is clear that the inhibitory effect of gemfibrozil *in vitro* is more potent than that of clofibric acid.

DISCUSSION

We studied the effect of gemfibrozil on peroxisomal density, the activity of individual enzymes of the fatty acid β-oxidation system, and HMG-CoA reductase.

Clofibrate reduces cholesterol synthesis and serum cholesterol levels, due to inhibition of HMG-CoA reductase.[1,2] Gemfibrozil has a similar structure to clofibrate but, unlike clofibrate, it stimulates the biosynthesis of cholesterol and bile acids.[14] We assayed serum cholesterol levels but found no significant change in normal rats (Table 1). This supports the results of previous reports.[31,32] Gemfibrozil is reported to reduce the serum cholesterol of rats with hyperlipidemia produced by a high fat high cholesterol diet.[33,34] However, gemfibrozil did not reduce the cholesterol level of rats with hyperlipidemia induced by Triton WR-1339 (Table 1). *In vivo*, gemfibrozil activates HMG-CoA reductase in hyperlipidemic rats (Table 3). In the present study, rats were fed a diet containing gemfibrozil for 2 weeks, and then injected intra-peritoneally with Triton WR-1339 2 d before sacrifice. Therefore, before the injection of Triton WR-1339, HMG-CoA reductase activity might have already been increased by gemfibrozil pretreatment. Furthermore, Triton WR-1339 is also known to stimulate HMG-CoA reductase activity.[35,36] Our results support the activation of reductase by Triton WR-1339 (Table 3). The cholesterol concentration of control rats is reported to be increased about 1.3 times by a high fat high cholesterol diet,[33] whereas we found it was increased about 10 times by injection of Triton WR-1339 (Table 1). Thus, the difference in the effects of gemfibrozil between the two types of hyperlipidemic rats may be due to the different conditions under which hyperlipidemia was induced. Unlike rats, human serum cholesterol levels are reported to be reduced by gemfibrozil.[37] Thus, there appear to be species differences in the effect of gemfibrozil on serum cholesterol. In any event, the serum cholesterol concentration is well regulated, and the effect of these agents on cholesterol levels seems to be very complex.

Triglyceride concentration was markedly reduced by gemfibrozil in both normal and hyperlipidemic rats (Table 1), suggesting that the agent is more effective against triglyceride hyperlipidemia than its cholesterol counterpart. This is common among clofibric acid derivatives.

High density peroxisomes have reportedly been prepared from the liver of clofibrate-treated rats.[38] From Fig. 2, it is clear that high density peroxisomes were also generated by gemfibrozil. Generally, the density of protein is higher than that of lipids. The cholesterol/protein content ratio of the peroxisomes of gemfibrozil-treated rats was approximately 45% that of controls (data not shown). This was probably a result of gemfibrozil-induced changes in protein and lipids, resulting in high density peroxisomes.

The activity of all enzymes in the fatty acid β-oxidation system was increased by gemfibrozil (Table 2) as well as clofibrate,[39,40] although the activation rates of both agents differed. Lalwani *et al.* reported that the activity of crotonase was increased by gemfibrozil,[7] and our results support this.

HMG-CoA reductase is considered to be localized only in microsomes. Recently however, the enzyme was found to be localized in peroxisomes as well as microsomes.[27,41] *In vivo* treatment with gemfibrozil increased HMG-CoA reductase activitiy in both the peroxisomes and microsomes of normal and hyperlipidemic rats (Table 3). We reported that the biosynthesis of cholesterol and bile acids is enhanced by gemfibrozil.[14] Further, we showed that the 4-methyl intermediate sterols in the cholesterol synthetic pathway accumulate in peroxisomes and microsomes after aminotriazol inhibition of cholesterol synthesis, and that the concentration of these intermediates is increased by gemfibrozil pretreatment.[42] It is clear that such phenomena, associated with cholesterol synthesis, are due to the increase in HMG-CoA reductase activity by gemfibrozil.

In rats, serum cholesterol levels are reported to be reduced by clofibrate.[2,32] However, gemfibrozil did not reduce cholesterol levels in either normal or hyperlipidemic rats (Table 1). This may be due, at least in part, to the activation effect of gemfibrozil on HMG-CoA reductase *in vivo*. However, if the enzyme is activated, the serum cholesterol would be expected to be increased. We could not detect any increase. The lack of change in the serum cholesterol may result from an increase in the activation of cholesterol metabolism (bile acid synthesis) in hepatocytes and excretion of cholesterol into bile by gemfibrozil.

In contrast to its effect *in vivo*, gemfibrozil exhibits an inhibitory effect on HMG-CoA reductase *in vitro* (Figs. 3—5). Therefore *in vivo*, gemfibrozil may not directly activate the enzyme but, rather, indirectly enhance activity through some unknown mechanism.

Thompson and Krisans demonstrated that the activity and protein content of peroxisomal HMG-CoA reductase are increased by gemfibrozil treatment (unpublished data).[43] Therefore, the *in vivo* increase of the HMG-CoA reductase activity of peroxisomes and microsomes (Table 3) seems to depend on enzyme induction by gemfibrozil. Nevertheless, we cannot rule out the possibility that the enzyme activity is increased by suppression of the enzyme degradation by gemfibrozil.

As shown in Figs. 3—5, when the concentration of gemfibrozil in the assay mixture is changed, the inhibition rates by the agent differ between peroxisomes and microsomes. Peroxisomes may be more resistant to damage by the agent than microsomes. From these results, it seems that the characteristics of the HMG-CoA reductase of peroxisomes are different from those of microsomes.

The activity of HMG-CoA reductase was reduced by gemfibrozil *in vitro*, but was increased *in vivo*. One

explanation for this is as follows. Gemfibrozil is degraded *in vivo*, into another product which does not show any inhibitory effect on the enzyme. However, there is a report that 48% of incorporated gemfibrozil is excreted unchanged in urine.[44] Therefore, this possibility can be ruled out. Clofibric acid inhibited HMG-CoA reductase activity at a relatively high concentration (Figs. 3—5). Clofibric acid has been reported to inhibit microsomal HMG-CoA reductase at 1.35 mM[2] but, the assay conditions of this report differed from ours. For example, we added 133 mM NaCl to the assay mixture. Therefore, we cannot directly compare both experiments. In any event, inhibition of HMG-CoA reductase is the mechanism by which clofibrate reduces serum cholesterol,[1,2] so clofibric acid may be present *in vivo* in effective concentrations. The concentration of gemfibrozil in liver cells after *in vivo* treatment has yet to be determined, however, gemfibrozil affected HMG-CoA reductase at lower concentrations than clofibric acid (Figs. 3—5). Therefore, gemfibrozil may also be present in effective concentrations *in vivo*, and inhibit the enzyme. Nevertheless, HMG-CoA reductase activity was not reduced by *in vivo* treatment, but was actually increased (Table 3). Compactin, a competitive inhibitor of HMG-CoA reductase, is reported to induce the reductase in rats.[45] The mechanism for this is considered to be as follows. Hepatic levels of cholesterol should first be reduced because of the inhibition of HMG-CoA reductase following administration of compactin. Therefore, in order to compensate for the reduced levels of hepatic cholesterol, the rate of hepatic sterol synthesis, *i.e.* HMG-CoA reductase activity, might be elevated. The increase in the reductase by *in vivo* treatment with gemfibrozil may be due to induction of the enzyme, similarly to compactin. Consequently, even if the enzyme was inhibited, the reduction in activity may be compensated by induced enzyme, so that only the activation effect is detected. Serum cholesterol levels in rats were unchanged by gemfibrozil as well as compactin.

From these results, it is clear that the effect of gemfibrozil is different from that of clofibric acid, the main difference being the effect on HMG-CoA reductase. Gemfibrozil increased the reductase activity *in vivo* unlike clofibric acid, but inhibited the enzyme *in vitro* more strongly than clofibric acid. As the two agents have a similar structure, the cause of such differences in effect is of particular interest. Experiments using gemfibrozil and clofibric acid are expected to help clarify the mechanism of cholesterol and bile acid synthesis.

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