Biochemical Pharmacology, Vol. 26, pp. 1697–1702. Pergamon Press, 1977. Printed in Great Britain.

DIFFERENTIAL INCREASE OF HEPATIC PEROXISOMAL, MITOCHONDRIAL AND MICROSOMAL CARNITINE ACYLTRANSFERASES IN CLOFIBRATE-FED RATS*

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(*Received* 30 *August* 1976; *accepted* 14 *January* 1977)

Abstract—Hepatic peroxisomes, mitochondria and microsomes from control and clofibrate-treated animals were separated by isopycnic sucrose gradient centrifugation and the carnitine acyltransferase system studied in each of these organelles. Clofibrate treatment produced a 13-fold increase in the total activity of carnitine acetyltransferase and a 5-fold increase in carnitine octanoyl- and palmitoyltransferase activities. The specific activities of the transferases in all three subcellular locations increased, but to different extents. Peroxisomal and microsomal carnitine acetyltransferases doubled in specific activity; the mitochondrial enzyme increased 10-fold. Peroxisomal, mitochondrial and microsomal carnitine octanoyltransferases all increased 3-fold in specific activity. Carnitine palmitoyltransferase, which is found only in mitochondria, increased 3-fold in specific activity. These differential increases changed the per cent distribution of total carnitine acetyltransferase from 50 per cent in the mitochondria of control livers to 90 per cent in treated livers. Peroxisomes from clofibrate-treated livers had a consistently greater isopycnic density in sucrose gradients. Total catalase activity increased 2-fold upon treatment and a greater percentage of it was found in the particulate fractions. The specific activity of peroxisomal catalase and urate oxidase remained the same as in controls. Carnitine acetyl- and octanoyltransferases are the first reported enzymes whose peroxisomal specific activity increases with clofibrate treatment. Preliminary results of treatment with another membrane-inducing drug, phenobarbital, indicated no change in peroxisomal density, catalase distribution and activity, and no effect on the specific activities of the peroxisomal, mitochondrial and microsomal carnitine acyltransferases.

Administration of the hypolipidemic drug clofibrate (ethyl-*p*-chlorophenoxy isobutyrate) has been shown to induce a rapid and marked proliferation of hepatic peroxisomes [1]. A moderate increase in the amount of smooth endoplasmic reticulum and mitochondrial protein content has also been observed in the liver of clofibrate-treated male rats [2, 3]. Each of these subcellular structures—the peroxisomes, mitochondria and endoplasmic reticulum—contains a carnitine acyltransferase system [4, 5] composed of two or more enzymes capable of using short- and medium-chain acyl-CoA substrates [6]. In addition, the mitochondrial acyltransferase system has the unique capability of using long-chain acyl-CoA substrates such as palmitoyl-CoA.

The importance of acyl-CoA derivatives as substrates and regulators of cholesterol synthesis and fatty acid metabolism has prompted investigators to study the effect of hypolipidemic drugs such as clofibrate on the carnitine acyltransferases. Increases in the activity of one or more of the transferases in liver homogenates from clofibrate-fed rats or in a mitochondrial fraction isolated by differential centrifugation have been reported by several groups [7–11]. A comparative study of the effect of clofibrate feeding on the different peroxisomal, mitochondrial and microsomal transferase systems is the subject of this report.

After this report was completed, a similar study was published by Kahonen [12]. Kahonen used rats that had been fasted for 2 days prior to sacrifice. We used fed rats because a rapid drop in peroxisomal enzyme activity occurs after 2 days of starvation or drug alteration. The two investigations establish a remarkable change in the distribution of the carnitine acyltransferases within the hepatic cells, when the rat is given clofibrate, which must implicate a change in the overall balance between metabolism and synthesis. Our data represent the first detailed comparison between these metabolic subcellular organelles isolated by zonal sucrose gradients from non-starved animals.

Recently Lazarow and De Duve [13] have implicated liver peroxisomes as a site for some fatty acyl-CoA oxidation. This discovery supports further the concept of two respiratory systems, mitochondria for energy conservation and peroxisomes where the energy is wasted. One role for the carnitine acyltransferases in this dichotomy would be to participate in shuttling the substrates between the different compartments.

* This work was aided by National Institutes of Health Grant HD 06441-05. During the course of the work, M. A. K. M. was supported by a Public Health Service Research Fellowship from the Heart and Lung Institute (1 F22 HL00259-01). This paper is journal article No. 7787 from the Michigan Agricultural Experiment Station. Part of this work has been reported in abstract form [M. A. K. Markwell, R. Gee and N. E. Tolbert, *Fedn Proc.* **35**, 1406 (1976)].

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Table 1. Effect of clofibrate on weight gain and liver weights*

	Control	Clofibrate
Daily weight increase (g/day)	6.95 ± 1.07	6.89 ± 0.65
Liver weight (g)	17.1 ± 1.6	21.8 ± 1.3†

* Mean values ± S. D. are from six rats.
† Statistically different from control, $P < 0.001$.

EXPERIMENTAL PROCEDURES

Drug administration and preparative procedures. Male Sprague–Dawley rats (200–225 g) which had been acclimated to ground Wayne Lab-Blox Stock diet were fed 0.25% (w/w) clofibrate (Ayerst Laboratories, N.Y.) in the diet *ad lib.* for 2–3 weeks. Control rats were fed on the basal diet for the same period. During the study, the animals were individually housed in metabolic cages and had free access to tap water. Food intake was monitored daily and weight gain weekly. Phenobarbital (sodium, Merck) pretreatment was performed in a similar manner by including this drug in the drinking water (0.1%, w/w) for 10 days prior to sacrifice.

For each experiment two animals were sacrificed between 9:00 and 10:00 A.M. by decapitation without previous fasting. The livers were each perfused, minced and washed with 300 ml of 0.25 M sucrose in 20 mM glycylglycine at pH 7.5 before homogenization in the same medium. One stroke of a loose-fitting Potter–Elvehjem homogenizer (clearance ≃ 0.016 in.) was used to prepare a 20% homogenate. The homogenate was filtered through one layer of Miracloth (Chicopee Mills, Inc.) and centrifuged for 10 min at 270 *g*. The resulting supernatant was subjected to isopycnic sucrose density centrifugation in an IEC B-29 or B-30 zonal rotor for 3 hr as previously described [14]. Sixty gradient fractions of 20 ml each from the B-29 rotor or 10 ml each from the B-30 were collected and immediately assayed for catalase and cytochrome *c* oxidases. The gradient fractions were then stored frozen for the rest of the determinations.

Biochemical determinations. The activities of catalase and cytochrome *c* oxidase and the sucrose density and protein profiles were determined in gradient fractions as previously described [14] except that 0.01% Triton X-100 was included in the catalase assay. Urate oxidase activity was measured in the presence of 0.05% Triton X-100 by the decrease in absorption at 293 nm [15, 16]. The microsomal marker NADPH–cytochrome *c* reductase was assayed as referenced [5, 17]. Carnitine acetyl-, octanoyl- and palmitoyltransferase activities were determined using the general thiol reagent method with corrections for carnitine-independent activity [18] at an acyl-CoA concentration of 200 μM. Pretreatment of the fractions by freezing reduced background levels without affecting transferase activities.

Results are reported as the arithmetic mean ± standard deviation. Two group comparisons of means were made applying the Student's *t*-test. The Range-STP (Tukey's) test was applied to multigroup comparisons.

RESULTS

Body and liver growth. As expected from the well-known hepatomegalic effect of clofibrate, the livers of the treated animals did increase significantly in weight as compared to the controls. On the average they were 27 per cent larger, but normal in appearance (Table 1). The normal rate of body growth was not changed by placing the animals on a diet of 0.25% (w/w) clofibrate for 2–3 weeks.

Centrifugal behavior of subcellular particles. Sedimentation patterns of marker enzymes (Fig. 1) on isopycnic sucrose density gradients indicate one particulate peak for each of the three subcellular structures: peroxisomes, mitochondria and microsomes. Perfusion of the liver and washing of the subsequently minced tissue were necessary steps to prevent aggregation of subcellular structures in homogenates from fed animals. The type and extent of cross-contamination of subcellular peaks did not alter with clofibrate treatment.

Peroxisomes from clofibrate-treated rats consistently sedimented at a slightly greater isopycnic density than from controls (Table 2). The shallow sucrose gradient in the peroxisomal region (Fig. 1) was necessary to resolve this difference. No apparent change was observed in the densities of the mitochondria and microsomes.

The percentage of total catalase found in the particulate fractions increased with clofibrate treatment ($P > 0.01$) from 35.7 ± 3.8 per cent in controls to 50.8 ± 5.3 per cent after drug treatment. It was not possible to distinguish whether this was the result of reduced peroxisomal fragility or a preferential increase of peroxisomal catalase over cytosolic catalase with clofibrate treatment.

Hepatic enzyme levels. Total catalase activity (Table 3) increased 2-fold on administration of clofibrate to the male rat. Increases in catalase were seen both in the particulate and soluble fractions, but were slightly greater in the peroxisomal fraction. Total urate oxidase and NADPH–cytochrome *c* reductase activities remained unchanged. The greatest biochemical change was observed in the amount of carnitine acyltransferase activities. Total carnitine acetyltransferase activity increased 13-fold; carnitine octanoyl- and palmitoyltransferase activities both increased 5-fold.

Because the carnitine acyltransferases have a multilocular distribution in the hepatocyte, the effect of clofibrate upon the amount of these enzymes in each sub-cellular location was studied. In the peroxisomes, the amount of catalase, a matrix enzyme of the particle, kept pace with proliferation of the organelle, maintaining a constant specific activity. The specific activity of urate oxidase, a core enzyme, remained the same or might have slightly decreased with treatment. In contrast, carnitine acetyl- and octanoyltransferases of the peroxisomal matrix actually increased 2- to 3-fold in specific activity. These are the first reported enzymes whose specific activities increase in isolated peroxisomes with clofibrate treatment [19].

The mitochondrial carnitine acyltransferases also increased with drug treatment, but in a different pattern of change from that in peroxisomes ($P > 0.01$). The specific activity of mitochondrial carnitine acetyltransferase was 10-fold greater from treated livers

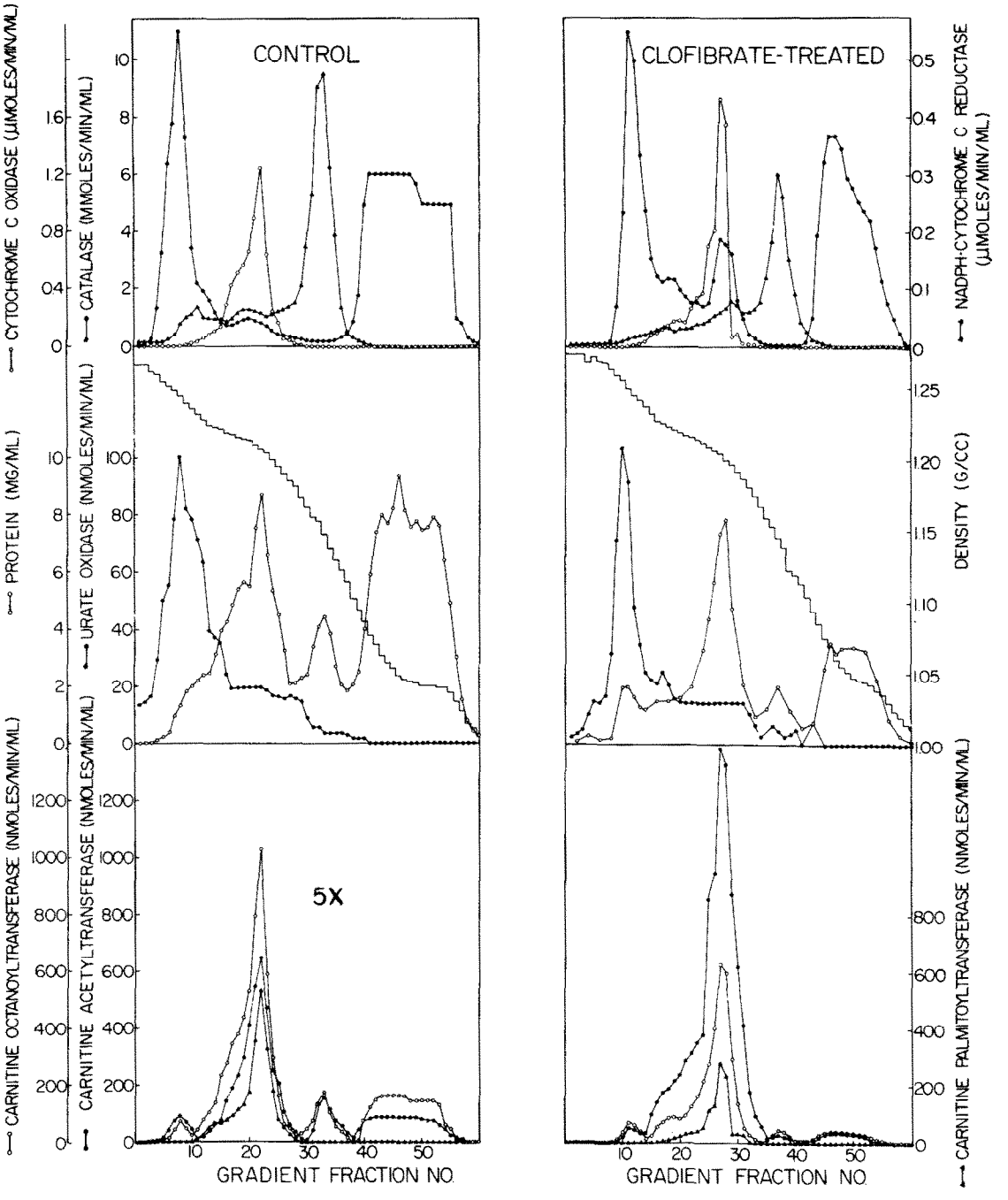

Fig. 1. Isopycnic sucrose density profiles of the carnitine acyltransferases and marker enzymes from control and clofibrate-treated rat liver. Catalase (●——●), cytochrome *c* oxidase (○——○) and NADPH-cytochrome *c* reductase (▲——▲), respectively, mark the peroxisomal, mitochondrial and microsomal peaks (top panels). The peroxisomal core enzyme urate oxidase (●——●), protein (○——○), and sucrose density profiles are seen in the middle panels. Activities of carnitine acetyl- (●——●), octanoyl- (○——○) and palmitoyl- (▲——▲) transferases are shown corrected for background activity (bottom panels). Transferase values for the control gradient were multiplied by 5 to maintain the same scale as for the treated. A 600-ml sucrose gradient was used for the control and a 1200-ml gradient for the treated livers. Two livers were used per gradient.

than from controls. For this enzyme the maximum increase in activity occurred in the mitochondria, with smaller increases in the peroxisomes and microsomes. Mitochondrial carnitine octanoyl- and palmitoyltransferases both increased about 3-fold over control values.

The response of the microsomal transferase system to clofibrate closely resembled the peroxisomal system. Microsomes, like peroxisomes, contain the acetyl- and octanoyltransferase, and both of these microsomal activities increased 2- to 3-fold in specific activity. However, these two enzymes are tightly as-

Table 2. Effect of clofibrate on the isopycnic density of subcellular fractions*

Subcellular fraction	Control (density of peak in g/cc)	Clofibrate
Peroxisomes	1.240 ± 0.003	1.251 ± 0.002†
Mitochondria	1.207 ± 0.002	1.206 ± 0.002
Microsomes	1.152 ± 0.005	1.146 ± 0.003

* Mean values ± S. D. are from three experiments.
† Statistically different from control, $P < 0.01$.

sociated with the reticular membrane, whereas the peroxisomal enzymes are soluble [6]. Another membranous enzyme of the microsomes, NADPH–cytochrome *c* reductase, remained constant or might have slightly increased upon drug treatment ($P < 0.05$).

The differential responses of the three subcellular carnitine acyltransferase systems to clofibrate resulted in a larger percentage of the transferases being mitochondrial (Table 4). Carnitine palmitoyltransferase and cytochrome *c* oxidase were used as markers for the mitochondrial region, since they are found only in that organelle. The greatest change was seen in carnitine acetyltransferase distribution. In control livers, half of the carnitine acetyltransferase activity was extramitochondrial, i.e. peroxisomal and microsomal. In treated livers, only 10–15 per cent was found outside mitochondria. The change in carnitine octanoyltransferase distribution was less because it increased 2- to 3-fold in all of its subcellular locations.

Comparison with phenobarbital. Like clofibrate, phenobarbital is known to cause a preferential induction of smooth endoplasmic reticulum, but without significantly changing the volume of rough endoplasmic reticulum, mitochondria or peroxisomes [20]. Preliminary results indicated that, in contrast to clofibrate, the effects of phenobarbital were limited to the microsomes and its drug-metabolizing system. The isopycnic density of peroxisomes (1.238 g/cc) and per cent of total catalase in particulate fractions (36.5%) were the same as in controls. Total and specific activities of catalase and urate oxidase in the peroxisomes also remained the same (Table 3). No apparent changes in the specific activities of the peroxisomal, mitochondrial or microsomal carnitine acyltransferases were observed. The effect of phenobarbital on NADPH–cytochrome *c* reductase, a component of the drug-metabolizing system, was evident from a 2-fold increase in its total activity to 3.1 μmoles/min/g of liver and in its increase in specific activity (see Table 3).

DISCUSSION

All three carnitine acyltransferase systems of the liver—peroxisomal, mitochondrial and microsomal—respond to clofibrate treatment with an increase in the specific activity of each of their transferase enzymes. Because of the extent of this increase (10-fold for mitochondrial carnitine acetyltransferase) and the fact that the soluble peroxisomal transferase system is affected to the same extent as the membranous microsomal one, it is difficult to attribute

Table 4. Effect of clofibrate on the subcellular distribution of the hepatic carnitine acyltransferases*

Enzyme	Control (% of total activity in mitochondrial region)	Clofibrate
Carnitine acetyltransferase	51 ± 9	87 ± 3†
Carnitine octanoyltransferase	61 ± 3	77 ± 5†
Carnitine palmitoyltransferase	95 ± 3	96 ± 3
Cytochrome *c* oxidase	95 ± 1	95 ± 3

* Mean values ± S. D. are from three experiments.
† Statistically different from control, $P < 0.01$.

Table 3. Effect of clofibrate and phenobarbital on the activity of liver enzymes*

	Control	Clofibrate	Phenobarbital
Total activity		(μmoles/min/g liver)	
Catalase	57,000 ± 3,000	122,000 ± 23,000†	61000
Urate oxidase	0.351 ± 0.068	0.480 ± 0.092	
NADPH–cytochrome *c* reductase	1.508 ± 0.167	1.979 ± 0.449	3.1
Carnitine acetyltransferase	0.420 ± 0.047	5.405 ± 1.225‡	
Carnitine octanoyltransferase	0.699 ± 0.058	3.362 ± 0.818†	
Carnitine palmitoyltransferase	0.195 ± 0.021	1.018 ± 0.275†	
Specific activity		(nmoles/min/mg protein)	
Peroxisomal peak			
Catalase	(5.93 ± 2.61) × 10^6	(5.04 ± 0.46) × 10^6	7.8 × 10^6
Urate oxidase	60.3 ± 18.7	43.4 ± 7.3	63.5
Carnitine acetyltransferase	14.0 ± 1.0	24.6 ± 3.8†	14.0
Carnitine octanoyltransferase	11.1 ± 1.0	37.4 ± 7.7†	12.4
Mitochondrial peak			
Carnitine acetyltransferase	14.1 ± 0.6	137.1 ± 46.2†	15.0
Carnitine octanoyltransferase	26.8 ± 2.9	84.9 ± 9.2†	28.0
Carnitine palmitoyltransferase	12.9 ± 0.9	36.3 ± 4.7‡	12.6
Microsomal peak			
NADPH–cytochrome *c* reductase	109 ± 8	160 ± 22	232†
Carnitine acetyltransferase	7.0 ± 0.3	10.6 ± 1.0†	6.4
Carnitine octanoyltransferase	8.0 ± 0.5	20.9 ± 3.9†	7.6

* Mean values ± S. D. are from three experiments for control and clofibrate, and one experiment for phenobarbital.
† Statistically different from control, $P < 0.01$.
‡ Statistically different from control, $P < 0.001$.

these changes solely to the membrane-proliferating effect of clofibrate. Alternative explanations such as a specific induction or slower turnover of these enzymes must be considered. That the microsomal transferase system was not changed by the microsomal membrane-proliferating drug phenobarital suggests that clofibrate had a specific effect on the transferases.

The carnitine acetyltransferase in the mitochondrial system appears to be under a different control from that in the peroxisomal and microsomal systems. In the latter two systems, the carnitine acetyl- and octanoyltransferase activities both increased the same amount (2- to 3-fold). In the mitochondrial system, the acetyltransferase clearly increased to a greater extent than the other two components, the carnitine octanoyl- and palmitoyltransferases. That the peroxisomal and microsomal transferase systems respond similarly to clofibrate is consistent with the concept that peroxisomes originate from smooth endoplasmic reticulum. Both the peroxisomes and microsomes contain the carnitine acetyl- and octanoyltransferases, but not carnitine palmitoyltransferase, a feature which distinguishes them from the mitochondrial transferase system [4, 5].

Although the clofibrate diet shifted the distribution of carnitine acetyltransferase toward a higher percentage in the mitochondria, the activity of this enzyme actually increased also in the peroxisomes. Carnitine acetyl- and octanoyltransferases are the first reported enzymes whose specific activity in isolated peroxisomes increases with clofibrate treatment [12, 19]. Catalase, a matrix enzyme like the transferase, maintained a constant specific activity, its synthesis keeping pace with the proliferation of the peroxisomes and its complement of protein. The specific activities of other matrix enzymes of the peroxisomes, such as D-amino acid oxidase and L-α-hydroxy acid oxidase, are reportedly depressed by clofibrate treatment [21]. The core enzyme of the peroxisomes, urate oxidase, remained the same in specific activity or was slightly lower than in controls as previously reported [22, 23].

In addition to altering levels of individual peroxisomal enzymes, clofibrate affected the centrifugal behavior of the whole organelle by increasing its isopycnic density in sucrose gradients. Although Kahonen's data with tube gradients suggested this [12], our use of large sucrose gradients in zonal rotors was necessary to establish a statistical significance to this increase. The different centrifugal behavior of these particles in Ficoll gradients has been previously suggested to depend on some structural alteration caused by clofibrate treatment [22]. Similarly, the increase in the per cent of total catalase found in peroxisomal fractions of liver of rats on clofibrate could be due to reduced peroxisomal fragility with less leakage of matrix enzymes into the supernatant. However, this increase in the percentage of particulate catalase could also be explained by a differential response to clofibrate of particulate and cytosolic pools of this enzyme.

Kahonen [12] found a lower percentage of the catalase in the liver peroxisomes of rats on clofibrate, and thought these particles might be less stable because of the treatment. In considering this difference between the two investigations, it must be noted that Kahonen used rats that had been starved for 2 days before sacrifice, while we used non-starved rats. Previously, investigators have used starved rats to facilitate organelle separation in sucrose gradients. Since we found that starvation was not essential for obtaining adequate organelle separation, and since rapid changes in peroxisomal properties occur with starvation, we used animals fed *ad lib.*

A similar but more complex problem exists in trying to determine if there are cytosolic carnitine acyltransferases. Some carnitine acetyl- and octanoyltransferase activity, but no carnitine palmitoyltransferase activity, was found in supernatant fractions (Fig. 1). The amount was slightly more than could be accounted for by peroxisomal breakage [21] if one assumes that catalase is totally peroxisomal and uses it as a measure of breakage. The problem is further complicated by the report that the outer mitochondrial transferase pool is easily lost to the supernatant fractions during homogenization [23]. Also, although we have shown that in isolated microsomal fractions the transferases present are membrane-bound [5], we do not know how much, if any, of the carnitine acetyl- and octanoyltransferase activities were in the reticular lumen before the endoplasmic reticulum was disrupted by homogenization and thus solubilized.

The possible causal increases in hepatic carnitine acyltransferase activities by clofibrate is consistent with its hypolipidemic influence. The ability of aryloxyisobutyrate derivatives such as clofibrate to decrease levels of triglycerides and neutral steroids in mammalian tissues has been known for some time [24]. Their mode of action is unclear, but has been ascribed to blockage of cholesterol synthesis at pre-mevalonic [25] and possibly post-mevalonic sites [26], and inhibition of fatty acid synthesis at both the acetyl-CoA carboxylase [27], and acyl-CoA-α-glycerophosphate acyltransferase [28] steps. Our data add the possibility that clofibrate also affects the availability of the acyl-CoA derivatives, which act as substrates and regulators for many of the above enzymes by changing the levels of carnitine acyltransferase activities.

Increases in the total hepatic activities of one or more of the carnitine acyltransferases have been observed in animals treated with other hypolipidemic drugs (such as nafenopin and methyl clofenapate), which are structural analogs of clofibrate [10], and with others (such as tibric acid) which are structurally unrelated [29]. All of the above compounds also induced peroxisome proliferation in the male rat, but this membrane-proliferating effect is not a necessary prerequisite for the hypolipidemic influence of these drugs. Proliferation of peroxisomes does not occur in female rats treated with clofibrate, yet its hypolipidemic effect is present [30]. Clofibrate, however, did cause a significant increase in total hepatic carnitine acetyltransferase in female rats, but to a lesser extent than in male rats [10].

During clofibrate treatment, the levels of CoA and its thioesters markedly increased [31]. Mitochondrial oxidation of medium- and long-chain fatty acids also increased [32, 33]. In normal mitochondria carnitine had little effect on octanoate oxidation. In mitochondria from clofibrate-treated animals, a 3-fold increase in octanoate oxidation was observed upon the addi-

tion of carnitine [33]. The appearance of this partial carnitine dependence under conditions of high rates of fatty acid oxidation implicates the involvement of carnitine octanoyl- and possibly acetyltransferases in the drug-treated animal.

Acknowledgement—The authors wish to thank Nancy Gerber and Harry Holmes for their technical assistance.

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