Effect of Clofibrate on *Tetrahymena*

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SUMMARY

The effects of clofibrate on the growth, glycogen content, triglyceride content, and levels of some peroxisomal enzymes of the ciliated protozoan *Tetrahymena pyriformis* have been studied. Clofibrate sensitivity inhibited growth and depleted the cell glycogen. The triglyceride content of *Tetrahymena* in Ringer's solution in the absence of an exogenous carbon source was doubled by exposure to clofibrate for 2 hr. Cells grown in the presence of clofibrate for 17 hr showed a 30% increase in isocitric dehydrogenase activity, a 30% decrease in isocitric lyase activity, and a 20% increase in catalase activity. The results are consistent with the hypothesis that clofibrate inhibits gluconeogenesis or activates glycogenolysis.

INTRODUCTION

Peroxisomes (microbodies) are a class of cell organelles containing peroxide-producing oxidases and catalase. They are found in liver and kidney and in the ciliated protozoan *Tetrahymena pyriformis* (1). Their physiological functions are unknown, but it is likely that they play some role in gluconeogenesis (1).

Clofibrate (Atromid-S; ethyl 2-(*p*-chlorophenoxy)-2 methyl- propionate) is a hypolipidemic agent used for the amelioration of disorders of lipid metabolism associated with atherosclerotic vascular disease (2). Its mechanism of action is unknown. Recently, Svoboda *et al.* (3) reported that treatment of mice and male rats with clofibrate caused a large increase in the number of peroxisomes in the liver and a parallel increase in catalase activity. *Tetrahymena* resemble mammalian cells, containing not only peroxisomes, but also epinephrine (4) and serotonin (5), and are sensitive to a variety of drugs known to alter the metabolism of these biogenic amines (6, 7). It therefore seemed possible that *Tetrahymena* might be sensitive to clofibrate. The finding that clofibrate strongly inhibited the growth of *Tetrahymena* led us to examine the effects of clofibrate on some aspects of the growth and metabolism of *Tetrahymena*.

MATERIALS AND METHODS

Tetrahymena pyriformis, strain HSM, were grown axenically in a medium consisting of 1% proteose peptone and 0.05% liver extract in 0.02 M potassium phosphate at pH 6.5. Cells were counted with a Coulter counter (Coulter Co., Hialeah, Florida). In some experiments, cells were grown in Erlenmeyer flasks with Morton closure tops in an incubator at 25°, without shaking, and containing less than one-tenth their nominal capacity. In other experiments, as specified below, the Erlenmeyer flasks were placed in a shaker bath at 27.5°. For experiments in which cells were to be studied for relatively short periods of time in the absence of proteose peptone, the cells were collected by centrifugation and resuspended in a "Ringer's" solution containing the following salts, in g/l: NaCl, 2.750; KCl, 0.149; $MgSO_4 \cdot 7$

H_2O, 0.246; Na_2HPO, 1.37; KH_2PO_4, 0.32; $CaCl_2$, 0.00163. This Ringer's solution was identical to that of Wagner (8) except for the addition of calcium chloride. Its pH was 7.4.

Glycogen was assayed by the phenol-sulfuric acid method of Dubois *et al.* (9), as described in detail elsewhere (6). Protein was measured by the method of Lowry *et al.* (10).

For the estimation of triglycerides, about 15×10^6 cells were collected by centrifugation at 300 *g* for 10 min at 0°. The cellular pellet was resuspended in 5% cold trichloroacetic acid. The resulting precipitate was collected by centrifugation at 300 *g* for 5 min at 0° and extracted with chloroform–methanol–water (27:13:10, v/v). The organic phase was separated by centrifugation, evaporated to dryness under nitrogen, and the remaining lipids were dissolved in benzene. Duplicate aliquots of this solution and standard tripalmitin were applied to thin-layer chromatography plates (TLC plates, silica gel F_{254}, E. Merck AG, West Germany) and developed in *n*-hexane–diethylether–acetic acid–methanol (85:15:1:0.5, v/v). After drying in air, chromatograms were exposed to iodine vapor and the areas corresponding to that of tripalmitin were marked. The iodine color was allowed to fade, the triglyceride spots were recovered from the plate by scraping and measured by the method of Snyder and Stephens (11).

For the preparation of homogenates, about 5×10^6 cells were chilled in ice, collected by centrifugation in the cold, and washed twice with a buffer consisting of 0.25 M sucrose in 0.0667 M phosphate, pH 7.6. All subsequent steps were at 0–4°. The cells were resuspended in about 10 ml of this buffer and treated with ultrasound twice for 30 sec, using a Branson Model LS-75 ultrasonic generator. The sonicate was used without further centrifugation.

Isocitric lyase activity was measured by the method of Kornberg (12). Isocitric dehydrogenase activity was measured by adding 0.3 ml of homogenate (diluted if necessary with sucrose-phosphate) to a cuvette containing 0.2 ml each of 0.05 M DL-sodium isocitrate, 1 mM NADP, 1 mM $MnCl_2$, and 0.3 M Na_2HPO_4, pH 8.0. The isocitric lyase and the isocitric dehydrogenase activities were computed from the linear rates of change of absorbance at 324 mμ and 340 mμ, respectively, using 1 cm pathlength cuvettes and a Gilford model 2000 absorbance recorder. The sample chamber was maintained at 30° by circulating water through thermospacers.

Catalase activity was measured at 0° by the method of Baudhuin *et al.* (13), using 0.1 ml of 2% (v/v) Triton X-100, 0.2 ml of homogenate, and 5 ml of 0.08 M imidazole, pH 7.0, containing 1 g of bovine serum albumin and 0.16 ml of 30% hydrogen peroxide per liter of the imidazole buffer. The first order rate constant is reported in units of mg^{-1} min^{-1} and was computed as though the assay had been performed in a volume of 50 ml.

It was established that clofibrate added to sonicates of control cells had no effect on the isocitric dehydrogenase, isocitric lyase, or catalase activities.

Clofibrate was a gift of Ayerst Laboratories, Inc., New York, New York. Its density, determined by weighing 0.20 ml, was 1.11 g/ml. It was prepared by dissolving 0.10 ml in 5 ml ethanol and then adjusting the volume to 25 ml with distilled water. The suspension was then boiled for 2 min and transferred to a sterile vial. Immediately before use, the suspension was thoroughly mixed on a Vortex mixer. Identical quantities of the same concentration of ethanol were added to control cultures. NADP was purchased from Pabst, Milwaukee, Wisconsin; the sodium salt of DL-sodium isocitrate (allo-free) from Sigma Chemical Co., St. Louis, Missouri; tripalmitin from Applied Science Laboratories, State College, Pennsylvania, titanium sulfate from Fisher Scientific Co., Fair Lawn, New Jersey; ferric perchlorate, from G. F. Smith Chemical Co., Columbus, Ohio; Triton X-100, Rohm and Haas, West Philadelphia, Pennsylvania; Bovine serum albumin, fraction V, Armour Pharmaceutical Co., Kankakee, Illinois.

RESULTS

The growth of *Tetrahymena* is sensitively inhibited by clofibrate (Fig. 1). At 0.124

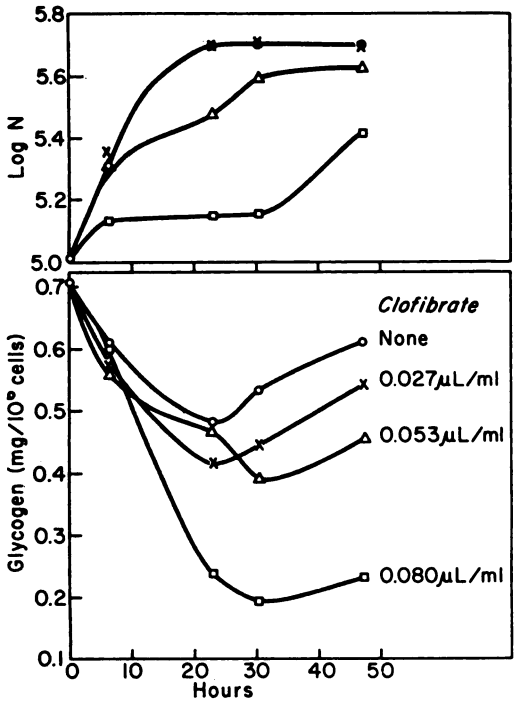

FIG. 1. *Effect of clofibrate on growth rate and glycogen content of Tetrahymena pyriformis*

At zero time 0, 0.2, 0.4, and 0.6 ml amounts of clofibrate suspension were added to Erlenmeyer flasks (500-ml capacity) containing 25 ml of an exponentially growing culture of *Tetrahymena*, and medium to make the final volume in each flask 30 ml. The final concentration of clofibrate in each flask was: ○, none; X, 0.027 μl/ml (1.24×10^{-4} M); △, 0.053 μl/ml (2.43×10^{-4} M); □, 0.080 μl/ml (3.66×10^{-4} M). The control flask (○) also contained 0.6 ml of 20% ethanol.

mM growth is not inhibited, but at 0.366 mM growth is almost completely inhibited for 30 hr. At higher concentrations such as 0.550 mM growth is completely inhibited and cell death occurs by 110 hr of exposure. If, however, the cells are diluted into fresh medium after 30 hr of complete growth inhibition, growth resumes at a near normal rate (data not shown).

Clofibrate rapidly depletes the glycogen content of *Tetrahymena* (Fig. 1). Some glycogen depletion occurs even at concentrations of clofibrate where no growth inhibition is observed, thus suggesting that glycogen depletion is not a consequence of growth inhibition. At high concentrations of clofibrate, the cell glycogen content is drastically depleted. In cultures in which growth resumes after a period of complete growth inhibition, a small increase in glycogen content is seen (Fig. 1). The glycogenolytic effect of clofibrate is also seen under anaerobic conditions.

In initial experiments on the effect of clofibrate on the triglycerides of *Tetrahymena* growing in proteose peptone with shaking, it was found that there was an initial rise in the triglyceride content followed by a decline. Since little is known about the physiology of fat synthesis in *Tetrahymena*, it was of interest to examine the effect of glucose, acetate, and phenylalanine on the fat content of washed cells. In the absence of an exogenous carbon source, there was little change in the triglyceride content in 2 hr (Table 1). The presence of acetate did not lead to an increase in the triglyceride content of washed cells during a 2-hr incubation, but the presence of phenylalanine or glucose increased the triglyceride content significantly. Clofibrate almost doubled the triglyceride content of the cells in the absence of an exogenous carbon source, probably indicating a rapid mobilization of endogenous glycogen or protein. Clofibrate also markedly increased the triglyceride content when acetate or phenylalanine was present, but slightly decreased the net synthesis of triglyceride in the presence of glucose. Thus, under circumstances where the exogenous carbon source could give rise to glucose units only via gluconeogenesis, triglyceride synthesis was enhanced by clofibrate, but when glucose was supplied exogenously and triglyceride synthesis was very rapid, clofibrate slightly inhibited net triglyceride formation.

Because of the findings of Svoboda *et al.* (3) it was decided to examine the effect of clofibrate on the activity of certain enzymes of *Tetrahymena*. The isocitric dehydrogenase of *Tetrahymena* is an NADP-linked enzyme, some of which is located in the peroxisome, the remainder being in the nonsedimentable supernatant fraction of the homogenate. The other enzyme which utilizes isocitrate is isocitric lyase, which has recently been shown to be localized in *Tetrahymena* in the peroxi-

TABLE 1

Effect of clofibrate on triglyceride content of Tetrahymena

Late log phase cells growing in a shaker bath at 27.5° were collected by centrifugation at 300 *g* for 5 min at 15° and resuspended in Ringer's solution. At zero time appropriate amounts of clofibrate, glucose, acetate, L-phenylalanine, or water were added to 45-ml aliquots of cell suspension in a 125-ml culture flask with a surface area of 39 cm^2. Volumes were adjusted to 50 ml with Ringer's solution so that final concentrations of additions were as shown above and cell density was 388,000 cells/ml. The flasks were then placed in a shaker bath at 27.5°. At zero time an aliquot of cell suspension in Ringer's solution was assayed for triglyceride as described in Materials and Methods. After 2 hr, cells were collected and assayed for triglyceride.

Experiment	Additions	Initial triglyceride ($\mu g/10^6$ cells)	Final triglyceride ($\mu g/10^6$ cells)	
			Clofibrate	
			None	3.11×10^{-4} M
A	None	14.3	14.7	27.0
	L-Phenylalanine, 5 mM		20.5	31.7
	Glucose, 10 mM		35.4	27.8
B	None	16.1	14.7	23.8
	L-Phenylalanine, 5 mM		18.6	21.5
	Glucose, 10 mM		20.7	19.2
C	None	8.0	10.1	16.5
	Acetate, 10 mM		10.6	19.0

somes (14). Catalase is also a peroxisomal enzyme in *Tetrahymena* (1). When cells were exposed for 17 hr to concentrations of clofibrate which caused mild growth inhibition, there was no significant change in the protein content, but there was about a 30% increase in the isocitric dehydrognease activity, a 30% decrease in the isocitric lyase activity, and a 20% increase in the catalase activity (Table 2). At slightly higher concentrations of clofibrate, where growth inhibition was almost com-

TABLE 2

Effect of clofibrate on isocitric dehydrogenase, isocitric lyase, and catalase activities of Tetrahymena

Cells from young cultures growing in 500 ml capacity Erlenmeyer flasks with about 150 ml culture fluid and at densities of about 73,000 and 118,000 cells/ml for experiments I and II, respectively, were used in these experiments. Suitable aliquots were put into Erlenmeyer flasks of 1-liter capacity, and the volume was adjusted to 40 ml by the addition of fresh culture medium. Clofibrate and 20% ethanol were added so that each flask had the indicated concentration of clofibrate and 0.4 ml of 20% ethanol. Initial cell counts were taken, and the cultures were incubated without shaking at 25° for 17 hr, counted, and collected by centrifugation. After washing twice in sucrose-phosphate buffer, duplicate aliquots of the washed cells were taken for the measurement of cell protein content. The remaining washed cells were then sonicated and the enzyme activities were assayed as described in the section in methods.

Expt. No.	Clofibrate ($M \times 10^4$)	Cells/ml		Isocitric dehydrogenase (μmoles/min/mg)	Isocitric lyase (μmoles/hr/mg)	Catalase ($min^{-1}mg^{-1}$)	Mg protein per 10^6 cells
		Initial	Final				
I	0	36,400	158,000	0.40	0.49	0.157	1.42
	1.36	72,800	228,000	0.54	0.30	0.178	1.24
	1.81	72,800	218,000	0.56	0.30	0.188	1.25
II	0	29,200	145,000	0.34	0.80	0.120	1.39
	1.36	58,400	150,000	0.44	0.56	0.143	1.40
	1.81	73,000	209,000	0.44	0.56	0.147	1.39

plete, the catalase activity generally increased by at least 30%.

DISCUSSION

Although clofibrate has been used in humans for the control of hyperlipemic states, remarkably little is known about its mode of action. One school of thought holds that it acts by competing with anions such as thyroxine for an anion binding site on plasma proteins (15). These displaced hormones are then thought to influence liver metabolism via normal physiological control mechanisms and thus to decrease the plasma content of triglycerides and cholesterol. Such a mechanism is highly improbable for *Tetrahymena*. Alternatively, Avoy *et al.* (16) suggested that clofibrate acts at a site between acetyl-CoA and mevalonic acid, since in their experiments on rat liver slices the rate of conversion of mevalonate-^{14}C to cholesterol was only slightly decreased by clofibrate whereas the rate of conversion of acetate-^{14}C to cholesterol was considerably inhibited. Azarnoff *et al.* (17), also working with rat liver, concluded that the site of inhibition due to clofibrate is between mevalonic acid and isopentonyl pyrophosphate. *Tetrahymena* do not contain cholesterol but instead have a closely related sterol called tetrahymanol (18), which is present to the extent of 0.4% (19) to 5% (20) of the total lipid content. Thus, even if the action of clofibrate were assumed to be between acetyl-CoA and isopentenyl pyrophosphate, the increase in triglycerides in washed *Tetrahymena* could not be explained by the diversion of acetyl units from the pathway of tetrahymanol biosynthesis.

Although great stress is laid on the hypolipidemic effects of clofibrate, it was observed by Platt and Thorp (15) that clofibrate also lowered the glycogen content of rat liver by almost 50%. *Tetrahymena* has a very high capacity for gluconeogenesis. In proteose peptone–liver extract culture medium, glycogen is synthesized primarily from amino acids, but it has been reported (8, 21) that these cells can synthesize glycogen from their endogenous lipids as well as from amino acids. The high capacity for the utilization of the carbon of at least some amino acids is further indicated by the present finding that phenylalanine serves as a source for the net synthesis of triglycerides. The effect of clofibrate in depleting cell glycogen content in *Tetrahymena* [and in rat liver (15)] suggests that clofibrate either interferes with a step in gluconeogenesis or activates glycogenolysis. If glyconenogenesis were inhibited by clofibrate, the increase in triglyceride content either in the presence of exogenous acetate or phenylalanine or in the absence of an exogenous carbon source would be a natural consequence of the shunting of the acetyl groups into the fatty acid synthesis pathways. This hypothesis does not explain the slight decrease in net triglyceride synthesis from exogenous glucose. It is interesting to note, however, that clinically clofibrate appears to be most useful in the treatment of those hyperlipemias associated with disorders of carbohydrate metabolism (22).

It is generally agreed that clofibrate induces hepatomegaly in rats, but its effect on liver protein concentration is variable. Platt and Thorp (15) reported a 20% increase in the protein concentration of rat liver after 5 days of drug treatment, but Azarnoff *et al.* (17) observed no significant increase. In *Tetrahymena* there was no significant effect of clofibrate on cell protein content, but there were significant changes in the activities of the three enzymes studied. These changes, though relatively small, were not merely due to growth inhibition since in other studies (Blum, to be published) growth inhibition caused by various adrenergically active drugs is accompanied by a variety of patterns of response of these three enzymes. It should also be noted that in the experiments reported in Table 2 the concentrations of clofibrate were chosen to give only partial inhibition of growth, so that the precise enzymatic state of the initial culture did not affect the results.

It is difficult to interpret the meaning of the changes in these enzyme activities,

and there is no reason to suppose that clofibrate's action is directly on any of these enzymes. In liver, Platt and Cockrill (23) reported changes in the enzymatic activities of several dehydrogenases, and it is probable that the increase in NADP-linked isocritic dehydrogenase observed in *Tetrahymena* is merely part of a general shift in enzyme levels in response to an altered pattern of metabolism. In the liver of male rats, both the number of peroxisomes and the catalase activity were increased by clofibrate, the latter increasing by about 80%. In *Tetrahymena* the peroxisome content has not yet been studied with the electron microscope, but the catalase content was increased by clofibrate, though to a lesser extent than in liver. It should be noted that the increase in catalase activity occurred in conjunction with a decrease in the isocitric lyase activity, thus suggesting that the peroxisome does not respond as a unit. Recent experiments of Levy and Hunt (24) show that isocitric lyase levels of *Tetrahymena* are repressed by glucose, which also acts to prevent gluconeogenesis, whereas the L-α-hydroxy-acid oxidase activities are increased by growth in the presence of glucose. Thus the experiments of Levy and Hunt (24) also indicate that the peroxisomal enzymes may respond differently to growth conditions. Since isocitric lyase is a key enzyme in gluconeogenesis in *Tetrahymena*, the decrease in its activity caused by clofibrate may account for part of the loss of glycogen.

Although neither in liver nor *Tetrahymena* is there any evidence to indicate a direct effect of clofibrate on peroxisomes, it may be more than a coincidence that liver and *Tetrahymena* have in common a high capacity for gluconeogenesis, an adrenergic metabolic control mechanism, and a sensitivity to the drug clofibrate which is manifested by changes in peroxisomal enzymes. Further work on the mechanism of action of clofibrate and on the physiology of peroxisomes is needed before the meaning of these coincidences can be assessed. Because of the great simplicity of *Tetrahymena* in comparison to even an isolated perfused liver, studies with *Tetrahymena* may provide new insights into the hypolipidemic effects of clofibrate in mammals.

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REFERENCES

1. C. de Duve and P. M. Baudhuin, *Physiol. Rev.* **46**, 323 (1966).
2. Symposium on Atromid, *J. Atherosclerosis Res.* **3**, Nos. 5 and 6 (1963).
3. D. Svoboda, H. Grady and D. Azarnoff, *J. Cell Biol.* **35**, 127 (1967).
4. K. Janakidevi, V. C. Dewey and G. W. Kidder, *J. Biol. Chem.* **241**, 2576 (1966).
5. K. Janakidevi, V. C. Dewey and G. W. Kidder, *Arch. Biochem. Biophys.* **113**, 758 (1966).
6. J. J. Blum, *Proc. Natl. Acad. Sci. U.S.* **58**, 81 (1967).
7. C. G. Rogers, *Can. J. Biochem.* **44**, 1493 (1966).
8. C. Wagner, "The Glycogen Metabolism of *Tetrahymena pyriformis*." Thesis, Univ. of Michigan, 1956.
9. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.* **28**, 350 (1956).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
11. F. Snyder and N. Stephens, *Biochim. Biophys. Acta* **34**, 244 (1959).
12. H. L. Kornberg, *Colloques Intern. Centre Natl. Rech. Sci. (Paris)*, p. 193. (1963).
13. P. Baudhuin, H. Beaufay, Y. Rahman-Li, O. Z. Sellinger, R. Wattiaux, P. Jacques and C. de Duve, *Biochem. J.* **92**, 179 (1964).
14. M. Muller and J. F. Hogg, *Federation Proc.* **26**, 284 (1967).
15. D. S. Platt and J. M. Thorp, *Biochem. Pharmacol.* **15**, 914 (1966).

16. D. R. Avoy, E. A. Swyryd and R. G. Gould, *J. Lipid Res.* **6**, 369 (1965).
17. D. L. Azarnoff, D. R. Tucker and G. A. Barr, *Metabolism* **14**, 959 (1965).
18. F. B. Mallory, J. T. Gordon and R. L. Conner, *J. Am. Chem. Soc.* **85**, 1362 (1963).
19. M. S. Shorb, B. E. Dunlap and W. O. Pollard, *Proc. Soc. Exptl. Biol. Med.* **118**, 1141 (1964).
20. G. A. Thompson, Jr., *Biochemistry* **6**, 2015 (1967).
21. J. F. Hogg and H. L. Kornberg, *Biochem. J.* **86**, 462 (1963).
22. R. P. Howard, P. Alaupovic, O. J. Brusco, and R. H. Furman, *J. Atherosclerosis Res.* **3**, 482 (1963).
23. D. S. Platt and B. L. Cockrill, *Biochem. Pharmacol.* **15**, 927 (1966).
24. M. R. Levy and A. E. Hunt, *J. Cell Biol.* **34**, 911 (1967).