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EFFECT OF CLOFIBRATE ON CO_2 FIXATION INTO GLYCOGEN AND FATTY ACIDS VIA THE LEUCINE CATABOLISM PATHWAY IN *TETRAHYMENA*

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Summary

Tetrahymena pyriformis were grown to stationary phase and then incubated for 17 h with 0.21 mM clofibrate, a concentration that causes considerable growth inhibition when added to exponentially growing cells. After the clofibrate treatment, the cells were resuspended in a salt solution and the incorporation of label from [1-^{14}C]leucine, [1-^{14}C]tyrosine, [1-^{14}C]pyruvate, and [^{14}C]bicarbonate into glycogen and into the fatty acid and glycerol moieties of lipids was measured. Each of these substrates yields $^{14}CO_2$ at an early step of its catabolism, so that incorporation of label into these products is a measure of CO_2 fixation. Clofibrate-treated cells incorporated a 2- or more-fold label from leucine, tyrosine, and bicarbonate into the fatty acid moieties of the lipids than did control cells, but only slightly more into the glycerol moiety. Because the only pathway for CO_2 fixation into fatty acids in *Tetrahymena* is via leucine degradation, these results demonstrate that clofibrate increases CO_2 fixation via the leucine degradative pathway.

Clofibrate treatment reduced $^{14}CO_2$ formation from [1-^{14}C]-labeled glucose, ribose, and glycerol by about 30—40%, but not from [1-^{14}C]-labeled glyoxylate, acetate, hexanoate, or octanoate. Incorporation of label from each of these substrates (and from tyrosine and leucine) into glycogen was increased (1.2-fold for glucose, up to 3.2-fold for octanoate) by clofibrate treatment. In addition to the increase in $^{14}CO_2$ fixation via the leucine catabolic pathway, these results show that clofibrate does not appreciably alter flux through the Krebs cycle or the glyoxylate bypass, but increases glyconeogenic capacity and inhibits glycolytic capacity.

Introduction

There are two major pathways of CO_2 fixation in *Tetrahymena.* $^{14}CO_2$ fixed in the step catalyzed by β-methyl crotonyl-CoA carboxylase, part of the leucine degradative pathway, appears in C_1 of acetoacetate and hence in C_1 of acetyl-CoA, where it may be used for lipogenesis or, via the glyoxylate bypass, for glyconeogenesis [1]. $^{14}CO_2$ may also be fixed via phospho*enol*pyruvate carboxylase, forming [4-^{14}C]oxaloacetate. After randomization of the label by reversal of the Krebs cycle to form the symmetric compound 1,4-succinate, and thus [1-^{14}C]oxaloacetate, label will appear in glycogen via the usual pathway beginning with phospho*enol*pyruvate caboxykinase. In *Tetrahymena,* $^{14}CO_2$ released from the decarboxylation of α-ketoisocaproate (the first step of the leucine degradative pathway) is preferentially fixed in comparison to added bicarbonate. This, plus a difference in the ratio of label incorporated from [1-^{14}C]leucine into lipid as compared to CO_2 demonstrates that there are at least two pools of CO_2 in *Tetrahymena* [1], but details of pool structure are lacking.

A number of years ago we reported [2] that low concentrations of clofibrate sensitively inhibited growth and depleted the glycogen content of *Tetrahymena.* It was also found that cell triacylglycerol content was increased, which was of interest in view of the well-known hypolipidemic effect of clofibrate in higher animals including man [3]. Nozawa [4] subsequently showed that an increased incorporation of label from [1-^{14}C]acetate into the triacylglycerol was accompanied by a corresponding decrease in phospholipid biosynthesis and in the synthesis of the sterol-like lipid, tetrahymenol, a component of the membranes of this cell. In view of these reports it appeared that not only was clofibrate a useful drug for studying lipid metabolism in *Tetrahymena,* but, more particularly it might be useful as a probe to examine the control of CO_2 fixation into lipids in this organism.

In this paper we report on a series of experiments in which [1-^{14}C]leucine, L-[1-^{14}C]tyrosine, [1-^{14}C]pyruvate, and [^{14}C]bicarbonate were used as substrates in stationary phase *Tetrahymena* that had been treated with clofibrate. Each of these substrates produces $^{14}CO_2$ at a very early step of its metabolism, so that appearance of label in glycogen or in lipids must result from CO_2 fixation. Because clofibrate affects both lipogenesis and glycogenesis in clofibrate-treated as compared to control cells, such experiments might provide further information on the compartmentation of the CO_2 pools as well as insight into the mode of action of clofibrate.

Materials and Methods

Tetrahymena pyriformis, strain HSM, were grown axenically in a medium consisting of 1% proteose peptone and 0.05% liver extract in 20 mM potassium phosphate at pH 6.5. Stock cultures were transferred daily, 11 ml of a 24 h stock being used to inoculate 100 ml of medium in a 500 ml capacity Erlenmeyer flask. Experimental flasks containing 100 ml medium were inoculated with 10 ml of a 24 h stock and kept at 25°C in a gyratory shaker for 24 h, i.e. until the beginning of stationary phase (approx. $9 \cdot 10^5$ cells/ml). Clofibrate (1.3 ml of 18.3 mM in 20% (v/v) ethanol) was then added to experimental

flasks and an equal volume of 20% (v/v) ethanol to control flasks. 17 h after addition of clofibrate or ethanol to the cells, during which time there was less than a 5% increase in cell density even in the control flasks, the cultures were counted, centrifuged at room temperature for 3 min at 200 × *g* and resuspended in Wagner's solution [5] at room temperature.

Labeled substrates. Substrates were used at the following concentrations and specific activities: [1-^{14}C]leucine, 3 mM, approx. 200 dpm/nmol; $NaH^{14}CO_3$, 6 mM, approx. 500 dpm/nmol; [1-^{14}C]pyruvate, 3 mM, approx. 250 dpm/nmol; L-[1-^{14}C]tyrosine, 0.75 mM, approx. 1500 dpm/nmol; [1-^{14}C]glucose, 6 mM, approx. 25 dpm/nmol; [1-^{14}C]ribose, 3 mM, approx. 120 dpm/nmol; [1-^{14}C]glycerol, 3 mM, approx. 20 dpm/nmol; [1-^{14}C]acetate, 3 mM, approx. 100 dpm/nmol; [1-^{14}C]hexanoate, 0.67 mM, approx. 100 dpm/nmol; [1-^{14}C]-octanoate, 0.67 mM, approx. 150 dpm/nmol.

For measurement of label incorporation into CO_2, 3 ml of cells suspended in Wagner's solution at a density of about 0.8—1.1 · 10^6 cells/ml were pipetted into 50-ml Erlenmeyer flasks equipped with removable center wells and containing 0.4 ml of substrates dissolved in water. After 1 h at 25°C in the gyratory shaker, $^{14}CO_2$ was collected and counted as described elsewhere [1]. The average value of $^{14}CO_2$ production from three identical flasks constituted a single datum.

For measurements of label incorporation into lipid or glycogen, 9 ml of cells in Wagner's solution were pipetted into 125-ml capacity Erlenmeyer flasks containing 1.2 ml of substrates dissolved in water and incubated at 25°C in a shaker bath for 1 h. Three 3-ml samples were then pipetted into 7 ml of 2% (v/v) trichloroacetic acid (for measurement of ^{14}C incorporation into lipids) or into 7 ml of ice-cold 95% (v/v) ethanol (for measurement of incorporation into glycogen).

Samples to be analyzed for glycogen content and for label incorporation into glycogen were treated by the glucoamylase procedure described elsewhere [1].

Samples to be analyzed for label incorporation into the fatty acid and glycerol moieties of lipids were extracted using a modification of the procedure described earlier [6]. The combined $CHCl_3/CH_3OH$ supernatants were washed with saline and then dried under N_2 in 12-ml screw-cap centrifuge tubes, and 1 ml of warm 7.5% (w/v) KOH in 25% (v/v) methanol was added. The tubes were capped tightly and heated for 4 h at 110°C. After cooling, 1.0 ml of 6 N HCl was added and approx. 10^6 dpm each of [^{3}H]oleate and [^{3}H]palmitate were added in 0.2 ml of benzene/hexane (1 : 1, v/v) to serve as recovery standards. Three 3-ml diethyl ether extractions were then performed. The aqueous phase was counted to determine radioactivity in lipid glycerol. The pooled ether phase was evaporated under N_2 and processed as described by Borgstrom [7], and the extracted fatty acids counted on double-label settings. All radioactive counts were performed in a Packard scintillation counter equipped with an automatic external standard.

Enzyme assays. After the 17 h exposure to 0.21 mM clofibrate in stationary phase, the cultures were chilled, centrifuged for 3 min at 200 × *g* at 0°C, and the cells resuspended in a buffer consisting of 0.5 mM reduced glutathione, 0.25 mM sucrose, 66.7 mM NaH_2PO_4, adjusted to pH 7.6. The cells were washed and disrupted by ultrasound and the homogenate assayed for catalase

and isocitrate lyase as described earlier [2]. The homogenate was also assayed for glutamate dehydrogenase by the method of Balinsky et al. [8] and for β-hydroxybutyryl-CoA dehydrogenase and thiolase as described by Lazarow [9].

Reagents. Clofibrate was a gift of Ayerst Laboratories, Inc., New York, NY, and was sterilized and handled as described earlier [2]. All radioactive chemicals were purchased from Amersham and other compounds from Sigma Chemical Co., St. Louis, MO.

Results

The concentration of clofibrate used in these experiments (0.047 μl/ml; 0.21 mM) was enough so that, had it been added to cells in early logarithmic growth phase, a considerable inhibition of growth would have been observed [2]. When added to cells that had already entered stationary phase, there was, of course, no effect on growth rate, and there was no evidence of any deleterious effect on cell motility as observed microscopically, nor were the cells noticeably more fragile during centrifugation than the controls.

Effect of clofibrate on glycogen content

In our earlier work, clofibrate caused a large reduction in glycogen content [2]. This, however, could have been a consequence of the inhibition of growth. In the present work, where clofibrate was added to cells that had already entered the stationary phase of growth, there was no difference in glycogen content between clofibrate-treated cells and controls at the end of the 17 h incubation; glycogen content ranged from 200 to 400 mg/10^6 cells, but the ratio of glycogen content in clofibrate-treated cells to that of controls was 1.05 ± 0.08 (n = 7).

Effect of clofibrate on CO_2 fixation from [1-^{14}C]leucine

Table I shows the results of three sets of experiments in which L[1-^{14}C]-leucine was incubated with or without the addition of unlabeled bicarbonate, pyruvate, or tyrosine, with clofibrate-treated and control cells. The amount of leucine entering into the metabolic pathway was measured by the production of $^{14}CO_2$, released in the first step after deamination to α-ketoisocaproate. The amount of $^{14}CO_2$ refixed was measured by the appearance of label in glycogen and in the glycerol and fatty acid moieties of the lipids. In each of the three sets of experiments the reciprocal incubations were also performed, e.g. in Expt. A the cells were incubated with labeled bicarbonate, with and without the addition of unlabeled L-leucine.

Examination of the results obtained when [1-^{14}C]leucine was the only substrate shows that about 80—100 nmol of leucine were decarboxylated per 10^6 cells in the hour incubation, and that the amount utilized increased only slightly (1.16—1.35-fold) in clofibrate-treated cells as compared to controls (Expts. B and C). CO_2 fixation into glycogen increased 1.4-fold (Expts. A and C) but CO_2 fixation into glycerol averaged only 1.1-fold higher in the clofibrate-treated cells. Assuming a single pool of triose phosphates and that they are almost fully equilibrated isotopically [6], this implies an increase in

TABLE I

EFFECT OF CLOFIBRATE ON CO_2 FIXATION FROM L-[1-^{14}C]LEUCINE, [1-^{14}C]PYRUVATE, [1-^{14}C]TYROSINE, AND $H^{14}CO_3$

Cells were treated with 0.21 mM clofibrate (CPIB) or ethanol only (controls) as described in Materials and Methods. They were washed and resuspended in Wagner's solution containing leucine, pyruvate, tyrosine, and $NaH^{14}CO_3$, as indicated. Unlabeled substrates, when present, were at the same concentrations. After incubation for 1 h label appearing in the indicated products was measured as described in Materials and Methods. For Expt. A, the means for the ratios of label incorporation into glycogen and into fatty acids are compared with the respective means for incorporation into glycerol. For Expt. C, the means of the CO_2 data and of the glycerol data are compared with the fatty acid data.

	Substrates		Products: CO_2		Glycogen		Fatty acids		Glycerol	
	Labeled	Unlabeled	Control range (nmol/10^6 cells per h)	+CPIB/—CPIB	Control range (nmol/10^6 cells per h)	+CPIB/—CPIB	Control range (nmol/10^6 cells per h)	+CPIB/—CPIB	Control range (nmol/10^6 cells per h)	+CPIB/—CPIB
A	Leucine	—			5—9	1.36 ± 0.16 ** (n = 5)	0.63—5.8	2.27 ± 0.96 *** (n = 9)	0.41—1.4	1.09 ± 0.20 (n = 9)
	Leucine	HCO_3			4—7	1.41 ± 0.45 *	0.13—1.8	2.02 ± 0.52 ***	0.33—1.4	1.06 ± 0.17
	HCO_3	—			8—20	1.72 ± 0.26 ***	0.20—4.6	2.23 ± 0.89 ***	0.13—1.5	1.07 ± 0.25
	HCO_3	Leucine			24—42	1.48 ± 0.24	2.1—44	7.39 ± 7.6 *	0.29—2.6	1.23 ± 0.34
B	Leucine	—	84—105	1.35 ± 0.12 (n = 3)			0.96—5.9	1.85 ± 0.99 (n = 4)	0.48—1.9	1.16 ± 0.19 (n = 4)
	Leucine	Pyruvate	81—89	1.28 ± 0.04			1.07—7.9	1.46 ± 0.49	0.30—1.9	1.31 ± 0.40
	Pyruvate	—	164—178	1.22 ± 0.08			0.25—0.62	1.25 ± 0.15	0.11—0.51	1.05 ± 0.16
	Pyruvate	Leucine	192—220	1.18 ± 0.07			1.2—3.5	1.67 ± 0.42	0.18—0.67	0.97 ± 0.11
C	Leucine	—	82—90	1.16 ± 0.20 ** (n = 3)	2—5	1.45 ± 0.49 (n = 3)	0.36-1.3	2.54 ± 0.48 (n = 3)	0.56—0.57	1.60 ± 0.07 (n = 3)
	Leucine	Tyrosine	82—95	1.09 ± 0.18	2—4	1.40 ± 0.35	0.64—1.4	1.76 ± 0.96	0.56—0.53	1.04 ± 0.31
	Tyrosine	—	28—37	1.22 ± 0.20 *	0.31—0.89	1.10 ± 0.06	0.01—0.02	3.67 ± 1.45	0.16—0.17	1.10 ± 0.15 ***
	Tyrosine	Leucine	3.5—3.8	1.15 ± 0.07 **	0.12—0.47	1.25 ± 0.27	0.01—0.05	5.25 ± 1.80	0.08—0.11	0.83 ± 0.11 ***

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.005$.

activity of the glycogenic pathway at a step above the level of the triose phosphates. CO_2 fixation into fatty acids increased about 2.3-fold in clofibrate-treated cells as compared to controls. Thus clofibrate treatment increases CO_2 fixation into glycogen and, especially, into lipid more than can be accounted for by the slight increase in total leucine utilization.

The presence of unlabeled bicarbonate caused a slight decrease in the amount of label from [1-^{14}C]leucine incorporated into glycogen and into glycerol, but a 3-fold decrease in CO_2 fixation into fatty acids (Table I), as expected from earlier results [1]. The presence of the bicarbonate did not, however, change the relative incorporation into any of the products in clofibrate-treated as compared to control cells. Neither pyruvate nor tyrosine caused any changes in the amounts of [1-^{14}C]leucine utilized nor in the incorporation of the $^{14}CO_2$ into any of the products measured in either control of clofibrate-treated cells (Table I).

Effect of leucine on CO_2 fixation from labeled bicarbonate

The incorporation of [^{14}C]bicarbonate into glycogen and into the glycerol moiety of lipid was increased about 2—3-fold by leucine in clofibrate-treated cells, while incorporation into fatty acids increased about 10-fold as expected from earlier results [1]. The ratio of $^{14}CO_2$ fixation into glycogen in clofibrate-treated relative to control cells was about 1.7, and independent of the addition of unlabeled leucine. This is significantly larger than the comparable ratio of about 1.1-fold for $^{14}CO_2$ fixation into glycerol, again indicating an increased flux through the glycogenic pathway above the level of the triose phosphates. The ratio of $^{14}CO_2$ fixation into fatty acids in clofibrate-treated cells relative to control was approx. 2.2-fold, i.e clofibrate preferentially increased bicarbonate fixation into fatty acids. It should be noted that this occurred even in the absence of any added metabolizable substrate. Since the primary pathway for incorporation of [^{14}C]bicarbonate into fatty acids in *Tetrahymena* is via the leucine degradation pathway, this suggests not only that clofibate treatment increased flux through this pathway but also that there was an adequate supply of leucine, which in turn requires that some protein degradation occurs during the hour incubation in Wagner's solution. We have shown elsewhere [5] that the requisite amount of protein degradation is likely to occur and comprises at most 1% of the dry weight of the cell. The presence of unlabeled leucine increased label incorporation from [^{14}C]bicarbonate into fatty acids over 3-fold, the ratio 7.39 to 2.23 (Expt. A) being significant at the $P < 0.05$ level. Thus both in the presence and absence of exogenous leucine, clofibrate preferentially increases the incorporation of label from [^{14}C]bicarbonate into fatty acids and, to a lesser extent, into glycogen, as compared to glycerol.

Effect of leucine on CO_2 fixation from L-[1-^{14}C]tyrosine

Addition of leucine to cells metabolizing carboxyl-labeled tyrosine causes a profound inhibition of tyrosine utilization (Table I). This requires either that the entrance of tyrosine into the cell be inhibited by leucine or that tyrosine transaminase or *p*-hydroxyphenylpyruvic acid oxidase be inhibited by leucine or a catabolite of leucine. Because phenylalanine and leucine appear to share a common transport system in *Tetrahymena* [10], it is likely that the inhibition

of the tyrosine utilization by leucine is an inhibition of its entry into the cell. Since only a small fraction of the $^{14}CO_2$ from tyrosine decarboxylation is fixed, the inhibition of tyrosine utilization is scarcely noticed in $^{14}CO_2$ fixation into glycogen or the lipid moieties.

Although leucine strongly inhibits tyrosine utilization, there was no significant difference betweeen the ratios of $^{14}CO_2$ fixation into glycogen or into glycerol in clofibrate-treated versus control cells (Expt. C, Table I). Clofibrate-treated cells, however, incorporated 3—5-fold more $^{14}CO_2$ from [1-^{14}C]tyrosine into fatty acids than did control cells, as observed with labeled bicarbonate.

Whereas addition of unlabeled leucine increased fixation of $^{14}CO_2$ from labeled bicarbonate into glycogen by 2—3-fold, unlabeled leucine decreased the incorporation into glycogen of $^{14}CO_2$ produced by tyrosine decarboxylation. This may indicate that $^{14}CO_2$ released from [1-^{14}C]tyrosine is diluted with CO_2 produced from oxidation of the unlabeled fumarate made available from the further degradation of the tyrosine.

Effect of leucine on $^{14}CO_2$ fixation from [1-^{14}C]pyruvate

Addition of unlabeled pyruvate to cells metabolizing [1-^{14}C]leucine did not change the amount of $^{14}CO_2$ produced nor the amount fixed into any of the products measured. The addition of unlabeled L-leucine to cells oxidizing [1-^{14}C]pyruvate, however, caused a 5-fold increase in $^{14}CO_2$ fixation into fatty acids and a slight increase of $^{14}CO_2$ fixation into the glycerol moiety of the lipids. Thus additional leucine affects CO_2 fixation from [1-^{14}C]pyruvate in a manner similar to the way it affects CO_2 fixation from [^{14}C]bicarbonate, but differently than it affects CO_2 fixation into the glycerol and fatty acid moieties from [1-^{14}C]leucine. In contrast to the appreciable enhancement of incorporation of $^{14}CO_2$ from [1-^{14}C]leucine, [^{14}C]bicarbonate, and [1-^{14}C]tyrosine into fatty acids by clofibrate, there was relatively little effect of clofibrate treatment on CO_2 fixation from [1-^{14}C]pyruvate into fatty acids.

Effect of clofibrate on utilization of [1-^{14}C]glyoxylate

Although the experiments shown in Table I did not indicate any appreciable increase in glyoxylate bypass activity as a result of clofibrate treatment, it was important to check this directly. When [1-^{14}C]glyoxylate was added to clofibrate-treated cells, there was only a 1.2-fold increment in $^{14}CO_2$ production and a 1.3-fold increment in $^{14}CO_2$ fixation into glycogen as compared to control cells (Table II). This rules out any large change in glyoxylate bypass activity as a result of clofibrate treatment.

Effect of clofibrate on oxidation of and glycogen synthesis from glucose, ribose, and glycerol

The formation of $^{14}CO_2$ from [1-^{14}C]glucose, [1-^{14}C]ribose, or [1-^{14}C]-glycerol requires flux through parts of the glycolytic pathway and, in the case of ribose, through the non-oxidative steps of the pentose phosphate pathway. For each of these substrates there was an appreciable inhibition of oxidative metabolism (Table II) as a result of clofibrate treatment. Since this was not observed with [1-^{14}C]glyoxylate, which is also oxidized via the Krebs cycle after condensing with acetyl-CoA in the peroxisomes to form [1-^{14}C]malate

TABLE II

EFFECT OF CLOFIBRATE ON $^{14}CO_2$ PRODUCTION AND CO_2 FIXATION INTO GLYCOGEN

Cells were treated as in Table I but resuspended in Wagner's solution containing labeled substrates as indicated. After incubation for 1 h label appearing in CO_2 and in glycogen was measured as described in Materials and Methods. Results are shown as mean ± S.D., with $n = 3$ for the experiments with glyoxylate, ribose, glucose, and glycerol, and $n = 5$ for the experiments with acetate, hexanoate, and octanoate. CPIB, clofibrate.

(1-^{14}C)-labeled substrate	CO_2		Glycogen	
	Control range (nmol/10^6 cells per h)	+CPIB/—CPIB	Control range (nmol/10^6 cells per h)	+CPIB/—CPIB
Glyoxylate	15.2—15.4	1.20 ± 0.14	5.5—6.5	1.30 ± 0.10
Glucose	25—34	0.71 ± 0.12	420—514	1.25 ± 0.14
Ribose	2.3—3.8	0.69 ± 0.25	12—15	1.40 ± 0.09
Glycerol	37—42	0.60 ± 0.06	27—29	1.65 ± 0.17
Acetate	811—911	1.06 ± 0.14	33—63	1.57 ± 0.21
Hexanoate	60—88	1.10 ± 0.13	1.3—5.4	2.18 ± 0.52
Octanoate	162—183	1.07 ± 0.17	3—10	3.16 ± 2.07

[5], the inhibition of oxidation of glucose, ribose, and glycerol cannot result from an inhibition of the Krebs cycle. It is required, therefore, that at least one step of glycolysis be reduced in clofibrate-treated cells. If only a single step is inhibited, it must be one common to the oxidation of glucose, glycerol, and ribose. Because $^{14}CO_2$ production from [1-^{14}C]pyruvate was slightly increased by clofibrate treatment (Table I, Expt. B), pyruvate dehydrogenase activity cannot have been inhibited by clofibrate treatment. The inhibition is thus probably localized between the level of the triose phosphates and of pyruvate.

Most of the glucose that enters into this cell is used directly for glycogen syntheis (Ref. 6; Stein, R.B. and Blum, J.J., unpublished results). The small increase in label incorporation from [1-^{14}C]glucose into glycogen in clofibrate-treated as compared to control cells (Table II) shows that clofibrate treatment did not cause any inhibition of UDP-glucose pyrophosphorylase or of glycogen synthetase, and also implies that there was adequate ATP to support near maximal glycogen deposition. The larger increase in label incorporation from [1-^{14}C]ribose and [1-^{14}C]glycerol into glycogen in clofibrate-treated cells implies that some step in the glyconeogenic pathway above the level of the triose phosphates had increased activity as compared to control cells. An increase in some step of the glycogenic pathway, presumably fructose diphosphatase, is also consistent with the data shown in Table I.

Effect of clofibrate on CO_2 fixation into glycogen from [1-^{14}C]-labeled acetate, hexanoate, and octanoate

Utilization of acetate, hexanoate, and octanoate, as measured by $^{14}CO_2$ release, was not affected by clofibrate (Table II), indicating no change in Krebs cycle activity or in the overall capacity for β-oxidation. $^{14}CO_2$ fixation into glycogen from these substrates, however, was increased by 1.5-fold or more.

Effect of clofibrate treatment on some peroxisomal enzymes

Because clofibrate treatment markedly increases the activity of several

peroxisomal and mitochondrial enzymes in liver [11], it was of interest to ascertain whether there was any change in the activity of a number of peroxisomal enzymes in *Tetrahymena*. We found no change in the activities of isocitrate lyase or catalase in clofibrate-treated cells as compared to controls, nor did we find any changes in thiolase or β-hydroxybutyryl-CoA dehydrogenase, which are localized in both the mitochondria and the peroxisomes in *Tetrahymena* [12]. Finally, there was no change in glutamate dehydrogenase, which is localized in the mitochondria of this cell [13]. Thus the earlier report [3] of small increases in some peroxisomal enzymes in *Tetrahymena* treated with clofibrate was probably a consequence of the inhibition of growth rather than a direct effect of clofibrate.

Discussion

In these experiments a relatively low concentration of clofibrate was added to cells that had already finished the logarithmic phase of growth, so that if any effects of the drug were detected, they would probably reflect a direct action on metabolism and not an indirect effect resulting from growth inhibition. An increase in the flux of label does not necessarily correspond to an increase in the flux of substrate, because precursor pool specific activities might vary. Because the cells were taken from stationary phase cultures and washed free of growth medium before incubation with the labeled compound, it is unlikely that there were any marked differences in pool sizes between clofibrate-treated and control cells. Thus while the magnitude of label flow varied considerably between experiments (and this could be due in part to differences in pool sizes in different experiments), the ratio of label flow in clofibrate-treated cells to that in control cells is probably a good estimate of the ratio of fluxes.

The results showed a small but definite enhancement in clofibrate-treated cells of carbon flow into glycogen from the level of the triose phosphates and an inhibition of carbon flux down the glycolytic pathway. Because phosphofructokinase and fructose-1,6-diphosphatase are the rate-limiting steps in this portion of metabolism in *Tetrahymena* [6], it is likely that these are the steps influenced by clofibrate treatment. Because these effects are relatively small, the changes in activity may reflect a clofibrate-induced change in some modifier such as AMP which affects both enzymes reciprocally in vitro for some species [14]. This effect of clofibrate on the glycolytic pathway in *Tetrahymena* may be similar to that observed in rat liver, where clofibrate treatment caused a small reduction in phosphofructokinase activity and a halving of pyruvate kinase activity [15].

A larger effect, and possibly one more closely linked to the primary action of clofibrate, is the enhancement of CO_2 fixation via the leucine degradation pathway. This was observed whether the label originated from the carboxyl group of leucine itself or from the carboxyl group of tyrosine, pyruvate, or bicarbonate, and must reflect an increase in flux through the leucine degradative pathway at some step(s) beyond the formation of β-methyl crotonyl-CoA. An increase in this pathway at steps prior to the formation of β-methyl crotonyl-CoA may also occur. Flux through this pathway appears to increase as a result of clofibrate treatment even in the absence of added leucine (Table I),

which may indicate that clofibrate enhances protein turnover. The observation that $^{14}CO_2$ fixation from [1-^{14}C]pyruvate did not increase appreciably in clofibrate-treated cells (Table I) is then of interest because it demonstrates a preferential effect of clofibrate treatment on $^{14}CO_2$ fixation from leucine (and tyrosine) as compared with pyruvate. Since both pyruvate dehydrogenase [16] and α-ketoisocaproate dehydrogenase [17] and the remaining enzymes of the leucine degradative pathway are localized in the mitochondria of rat liver [18], it is unlikely that the explanation of the difference in effect of clofibrate on $^{14}CO_2$ fixation into fatty acids from [1-^{14}C]leucine as compared to [1-^{14}C]-pyruvate can result from separate pools of CO_2 in the mitochondria. It appears, therefore, that clofibrate treatment preferentially increases the activity of the leucine degradative in *Tetrahymena.* There is no necessity to invoke a similar increase in the tyrosine degradative pathway, since the increase in $^{14}CO_2$ fixation into fatty acids caused by the clofibrate treatment can entirely be explained by the increase in the leucine pathway. Similarly, the enhanced fixation of $^{14}CO_2$ generated mitochondrially from the oxidation of [1-^{14}C]acetate, hexanoate, and octanoate may be explained as a consequence of the increased activity of the leucine degradative pathway.

These experiments, including the failure to observe any changes in several peroxisomal enzymes, do not support the view that clofibrate has any direct effect on peroxisomal content of stationary phase *Tetrahymena,* although this is clearly an important part of the response to clofibrate in liver [11,19]. There is also no similarly between the long-term effects of clofibrate treatment in the present experiments and the marked inhibition of uptake of [U-^{14}C]leucine during a 60 min incubation of rat adipocytes with 30 mM clofibrate [20]. Thus the increase in CO_2 fixation via the leucine degradative pathway appears to be a hitherto undescribed effect of clofibrate and deserves further study both in *Tetrahymena* and in liver, if, indeed, this effect occurs in liver.

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References

1 Borowitz, M.J., Raugi, G.J., Liang, T. and Blum, J.J. (1977) J. Biol. Chem. 252, 3402–3407
2 Blum, J.J. and Wexler, J.P. (1968) Mol. Pharmacol. 4, 155–161
3 Witiak, D.T., Newman, H.A.I. and Feller, D.R. (1977) Clofibrate and Related Analogs, Marcel Dekker, Inc., New York
4 Nozawa, Y. (1973) J. Biochem. 74, 1157–1163
5 Raugi, G.J., Liang, T. and Blum, J.J. (1975) J. Biol. Chem. 250, 5866–5876
6 Borowitz, M.J., Stein, R.B. and Blum, J.J. (1977) J. Biol. Chem. 252, 1589–1605
7 Borgstrom, B. (1952) Acta Physiol. Scand. 25, 111–119
8 Balinksy, J.B., Shambaugh, G.E., III and Cohen, P. (1970) J. Biol. Chem. 245, 128–137
9 Lazarow, P.B. (1978) J. Biol. Chem. 253, 1522–1528
10 Hoffman, E.K., Rasmussen, L. and Zeuthen, E. (1970) C. R. Trav. Lab. Carlsberg 38, 133–143
11 Lazarow, P.B. and DeDuve, C. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2043–2046
12 Blum, J.J. (1973) J. Protozool. 20, 688–692
13 Porter, P., Blum, J.J. and Elrod, H. (1972) J. Protozool. 19, 375–378

14 Newsholme, E.A. and Start, C. (1973) Regulation in Metabolism, p. 123, John Wiley and Sons, New York
15 Wilkening, J. and Schwandt, P. (1977) Horm. Metab. Res. 9, 132—136
16 Reed, L., Pettit, F. and Yeaman, S. (1978) in Microenvironments and Metabolic Compartmentation (Srere, P.A. and Estabrook, R.W., eds.), pp. 305—314, Academic Press, New York
17 Parker, P.J. and Randle, P.J. (1978) FEBS Lett. 90, 183—186
18 Noda, C. and Ichihara, A. (1974) J. Biochem. 76, 1123—1130
19 Markwell, M.A.K., Bieber, L.L. and Tolbert, N.E. (1977) Biochem. Pharmacol. 26, 1697—1702
20 Greenspan, M.D., Germerhausen, J.I. and Mackow, R. (1975) Biochim. Biophys. Acta 380, 190—198