Characteristics of Changes in Fatty Acid Metabolism by Suppression of the Activities of Peroxisomal β-Oxidation System and Glyoxylic Acid Cycle in *Tetrahymena pyriformis*

Ken-ichi GOTOH, Mineo TAKEI, Takafumi WATANABE* and Tetsuya SUGA

Department of Clinical Biochemistry, Tokyo College of Pharmacy, Horinouchi 1432-1, Hachioji, Tokyo 192-03, Japan. *Received November 16, 1989*

The effects of inhibitors on peroxisomal enzymes were examined in order to clarify the role of peroxisomes in the growth of *Tetrahymena pyriformis*. With the supplementation of chlorpromazine, known as an inhibitor of the peroxisomal β-oxidation system, to the culture medium, the growth of *T. pyriformis* was suppressed and the activity of the glyoxylate cycle which is linked to β-oxidation, was reduced. Supplementation of itaconic acid to the medium, a competitive inhibitor of isocitrate lyase of the glyoxylic acid cycle, also inhibited the growth. In these cases, no change in the activity of mitochondrial β-oxidation was observed. On the other hand, in an experiment concerning the fatty acid metabolism using [U-^{14}C]palmitic acid conversions to glycogen and proteins were significantly inhibited by these inhibitors. Thus, a decrease in the supply of carbon atoms, which are necessary for energy production and the biosynthesis of materials used in cell construction, was suggested. The acyl-coenzyme A not subjected to β-oxidation might be used for lipid synthesis, resulting in an accumulation of triglyceride and phospholipids.

From these results, it was concluded that in *Tetrahymena* peroxisome is not merely an assistant in the mitochondrial metabolic system, but has an important role in the production of energy essential for cell movement and cell growth, and in the conversion of fatty acids to carbohydrates and proteins.

Keywords *Tetrahymena pyriformis*; peroxisomes; enzyme activity; inhibitor; chlorpromazine; itaconic acid; fatty acid metabolism

Peroxisomes of *Tetrahymena pyriformis* house a β-oxidation system[1] differing from the mitochondrial one and enzymes related to the glyoxylic acid cycle.[2,3] In the metabolism of acetyl-coenzyme A (acetyl-CoA) produced by β-oxidation of fatty acids, two pathways are considered by which the acetyl-CoA is transferred to mitochondria, followed by further metabolism and is then used as a substrate of malate synthetase in glyoxylic acid. Because of the lack of pyruvate carboxylase in *Tetrahymena*,[4] high activity of the glyoxylic acid cycle is essential for gluconeogenesis.[5] Considering the localization of the enzymes, main physiological function of peroxisomes in *Tetrahymena* might thus be in transforming of lipids into sugars. However, addition of acetic acid to a medium where cells were in log phase did not enhance gluconeogenesis, although the activities of the glyoxylic acid cycle-related enzymes were increased.[6] The acetic acid was mainly converted to citric acid and glutamic acid.[7] Therefore, in log phase the glyoxylic acid cycle appears closely related to the mitochondrial tricarboxylic acid (TCA) cycle.

In a preceding paper we examined the relationship between the growth and peroxisomes of *T. pyriformis*.[8] In order to clarify the physiological role of these peroxisomes in more detail an examination using an inhibitor of peroxisomal enzyme seemed meaningful. Thus, in this report we examined the effects of chlorpromazine known as an inhibitor of peroxisomal β-oxidation[9] and itaconic acid, a competitive inhibitor of isocitrate lyase in the glyoxylic acid cycle, on growth and peroxisomal enzymes of *T. pyriformis*. Using [U-^{14}C]palmitic acid we also examined what changes in the metabolism of fatty acid were induced by these inhibitors.

Materials and Methods

Materials L-Carnitine-HCl was a kind gift from Earth Pharmaceutical Co., Ltd., Osaka, Japan. Acetyl-CoA, palmitoyl-CoA, CoA and nicotinamide adenine dinucleotide (NAD) were obtained from Sigma Chemicals Co., Ltd., St. Louis, Mo., U.S.A. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) and dithiothreitol (DTT) were obtained from Wako Pure Chemicals, Osaka, Japan. Proteose peptone and yeast extract were obtained from Difco Laboratories, Detroit, Mich., U.S.A. Other chemicals, all of analytical grade, were obtained from commercial sources.

Cell Cultures A preculture of *T. pyriformis* GL strain grown in the medium (20 g proteose peptone/10 g yeast extract/6 g glucose/l distilled water, adjusted to pH 7.0 with diluted NaOH) was added to synthetic medium at a final concentration of approx. 2×10^4 cells/ml and cultured at 26 °C. Chlorpromazine and itaconic acid were solubilized in the sterilized distilled water, the pH was adjusted to 7.0, and added to the medium. Then, the cells were harvested by centrifugation at $600 \times g$ for 5 min at 4 °C and washed. Washed cells were finally suspended in 0.25 M sucrose to a final concentration of 10^6 cells/ml. The cell suspensions were homogenized in a Potter-Elvehjem type homogenizer with a Teflon pestle (40 strokes) at 4 °C.

Assay Methods The enzyme activity of catalase was determined by the method of an earlier report.[10] One unit of the enzyme activity was defined as the amount of the enzyme giving $K=1$, where K is the rate constant of the enzyme. Fatty acyl-CoA oxidase (FAO) activity was determined by measuring the acyl-CoA dependent H_2O_2 generation by using palmitoyl-CoA as a substrate according to the method of Horie *et al.*[11] Carnitine acetyl- and palmitoyl-transferase (CAT and CPT) activities were determined spectrophotometrically using DTNB, and acetyl-CoA and palmitoyl-CoA as substrates according to the method of Fritz and Schultz.[12] Isocitrate lyase (ICL) activity was determined spectrophotometrically by measuring the phenylhydrazone according to the method of Dixon and Kornberg.[13] Malate synthetase (MS) activity was determined by a modification of the method of Cooper and Beevers.[14] One unit of the above enzyme activity corresponds to the conversion of 1 nmol/min. Protein was determined by the method of Lowry *et al.*[15] with bovine serum albumin as a standard.

Sucrose Density Gradient Centrifugation of the Large Granule Fraction of *T. pyriformis* The large granule fractions prepared from cell homogenates by the method of de Duve *et al.*[16] were centrifuged in the discontinuous sucrose density gradient. The sucrose density gradient was prepared in 60 ml tubes with the following components from bottom to top: 10 ml of 55% (w/v, density 1.26 g/ml), 17 ml of 46% (1.22 g/ml), 11 ml of 38.2% (1.17 g/ml), and 10 ml of 23.3% (1.11 g/ml) in 20 mM glycylglycine buffer, pH 7.2. Finally, 4 ml of the large granule fraction (corresponding to 15 mg protein) was laid on the gradient. Centrifugation was performed at 24000 rpm for 2.5 h at 4 °C in a Hitachi RPS 25-2 rotor. After the centrifugation, 13 fractions were collected from bottom to top.

Metabolic Fate of [U-^{14}C]Palmitic Acid a) Culture Condition: The cells in logarithmic phase ($2—4 \times 10^5$) were seeded in 9.5 ml of synthetic medium[17] with or without chlorpromazine or itaconic acid at 10^5 cells/ml in Erlenmeyer flasks of 100 ml volume and precultured for 1 h. After the preculture 0.5 ml of 1% (w/v) palmitic acid containing 0.7 μCi of [U-

^{14}C]palmitic acid was added at the final concentration of 0.05% (w/v) and the final specific radioactivity of 0.0138 μCi/μmol), and then incubated for 2 h unless otherwise stated at 26 °C.

b) CO_2 Trapping: After the incubation 1 ml of 60% perchloric acid was added to the medium and then released CO_2 was trapped in phenetylamine by allowing it to stand for 1 h in ice-cold.[18]

c) Lipids Extraction and Separation: After trapping CO_2 lipids were extracted from a 1 ml aliquot of the medium by the method of Folch *et al.*[19] The obtained chloroform layer was dried up, solubilized in 0.5 ml of chloroform and then 50 μl of the aliquot was applied to thin layer chromatography (TLC) to separate lipids (Merck $60F_{254}$, *n*-hexane : diethylether : acetic acid = 80 : 30 : 1, v/v). After developing, spots were identified by iodine vapor, scraped, and the radioactivities were determined. As standard compounds tripalmitin, 1,2-dipalmitine, 1,3-dipalmitine, 1-monopalmitin and lecithin were used.

d) Separation of Glycogen and Protein: The residual medium was centrifuged at 15000 × *g* for 20 min and the pellet was fully washed with ethanol. After recentrifugation the pellet was subjected to separation of glycogen and protein.[20]

e) Radioactivity Measurement: The samples were mixed with 10 ml of a scintillation cocktail (Aquazol-2) and then the radioactivities were measured in a liquid scintillation counter, Aloka 903 (Aloka, Japan).

Results

Effect of Chlorpromazine on Growth and Peroxisomal Enzyme Activities of *T. pyriformis* The growth curves of *T. pyriformis* in the medium supplemented with chlorpromazine at 10, 20 and 50 μM are shown in Fig. 1. Growth was inhibited dose-dependently, and decreased to 50% of the control at 20 μM. At more than 200 μM all of the cells were dead. Changes in the activities of peroxisomal enzymes, FAO, ICL, MS, CAT, catalase and mitochondrial CPT after growing in the presence of chlorpromazine are shown in Fig. 2. Although no change in the activity of mitochondrial CPT was observed, FAO, which is the first and rate-limiting enzyme in the peroxisomal β-oxidation system was suppressed by chlorpromazine dose-dependently. A significant decrease in the activities of ICL and MS in the glyoxylic acid cycle linked with peroxisomal β-oxidation was also observed. Although there was a slight decrease, the extent of the change was much smaller than ICL and MS. No change in catalase activity was seen. These changes in the enzyme activities were also confirmed by sucrose density gradient centrifugation of large granule fractions obtained from the cells grown in chlorpromazine-supplemented medium (Fig. 3). The activity of FAO in fraction 3, a peroxisomal fraction, was decreased 50% compared with control and the activities of ICL and MS were also decreased significantly. The activity of CAT in the 3rd and 7th fractions (mitochondrial fraction) was reduced.

The effect of chlorpromazine on the growth and the enzyme activities of the cells grown in medium with or without oleic acid is shown in Fig. 4. Increase in the activities of peroxisomal fatty acid metabolism-related enzymes by oleic acid was significantly suppressed by this inhibitor. On the other hand, the mitochondrial CPT activity was not changed by chlorpromazine. The results showed that regardless of the presence or absence of oleic acid the changes in cell growth were similar to those in peroxisomal enzyme activities.

Effect of Itaconic Acid on the Growth and Peroxisomal Enzyme Activities Regardless of the presence or absence of oleic acid, the growth of *T. pyriformis* was significantly suppressed when itaconic acid was added to the medium (Fig. 5). The activities of peroxisomal enzymes and mitochondrial CPT are shown in Fig. 6. Although ICL activity was suppressed by itaconic acid even in the absence of oleic acid, the activities of FAO and CPT were not affected whether oleic acid was present or not. However, the increase in MS activity by oleic acid was suppressed by itaconic acid accompanied by the suppression of ICL activity. Although a decreasing tendency in the activity of CAT was also observed, the change was not statistically significant. The changes in these enzymes in the cells cultured in these medium were also confirmed by sucrose

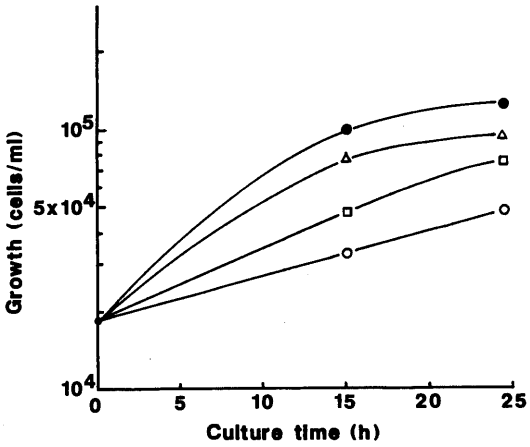

Fig. 1. Effect of Chlorpromazine on the Growth of *T. pyriformis*

Cells were cultured in chlorpromazine-supplemented medium. (●) control, (△) 10 μM, (□) 20 μM, (○) 50 μM.

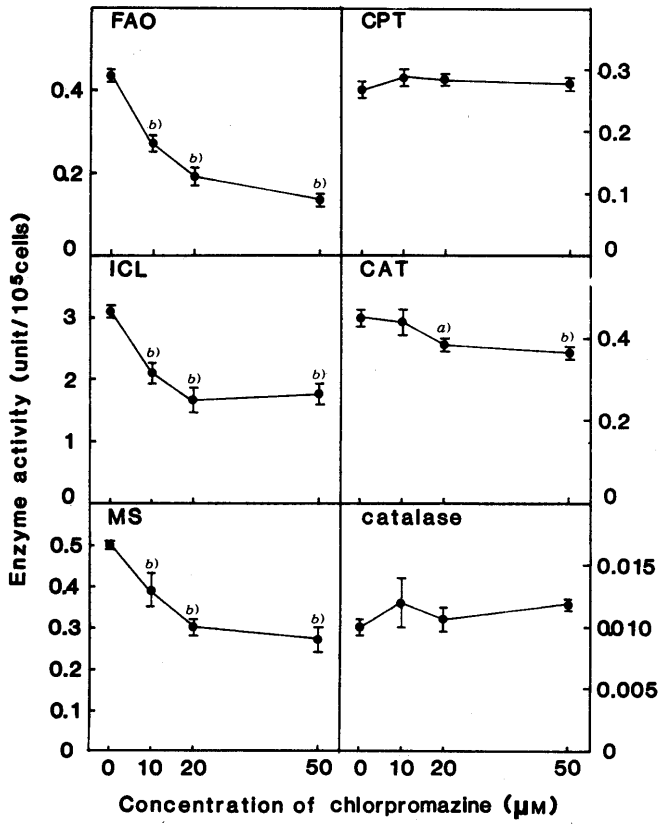

Fig. 2. Changes in Peroxisomal Enzyme Activities of *T. pyriformis* in the Presence of Chlorpromazine

Cells were cultured for 24 h at 26 °C in chlorpromazine-supplemented medium. Data are expressed as the means of 3 separate samples and the bars are S.D. Statistical significance is evaluated by Student's *t*-test: *a*) $p < 0.05$, *b*) $p < 0.01$.

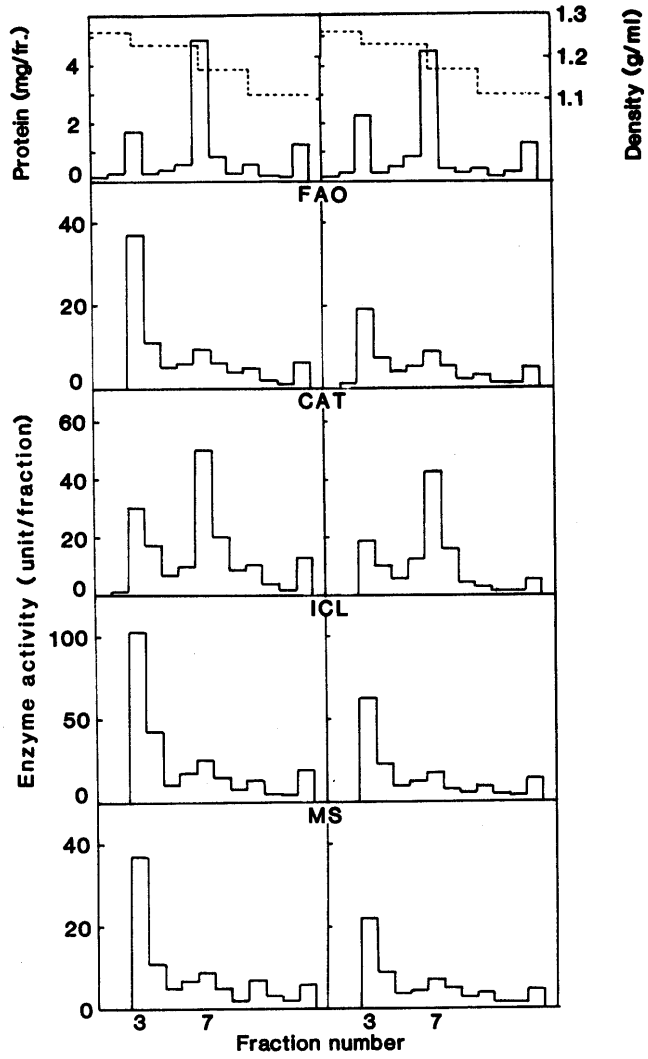

Fig. 3. Sucrose Density Gradient Centrifugation of the Large Granule Fraction of the Cells Grown in Medium with or without Supplementation of 20 μM Chlorpromazine

Left and right columns show the results of the control (without chlorpromazine) and the chlorpromazine-supplemented, respectively. Recovery of the enzyme activity: FAO (98% in the control and 96% in chlorpromazine-supplemented), CAT (91% and 98%), ICL (95% and 97%) and MS (104% and 110%).

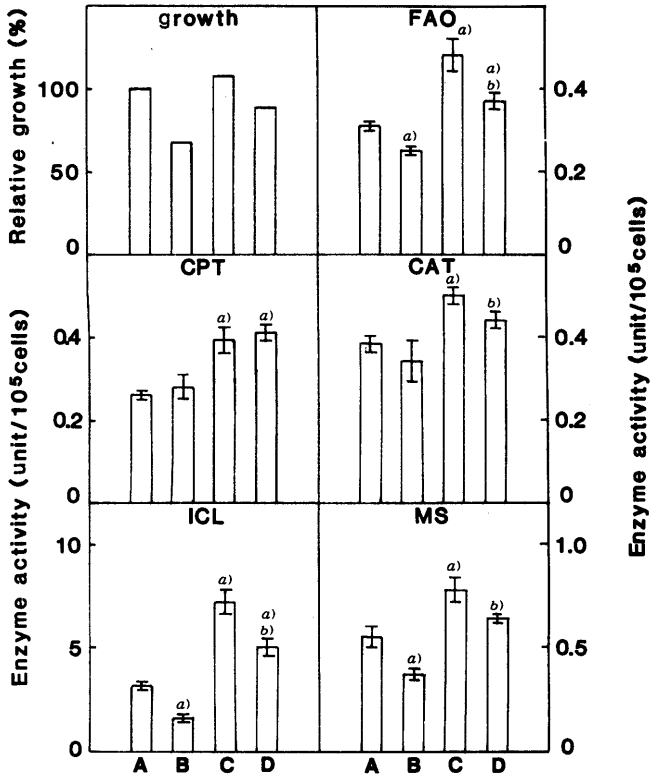

Fig. 4. Effect of Chlorpromazine on the Growth of Cells and the Peroxisomal Enzyme Activities under the Condition with or without Oleate

Oleate was supplemented at a concentration of 0.05% (w/v). Cells were cultured for 20 h at 26 °C in each medium. The growth of cells is expressed as the relative value of the control (no addition). Data of enzyme activities are expressed as the means from 3 separate samples ± S.D. Statistical significance was evaluated by Student's *t*-test: *a*) $p<0.05$ *vs.* control, *b*) $p<0.05$ *vs.* oleate-supplemented. A, control (no addition); B, 20 μM chlorpromazine; C, 0.05% (w/v) oleate; D, 20 μM chlorpromazine + 0.05% (w/v) oleate.

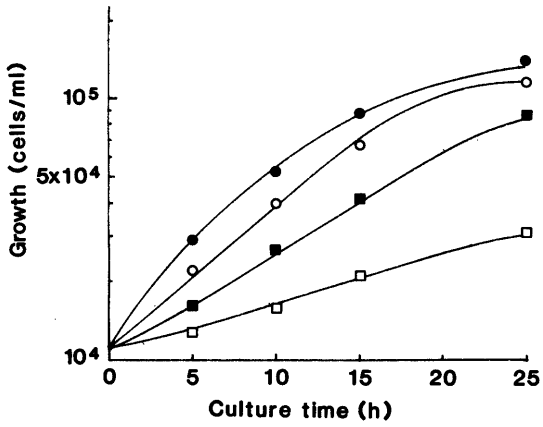

Fig. 5. Effect of Itaconate on the Growth of Cells of the Control and Oleate-Grown *T. pyriformis*

Oleate was supplemented at a concentration of 0.05% (w/v). Cells were cultured for 24 h at 26 °C in each medium. (○) control, (□) 2 mM itaconate, (●) 0.05% oleate, (■) 2 mM itaconate + 0.05% oleate.

density gradient centrifugation (data not shown).

Change in Fatty Acid Metabolism by Suppression of Peroxisomal Enzymes A time-dependent change in palmitic acid metabolism is shown in Fig. 7. ^{14}C-Palmitic acid was incorporated mainly into lipids, especially triglycerides. Incorporation into phospholipids increased with time and that into diglycerides reached a plateau within 2 h. The rate of the alteration of palmitic acid to proteins through the β-oxidation pathway was remarkably high. Palmitic acid incorporation into glycogen and CO_2 increased with time. MeOH–H_2O layer of Folch's extraction method might include primarily ketone bodies and organic acids, and incorporation into this fraction reached plateau after 2 h. With supplementation of chlorpromazine or itaconic acid to the culture medium the incorporation of palmitic acid into cells was increased (Table I), while its incorporation into lipids such as diglycerides and others (tetrahymanol, etherphospholipids) was not affected; an increase in the incorporation into triglycerides and phospholipids was observed. On the other hand, the incorporation of palmitic acid into glycogen and protein was significantly decreased (Table II).

These findings are summarized in Table III, and suggest that with suppression of peroxisomal enzyme activities the synthesis of materials through β-oxidation is significantly inhibited, and palmitic acid incorporated into cells may be mainly used for the synthesis of lipids.

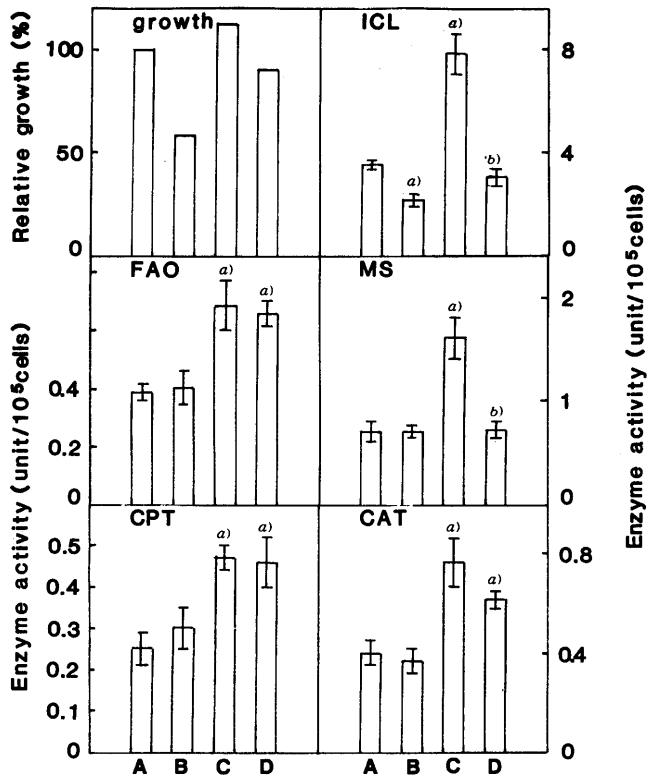

Fig. 6. Effect of Itaconate on the Growth of Cells and the Peroxisomal Enzyme Activities of the Control and Oleate-Grown *T. pyriformis*

Oleate was supplemented at a concentration of 0.05% (w/v). Cells were cultured for 20 h at 26 °C in each medium. The growth of cells is expressed as the relative value of control (no addition). The data of enzyme activities are expressed as the means of 3 separate samples ± S.D. Statistical evaluation was evaluated by Student's *t*-test: *a*) $p<0.05$ *vs.* control, *b*) $p<0.05$ *vs.* oleate-supplemented. A, control (no addition); B, 2 mM itaconate; C, 0.05% (w/v) oleate; D, 2 mM itaconate + 0.05% (w/v) oleate.

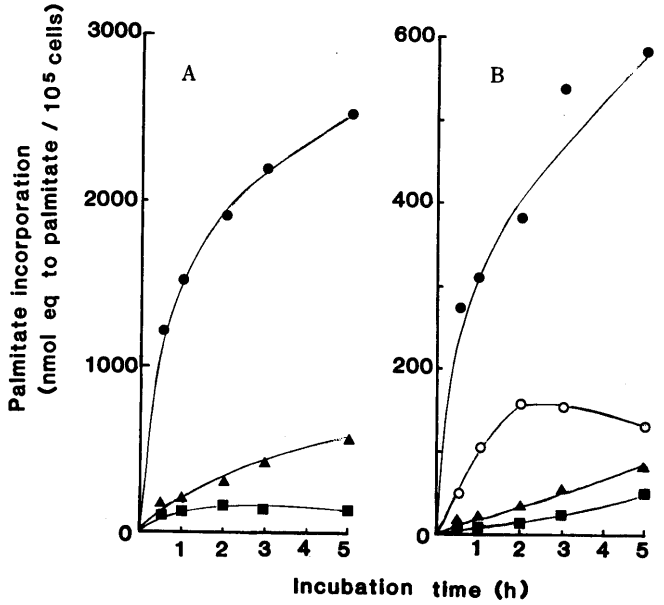

Fig. 7. Incorporation of the Radioactivity from [^{14}C]Palmitate into Lipids and β-Oxidation Products in *T. pyriformis*

Cells were incubated as described in Materials and Methods and samples were taken at various times. A: (●) TG, (▲) PL and MG, (■) DG, B: (●) Protein, (○) MeOH–H_2O extract, (▲) glycogen, (■) CO_2.

Discussion

Chlorpromazine HCl, a phenothiazine type major tranquilizer, is widely used clinically because of its wide pharmacological activities. Supplementation of the drug to a culture of *T. pyriformis* inhibited the incorporation of glucose into cells,[21] and the accumulation of phospholipids and suppression of cell growth were observed.[22] These effects have been considered to be due to the enhancement of cell permeability.[23] Recently, Leighton *et al.* reported that chlorpromazine specifically inhibited peroxisomal β-oxidation in hepatocytes,[24] and this inhibition might relate to the inhibition of growth of *T. pyriformis*. We therefore examined the activities of peroxisomal enzymes and the growth of the cells when chlorpromazine was supplemented to culture medium.

Table I. Effects of Chlorpromazine and Itaconate on the [U-^{14}C]-Palmitate Uptake into Cells of *T. pyriformis*

	Number of cells (cells/ml) 0 h	2 h	^{14}C-Palmitate uptake into cells (dpm)[a]
Control	1.00×10^5	1.11×10^5	148615 ± 13077 (24.8)
CPZ (20 μM)	1.00×10^5	1.04×10^5	213262 ± 12800 (35.5)[b]
Itaconate (2 mM)	1.00×10^5	1.02×10^5	159415 ± 10431 (26.6)

Parentheses are the percentage of the amount of added ^{14}C-palmitate. *a*) Values are represented as the means ± S.D. of three experiments. Statistical significance: *b*) $p<0.01$ *vs.* control.

Table II. Effects of Chlorpromazine and Itaconate on the Incorporation of the Radioactivity from [U-^{14}C]Palmitate into Lipids and β-Oxidation Products in *T. pyriformis*

	Palmitate incorporation (nmol/10^5 cells/h) Control	Chlorpromazine (20 μM)	Itaconate (2 mM)
Triglycerides	1264 ± 142 (60.2)	2514 ± 207[b] (77.6)	1889 ± 153[b] (73.6)
Diglycerides	69 ± 7 (3.3)	64 ± 16 (2.0)	66 ± 29 (2.6)
Phospholipids and monoglycerides	178 ± 26 (8.5)	211 ± 8[a] (6.5)	285 ± 19[b] (11.1)
Others	33 ± 14 (1.6)	29 ± 8 (0.9)	25 ± 6 (1.0)
MeOH–H_2O extracts	63 ± 11 (3.0)	70 ± 18 (2.2)	53 ± 9 (2.1)
CO_2	6 ± 1 (0.3)	8 ± 1[b] (0.3)	2 ± 1[b] (0.1)
Glycogen	29 ± 3 (1.4)	21 ± 1[b] (0.6)	11 ± 1[b] (0.4)
Protein	455 ± 28 (21.7)	321 ± 22[b] (9.9)	234 ± 50[b] (9.1)

Values are represented as the means ± S.D. of three experiments. Parentheses are the percentage of the amount of the added ^{14}C-palmitate. Statistical significance: *a*) $p<0.05$, *b*) $p<0.01$ *vs.* the control.

Table III. Summary of the Effects of Chlorpromazine and Itaconate on the Palmitate Utilization

	Palmitate utilization (%) Lipids	β-Oxidation products
Control	73.6 ± 9.0	26.4 ± 2.1
Chlorpromazine (20 μM)	87.0 ± 7.3	13.0 ± 1.3[a]
Itaconate (2 mM)	88.3 ± 8.0	11.7 ± 2.4[a]

Values are represented as the means ± S.D. of three experiments. Statistical significance: *a*) $p<0.01$ *vs.* the control.

This supplementation resulted in the activity of peroxisomal β-oxidation being inhibited in a dose-dependent manner, accompanied by suppression of the growth, while mitochondrial activity was not affected. Similarly, the activities of ICL and MS of the glyoxylate cycle, which participates in the metabolism of acetyl-CoA derived from peroxisomal fatty acid β-oxidation, and of CAT were also reduced. However, at the dose used in the present experiment the drug did not show direct an inhibitory effect on ICL, MS or CAT activity (data not shown). Therefore, changes in the activities of these enzymes might be the result of adaptation to the decreased supply of acetyl-CoA as a substrate brought about by the drug's suppression of the β-oxidation pathway. However, the extent of inhibition of CAT was less than those of ICL and MS, suggesting that the glyoxylate cycle might relate more to acetyl-CoA metabolism in *Tetrahymena* peroxisomes greater than CAT. The same results were found when oleic acid was added to the culture medium. These findings suggest that chlorpromazine inhibited peroxisomal β-oxidation reaction and decreased the supply of acetyl-CoA which was a substrate for various important biochemical reactions in the cells, thus causing an inhibition of the growth.

We further examined the effect of itaconic acid, a competitive inhibitor of ICL activity of the glyoxylate cycle, on the growth of *T. pyriformis* and peroxisomal enzyme activities. With the supplementation of 2 mM of itaconic acid to the medium the activity of ICL was reduced to one half and the growth was also significantly inhibited. In the absence of oleic acid no changes in any other enzyme activities were observed. The reason was thought to be that the activity of ICL was markedly higher than that of MS, and that even when ICL activity was decreased the supply of glyoxylic acid as a substrate of MS might be sufficient under this experimental condition. On the other hand, in the presence of 0.05% oleic acid the activity of MS which was metabolically linked to ICL was significantly decreased by itaconic acid. In this case where the supply of acetyl-CoA derived from β-oxidation was greater, the supply of the corresponding glyoxylic acid was insufficient, causing a decrease in the MS activity. There was no interruption in the production of acetyl-CoA but in the following metabolism, and considering the change in the activity of CAT the important role of the glyoxylic acid cycle in the metabolism of acetyl-CoA derived from peroxisomal β-oxidation and the relation to the growth were suggested.

A study of the effect of peroxisomal enzyme inhibitors on [U-^{14}C]palmitic acid metabolism showed the ratio of the conversion to glycogen or protein to be significantly decreased. Glycogen and protein were synthesized through peroxisomal β-oxidation and glyoxylic acid cycle. Therefore, the inhibition of peroxisomal fatty acid metabolism might cause a reduction of energy production or a decrease in the synthesis of protein essential for the construction of cells, resulting in an inhibition of growth. The incorporation of palmitic acid into triglycerides and phospholipids might be due to cellular acyl-CoAs not oxidized by β-oxidation being used for the synthesis of each lipid, resulting in an excess of lipids; this hypothesis is supported by the reports of Rogers[25] and Blum.[26]

In conclusion, peroxisomes in *T. pyriformis* participate in fatty acid β-oxidation and the synthesis of sugars and proteins from acetyl-CoA derived from the β-oxidation pathway. Furthermore, the fact that the inhibition of this metabolic pathway induced an inhibition of the growth shows that peroxisomes have an important role in the growth process of *Tetrahymena*.

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