Wax ester fermentation in *Euglena gracilis*

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Aerobically grown *Euglena gracilis*, a bleached mutant, shows a prompt synthesis of wax esters with the concomitant fall of the paramylon (a β-1,3-glucan) content upon exposure to anaerobiosis. Bringing the anaerobic cells back to aerobiosis causes the reverse conversions. The anaerobic wax ester formation is accompanied by a net synthesis of ATP. The transition between the fermentation and respiration occurs at 10^{-5}–10^{-7} M of the O_2 concentration.

Fermentation *Wax esters* Euglena gracilis *Paramylon* *O_2 concentration*

1. INTRODUCTION

Wax esters are formed in *Euglena gracilis* when grown in an organic carbon-rich medium under adverse conditions [1–5]. We have found that transfer of aerobically grown *E. gracilis* to anaerobic conditions causes a prompt formation of wax esters at the expense of paramylon, the reserve polysaccharide. The anaerobic wax ester formation is accompanied by a net synthesis of ATP.

2. MATERIALS AND METHODS

2.1. *Cells and cultures*

Euglena gracilis SM-ZK, a streptomycin-bleached mutant [6] derived from strain Z, was used throughout experiments. Cells were cultured in a medium as in [7] under illumination (2000 lux) with shaking (90 strokes/min) at 27°C. The cells employed in chase label experiments, containing labelled paramylon, were obtained by culturing *Euglena* in the medium containing [U-^{14}C]glucose (4 μCi/150 ml); of the incorporated radioactivity 75% was present in the paramylon fraction.

2.2. *Anaerobiosis*

Cells were harvested by centrifugation (1500 × *g*, 2 min) and suspended at 27°C in 2 ml potassium phosphate buffer (pH 6.8) to 2×10^7 cells/ml. Anaerobiosis was applied by bubbling N_2 which had passed through 15% potassium hydroxide solution containing 10% pyrogallol and then distilled water. O_2 in the suspension was $\sim 10^{-8}$ M. For obtaining N_2–O_2 mixtures with different, precise O_2 concentrations, an oxygen pump (Toray, model SEP-104) was employed.

2.3. *Analyses of cells components*

Cells in the 2 ml suspension were collected by centrifugation (5000 × *g*, 2 min) and washed with distilled water, and added into 4.75 ml of a mixture of chloroform–methanol–water (10:20:8, by vol.). After thorough agitation the mixture was centrifuged to remove cells and debris. The extraction was repeated, and to the combined supernatants were added 2 ml each of chloroform and water, and the whole was shaken vigorously. The chloroform layer was separated and used as the lipid fraction. In a short time (~15 min) tracer experiments, cell collection and washing were omitted and lipids were extracted directly by adding 7.5 ml chloroform–methanol mixture (1:2) to 2 ml cell suspension. After adding 2 ml each of chloroform and water and shaking, the chloroform phase was used. The lipid fractions were evaporated, dissolved in chloroform and submitted to thin-layer chromatography with a solvent system of petroleum ether–ethyl ether–acetic acid

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(80:20:1) to separate lipid classes. For the determination of wax esters an aliquot of the chloroform solution was subjected to gas–liquid chromatography (Yanaco, model GCG-550F) equipped with a hydrogen detector, on a glass column (100 × 0.3 cm) packed with 5% Thermon-100 at 230°C. Myristyl myristate or myristyl palmitate, prepared as in [8], was used as an internal standard.

The residue of the lipid extraction was dried and stirred in 5 ml 0.45 N perchloric acid. After centrifugation at 10000 × *g* for 10 min, the perchloric acid-soluble fraction was neutralized with potassium carbonate and the precipitate removed by centrifugation at 4°C. The supernatant was applied onto columns of Dowex 50 and 1 to separate amino acids, organic acids and sugars [9]. The perchloric acid-insoluble fraction was heated with 2 ml 1% SDS at 100°C for 15 min; the soluble and insoluble fractions were proteins and paramylon, respectively. Paramylon was solubilized with 1 N sodium hydroxide and determined by the phenol–sulfuric acid method [10]. Protein was determined as in [11]. CO_2 was trapped with 1 N potassium hydroxide.

For the determination of ATP, 0.1 ml 70% perchloric acid was added to the incubated cell suspension (2 ml) and cooled to 4°C. Cells were homogenized by sonication (10 kHz, 1 min) and the homogenate neutralized with potassium carbonate. After removing precipitate by centrifugation the supernatant was used for the ATP determination as in [12].

Radioactivity was measured with a liquid scintillation photometer (Aloka, model LSC-602).

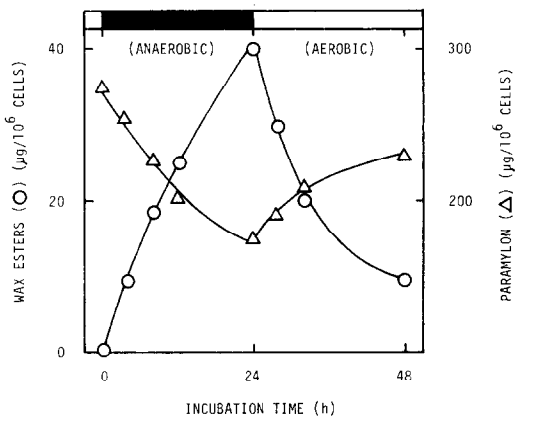

Fig. 1. Variation of the contents of wax esters and paramylon due to transfer of aerobically grown *E. gracilis* to anaerobiosis and then back to aerobiosis.

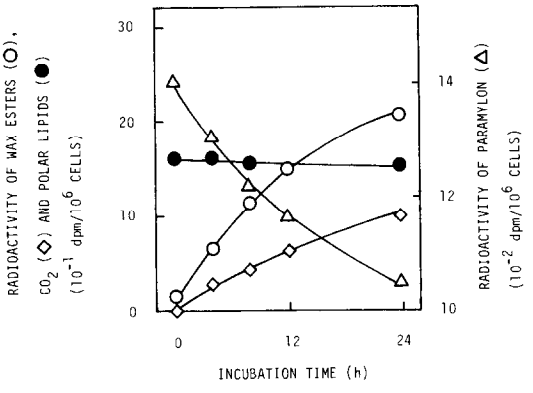

Fig. 2. Migration of radioactivity from labelled paramylon after transfer of *Euglena* cells to anaerobiosis.

3. RESULTS AND DISCUSSION

3.1. *Formation of wax esters in anaerobiosis*

The content of wax esters in *E. gracilis* (strain SM-ZK) grown to the stationary phase with shaking was 1 $\mu g/10^6$ cells and, as shown in fig. 1, transferring the cells to anaerobiosis caused a prompt synthesis of wax esters with a concomitant decrease of the paramylon content. The synthesis of 39 $\mu g/10^6$ cells of wax esters and loss of 105 $\mu g/10^6$ cells of paramylon after 24 h of anaerobic incubation in the buffer correspond to 1.287 and 1.296 $\mu mol/10^6$ cells, respectively, of acetyl-CoA, indicating that all carbons in the wax esters are supplied from paramylon. When the cells were brought back to aerobiosis by bubbling air, the reverse changes took place; wax esters were decomposed rapidly while paramylon synthesized.

Fig. 2 shows distribution of radioactivity among CO_2, wax esters and polar lipids when aerobically grown *Euglena,* containing labelled paramylon (see section 2), was placed in anaerobiosis. Along with the prompt fall of radioactivity in paramylon, the activity in wax esters and CO_2 was increased, and the increased radioactivity in these fractions in sum was equivalent to more than 90% of the decreased radioactivity in paramylon; the ratio of radio-

activity in wax esters and CO_2 was 2:1 at any time of incubation. The radoactivity in polar lipids and in other cell components was not changed by the transfer; no leak of radioactivity was detected into medium both from aerobic and anaerobic cells. The results suggest that CO_2 evolves by the action of pyruvate dehydrogenase in the course of glucose degradation and that acetyl-CoA simultaneously formed is utilized in the wax ester synthesis.

Aerobic *Euglena* cells were placed in anaerobiosis for different times, and then incubated anaerobically with [1-^{14}C]acetate for 15 min. The activity of the cells to synthesize wax esters, as assayed by the incorporation of radioactivity into wax esters, increased rapidly with the adaptation period reaching a maximum after 15 min adaptation to anaerobiosis; the maximum activity was ~40-times higher than the activity in the aerobic cells. The addition of 2.5 μg cycloheximide/ml, the concentration effective to inhibit synthesis of some enzymes (unpublished), influenced neither the wax ester synthesis nor the paramylon degradation, indicating that the activation of wax ester synthesis does not involve an enzyme induction.

While the wax esters in aerobic cells are composed of saturated esters mainly with carbon number 28 and 30 and ~30% of unsaturated esters, those in anaerobic cells contain mainly saturated C_{28} ester with considerable amounts of saturated C_{26} and C_{27} esters but none of unsaturated ones.

3.2. *Anaerobic ATP generation*

Fig. 3 shows that placing aerobically grown *Euglena* cells in anaerobiosis causes a prompt fall in the cellular ATP level, but after 2 min, when the synthesis of wax esters becomes evident, the ATP level starts to increase gradually to regain the original level after 13 min, and this level is maintained during the anaerobiosis, showing that the synthesis of wax esters is coupled with the generation of ATP. Sodium arsenite and sodium fluoride, inhibitors of glycolysis, inhibited the ATP generation and wax ester synthesis in anaerobiosis.

The results heretofore obtained demonstrate that the phenomenon is a fermentation with wax esters as the end product, and is to be called the wax ester fermentation. ATP should be generated by the substrate-level phosphorylations in glycolysis. Thus, *E. gracilis* lives on respiration in the presence of sufficient amount of O_2 and on fermentation in anoxia by using paramylon as the reserve substance.

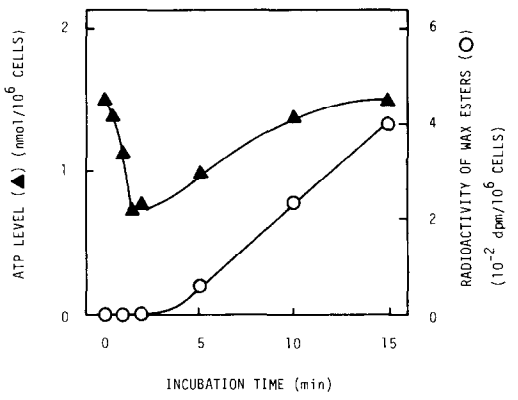

Fig. 3. The ATP level and synthesis of wax esters from [1-^{14}C]acetate after transfer of *Euglena* cells to anaerobiosis. [1-^{14}C]Acetate (0.1 mM, 0.2 μCi) was added to cell suspension (2 ml) just when cells were transferred to anaerobiotic condition.

The fermentation with wax esters as the end product is unique in that they are with relatively high M_r-values and quite insoluble in water. They are sometimes called crystalloids [13], present as a suspending solid in cytoplasm, and apparently do not affect other metabolisms. Products of ordinary fermentations, lower alcohols and organic acids, are certainly water-soluble and may be harmful when accumulated in the cell so that they must be deposed with the loss of carbon. In *Euglena* the fermentation product is retained in the cell with as little a loss of carbon as possible, and when aerobic conditions are restored the wax esters are rapidly converted back to paramylon, and more efficient respiration takes place. The phenomenon resembles, in a sense, the lactate formation in anaerobic muscle and gluconeogenesis from lactate in aerobic liver in animals, but in unicellular *Euglena* the interconversion between glucan and wax esters takes place in the same cells.

3.3. *Dependency of the energy metabolism on the O_2 concentration*

An oxygen pump was employed to obtain N_2–O_2 mixtures with desired O_2 concentrations, and cells were adapted to different O_2 concentrations by bubbling cell suspension with the mixed

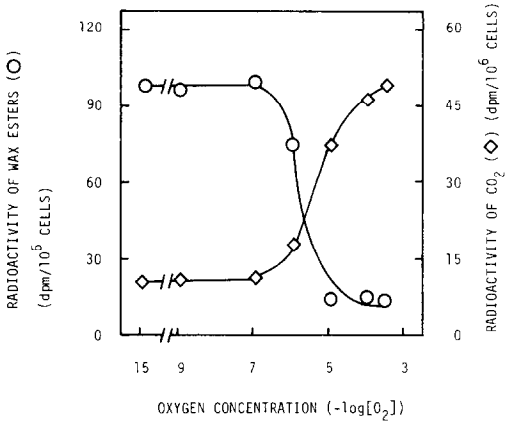

Fig. 4. Effect of oxygen concentration on the migration of radioactivity from [2-^{14}C]pyruvate to wax esters and CO_2. [2-^{14}C]Pyruvate (0.1 mM, 0.2 μCi) was fed into cell suspension (2 ml) for 15 min.

gases with different O_2 concentrations for 15 min. The adapted cells were incubated with [2-^{14}C]pyruvate in potassium phosphate buffer for 15 min, and then the radioactivity in wax esters formed by fermentation and CO_2 evolved by respiration was determined. The results are given in fig. 4. Along with the fall of O_2 concentration, the radioactivity in wax esters was promptly increased at 10^{-5}–10^{-7} M while the radioactivity in CO_2 promptly decreased at these concentrations. At $<10^{-7}$ M O_2 the incorporation of radioactivity into CO_2 was negligible, and that into wax esters was constant. These data demonstrate that transition between respiration and fermentation in *E. gracilis* occurs at 10^{-5}–10^{-7} M O_2. The ATP level in *Euglena* adapted to O_2 at 10^{-3}–10^{-15} M, was quite identical.

In the degradation of paramylon in *E. gracilis,* both β-1,3-glucanases and β-1,3-glucan phosphorylases are active [14], and depending on the extent of participation of these enzymes, net 2–3 molecules of ATP and 4 molecules of NADH are produced/molecule of glucose by glycolysis and the action of pyruvate dehydrogenase from paramylon. Acetyl-CoA produced from pyruvate is utilized for the synthesis of fatty acids and then wax esters. If the ordinary pathway of fatty acid synthesis involving malonyl-CoA intermediate is responsible for the wax ester synthesis in anaerobic *Euglena* cells, the acetyl-CoA formed by pyruvate dehydrogenase located in mitochondria [15] must be translocated to cytoplasm, since bleached *E. gracilis* contains the malonyl-CoA-involving system of fatty acid synthesis in cytoplasm [16–19] and microsomes [20,21]. In the course of translocation of acetyl-CoA and synthesis of malonly-CoA, ATP is consumed, and therefore no net synthesis of ATP will ensue in the wax ester fermentation in *Euglena.* It is probable that a novel system of fatty acid synthesis with little or no loss of ATP is operative in *Euglena,* and this will be the subject of our forthcoming papers.

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