Utilization of Pyrophosphate and Tripolyphosphate by Euglena gracilis Z

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Utilization of Pyrophosphate and Tripolyphosphate by *Euglena gracilis* Z

Kazutaka MIYATAKE, Toshikazu NAKANISHI, and Shozaburo KITAOKA

Nutrition Laboratory, College of Agriculture

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Abstract

Euglena gracilis, strain z, and a SM-bleached mutant therefrom, utilized pyrophosphate and tripolyphosphate for growth as evidenced by the effects on cell multiplication, the consumption of phosphates in the growth media and the incorporation of phosphates into cells.

Pyrophosphate- and tripolyphosphate-hydrolyzing activities were detected in the *Euglena* cells and the these activities were induced by the addition of these inorganic phosphates indicating that these phophate compounds are assimilated also.

The hydrolyzing activities, probably due to pyrophophatase and triphosphatase, were localized in different subcellular fractions in *E.gracilis.*

Many algal species can grow even when orthophosphate are replaced by various phosphorous containing substrates. *Chlorella* can utilize inorganic polyphosphate (up to a chain length of 55 phosphate units) yielding the same growth rate as that with potassium phosphate[1]. Albaum *et al.* identified about a dozen phosphates contained in *Euglena,* including several phosphorylated intermediates of glycolysis as well as metaphosphate and inorganic pyrophosphate[2].

However the utilization of inorganic polyphosphates, including pyrophosphate and tripolyphosphate, have not been examined in *Euglena.*

In the present paper, we wish to report on utilization of the above two inorganic phosphates by *E. gracilis* z and occurence of enzymatic activities of hydrolyzing these compounds and their subcellular localization in the *Euglena* cells.

Materials and Methods

Materials. All Chemicals were analytical grade reagents prepared in Japan.

Culture. E. gracilis z and a streptomycin mutant were cultured in Cramer and Myers medium[3] which contained 0.25% (v/v) ethanol as a carbon source; the initial pH was 3.4. Cultivation was conducted under illumination (2,000 lux) or in the dark at 27°C with aeration. For experiments, orthophosphate was replaced by potassium pyrophosphate or potassium tripolyphosphate. These phosphates were acid and heat labile substances. Hence, orthophosphate containing and orthophosphate-free media were autoclaved, but pyro- and tripolyphosphate were added asceptically to the medium by using membrane filter (Toyo-Roshi, Tokyo). A orthophosphate-free medium was used as the control. Growth was measured by counting cell number with a hemocytometer. Orthophosphate, in the medium or in the cells after complete hydrolysis with 60% perchloric acid was determined by the method of Nakamura[4]. Protein was determined by the method of Lowry *et al.* using bovine albumin as a standard[5].

Preparation of crude enzyme. E.gracilis cells were washed with 20mM glycyl-glycine buffer, pH 7.4, containing 0.25M sucrose and disrupted in the same buffer by sonication (10Kc) for 1 min. The supernatant obtained by centrifuging the sonicate at 10,000 x *g* for 10 min was used as a crude enzyme.

Disruption of cell by the digestion techniques and fractionations of cell homogenate by different centrifugation. Partial digestion with trypsin followed by mechanical disruption of *E.gracilis* cells was performed by the method of Tokunaga *et al.*[6]. Fractionation of cell homogenate by differential centrifugation was carried out according to the method of Shigeoka *et al.*[7].

Enzyme assays. NADP-dependent succinic semialdehyde dehyrogenase was assayed according to Tokunaga *et al.*[8], and glucose-6-phosphatase was assayed as described by Yokota and Kitaoka[9]. Pyrophosphatase and tripolyphospahtase activities were measured according to the method of Deuel *et al.*[10].

Results and Discussion

Growth of E.gracilis on pyrophosphate or tripolyohosphate as the sole phosphorous sources. Utilities of pyrophosphate and tripolyphosphate as a sole phosphorous source for the growth of *E.gracilis* under illumination were studied by observing their effects on cell multiplication (Fig. 1). Pyrophosphate and tripolyphosphate allowed cells to grow well to reach the stationary phase after 7 days (Fig. 1). The cell number increased from

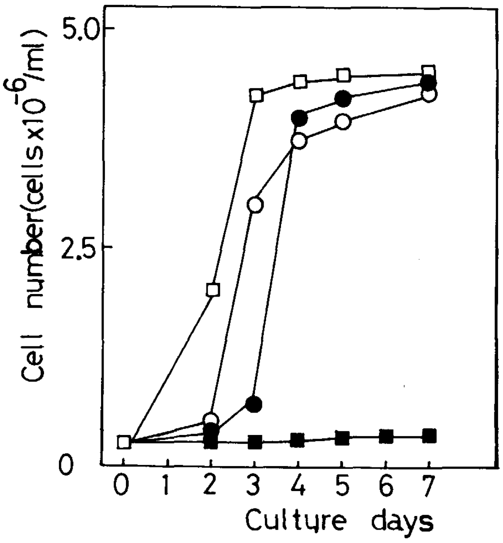

Fig. 1 Growth of ***E.gracilis*** on inorganic phosphates.
E.gracilis was grown in media made by replacing the orthophosphate in the Cramer and Myers medium by such amounts of inorganic phosphates tested as corresponding to 2 μmole as orthophosphate.
Symbols: ○; Pyrophosphate, ●; Tripolyphosphate, □; Orthophosphate, ■; Phosphate free.

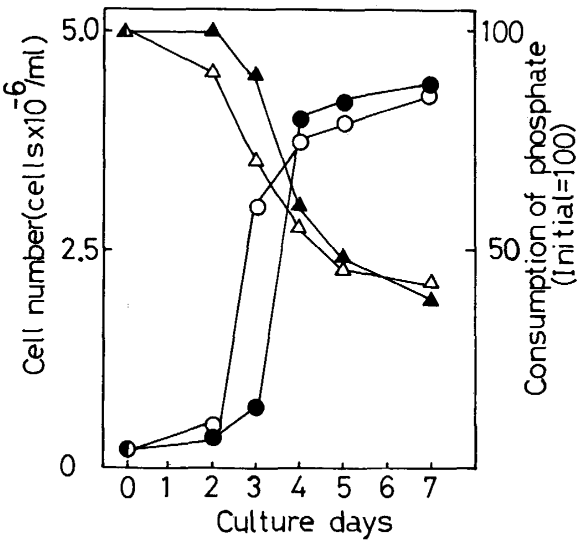

Fig. 2 Consumption of inorganic phosphorous sources by ***E. gracilis.***
Consumption of pyrophosphate and tripolyphosphate in the growth media was shown by percentage against the amounts contained initially in the media after intervals of culturing. Initail concentration of inorganic phosphates were adjusted to 2 μmoles as orthophosphate.
Symbols: △; Consumption of pyrophosphate, ▲; Consumption of tripolyphosphate, ○; Cell number of pyrophosphate, ●; Cell number of tripolyphosphate.

2.0×10^4 cells to 4.2×10^6 cells during 7 days of cultivation, while the attained cell number in the phosphate-free medium was 3.0×10^4. The slight increase of cell number of the phosphate-free cultivation was due to the phosphorous sources available in the inocula. Similar results were obtained in the dark-grown, wild starin and streptomycin-bleached mutant cells. The generation time of cells grown on pyrophosphate and tripolyphosphate were 13.8 hr and 14.5 hr, respectively and these values were slightly longer than that for orthophosphate-grown cells (12.5 hr).

In Fig. 2 is shown that pyrophosphate and tripolyphosphate in the growth media are consumed well by *E.gracilis.* It shows that 0.57μ mole (1.14 μmole as orthophosphate) of pyrophosphate and 0.41 μmole (1.23 μ mole as orthophosphate) of tripolyphosphate were removed from the medium after 7 days of culturing. Analyses after perchloric acid hydrolysis showed that all of the incorporated phosphate in the cells were in the organic form. Blum has shown, using ^{32}p- labelled phosphate, that within one minutes of uptake at 25°C, more than 95% of uptaken inorganic phosphate was converted to organic compounds in *Euglena*[11]. Incorporated phosphates in the cells were recovered stoichiometrically as orthophosphate with the concomitant elimination of pyrophosphate or tripolyphosphate in the medium.

Smillie and Krotkov found that autotrophic *Euglena* contained 5.1pg phosphorous per cell, heterotrophic *Euglena* 7.1pg phosphorous per cell, and cells grown in the light in the presence of organic carbon sources 6.0pg phosphorous per cell[12]. The present results show that *E.gracilis* grown in the two inorganic phosphate-containing media contained 5.6-6.1pg phosphorous per cell, and the results clearly show the ability of *Euglena* to utilize pyrophosphate and tripolyphosphate for growth as well as orthophosphate.

Induction of pyrophosphatase and tripolyphosphatase. Pyrophosphate- and tripolyphosphate-hydrolyzing activites in *E.gracilis* were assayed in the crude extract from the cells grown on pyrophosphate, tripolyphosphate and orthophosphate as the sole phosphorous sources for 5 days. As shown in Table 1, pyrophosphate- and tripolyphosphate-hydrolyzing activities or pyrophosphatase and tripolyphosphatase activities are inducible by the substrates; pyrophosphatase was more induced by pyrophosphate while tripolyphosphatase by tripolyphosphate than other phosphates respectively.

Table 1. Induction of Pyrophosphatase and Tripolyphosphatase in ***E.gracilis*** on various phosphorous sources after 5 days culturing

Phosphorous source	Activity (nmole/10^6 cells/min)	
	Pyrophosphatase	Tripolyphosphatase
Pyrophosphate	64.5	4.2
Tripolyphosphate	42.6	12.7
Orthophosphate	39.1	4.1

Subcellular localization of pyrophosphatase and tripolyphosphatase. Table 2 shows distribution of pyrophosphatase and tripolyphosphatase activities together with those of some marker enzymes in the subcellular fractions from differential centrifugation of *E.gracilis* grown under illumination. Pyrophosphatase was mainly localized in chloroplast and slightly in mitochondria fractions. Smillie reported that part of pyrophosphatase activity in autotrophic cells was localized in chloroplasts[13]. The tripolyphosphatase was mainly localized in mitochondria and sligtly in microsome fractions; it was absent in chloroplasts.

Table 2. Distribution of Pyrophosphatase, Tripolyphosphatase and Marker Enzymes in Subcellular Fractions of *E.gracilis.*

Enzymes	Enzyme activities (% of the activities in the crude extracts)			
	Chloroplast	Mitochondria	Microsomes	Cytosol (100,000 xg supernatant)
Pyrophosphatase	71.5	18.5	9.7	0
Tripolyphosphatase	0	77.5	12.8	9.7
Glucose-6-phosphatase	0	4.7	78.2	17.1
Succinic semialdehyde dehydrogenase	0	81.0	2.5	16.3
Glutamate dehydrogenase	0	0	0	95.3

Some properties of pyrophosphatase and tripolyphosphatase. Crude *Euglena* pyrophosphatase showed an optimum pH 7.5 and optimum temperature of 40°C, and was activated by Mg^{2+}. The Km value of the enzyme for pyrophosphate was 0.42mM under optimum conditions. Smillie reported that the optimal pH of pyrophosphatase in extracts of either autotrophic cells or streptomycin-bleached cells of *E.gracilis* strain z was about 8.0, and although the level of activity in both types of cells were similar.[13,14) He also described that metaphosphate was split at the same rate as pyrophosphate and Mg^{2+} and EDTA were required for the maximum enzyme activity.[13,14) Recently, Piccinni and Coppellotti reported that enzymes hydrolyzing inorganic polyphosphates were detected in a streptomycin-belached mutant of *E.gracilis:* one of them was a pyrophosphatase and it was most active at pH 7.5.[15,16)

Crude tripolyphosphatase showed an optimum pH 7.8 and an optimum temperature of 40°C and was activated also by Mg^{2+}; the Km value for tripolyphosphate was 0.24mM. Optium Mg^{2+} concentration of pyrophosphatase was 10mM while that of tripolyphosphatase was 5mM, and higher concentrations were inhibitory. Different subcellular localization and properties show that pyrophosphatase and tripolyphosphatase in *E.gracilis* are distinct enzymes.

Data presented in the present paper show that *E.gracilis* is able to utilize pyrophosphate and tripolyphosphate for the growth by the action of two distinct enzymes for assimilating these compounds.

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