Effect of Ammonium Ion on A Rapid Breakdown of Paramylon (β -1, 3-glucan) in Protist Euglena gracilis

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Effect of Ammonium Ion on A Rapid Breakdown of Paramylon (β-1, 3-glucan) in Protist *Euglena gracilis*

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Abstract

Ammonium ion induced a rapid breakdown of paramylon in carbon-starved cells of the bleached mutant *Euglena gracilis* SM-ZK. Cells showing a rapid breakdown of paramylon contained the induced high activities of the paramylon degrading enzymes; β-1, 3-phosphorylase (EC 2.4.1.97) and laminarinase (EC 3.2.1.16). The ammonium ion induced breakdown of paramylon appeared to be associated with the increase of amino acid pools in the cells. Co-existence of ammonium ion and cycloheximide, an inhibitor of cytoplasmic protein synthesis, greatly delayed the breakdown of paramylon. We suggest that the main role of paramylon in *E. gracilis* is to provide the necessary energy for surviving periods in the absence of suitable food sources and to provide the carbon necessary for amino acids in cytoplasmic metabolisms.

Introduction

A wide range of organisms store carbohydrate as glucan. These glucans, including starch and glycogen, are usually degraded under conditions of carbon shortage, *e.g.* in many seeds during germination[1)] and during periods of starvation in mammals.[2)] In the unicellular organisms *Euglena gracilis* the carbohydrate reserve is paramylon, a β-1, 3-glucan.[3)] Blum and Buetow[4)] have shown that in a streptomycin-bleached strain of *E. gracilis*, paramylon is slowly degraded under starvation conditions and Dwyer and Smillie[5)] have demonstrated a light-induced breakdown of paramylon associated with the differentiation of chloroplasts in *Euglena.* The breakdown of paramylon by light has also been shown providing carbon and energy for plastid development, in dark-organotrophically grown *Euglena* cells exposed to light.[6,7)] Recently Sumida *et al.*[8)] reported that the ammonium-induced breakdown and light induced paramylon breakdown in *Euglena* cells.

However, there is little information available on the importance of paramylon for the growth or survival of this organism or its actual physiological role and on the enzymes involved in the breakdown of paramylon. Recently Inui *et al.*[9–11)] have reported that paramylon is rapidly degraded under anaerobic conditions and have demonstrated wax ester fermentation, a kind of survival strategy of *Euglena* under anaerobic conditions. This report prompted us to reinvestigate the degradation mechanism of paramylon in *Euglena.*

We examined changes in the paramylon content and the activities of the enzymes

involved in the breakdown of paramylon during carbon depletion after additon of exogeneous ammonium ion. Some possible control mechanisms were considered and it is suggested that paramylon is not only a general reserve of endogenous carbohydrate but also has a specific role to play in the cells for assimilation of exogenous ammonium ion.

Materials and Methods

Organisms and culture

A streptomycin-bleached mutant SM-ZK[12] derived from *E. gracilis* was employed. The cells were grown heterotrophically in the darkness at 27°C in Hutner's medium[13] with aeration (90 strokes/min). Experiments in which the level of paramylon was studied under conditions of carbon depletion were performed. Stationary phase cells (2.0×10^7 cells) were harvested by centrifugation at $200 \times g$ for 3 min. They were washed twice by suspension and centrifuged in Hutner's medium[13] from which all the carbon containing compounds except thiamin, sodium ethylenediaminetetraacetate, and vitamin B_{12} had been omitted. The washed cells were suspended in the same medium to a density of about 2.0×10^7 cells and were shaken for a further 2 days 90 strokes/min in the darkness. All manipulations were carried out using aseptic techniques and in the dark. The starved cells were then added to $(NH_4)_2HPO_4$ at 10 mM and were shaken for 24 hr in the light (2000 lux) or in the dark. The control flasks were kept under the same conditions without $(NH_4)_2HPO_4$. Where inhibitors or glucose (10 mM/ml) were added to the medium, these were added immediately prior to adding the ammonium ion. Cell density was determined using a haemocytometer.[12]

Determination of paramylon, cell proteins and soluble sugars.

Cell protein was determined in 1M NaOH by suspending *E. gracilis* for 1 hr at room temperature. After centrifugation at $10{,}000 \times g$ for 10 min, the protein in the supernatant was determined.[14] The determination of paramylon was assayed using the phenol-sulfuric acid method with glucose as a standard.[14] The soluble sugars in the protein fraction were determined by the methods of phenol-sufric acid and Somogyi-Nelson reagent.[15]

Analysis of the amino acid pool.

The determination of amino acid pools in *Euglena* cells was performed by the method of Park *et al.*[16] Trichloroacetic acid extracts of *Euglena* cells were extracted with ether and were evaporated to dryness. The residue was dissolved in 2 ml of distilled water and the suspension was directly used for the determination of the amino acid pool which had not yet undegone hydrolysis with a Hitachi KLA-5A amino acid analyzer. Since tryptophan was not detected in the *Euglena* trichloroacetic acid soluble fraction it was not assayed.[16]

Enzyme assays and measurement of metabolites.

Preparation of the cell-free extracts[14] and β-1, 3-glucan phosphorylase (EC 2.4.1.97)[17], laminarinase (EC 3.2.1.16)[5], β-1, 3-glucan synthetase (EC 2.4.1.12)[18] and hexokinase (EC 2.7.1.1)[5] were assayed by the method of the references cited. Concentration of ATP[19], ADP and AMP[20], glucose 6-phosphate and fructose 6-phosphate[21]

were measured enzymatically in neutralized HCO_4 extracts as indicated. Biochemicals used for these measurements were from Boehringer. The protein was determined by the method of Lowry *et al.*[22]

Results

Ammonium ion-induced breakdown of paramylon.

To eliminate the effect of light, chloroplasts or plastids development, the streptomycin bleached mutant SM-ZK was employed the throughout the experiemnts. SM-ZK cells lack detectable ribulose-1, 5-bisphosphate carboxylase/oxigenase activity (EC 4.1.1.39). Figure 1 illustrates the ammonia-induced breakdown of paramylon organotrophically accumulated in the mutant cells. In the control experiments, paramylon was utilized only slowly in both the cells kept in the light or in the darkness. In contrast, paramylon was rapidly deleted if the cells were exposed to ammonium ion. Most of the paramylon was broken down within 24 hours and was linear for 24 hours. Microscopic examination confirmed the existence of small paramylon granules in the cells 24 hours after addition of ammonium ion and the average diameter and volume of the paramylon granules decreased 30% and 65%, respecrively. The results clearly indicate that ammonium ion (probably ammonia) induced the breakdown of accumulated paramylon, independent of light. It is very likely that after inducing this breakdown, ammonia also enhances the process by reacting with the degradation products to form the nitrogeneous

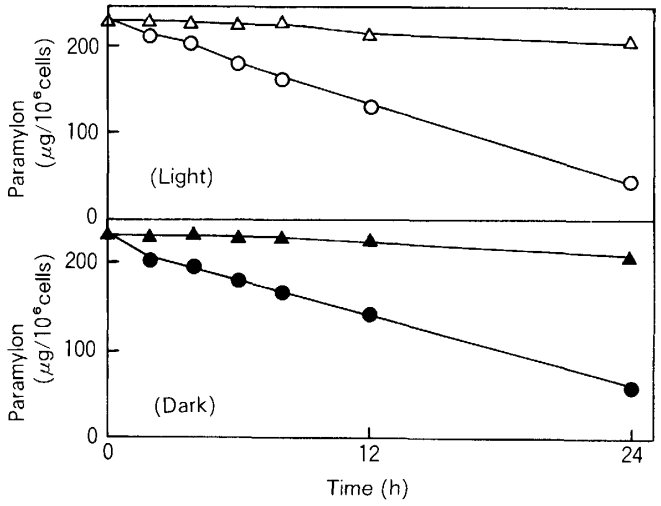

Fig. 1 Kinetics of ammonium ion induced degradation of paramylon contained in the dark grown bleached mutant cells of *Euglena* (SM-ZK). At 0 time, dark grown bleached mutant cells were incubated to ammonium containing medium or without ammonium and at appropriate times samples were with drawn for the determination of paramylon. Open and closed triangles represent paramylon content of *Euglena* with and without illumination in the absence of ammonium, respectively. Open and closed circles are in the presence of 10 mM $(NH_4)_2HPO_4$ with and without illumination, respectively. During the experiments, the cell number did not changed on different media (data not shown). Each point represents the mean of two separate experiments, the results of which agreed to within 12.2%.

Table 1 Amino acid analyses of pool material extracted from ammonium added and control *E. gracilis* cells
Cells of *E. gracilis* after addition of ammonium or without ammonium ion were incubated for 24 hr. Cells were washed, then extracted twice with 0.2M $HClO_4$. Amino acid analysis was carried out on a Hitachi amino acid analyzer. Each values are the mean of four experiments (S.D. ≤ 12%).

Amino acids	Amino acid composition (μmoles/10^9 cells)				
	Control cells (Time after without ammonium)		Ammonia added cells (Time after ammonium addition)		
	(hours)				
	0	24	2	6	24
Lysine	0.12	0.13	0.15	0.31	0.62
Histidine	0.07	0.07	0.09	0.14	0.20
Ammoina	15.51	16.02	197.52	214.92	178.25
Arginine	0.15	0.15	0.25	0.40	0.72
Aspartic acid	0.07	0.08	1.20	3.26	4.26
Threonine	0.02	0.02	0.06	0.10	0.16
Serine	0.03	0.03	0.07	0.12	0.21
Glutamic acid	0.25	0.28	15.46	24.28	23.28
Proline	0.08	0.08	0.12	0.18	0.25
Glycine	0.17	0.17	0.32	0.49	0.25
Alanine	0.27	0.26	3.46	3.52	3.42
1/2 Cystine	0.01	0.01	0.02	0.03	0.03
Valine	0.04	0.04	0.06	0.08	0.12
Methionine	0.01	0.01	0.01	0.02	0.02
Isoleucine	0.02	0.02	0.03	0.06	0.12
Leucine	0.03	0.03	0.03	0.08	0.15
Tyrosine	0.07	0.07	0.07	0.10	0.15
Phenylalanine	0.08	0.08	0.08	0.12	0.23

substances. $(NH_4)_2HPO_4$ caused only a minor transient change in the level of ATP and ADP after which the level of ATP increased slightly. Small transient changes apparently occured in the level of the sugar phosphates (glucose 6-phosphate and fructose 6-phosphate) just after the addition. While those intermediate changes were within the range of experimental error, they are not shown.

Upon addition of ammonium ion to the cells, there was a rapid increase in the amino acids pool, especially in glutamate, aspartic acid and alanine, as shown in Table 1.

The effect of ammonium ion on the activities involved in the catabolism of paramylon.

During the period of rapid breakdown of paramylon, the activities of several enzymes

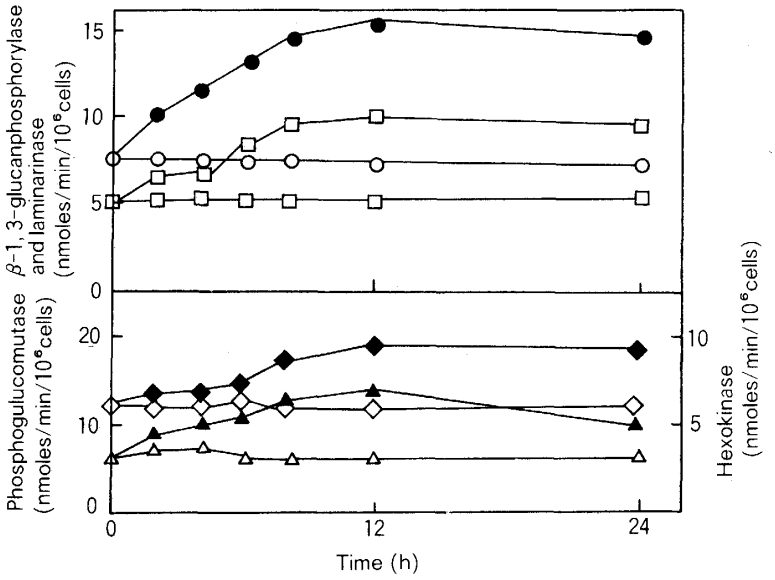

Fig. 2 Changes in levels of paramylon and enzymes catabolizing paramylon after addition of ammonium ion.
Open circles, squares, diamonds and triangles show the activities of laminarinase, β-1, 3-glucanphosphorylase, phosphoglucomutase and hexokinase, respectively, in the absence of ammonium. Closed circles, squares, diamonds and triangles represent the activities of laminarinase, β-1, 3-glucanphosphorylase, phosphogulucomutase and hexokinase, respectively, in the presence of ammonium. Each point represents the mean of three separate experiments with ± less than 10% of the mean.

involved in the catabolism of paramylon increased. Figure 2 shows changes in the activities of β-1, 3-glucan phosphorylases, β-1, 3-hydrolase and phosphogucomutase and hexokinase. The peaks of activity were usually attained during the first 4 hours of ammonium addition, but the changes in the activities of these enzymes in control experiments were kept constant. β-1, 3-glucan synthetase, which was readily detectable in growing cells, could be found in only trace amounts in ammonium added cells showing a rapid net breakdown of paramylon and this activity was the same as that of the control experiment.

Effect of inhibition of protein synthesis on the breakdown of paramylon.

The synthesis of proteins involved in the catabolism of paramylon in non-dividing dark-adapted cells after addition of ammonium ion is inhibited by cycloheximide (5 μg/ml). Figure 3 shows changes in the paramylon content of the cells treated with cycloheximide and the activities of paramylon degrading enzymes. The results show that cycloheximide inhibits the paramylon breakdown after addition of ammonium and it indicates that the decrease of paramylon degradation involves induced, *de novo* synthesis of enzymes in the cytosol. Treatment with storeptomycin, a specific inhibitor of translation of the plastid ribosomes in *Euglena,* did not block the breakdown of paramylon (unpublished).

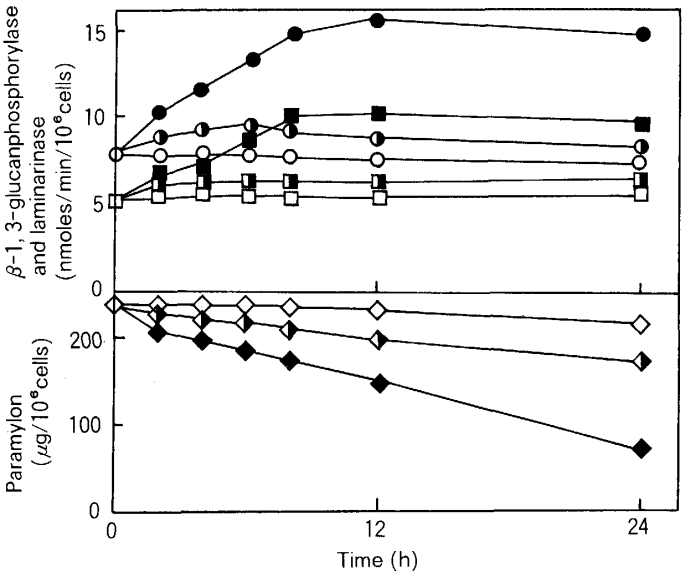

Fig. 3 Effect of cycloheximide on the breakdown of paramylon and paramylon degradating enzymes after addition of ammonium ion.
Open circles and open squares show the activities of laminarinase and β-1, 3-glucanphosphorylase, respectively, in the absence of ammonium. Closed circles and squares show the activities of laminarinase and β-1, 3-glucan phosphorylase, respectively, in the presence of ammonium. Half closed circles and half squares represent the activities of laminarinase and β-1, 3-glucanphosphorylase, respectively, in the presence of cycloheximide and ammonium. Open half and closed diamonds are the contents of paramylon in the absence, in the presence of cycloheximide and ammonium and in the presence of ammonium, respectively. Each point represents the mean of two separate experiments, the results of which agreed to within 11.5%.

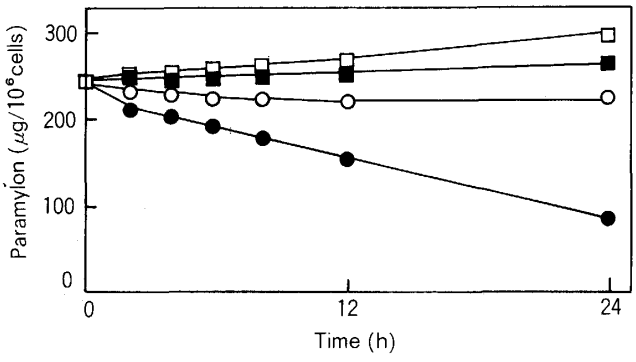

Fig. 4 Effect of added glucose on paramylon content during ammonium induced degradation of paramylon.
Open and closed circles represent the contents of paramylon without and with ammonium, respectively. Closed and open squares are the contents of paramylon in the co-presence of ammonium and glucose and in the presence of glucose, respectively. Cell growth was not found during the experiments even in the co-presence of ammonium and glucose (data not shown). Each experimental point represents the mean of three determinations (S.D. $\leqslant$ 11%).

Effect of added glucose on the cellular levels of paramylon after addition of ammonium.

Figure 4 shows the effect of addition of glucose to the medium prior to exposing the cells to ammonium ion. In the corpresence of glucose and ammonium there was little change in paramylon per cell, while in the cells kept without ammonium paramylon increased rapidly and by 12 hours had reached the level normally found in a growing culture of dark-adapated cells. Hence, even in the presence of an excess of exogeneous glucose, a marked difference between the paramylon kept in ammonium or not was preserved. This result shows that an exogeneous supply of glucose is utilized as well as endogeneous glucose from paramylon for the carbon skelton of amino acids, as shown in Table 1.

Discussion

Growing cells of dark-adapted *E. gracilis* or bleached mutant accumulate a large store of carbohydrate in the form of β-1, 3-glucan, paramylon which can account for as much as 50% of the dry weight of the cells. When these cells are transfered to medium lacking carbon growth sources, the paramylon is then utilized very slowly in dark or carbon starved conditions, as shown Fig. 1. Upon ammonium addition to these cells, most of the paramylon is depleted within 24 hours as shown in Fig. 1, a process which appears to be associated with ammonium assimilation of cytosol. We found that with the addition of NH_4Cl, the decomposition of wax esters was accelerated and proteins were formed.[23] We suggested that the wax esters are the auxiliary energy source, the major one being paramylon in *Euglnea.*[23] The ammonium-induced breakdown of paramylon is accompanied by an increase in activity of several enzymes catabolizing β-1, 3-glucan, including β-1, 3-glucan hydrolase and β-1, 3-glucan phosphorylase. Because cycloheximide does not inhibit respiration in *Euglena,*[24] the inhibition of paramylon breakdown shown by cycloheximide in the present study shows the *de novo* synthesis of paramylon degrading enzymes in the cytosol, while streptomycin, an inhibitor of protein synthesis on the plastid ribosomes in *Euglena,* did not show such an effect (data not shown). As shown in Fig. 4, the experiments with glucose also altered the importance of paramylon as a source of carbon and energy for amino acid pools in cytosol.

The increased rate of amino acid pools upon addition of ammonium provides clear evidence of the regulatory effect of ammonium ion on paramylon degradation. As shown in Table 1, free ammonia increased markedly after addition of ammonium ion, which indicates that the incorporated ammonium ion increases the carbon flow and accelerates the rate of the amino acids pool. This conclusion is supported by the sudden increase in the level of glutamate, aspartate and alanine, and these amino acid carbon skelton precusors are supplied by the tricarboxylic acid cycle through glycolytic enzymes. Two of them, glutamate and aspartate may be present in their amide forms or in conjugated forms with arginine as has been reported by Kempner and Miller.[25] They found that *Euglena* contained a considerable amount of peptides composed mainly of arginine, aspargine and glutamine. To confirm their observation, acid hydrolysis of amino acids pools should be necessary.

We have reported occurrence of two types of isocitrate dehydrogenases in *E. gracilis*: one is NAD (EC 1.1.1.41) and located mitochondria and the other is NADP-linked (EC 1.1.1.42) and located mitochondria and cytosol fractions.[26] Strong activities of NADP-

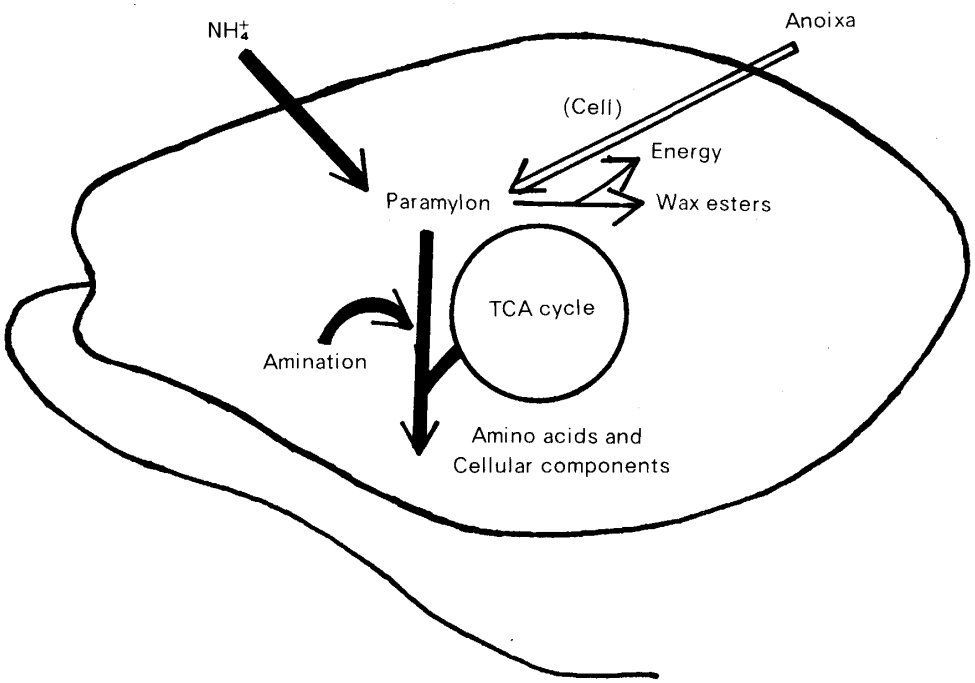

Fig. 5 Regulatory mechanism of paramylon breakdown by ammonium ion.

linked glutamate dehydrogenase (EC 1.4.1.4) are present in cytosol and glutamate-oxaloaetate transaminases (EC 2.6.1.1) in mitochondria and cytosol fractions, and we suggest that a cycle system of NADPH between glutamate NADP-dependent glutamate dehydrogenase and NADP-isocitrate dehydrogenase in this protozoon is actually operative.[26] The rate of glutamate increase upon addition of ammonium together with reductive amination of α-ketoglutaric acid is the primary route of incorporation of ammonium in *Euglena,* which shows that the regulatory effects of ammonium on carbon metabolism are responsible for the observed immediate increase of other amino acids, especially aspartate and alanine.

From these results, we suggest an *Euglena* paramylon role in a survival mechanism which is two-fold. Firstly, it provides the energy to allow the cell to survive for periods in the anaeribiosis in the absence of food sources; and secondly, it provides the energy and their carbon skelton to converse into amino acids by amination in the cells. Inui *et al.*,[9] have recently found that when *E. gracilis* is transfered from aerobic to anaerobic conditions in the absence of a carbon source, was esters are promptly formed from paramylon, accompanied by generation of ATP and they have named this phenomenon as wax ester fermentation. This strategy is categorized in the former. The present results belong to the later category and possible regulation of the ammonium is summarized in Fig. 5. In the initial step, the ammonium ion taken up is directly aminated reductively by glutamate dehydrogenase, and transaminated with tricaboxylic acid intermediates by tranaminases and accumulates as glutamate, aspartate and alanine. Then glutamate and asparate are further aminated to amide forms and these amides are conjugated with arginine through unknown pathways and are stored as a nitrogen source in *Euglena* cells. A part of these amino acids and peptides are metabolized for synthesis of cellular components and used for *de novo* protein synthesis including paramylon degrading enzymes.

References

1) VARNER, J.E., CHANDRA, G.R. and CHRISPEELS, M.J. (1965). Gibberellic acid-controlled synthesis of α-amylase in barley endosperm. *Journal of Cellular Comprehensive Physiology* **66**, 55–67.
2) SIE, H.G., HABLANIAN, A. and FISHMAN, W.H. (1964). Solubilization of mouse-liver glycogen synthetase and phosphorylase during starvation glycogenolysis and its reversal by cortisol. *Nature* **201**, 393–394.
3) BARRAS, D.R. and STONE, B.A. (1968). Carbohydrate composition and metabolism in *Euglena.* In "*The Biology of Euglena*", **Vol. II**, (Ed. BUETOW, D.E.). Academic Press, New York, 149–191.
4) BLUM, J.J. and BUETOW, D.E. (1963). Biochemical changes acetate deprivation and repletion in *Euglena. Exp. Cell Res.*, **29**, 407–421.
5) DWYER, M.R. and SMILLIE, R.M. (1970) A light induced β-1, 3-glucan breakdown associated with the differentiation of chloroplasts in *Euglena gracilis. Biochem. Biophys. Acta* **216**, 392–401.
6) SCHWARTZBSCH, S.D., SCHIFF, J.A. and GOLDSTEIN, N.H. (1975). Events surrounding the early development of *Euglena* chloroplasts. V. Control of paramylon degradation. *Plant Physiol.*, **56**, 313)–317.
7) SCHIFF, J.A. and SCHWARTZBACH, S.D. (1982). Photocontrol of chloroplast development in *Euglena.* In "*The Biology of Euglena*", **Vol. III**, (Ed. by BUETOW, D.E.) Academic Press, New York, 313–352.
8) SUMIDA, S., EHARA, T., OSAFUNE, T. and HASE, E. (1987). Ammonia- and Light-Induced Degradation of Paramylum in *Euglena gracilis. Plant Cell Physiol.*, **28**, 1587–1592.
9) INUI, H., MIYATAKE, K., NAKANO, Y. and KITAOKA, S. (1982). Wax fermentation in *Euglena gracilis. FEBS Lett.*, **150**, 89–93.
10) INUI, H., MIYATAKE, K., NAKANO, Y. and KITAOKA, S. (1984). Fatty acid synthesis in mitochondria of *Euglena gracilis. Eur. J. Biochem.*, **142**, 121–126.
11) INUI, H., MIYATAKE, K., NAKANO, Y. and KITAOKA, S. (1985). The physiological role of oxygen-sensitive pyruvate dehydrogenase in mitochondrial fatty acid synthesis in *Euglena gracilis. Arch. Biochem. Biophys.*, **237**, 423–429.
12) ODA, Y., NAKANO, Y. and KITAOKA, S. (1982). Utilization and toxicity of exogenous amino acids in *Euglena gracilis. J. Gen. Microbiol.*, **128**, 853–858.
13) HUTNER, S.H., BACH, M.K. and ROSS, G.I.M. (1956). A sugar-containing basal medium for vitamin B_{12}-assay with *Euglena*; application to body fluid. *J. Protozool.*, **3**, 101–112.
14) YOKOTA, A., HOSOTANI, K. and KITAOKA, S. (1982). Mechanism of metabolic regulation in photoassimilation of propionate in *Euglena gracilils* z. *Arch. Biochem. Biophys.*, **213**, 530–537.
15) SPIRO, R.G. (1966). Analysis of sugars found in glycoproteins. In "*Methods in Enzymology*", **Vol. 8**, (Eds. NEUFELD, N. and GINSBURG, V. Academic Press, New York, 3–25.
16) PARK, B.S., HIROTANI, A., NAKANO, Y. and KITAOKA, S. (1983). The physiological role and catabolism of arginine in *Euglena gracilis. Agric. Biol. Chem.*, **47**, 2561–2567.
17) MARECHAL, L.R. and GOLDEMBERG, S.H. (1963). Laminaribiose phosphorylase from *Euglena gracilils. Biochem. Biophys. Res. Commun.*, **13**, 106–109.

18) MARECHAL, L.R. and GOLDEEMBERG, S.H. (1964). Uridine-diphosphatae glucose-β-1, 3-glucaan β-3-glucosyl transferase from *Euglena gracilis*. *J. Biol. Chem.*, **239**, 3163–3167.
19) LAMPRECHT, W. and TRAUTCHOLD, I. (1974). Determination with hexokinase and glucose-6-phosphate dehydrogenase. In "*Methods of Enzymatic Analysis*", **Vol. 4**, (Ed. by BERGMEYER, H.U.). Academic Press, New York and London, 2101–2110.
20) JAWORK, D., GRUBER, W. and BERGMEYER, H.U. ((1974). Adenosine-5′-diphosphate and adenosine-5′-mono-phosphate. In "*Methods of Enzymatic Analysis*", **Vol. 4**, (Ed. by BERGMEYER, H.U.). Academic Press, New York and London, 2127–2131.
21) LANG, G. and MICHAL, G. (1974). D-glucose-6-phosphate and D-fructose-6-phosphate. In "*Methods of enzymatic Analysis*", **Vol. 3**, (Ed. by BERGMEYER, H.U.). Academic Press, New York and London, 1238–1242.
22) LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
23) INUI, H., OHOYA, O., MIYATAKE, K., NAKANO, Y. and KITAOKA, S. (1986). Assimilation and metabolism of fatty alcohols in *Euglena gracilis*. *Biochem. Biophys. Acta* **875**, 543–548.
24) KIRK, J.T.O. (1970). Failure to detect effects of cycloheximide on energy metabolism in *Euglena gracilis*. *Nature* **226**, 182.
25) KEMPNER, E.S. and MILLER, J.H. (1965). The molecular biology of *Euglena gracilis*: III. General carbon metabolism. *Biochemistry* **4**, 2735–2739.
26) ODA, Y., MIYATAKE, K., NAKANO, Y. and KITAOKA, S. (1981). Subcellular location and some properties of isocitrate dehydrogenase isozymes in *Euglena gracilis*. *Agric. Biol. Chem.*, **45**, 2619–2621.