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Inability of *Euglena gracilis* z to Utilize Nitrate, Nitrite and Urea as the Nitrogen Sources

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Abstract

Euglena gracilis, strain z, did not utilize nitrate, nitrite and urea for growth as evidenced by their effects on cell multiplication, consumption of the nitrogenous compounds from the growth media and change of pH in the media, and also for the chlorophyll synthesis. Nitrate reductase and urease were not detected in the cells indicating that these nitrogenous compounds are not enzymatically assimilated. The results establish a clear distinction of *E. gracilis*, a plant-like protozoon, from green algae and other lower plants in nutritional requirements.

While green algae and higher plants utilize nitrate and nitrite as the nitrogen sources, *Euglena gracilis*, a photosynthetic protozoon, was reported not to grow on these salts,[1, 2] although an early paper[3] described a contradictory result. On the other hand, urea, another efficient nitrogen source for plants, was reported to be utilized by *E. gracilis* according to Roon and Levenberg[4] whereas not utilized according to Birdsey and Lynch.[5] To have exact information on the availability of these compounds by *Euglena* is desirable for understanding the evolution of nutritional requirements in photosynthetic unicellular organisms. The present paper reports that growth and chlorophyll synthesis in *E. gracilis* are not supported by nitrate, nitrite and urea as the nitrogen sources and that the cells have no enzymes for assimilating these compounds.

Materials and Methods

Cultures. E. gracilis, strain z, was stock-cultured in the Hutner's medium[6] which contained (g/liter) 0.4 KH_2PO_4, 0.2 $(NH_4)_2HPO_4$, 0.5 $MgSO_4 \cdot 7H_2O$, 0.2 $CaCO_3$, 2.0 DL-malic acid and 5.0 sodium glutamate, and small amounts of minor salts and vitamins B_1 and B_{12}; the initial pH was 3.3. Cultivation was conducted under illumination (3000 lux) at 27°C with aeration. For experiments, the nitrogenous compounds were replaced by $NaNO_3$, $NaNO_2$, urea or some ammonium salts. $NaNO_3$ and ammonium salts were autoclaved in the test media, but $NaNO_2$ and urea were added asceptically to the medium by using membrane-filter (Toyo Roshi Co., Tokyo). A nitrogen-free medium was used as the control.

Growth was measured by counting cell number with a hemocytometer. Chlorophyll was determined by the of method MacKinney[7] after extracting cells with 80% acetone.

Analyses of nitrogenous compounds. Nitrate was determined by the ultraviolet

absorption method,[8] nitrite by the Griess-Ilosvay method,[9] ammonium by the Nessler method[10] and urea by the diacetylmonoxime method.[11] Protein was determined by the method of Lowry *et al.*[12] using bovine albumin as a standard.

Enzyme assays. *E. gracilis* cells were harvested, suspended in 50 mM phosphate buffer, pH 7.0, and disrupted by sonication. The sonicate was centrifuged at 20,000 x *g* for 15 min and the supernatant was used as the crude extract of enzymes.

Nitrate reductase was assayed by measuring nitrite to be formed by the method of Hageman and Huchesby.[13] For assaying urease, a reaction mixture was made from 50 mM phosphate buffer, pH 7.0, 2% (final concentration) urea and 0.1 ml of the crude extract (final volume 1 ml), and it was incubated at 25°C for 30 min with gentle shaking, when 0.8 ml of 10 N sodium hydroxide was added to terminate reaction. The mixture was aerated to drive off the ammonia formed into 0.2 N sulfuric acid, which was determined by the phenol-hypochlorite method.[14]

Results and Discussion

Growth of E. gracilis on nitrate, nitrite and urea as the sole nitrogen sources.

Utilities of nitrate, nitrite and urea as the sole nitrogen sources for the growth of *E. gracilis* were studied by observing their effects on cell multiplication (Fig. 1), removal of nitrogenous compounds from the growth media (Fig. 2) and change of pH in the media (Fig. 3) in comparison with ammonium salt.

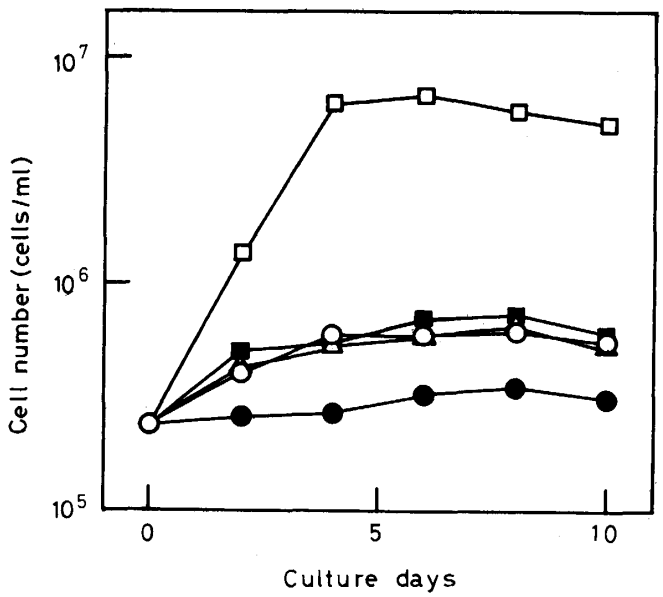

Fig. 1 Growth of *E. gracilis* on various nitrogen sources

E. gracilis was grown in media made by replacing the nitrogen compounds in the Hutner's medium by such amounts of various nitrogenous compounds tested as corresponding to 0.457 g N/liter, which is the original nitrogen content. Difference in the content of carbon source occurring by removing glutamate from the Hutner's medium was offset by correspondingly increased amount of malate.

Symbols: ○, $NaNO_3$, ●, $NaNO_2$, △, urea, □, $(NH_4)_2HPO_4$, ■, nitrogen-free.

$(NH_4)_2 HPO_4$ allowed cells to grow efficiently to reach the stationary phase after 4 days (Fig. 1). The cell number was increased from 2.4×10^5 to 6.8×10^6 during 6 days of cultivation. With $NaNO_3$, $NaNO_2$ and urea as the sole nitrogen sources, the attained cell number were 5.9×10^5, 3.2×10^5 and 6.3×10^5, respectively. Since the cell number in the nitrogen-free medium after 6 days of culturing was 7.0×10^5, nitrate, nitrite and urea did not support the cell multiplication at all. The slight increases of cell number by these compounds as well as in the nitrogen-free medium were surely due to the available nitrogen sources in the inocula.

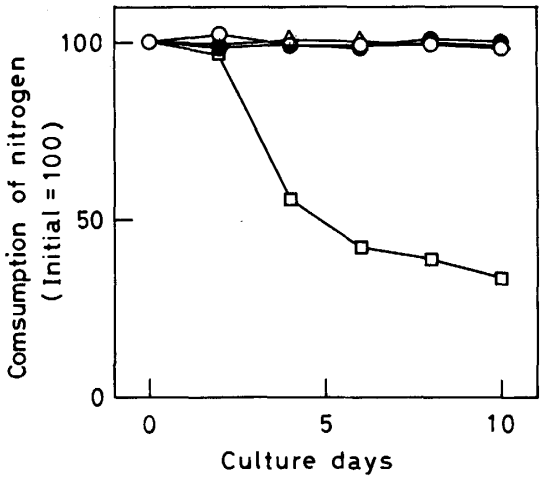

Fig. 2 Consumption of various nitrogen sources by *E. gracilis*

Consumption of various nitrogenous compounds in the growth media was shown by percentage against the amounts contained initially in the media after intervals of culturing.

Symbols are the same as in Fig. 1.

In Fig. 2 is shown that $NaNO_3$, $NaNO_2$ and urea in the growth media are not consumed by *E. gracilis*. While $(NH_4)_2HPO_4$ was removed from the medium by 67% after 10 days of culturing, $NaNO_3$, $NaNO_2$ and urea remained unconsumed over this period. When NH_4NO_3 was employed, the ammonium ion was removed after about 4 days but nitrate ion remained unchanged up to 10 days.

Fig. 3 shows that while pH of the culture broth rose along with cellular growth when $(NH_4)_2HPO_4$ was used as the nitrogen source, it remained unchanged up to 10 days of cultivation when $NaNO_3$, $NaNO_2$ or urea was employed. With NH_4NO_3 pH rose to 5.5 and remained unchanged thereafter.

These data show clearly that *E. gracilis* is unable to utilize nitrate, nitrite and urea for growth. The results were not different when the carbon source was changed from malate to glucose or ethanol.

Synthesis of chlorophyll on nitrate, nitrite and urea as the sole nitrogen sources.

After Harris and Kirk,[15] *E. gracilis* was grown in the dark for 4 days in a medium

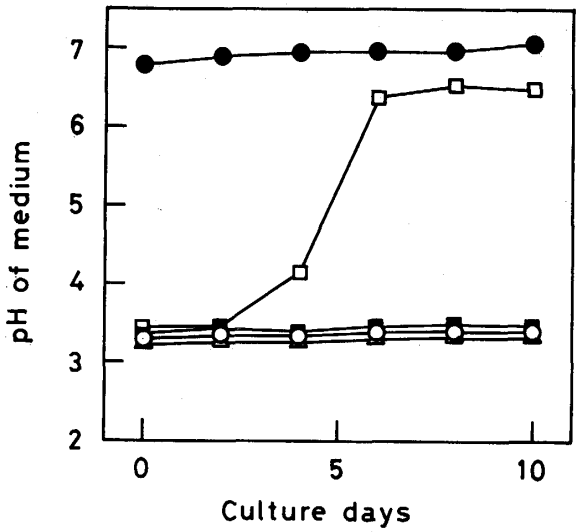

Fig. 3 Change of pH in the growth media along culturing of *E. gracilis* on various nitrogen sources

$NaNO_2$ was added to the growth medium adjusted to pH, 6.8 and culturing was started at this pH, since $NaNO_2$ was decomposed at lower pH's.

Symbols are the same as in Fig. 1.

containing 0.025% $(NH_4)_2HPO_4$ and 1.18% ethanol, and the harvested, washed cells were incubated in 40 mM phosphate buffer, pH 7.0, containing $NaNO_3$, $NaNO_2$, urea, $(NH_4)_2HPO_4$ or $(NH_4)_2SO_4$ in an amount corresponding to 100 μg N/ml at 27°C for 24 hours under illumination. The amounts of chlorophyll synthesized are shown in Table 1.

Table 1. Chlorophyll synthesis in *E. gracilis* on various nitrogen sources

Nitrogen source (100μg N/ml)	Chlorophyll synthesized (μg/10^6 cells/24 hr)	Relative yield (%) (against the yield on $(NH_4)_2SO_4$)
$NaNO_3$	0.48	24.6
$NaNO_2$	0.47	24.1
Urea	0.51	26.2
$(NH_4)_2HPO_4$	1.85	94.9
$(NH_4)_2SO_4$	1.95	100
None	0.48	24.6

Against the amount synthesized on $(NH_4)_2SO_4$, $NaNO_3$, $NaNO_2$ and urea gave 24–25% of the yield and these values are quite the same with the yield of chlorophyll in the nitrogen-free medium, indicating that these 3 nitrogenous compounds were not utilized at all for the chlorophyll synthesis by *E. gracilis*.

Absence of nitrate reductase and urease in E. gracilis.

Nitrate reductase was assayed in the crude extract from cells grown on NH_4NO_3 as the sole nitrogen source for 6 days. All ammonium ions had been consumed by 4 days remaining nitrate ion unconsumed. NH_4NO_3 was employed since nitrate reductase is a

typical enzyme inducible by the substrate,[16] although its formation is inhibited by ammonium ion in fungi[17] and green algae.[18] Nitrate reductase activity was not detected at all in *E. gracilis*.

Urease was not detected also in the crude extract of *E. gracilis* grown on ammonium and glutamate (Hutner's medium) as the nitrogen sources. In some yeasts and algae grown on urea as the sole nitrogen source, the presence of ATP: urea-amidolyase (ADP) has been reported instead of urease.[4] This amidolyase was not detected in *E. gracilis*.[4] The result of the present experiments and the reported fact indicate that *E. gracilis* has no enzyme catabolizing urea supporting the preceding results that it cannot grow urea.

Data presented in the present paper clearly show that *E. gracilis* is not able to utilize nitrate, nitrite and urea for the growth and chlorophyll synthesis, and that it lacks enzymes for assimilating these compounds. Huzisige and Satoh[2] reported that *E. gracilis* var. *bacillaris* had nitrite reductase activity. From the fact that the cells cannot grow on nitrite the result apparently should be reexamined.

The inability of *E. gracilis* to grow on these nitrogen sources provides a clear distinction of this plant-like protozoon from green algae and other lower plants in regard to nutritional requirements.

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