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RESEARCH PAPERS

Effect of the Form of Mineral Nitrogen on the Growth and Heterotrophic Fixation of ^{14}C-Bicarbonate in *Chlamydomonas reinhardtii*

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Abstract—The earlier-detected effect of the mineral nitrogen form in the nutrient medium (ammonium or nitrate) on the quantity of ribosomes in the cells of chlamydomonas and the callus of soybean requires experimental corroboration of the proposed interpretation of the discovered phenomenon. In order to elucidate the mechanism of ammonium's action on the formation of protein-producing structures (ribosomes) in the cells of green alga chlamydomonas (*Chlamydomonas reinhardtii,* strain *gr 21*), we compared the effect of the form of mineral nitrogen (nitrate and ammonium) on the growth and heterotrophic fixation of ^{14}C-bicarbonate. Our experiments have shown that the process of cell division (mitosis) was hampered at the beginning of the growing cycle (on days 1–3) when the cells were cultured on nitrate medium. By the end of the growing cycle (days 7–10), the number of cells and the content of dry matter in the cells equalized. It was found that the cells grown on TAP medium with ammonium fixed $H^{14}CO_3^-$ at a higher rate than the cells cultured on TAP medium with nitrate. The obtained results have shown that the rate of dark fixation of $H^{14}CO_3^-$ in the cells grown in the medium with ammonium was higher than in the cells on nitrate medium irrespective of the mode of calculation: on the number of cells or per dry weight unit. An assumption that ammonium present in the nutrient medium can activate synthesis of extra amino acids participating in the formation of protein components of ribosomes in the alga chlamydomonas is discussed.

Keywords: Chlamydomonas reinhardtii, ammonium, nitrate, growth, heterotrophic fixation of ^{14}C-bicarbonate

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INTRODUCTION

Ammonium and nitrate are the main forms of mineral nitrogen that prevail in the soil and are assimilated by photosynthesizing organisms. For the majority of plant crops, nitrate is a preferable form of mineral nitrogen, whereas ammonium nitrogen is often assimilated by plant objects only in conjunction with nitrate. Application of ammonium as a sole source of nitrogen is undesirable since it may cause plant death if its concentration exceeds a permissible limit [1].

Mineral nitrogen is necessary for cells to produce nitrogen-containing organic compounds: amino acids and proteins. Whereas ammonium nitrogen is ready for the synthesis of nitrogen-containing organic compounds [2], the assimilation of nitrate by plant cells requires its reduction to ammonium. For this purpose, plants employ a complex of enzyme systems ensuring transformation of oxidized form of mineral nitrogen into reduced form [2].

When we investigated the effect of the form of mineral nitrogen on the development of the structure of the photosynthetic apparatus in the cells of soybean's (*Glycine max* L.) callus culture [3], we observed that the lack of ammonium in the nutrient medium disturbed the organization of lamellar structure of the chloroplasts and mitochondria. Moreover, the number of ribosomes in the callus cells sharply (5–10 times) decreased if the nutrient medium lacked ammonium [3]. The disturbance of lamellar structure of chloroplasts associated with a lack of ammonium component of nutrition was also observed in native cells of higher plants: pea (*Pisum sativum* L.) [4] and wheat (*Triticum vulgare muticum* L.) [5].

However, among lower plants (algae) there exist organisms capable of growing and propagating on the nutrient media containing either nitrate or an ammonium form of mineral nitrogen. An example of such plant object is a green single-cell alga chlamydomonas (*Chlamydomonas reinhardtii*) [6].

Investigations into the effect of the forms of mineral nitrogen conducted on cell suspension of *C. reinhardtii* (strain *gr 21*) also showed a considerable reduction in the number of ribosomes in the cells of alga when nitrate was the single source of nitrogen [7]. However,

it is interesting that the reduction in the number of ribosomes in the cells of alga cultured on nitrate (ammonium-free) medium was less pronounced (twofold).

Since any membrane is a protein–lipid complex and ribosome is a nucleoprotein complex, the reduction in the number of ribosomes in the cells of soybean and imperfect organization of membrane structures of their organelles observed on the medium lacking or deficient in ammonium suggested a decrease in the activity of protein metabolism. On the other hand, the lamellar structure of chloroplasts in the cells of chlamydomonas essentially did not depend on the form of consumed nitrogen (nitrate or ammonium) [8] and only less numerous ribosomes in the cells grown on ammonium-free medium pointed to a possible modification of protein metabolism in the cell.

Intensity of protein metabolism in the cell may be altered in two ways: via modifying the activity of the existing pathway of protein macromolecules' formation or owing to emergence (or disappearance) of an additional pathway of their production.

When free ammonium is available, heterotrophic (dark) fixation of CO_2 is one of the ways for intensification of protein orientation of cellular metabolism. Stimulation of this process with ammonium was detected in cell suspension of maple (*Acer pseudoplatanus* L.) [9] and in leaf tissues of maize (*Zea mays* L.) [10].

Assuming that the activation of such a pathway of protein production by ammonium is also possible in the cells of green alga chlamydomonas, we conducted an investigation aiming to detect heterotrophic fixation of CO_2 in the cells of *C. reinhardtii* and to estimate the effect of the form of mineral nitrogen in the nutrient medium on this process.

MATERIALS AND METHODS

As a test subject, we chose mixotrophic single-cell alga *Chlamydomonas reinhardtii* (strain *gr 21*). Cell suspension was cultured on TAP nutrient medium [11] that, depending on the aim of the experiment, contained solely ammonium (NH_4Cl, type 1) or nitrate (KNO_3, type 2) mineral nitrogen. Elemental content of nitrogen in the nutrient medium with nitrate or ammonium components was equalized to a standard level (TAP medium) and corresponded to 7 mM nitrogen for both types of media.

Before the experiment, original cell suspension of *C. reinhardtii* was cultured at a continuous agitation in 300-mL cone flasks with 100 mL of nutrient medium at a temperature of 25°C and soft light (illuminance of approximately 1 klx) during 10–12 days on ammonium-free (nitrate) medium (type 2). Equal volumes of cell subculture (100 μL) from ammonium-free medium were then transferred to the media with different components of mineral nitrogen (types 1 and 2) and further cultured at the same conditions for 10–12 days. Only after such an adaptation, the cells were used in the experiment. For the purity of the experiment, ammonium-free medium (type 2) should be free of even micro quantities of ammonium; therefore, the micro component $Mo_7O_{24}(NH_4)_6 \times 4H_2O$ was replaced with $Na_2MoO_4 \times 2H_2O$. Other components and characteristics corresponded to the standard TAP medium.

Cell suspensions adapted to nitrate or ammonium medium (types 1 and 2) were cultured in 300-mL cone flasks in a controlled-climate chamber under the following conditions: 16/8 h of light/dark, light intensity of approximately 1 klx, and temperature of 25 ± 1°C. Cell suspensions were stirred using magnetic agitators. Rotation speed was determined empirically and maintained the cells in a suspended state throughout the whole growing cycle.

Heterotrophic (dark) fixation of CO_2 by the cells of *C. reinhardtii* was estimated by the quantity of ^{14}C-bicarbonate incorporated into acid-resistant metabolites. Cell suspension (2–5 mL) was incubated in 2 mM bicarbonate in phosphate buffer in the dark at room temperature and regular stirring for 1 h. The suspension was then supplemented with 2–4 MBq of ^{14}C-bicarbonate. The samples of suspension (by 100 μL) were regularly taken in the course of incubation (for 1 h) and transferred to scintillation flasks containing 100 μL of formic acid. After a 30-min-long incubation, the samples were examined using a Beckman LS 6500 scintillation counter (United States) in a Ready Safe scintillation cocktail for aqueous samples (Beckman). The quantity of carbon dioxide fixed during the initial 15 min after the addition of the label was calculated from specific radioactivity of ^{14}C-bicarbonate in the incubation medium.

In the course of the growing cycle, the number of cells was determined using a Fuchs-Rosenthal counting chamber (PAO Krasnogvardeets, St. Petersburg, Russia) according to a common technique.

In order to compare the effects of oxidized and reduced forms of nitrogen on size distribution of the cells in suspension, we processed microimages of cells using a Leica TCS SPE microscope (Leica, Germany). The size of cells was estimated by the section area. The images were processed using the ImageJ computer program. The obtained microscopic images were converted to a binary mask using Otsu's method [12]. On the obtained mask, open-ended sections of the cells were manually corrected and too close (stuck together) cells were separated. Cell areas were computed using an Analyze particles function. Each sample shows average sizes of 100–1000 cells depending on the number of cells in the visual field (in 7–10 pictures).

Dry matter of the cell suspension was determined after achieving the fixed weight of the sample of cells at 90°C.

The obtained data are typical results of one replication out of 3–5 biological experiments, each with three replicates, ± standard deviation. The ratio between the rate of bicarbonate fixation and growth parameters (10^5 cells or mg dry wt) is the mean of all the biological experiments, and standard deviation is the mean of all deviations.

RESULTS

Increase in the number of cells of *C. reinhardtii* in suspension cultured on media of types 1 and 2 was described by an S-shaped growth curve typical of such plant objects. However, in spite of the identical character of increase in the number of cells in suspensions cultured during the growing cycle on media 1 and 2, the speeds of passage through the initial growth phases (lag-phase and exponential phase) therein considerably differed (Fig. 1). The rate of increase in the number of cells in suspension lacking an ammonium component (type 2) in the exponential and linear phases of growth was much lower than in the cells of suspension cultured on exogenous ammonium (type 1). Such a delay in the rise in the number of cells could be accounted for by a reduced activity of mitotic process in the cells devoid of exogenous ammonium. Such interpretation of the obtained result is corroborated by the estimation of cell distribution by size (Table 1).

Since a single cell of chlamydomonas can divide into more than two daughter cells [6] (usually into four cells), only the smallest cells (the cells with cross section area of 20 μm^2 in our experiments) can be reliably considered just-divided cells. In this relation, the analysis was based on the smallest cell of the suspension (cross section area of 20 μm^2). Therefore, the cells with a cross section area of approximately 80 μm^2 could be considered parental and ready to divide because they completed their growth cycle. Cell clusters and dividing cells present in the suspension were disregarded because they occurred sporadically and accounted for no more than 1–3% of the total number of cells on the grid of the Fuchs-Rosenthal counting chamber.

Analysis of size distribution of the cells in suspension has shown that, in the beginning of culturing, the cells with small cross section area were much more numerous in cultures on the medium with ammonium (type 1) (Table 1, cross section area of 20 μm^2). At the end of the growing cycle on ammonium-free medium (type 2), the number of giant cells (Table 1, cross section area of more than 80 μm^2) considerably rose. Thus, the delay in growth of cell suspension on ammonium-free medium at the beginning of the growing cycle and cell gigantism at the end of the growing cycle (type 2) can point to a suppression of mitotic process of cell division by the deficiency of ammonium.

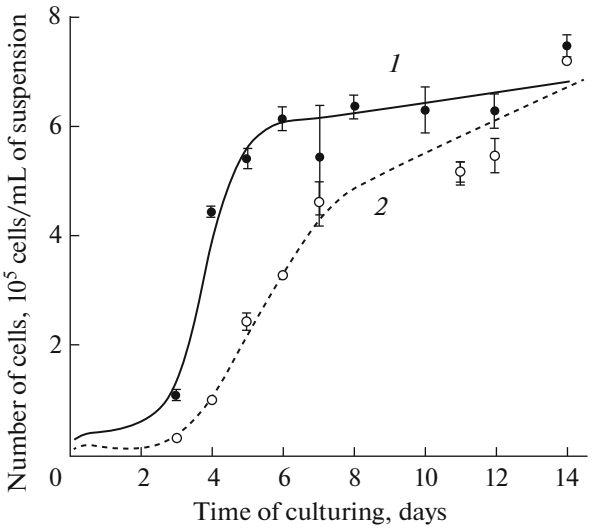

Fig. 1. Increase in the number of cells of *Chlamydomonas reinhardtii* during the cycle of culturing on TAP media with ammonium or nitrate. (*1*) Cells grown on ammonium medium; (*2*) cells grown on nitrate medium. Zero point is the beginning of culturing of 10–12-day-old adapted cells of the previous passage on fresh nutrient medium.

Determination of dry matter of the cells in the course of the growing cycle has shown that differences between the types of media were not so great, at least in the stages of linear and steady-state growth (Table 2). Low initial density of cell suspension at the beginning of the growing cycle (lag phase) does not allow us to suggest an effect of the form of mineral nitrogen on the accumulation of dry matter of the cells, since determination of dry mass by weighing could not reliably show differences in the accumulation of dry mass of the cells grown on ammonium and nitrate media.

Table 1. Number of small and big cells of *Chlamydomonas reinhardtii* in the samples* of suspensions, types 1 and 2, in the course of growing cycle

Time of culturing, days	Type 1		Type 2	
	20–40 μm^2	>80 μm^2	20–40 μm^2	>80 μm^2
3	434 ± 21	2 ± 0	86 ± 9	16 ± 3
5	212 ± 12	4 ± 2	173 ± 13	56 ± 7
7	130 ± 15	60 ± 4	126 ± 16	94 ± 10

* The size of a sample taken on the specified days of culturing depended on the density of the least dense suspension.

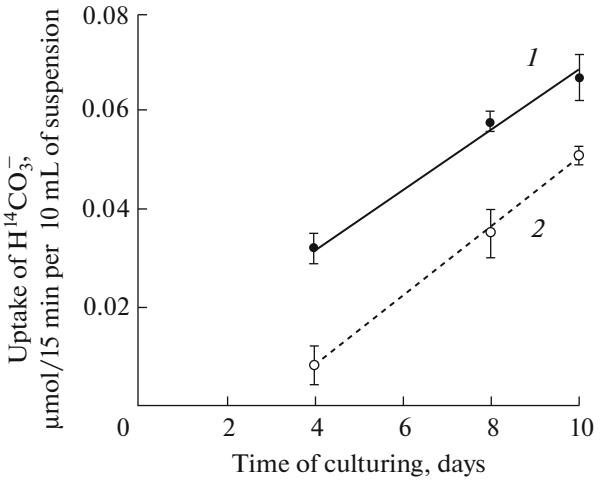

Fig. 2. Rates of heterotrophic (dark) fixation of $H^{14}CO_3^-$ by the cells of *Chlamydomonas reinhardtii* during the cycle of culturing on ammonium and nitrate TAP media. (*1*) Cells grown on ammonium medium; (*2*) cells grown on nitrate medium. Zero point is the beginning of culturing of 10–12-day-old adapted cells of the previous passage on fresh nutrient medium.

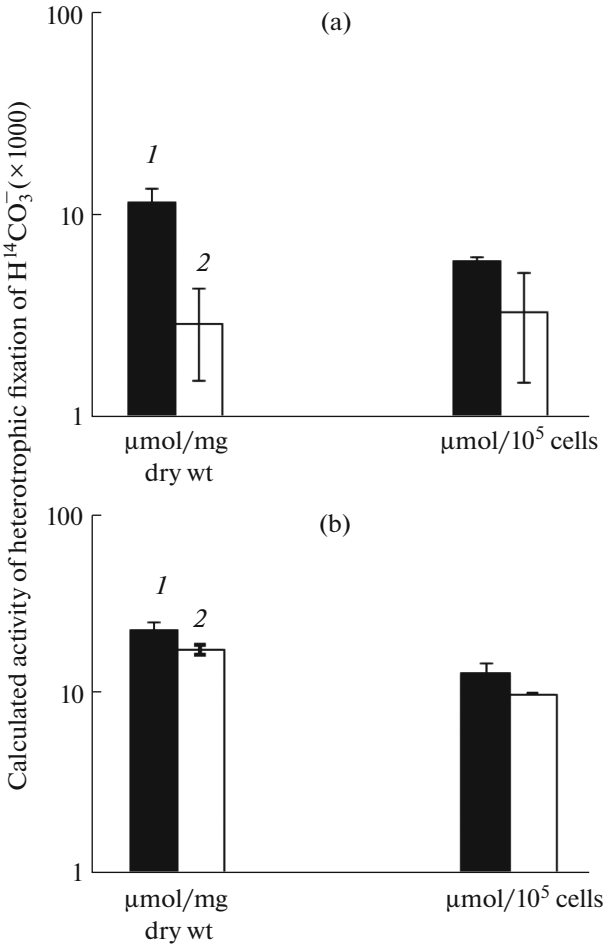

Fig. 3. Calculated characteristics of activity of heterotrophic $H^{14}CO_3^-$-fixation in the dark by the cells of *Chlamydomonas reinhardtii* grown on ammonium and nitrate TAP media relative to their physiological parameters in (a) 5-day-long and (b) 10-day-long cycles of culturing. (*1*) Cells grown on ammonium medium; (*2*) cells grown on nitrate medium. For the purpose of representation, the results were multiplied by 1000 and shown on a logarithmic scale.

Determination of the rate of dark fixation of $H^{14}CO_3^-$ by the cells of types 1 and 2 has shown that the presence of ammonium nitrogen in the nutrient medium intensified heterotrophic fixation of CO_2 (Fig. 2). Apparently, the greater values of the rate of heterotrophic fixation of $H^{14}CO_3^-$ in the cells grown in the presence of ammonium were observed right after the introduction of cell subculture to the respective nutrient medium, since elevated activity of this process in the cells of type 1 occurred both in the beginning and at the end of the growing cycle of alga.

Greater rate of dark fixation of $H^{14}CO_3^-$ in the cells grown on ammonium medium (as compared with ammonium-free medium) remained valid irrespective of the method of calculation: on the basis of 10^5 cells or per mg dry weight (Fig. 3).

DISCUSSION

Investigation into the effect of mineral nitrogen's form on alga chlamydomonas's growth of cells, strain *gr 21,* showed that total accumulation of cell biomass in types of treatment 1 and 2 at the end of the growing cycle was essentially the same (Table 2), whereas the shape of the curve describing the rise in the number of cells in suspension changed depending on the type of medium (Fig. 1). Size distribution of the cells showed that cell divisions were suppressed right after the addition of subculture to the fresh medium if the nutrient medium lacked an ammonium component (Table 1). This means that the reduced form of mineral nitrogen somehow promotes the onset of cell divisions. It is still unknown why there is a delay in cell divisions when an ammonium component is lacking. Scarcity of necessary information makes it impossible to interpret the obtained results. We assume that initiation of cell divisions and early stages of cell development require an ammonium factor participating in the generation of a

Table 2. Effect of the form of mineral nitrogen on the content of dry matter (mg/mL suspension) in the cells of *Chlamydomonas reinhardtii,* types 1 and 2, in the course of the growing cycle

Type of cells	Time of culturing, days			
	3	5	8	10
1	1.29 ± 0.35	2.76 ± 0.21	3.38 ± 0.28	3.00 ± 0.18
2	2.09 ± 0.30	2.69 ± 0.30	4.14 ± 0.53	2.90 ± 0.21

Type 1 are cells grown on TAP medium with ammonium; type 2 are cells grown on TAP medium with nitrate.

signal or acting as a signal to cell division. It is possible that a shortage of endogenous ammonium arises at the beginning of cell divisions as a result of a low nitrate-reducing activity associated with a low content of necessary enzymes, their low activity, or rapid conversion of arising free ammonium to nonsignal compounds.

As we found earlier, the reduced form of mineral nitrogen promoted the formation of more numerous protein-synthesizing structures in plant cells than its oxidized form did [3]. A similar result concerning the number of ribosomes in the cells of *C. reinhardtii* [8] and changes in the structure of the photosynthetic apparatus in the cells of soybean callus under the effect of an ammonium component [3] suggested that the ammonium form of mineral nitrogen contributes to protein orientation of metabolism.

Ribosomes are known to form a ribonucleoprotein complex composed of several tens of protein molecules and some molecules of rRNA [13]. Biogenesis of protein-synthesizing structures is a multistep process starting with expression of respective genes and occurring in three cellular compartments: cytoplasm, nucleoplasm, and nucleolus [14]. The majority of ribosomal proteins are synthesized on ribosomes in the cytoplasm, and the two other compartments are the place where the ribonucleic component of the ribosome is produced.

When the effect of the form of mineral nitrogen on the expression of some ribosomal genes was investigated, we found that expression of the genes encoding ribosomal protein rpS6 of the small subunit and 18S rRNA in the cells of *C. reinhardtii* grown on ammonium and ammonium-free media did not have any significant differences [7]. A similar result was obtained in the callus cells of *G. max* [8]. As was mentioned above, the structure of the ribosome comprises several tens of protein molecules and several molecules of RNA. As the formation of ribosomes requires the presence of structural components in equimolar quantities, one can assume that, in this stage of their biogenesis, ammonium did not influence the expression of other genes participating in the formation of the structure of these organelles either.

It is known that the uptake of a cation (exogenous ammonium) by a plant cell is accompanied by a release of H^+-ions out of it; as a result, cytoplasmic pH becomes more alkaline [1]. Discharge of H^+-ions from the cells associated with the uptake of ammonium may apparently cause the activation of PEP-carboxylase whose pH optimum is in the alkaline region [15]. Activation of this enzyme must intensify heterotrophic fixation of carbon dioxide [9] and production of organic acids whose subsequent transformations (in the presence of ammonium) may form another source of a number of amino acids [16]. Extra amino acids can ensure the production of additional protein. Such a phenomenon was observed in the cells of rose-bush, cv. Paul's Scarlet [17], and sycamore maple *(Acer pseudoplatanus*) [18].

However, heterotrophic assimilation of CO_2 may occur not only via PEP-carboxylase. At least several more enzymes are known, which can promote the incorporation of inorganic CO_2 into organic compounds under certain conditions: malic enzyme and others [19]. We do not know so far which enzyme participates in the assimilation of CO_2 in the cells of chlamydomonas of the examined strain.

Figure 4 shows a putative chart of assimilation of ammonium and nitrate arriving in the cell and participation of PEP-carboxylase in heterotrophic fixation of CO_2 as the most probable pathway of production of an additional source of amino acid precursors during the formation of ribosomes.

Our experiments with $H^{14}CO_3^-$ have shown that the rates of heterotrophic fixation of bicarbonate by the cells of *C. reinhardtii* considerably differed depending on the consumed form of mineral nitrogen and remained higher in the algal cells grown on the medium with ammonium nitrogen (Fig. 2).

When we estimate the effect of a factor on a process, it is important to choose a criterion for comparing the obtained results. Since the life cycle of chlamydomonas cells is much shorter than the cycle of cell suspension growing in enrichment culture, investigation of the effect of the form of mineral nitrogen always deals with the cells in different stages of development (Fig. 1). In young, just-divided cells and old cells, metabolic processes (or their rates) may considerably differ. For instance, the processes related to the formation of functional structures of the cell itself are more active in young cells, and the processes associated with accumulation of organic matter necessary for the coming division are more active in mature cells. Thus, on the same day of culturing the cells with different growth rates (Fig. 1), a volume unit of suspension may contain a different number of cells and a different quantity of dry matter. In this relation, elevated activity of dark fixation of ^{14}C-bicarbonate by ammonium was estimated relative to two parameters mentioned above: per cell (Fig. 1) and per dry weight unit (Table 2). Results shown in Fig. 3 suggest that a stimulatory effect of ammonium on the rate of heterotrophic fixation of ^{14}C-bicarbonate was valid relative to both parameters (per cell and per dry weight unit).

A lack or shortage of ammonium nitrogen in the cell must result in termination or suppression of synthesis of amino acid components. Amino acids and, subsequently, proteins may arise only after the reduction of nitrate nitrogen to ammonium form. Reduction of nitrate to ammonium requires the participation of reducing equivalents, such as NADH. Spending of the reductant on the production of assimilatory form of nitrogen could reduce photosynthetic assimilation of carbon and, therefore, suppress growth function of

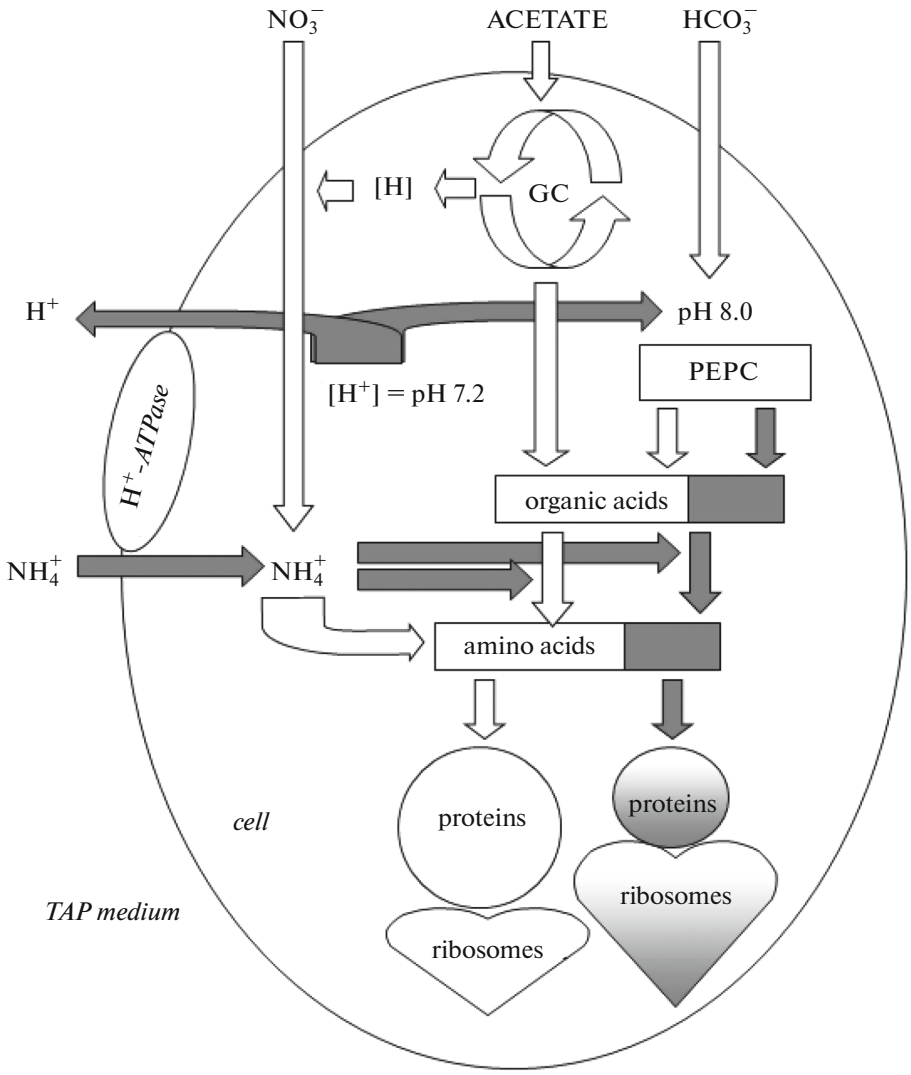

Fig. 4. Putative pattern of participation of exogenous ammonium in the production of protein upon formation of ribosomes in the cell of *Chlamydomonas reinhardtii*. Dark arrows are processes depending on exogenous ammonium.

the cells. However, in our experiments with low light intensity, photosynthesis apparently could not have a profound effect on metabolic activity of algal cells; therefore, only dark processes of carbon metabolism could considerably contribute to the synthesis of organic matter in the cell.

In the experiments with the cultured chlamydomonas cells, the main source of carbon was acetate. Metabolic transformations of this substrate are known as a variation of the Krebs cycle: glyoxylate cycle [20]. Organic acids produced in the course of this process are the fragments of C-skeleton out of which amino acids are synthesized (in the presence of ammonium, as a result of their amination) and protein molecules are subsequently produced.

If we assume that the main pathway of carbon in the course of amino acid components' formation runs through the Krebs cycle, in accordance with the feedback principle, this pathway may turn out to be limited in respect to reducing compounds (NADH) arising within the cycle, since the addition of acetate may cause an excess of organic acid molecules and a suppression of glyoxylate cycle operation in the absence of ammonium. As a result, the cell becomes deficient in reductant that should be partially spent on the reduction of nitrate nitrogen. Vice versa, the introduction of ammonium into the nutrient medium must lead to a conversion of arising organic acids to amino acids and elimination of organic acids from the glyoxylate cycle. Such a process would enable the cell to freely spend reductant on the reduction of arriving nitrate and supply amino acids to the production of the protein component of the ribosomal structure. Thus, intensification of the process of heterotrophic fixation of HCO_3^-

under the effect of exogenous ammonium (Fig. 2) is capable of providing the cell with additional amino acids necessary for the synthesis of protein component of ribosomes.

Diversion of the reductant on the process of nitrate reduction must inevitably lead to a suppression of the growth function of the cells. Lack of ammonium actually brought about a suppression of the increase in the number of cells at the beginning of the growing cycle (Fig. 1); however, by the end of the growth cycle, their number in the equal volumes of suspension became approximately the same. In the experiment with the accumulation of dry mass by the cells of chlamydomonas at the end of the growing cycle, we did not find considerable differences between the used forms of mineral nitrogen either (Table 2). At the same time, size distribution of the cells showed a much greater proportion of giant cells in suspension grown on nitrate medium (Table 1). The question arises: if the form of mineral nitrogen at the end of growing cycle did not affect the number of cells in suspension or dry mass of the cells, what is the reason for a sharp decrease in the number of ribosomes in the nitrate (type 2) cell as compared with ammonium (type 1) cells?

Comparative investigations conducted with the callus cells of soybean and the cells of chlamydomonas [8] showed that the chloroplasts of these cells accumulated numerous large starch grains on ammonium-free medium [3], which indicated an intensification of carbohydrate orientation of cellular metabolism. Under identical conditions, the chloroplasts of chlamydomonas cells did not accumulate much starch [7]. Therefore, one can assume either a low photosynthetic activity of the cells under such growing conditions or a rapid release of carbohydrates from the chloroplasts and their consumption in the processes not related to the synthesis of organic matter, for instance, in intensification of spatial mobility of chlamydomonas cells.

Under conditions when the main source of carbon is acetate and not CO_2 from the air, one cannot rule out that the final product of metabolic conversion of acetate in algal cells may not have a carbohydrate nature but arise and accumulate as lipid compounds. Lack of ammonium in the original nutrient medium does not imply its endogenous zero level since the mechanism of nitrate reduction operating in the cells permanently produces endogenous ammonium; therefore, nitrogen-containing compounds (including amino acids) may be synthesized. On the other hand, it is not known what amino acids and in what quantity arise in the cell and what proteins may be produced from these amino acids. The presence of a sufficient quantity of certain amino acids may directly influence the formation of primary structure of protein molecules of the ribosome; therefore, one can assume that heterotrophic fixation of CO_2 is the mechanism responsible for the necessary composition of precursors to required amino acids, i.e., of those organic acids whose production is insufficient when acetate is utilized. Such a metabolic variability is well-grounded and partly shown in the cells of chlamydomonas [21].

Thus, differences in the number of ribosomes in the cells of chlamydomonas grown on ammonium (type 1) and nitrate (type 2) media [7] may be related to changes in the quantity of protein molecules responsible for the structure of protein-synthesizing organelles under the effect of a specific form of mineral nitrogen. At present, one should not regard this assumption as an established fact since the quantitative ratio between protein components of the cells on media 1 and 2 and the changes in the content of amino and organic acids in the presence of oxidized and reduced forms of mineral nitrogen are not known. We hope that future investigations will elucidate the essence of changes occurring upon the use of oxidized and reduced forms of mineral nitrogen.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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