STIMULATORY AND INHIBITORY EFFECT OF LIGHT ON THE NITRATE ASSIMILATION BY *CHLORELLA*

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INTRODUCTION

The fact that the assimilation of nitrate by the green alga *Chlorella* is markedly accelerated by the effect of light was first demonstrated in 1920 by Warburg and Negelein¹⁾, who explained the phenomenon as being due to the photochemical formation of some hydrogen donor or reducing power required for the reduction of nitrate to ammonia. In view of the fact that the reduction of nitrate to ammonia involves several intermediate steps, a question was raised as to what step or steps are light-dependent or accelerated most strongly by light. Evans and Nason²⁾ have shown that light stimulates the reduction of nitrate to nitrite in a system consisting of nitrate reductase, TPN, and a preparation of grana isolated from soybean leaves. In the absence of any of these three components, the rate of the reduction dropped markedly, especially in the light. Their experimental results, however, do not exclude the possibility that light stimulates also the reduction of nitrite or other substances appearing as the intermediates in the nitrate reduction. According to the experiments by Kessler, nitrite was accumulated in the dark in the culture of *Ankistrodesmus* with nitrate as the nitrogen source, and the nitrite thus accumulated disappeared on illumination³⁾. Based on his further study⁴⁾ on the effect of temperature on the reduction of nitrate and nitrite by the green alga, he concluded that light accelerates the reduction of nitrite more directly than the reduction of nitrate. Whichever reduction step in the nitrate assimilation be stimulated by light, the overall assimilation of nitrate by algal cells will be photo-activated in conformity with the original observation made by Warburg and Negelein.

A completely different result has been reported by Mayer⁵⁾ who, using *Chlorella vulgaris*, investigated the effect of light upon the formation of nitrite and ammonia from nitrate. In his experiment, more nitrite and ammonia were accumulated in the light than in the dark, a finding which appears irreconcilable with the results reported by Kessler.

The present study was undertaken in order to clarify the cause of the contradictory findings reported by these workers. It was revealed that the

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effect of light upon the precesses of nitrate and nitrite assimilation by *Chlorella* varied considerably according to whether the cells had been previously placed under dark-adapted or light-adapted condition. Experiments performed under different conditions have led the writer to the concept that the modification of the light effect upon the nitrogen metabolism may be accounted for by the interrelation of photosynthetic process and the Krebs cycle occurring in the light and dark, respectively, in algal cells.

METHOD

Culturing of algae. The organism used was *Chlorella ellipsoidea* which was grown photo-autotrophically at 25°C. using the culture medium having the following composition, per liter:⁶⁾ KNO₃, 5.0 g., MgSO₄·7H₂O, 2.5 g., KH₂PO₄, 1.25 g., FeSO₄·7H₂O, 0.003 g., and 1 ml. of Arnon's A₅ solution. Cultures were constantly aerated with air containing 5% CO₂, and the illumination was furnished by incandescent lamps which yielded 10,000 lux at the surface of the culture flasks. After several days' culturing under continuous illumination, light was turned off, and under continued bubbling with CO₂-enriched air, the culture was kept in the dark for one or two days. These cells were harvested by centrifugation and resuspended in a new medium for each experiment. The cells were adapted to the darkness during the period of dark incubation. As will be shown later, the behaviour of the dark-adapted cells in nitrate assimilation was found to be completely different from that of the light-adapted cells.

Determination of nitrate. The concentration of nitrate in the medium was measured photometrically by modified Kolthoff's diphenylamine method⁷⁾. In this method, nitrate and nitrite oxidize diphenylamine to an intensely blue-colored quinoidal compound. By measuring the absorbance at 570 mμ of the colored solution, the sum of the concentrations of nitrate and nitrite was determined. The concentration of nitrate was calculated by subtracting the concentration of nitrite which was determined by the method described below.

Determination of nitrite and hydroxylamine. The concentration of nitrite was determined by using the coupling reaction between sulfanilic acid and α-naphthylamine. The amount of the diazo dye formed was measured photometrically by the absorption at 540 mμ. Hydroxylamine was determined, after being oxidized with iodine, by the same color reaction. The excess of iodine remained had been removed by sodium thiosulfate.

Determination of amino acids. The change of amino acid content in algal cells was followed by measuring the color developed by modified ninhydrin reaction⁸⁾. For the reaction, pyridine, ascorbic acid and ninhydrin solution were added to the cell suspension. The mixture was put into boiling water for 30 minutes and, after cooling, the suspension was shaken violently. The violet color developed in this process was estimated by the

absorption at 570 $m\mu$, using the opal glass method which has been developed by Shibata, Benson and Calvin⁹⁾ for the spectroscopic measurement of translucent suspensions. The use of the opal glass method enabled us to determine the amount of amino acids contained in cells, without performing the process of extraction. The advantage of the modified color reaction using pyridine and ascorbic acid is that we can obtain almost similar violet colors with nearly equal molar extinction coefficients for different kinds of amino acids, so that the value of absorbance measured is roughly proportional to the sum of the molar contents of the ninhydrin positive compounds.

For a few samples, amino acids in the cells were separated two-dimensionally by paper chromatographic technique. In the separation, the cells were first extracted with 80% hot ethanol and the concentrated extract was placed on a paper (Toyo Filter paper No. 51) and was developed with the following solvent systems; n-butanol-glacial acetic acid-water (4:1:1) for one dimension, and phenol-water (3:1) with the addition of a small amount (0.15%) of ammonia for the other dimension. The chromatogram thus developed was sprayed with ninhydrin solution, and the relative amount of the colored compound formed in the spots on the chromatogram was determined by the following procedure. The whole colored area of each spot was divided into small triangles so as to obtain uniform color in each triangle, and the absorbance at 570 $m\mu$ of each triangle was measured by the opal glass method^{9,10)}. The relative amount of the amino acid in the spot was calculated by summing up the products of the absorbance value and the area of the triangles. Thus, the rather great error due to the non-uniformity of the color on the spot could be eliminated.

RESULTS

Nitrate assimilation by dark-adapted cells. The cells adapted to the darkness were subjected to a series of experiments using nitrate, nitrite or hydroxylamine as the nitrogen source. As shown in Fig. 1, the dark-adapted cells assimilated more nitrate in the light than in the dark. During this process, no accumulation of nitrite or hydroxylamine was observed, suggesting that all the nitrate absorbed has been incorporated into amino acids.

The same acceleration by light

Fig. 1. Nitrate assimilation by dark-adapted cells at 25°C.

○: light (10,000 lux) ●: dark

Fig. 2. Nitrite assimilation by dark-adapted cells at 25°C.

○: light (10,000 lux) ●: dark

accelerated by light. Quite different were the results obtained with the light-adapted cells.

Nitrate assimilation by light-adapted cells. The dark-adapted cells were suspended in the complete medium containing nitrate as the nitrogen source, and under illumination (10,000 lux) the change of the concentration of nitrite in the culture was followed. The curves in Fig. 4 show the increase of the logarithm of packed cell volume and the change of nitrite concentration occurring during the incubation. The solid curve in the figure shows the change of nitrite in the light and the dashed curve shows the change in the dark. During the first 27 hours in the light, there occurred no appreciable accumulation of nitrite. After about 30

was observed when nitrite or hydroxylamine was given to the cell suspension. Figs. 2 and 3 show the changes of nitrite and hydroxylamine concentrations in the light and in the dark. In the experiment with nitrite (Fig. 2), there occurred no accumulation of nitrate, indicating that the back reaction from nitrite to nitrate did not take place. The experiment with hydroxylamine was run using a single sample which was subjected to alternate illumination and darkening. In this case, the accelerating effect of light was most distinct, practically no uptake occurring in the dark.

These results show clearly that in the dark-adapted cells the uptake of nitrate, nitrite and hydroxylamine was markedly

Fig. 3. Hydroxylamine assimilation by dark-adapted cells at 25°C.

○: light (10,000 lux) ●: dark

hours of incubation, by the time of which most of the cells entered the stage of the light-adapted cells, nitrite began to accumulate at a considerable rate and the increase continued at a steady rate.

At the 52nd hour of illumination, a part of the sample was placed in the dark. While the accumulation of nitrite continued in the sample kept in the light, the nitrite content dropped in the sample transferred to the darkness. As shown by the dashed curve in the figure, the decrease of nitrite stopped during 15 hours in the dark, after which the accumulation started slowly again. After 48 hours in the dark (100 hours from the beginning of the experiment), during which time the light-adapted cells transformed themselves into the dark-adapted cells, the sample was brought again in the light. As may be seen from the figure, there occurred a sudden drop of nitrite concentration, indicating that the light exerted opposite effects upon the accumulation of nitrite by the dark- and the light-adapted cells. The results presented in Fig. 4 are summarized in Table 1, which shows that the contradictory data reported by different workers^{3,5)} may probably be due to the difference in the prehistory of algal cells used in their experiments.

Fig. 4. Effect of light and dark on the nitrite level in *Chlorella* suspension at 17°C.

$\log V_p$: logarithm of packed cell volume.

L: light (10,000 lux). D: dark.

Table 1. Effects of light and dark on the nitrite level in the dark- and light-adapted cells of *Chlorella*.

	Nitrite level	
	in dark-adapted cells	in light-adapted cells
effect of light	decrease	increase
effect of dark	increase	decrease

An experiment with similar arrangement was performed and the amount of nitrite and free amino acids were followed. The results obtained are shown in Fig. 5. The increase of nitrite in the light and its decrease in the dark proceeded in a manner similar to those observed in the previous experiment. The amino acid content increased almost from the beginning

Fig. 5. Relation between the concentration of nitrite and free amino acids in *Chlorella* suspension. (17°C. 10,000 lux).

of the experiment in the light, whereas nitrite did not accumulate recognizably in the meantime. It may be noticed that, when nitrite began to accumulate at a considerable rate in the light, the rate of increase of amino acids dropped appreciably. On the other hand, while nitrite decreased in the dark, there occurred a considerable increase of amino acids. The antiparallelism observed between nitrite and amino acid contents may be taken as an evidence that nitrite is an intermediate in the regular path of the formation of amino acids from nitrate.

Effect of darkening on the amino acid content of light-adapted cells.

A suspension of the light-adapted cells containing nitrite was divided into two parts. One part was placed in the dark and the other was kept in the light. After 30 minutes, free amino acids in these cells were extracted with alcohol and separated two-dimensionally by the paper chromatographic technique. For both samples incubated in the light and in the dark, aspartic acid, glutamic acid, serine, glycine, alanine, threonine, lysine, arginine and cysteine were identified by ninhydrin spray. The relative amounts of the amino acids of the major spots were measured by the method described before and are listed in Table 2.

Table 2. Effect of darkening on the amino acid content of light-adapted cells.

Amino acid	Relative amino acid content		
	A (30 min., light)	B (30 min., dark)	B/A
Aspartic acid	24	34	1.42
Glutamic acid	644	1400	2.17
Serine	320	460	1.44
Glycine	88	112	1.27
Alanine	396	347	0.88
Sum	1472	2353	1.60

As may be seen from the table, all the amino acids listed, except only alanine, increased more or less markedly during the 30 minutes of dark

incubation. The increase in the dark of total ninhydrin-positive substances amounted to as much as 60%. The most remarkable increase was found in the content of glutamic acid, suggesting that α -ketoglutaric acid had been formed in an appreciable amount in the dark. The same may be true of oxaloacetic acid (precursor of aspartic acid)¹¹⁾, and glyoxylic acid (precursor of glycine,¹²⁾ which in turn may give rise to serine).

To investigate whether the high level of nitrite in the light-adapted cells was due to the lack of the keto acids functioning as precursors in the amino acids synthesis, the following experiment was carried out. Glucose, pyruvate, fumarate, α -ketoglutarate and oxaloacetate were added to the suspension of the light-adapted cells and the change of nitrite level occurring under continued illumination was followed. The results obtained are presented in Fig. 6. As may be seen from the figure, the addition of glucose and pyruvate had practically no effect on the accumulation of nitrite, while more or less remarkable decrease of nitrite took place in the presence of fumarate, α -ketoglutarate or oxaloacetate. The most striking effect in lowering the nitrite level was observed, in some experiments, with α -ketoglutarate and, in some other experiments, with oxaloacetic acid. Similar experiments carried out with dark-adapted cells showed no dropping of the accumulated nitrite, suggesting that the factor controlling the level of nitrite was not the keto acids in the case of dark-adapted cells.

Fig. 6. Effect of some metabolic substances which influence the concentration of nitrite in light-adapted cells. (22°C. 10,000 lux, the final concentration of metabolic substances was $4 \times 10^{-3} M$.)

DISCUSSION

The experimental results described above may be explained by assuming two factors controlling the processes of nitrogen and carbon assimilation in green algae. One factor is a photogenic reducing power or a group of reducing agents, which bring about the reduction of nitrate, nitrite and hydroxylamine. This factor, which may be designated by R in the following, may be the same as, or closely related to, that functioning in normal photosynthesis (reduction of phosphoglyceric acid to triosephosphate) as well as in the Hill reaction. The other factor is keto acids which react with

hydroxylamine or ammonia to form amino acids. Factor R is also required in this latter reaction¹³⁾. The course of events occurring in the process of amino acid synthesis from nitrate may thus be visualized as follows:

The generator of keto acids is the Krebs cycle which in algal cells operates in a specific way in connection with the photosynthetic cycle of Calvin and Benson. Using radioactive carbon as a tracer, Bassham, Shibata, Steenberg, Bourdon and Calvin¹⁴⁾ have shown that in the light carbon dioxide is assimilated mainly into sugars and the intermediates in photosynthetic cycle, and only a little C¹⁴ was incorporated into the members of the Krebs cycle. When light was turned off, there occurred a rapid and great increase of radioactivities of the members of the Krebs cycle, indicating that the transformation of photosynthetic products and intermediates into the keto acids of the Krebs cycle had been hampered by the effect of light.

In view of these findings it may be reasonable to assume that the operation of the Krebs cycle, and consequently the formation of keto acids, is more vigorous in dark-adapted cells than in light-adapted cells. Conversely, the light-adapted cells must contain a large amount of R than do dark-adapted cells. It is, therefore, plausible that the limiting factor in the process of nitrate assimilation is keto acids in light-adapted cells and R in dark-adapted cells. When dark-adapted cells are illuminated, the level of R will be enhanced and the assimilation of nitrate, nitrite and hydroxylamine will be accelerated as we have seen in the experiments presented in Figs. 1, 2 and 3. Kessler⁴⁾ found in his experiment that light accelerated nitrite reduction more strongly than the reduction of nitrate to nitrite, a finding which seems to account for our observation that the nitrite accumulated in dark-adapted cells decreased when the cells were illuminated (Fig. 4). When the dark-adapted cells are illuminated for a certain period of time, the reserve of keto acids will decrease, and eventually the amount of the keto acids will become the limiting factor of the process of nitrate assimilation. The fact that the light-adapted cells accumulated a large amount of nitrite (Fig. 4) may be due to the lack of keto acids. The lack of keto acids in the light-adapted cells was experimentally proved by the fact that the addition of some members of the Krebs cycle (fumaric,¹⁴⁾ α -ketoglutaric¹⁵⁾ and oxaloacetic acids) decreased the amount of accumulated nitrite (Fig. 6). The sudden drop in nitrite content in the dark after prolonged illumination may have been caused by the formation of keto acids in the dark (Fig. 4). When the cells are kept longer in the dark, the assimilation rate will depend again on the content of R left, so that nitrite will increase again as was observed in

the experiment presented in Fig. 4. On the other hand, when the cells are illuminated after prolonged dark-adaptation, the content of nitrite suddenly drops, because in dark-adapted cells the amount of R is the limiting factor of nitrate assimilation.

The data presented in Table 2 show that, among the five major amino acids found in algal cells, alanine is an exception in decreasing in amount when the algal cells were incubated in the dark. This fact may be attributed to the situation that pyruvate,¹⁶⁾ the precursor of this amino acid, is, unlike the precursors of other amino acids, a direct product formed in the photosynthetic cycle.

It should be added that by a series of other experiments not reproduced here it was demonstrated that the rate of uptake of nitrite and the formation of amino acids by algal cells were markedly decreased both on long continued illumination or long continued darkness. To induce an enhanced formation of amino acids in these cells, a certain period of darkness (after continued illumination) or light (after continued darkness) was required. In other words, a periodical alternate change of light and darkness is a necessary condition for the efficient assimilation of nitrate or nitrite. According to the data presented before, the time required for the transition between the dark-adapted and the light-adapted cells is of the order of a day and a night. This may be of interest from the view point of nitrogen metabolism occurring in algal cells living in nature.

SUMMARY

1. Using *Chlorella ellipsoidea* as material it was demonstrated that the process of assimilation of nitrate or nitrite was affected by light in quite different ways according as the cells had been placed under dark-adapted or light-adapted condition. The cells cultured with nitrate as the nitrogen source accumulated a large amount of nitrite on long continued illumination, and the nitrite thus accumulated rapidly disappeared when light was turned off (inhibitory effect of light). When the cells were kept in the dark for a sufficient length of time, re-accumulation of nitrite took place. However, this nitrite decreased markedly when the cells (dark-adapted cell) were illuminated (stimulatory effect of light). The rate of formation of amino acids decreased when nitrite was accumulated, and increased when the level of nitrite dropped, indicating that the change of nitrite level was closely related to the amino acid and follow-up protein syntheses.

2. The addition of keto acids to the light-adapted cells decreased the level of nitrite, while practically no effect of the addition was observed with the dark-adapted cells. This observation together with the fact that the amino acids content of light-adapted cells increased markedly when the cells were kept in the dark for a short period of time (30 minutes) led us to the inference that in the light-adapted cells keto acids form the

limiting factor for the nitrate assimilation and that the keto acids are accumulated in the dark conceivably owing to the enhanced operation of the Krebs cycle in the darkness.

3. Various experimental results were explained by assuming two factors controlling the process of amino acids synthesis from nitrate. One factor is a photogenic reducing agent(s), R, which is required for the reduction of nitrate and the intermediates between nitrate and amino acids, and the other is keto acids, which react with hydroxylamine or ammonia to form amino acids. The light-adapted cells contain a large amount of R but only a small amount of keto acids, while dark-adapted cells contain a small amount of R but a large amount of keto acids. Along the line of this reasoning, it was postulated that the assimilation of nitrate or nitrite by algal cells would proceed most efficiently when the cells are subject to an intermittent illumination of appropriate intervals.

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