NITRATE REDUCTION BY CHLORELLA

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During the course of metabolic studies with Chlorella it was found that oxygen was produced when cells were illuminated in modified Knop's solution containing glucose, without atmospheric carbon dioxide. This observation has a bearing on the problem of nitrate reduction by plants and forms the basis for this report.

Chlorella cells were grown on nutrient agar slants under continuous illumination of 140 fc at 23.5 ± 1.5° C. The medium contained: KNO_3, 1.21 gm.; $MgSO_4$, 1.20 gm.; KH_2PO_4, 1.22 gm.; A4 microelement solution (1), 1.0 ml.; iron solution, 1.0 ml.; tryptone, 4.5 gm.; yeast extract, 0.36 gm.; glucose, 10 gm.; agar, 10 gm.; distilled water, 998 ml. The iron solution supplied the medium with 10^{-5} M ferrous sulphate and 10^{-5} M potassium citrate.

Cells from cultures 11 days old were harvested, washed with distilled water, and suspended in the medium in which their ability to evolve oxygen was to be measured. The solution, subsequently referred to as a modified Knop's solution, contained the major salts, microelements, and iron in the concentrations listed above. This solution plus 1% glucose formed the basic medium for the following investigation. All solutions were adjusted to pH 5 with sodium hydroxide and hydrochloric acid.

Gas evolution was measured in ordinary conical Warburg vessels which were illuminated from below by seven 100-watt incandescent lamps. The light intensity on the bottoms of the vessels was about 1,300 fc, with less than 1% variation across the length of the tank. Each vessel contained 1.8 ml. of cell suspension in the main chamber and 0.2 ml. of 20% KOH in a side arm. The illuminated area of each suspension was about 7.1 $cm.^2$

Chlorella cells were suspended in 1% glucose-modified Knop's solution in the absence of atmospheric carbon dioxide and illuminated. Although initially little or no positive pressure change developed, the rate of oxygen production gradually increased, until after two or three hours it was fairly rapid. The data plotted in figure 1 A were obtained six hours after the beginning of illumination. In most of the following experiments the data plotted were obtained after two or three hours illumination, a long enough time to avoid the lag phase consistently found.

Since the cells were exposed to light but were not given carbon dioxide, photosynthesis should no more than compensate for respiration. Under these conditions, some other process must have been ultimately responsible

for the production of oxygen. The purpose of the following experiments was to establish the factors and process responsible for this extra oxygen.

The necessity of glucose for the oxygen production is indicated by figure 1 A. Cells in modified Knop's solution liberated only 6.2% as much oxygen as cells in glucose-modified Knop's solution. Some component or components of modified Knop's solution are also required (fig. 1 B). Although oxygen was produced when cells were illuminated in a glucose solution without salts it only amounted to 28.3% of that produced by cells given both glucose and salts.

To determine the components in the mineral nutrient solution responsible for the production of the extra oxygen, tests were made with the individual major salts in the presence of glucose (fig. 2 A). Cells in glucose-KNO_3

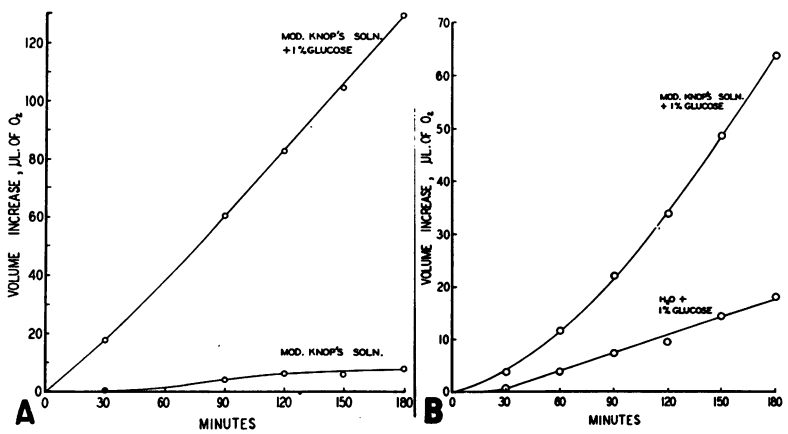

FIG. 1. A. The production of extra oxygen by Chlorella suspended in modified Knop's solution with and without glucose. Conditions: ca. 1,300 fc; KOH in side arm; 15.2 mm.^{3} cells/vessel; plot of data after six hours of illumination. B. The production of extra oxygen by Chlorella suspended in 1% glucose solution with and without nutrients of modified Knop's solution. Conditions: ca. 1,300 fc; KOH in side arm; 18.8 mm.^{3} cells/vessel; plot of data after two hours illumination.

solution produced 51.4% of the gas volume increase caused by cells in glucose-modified Knop's solution. Singly, KH_2PO_4 and $MgSO_4$ in the presence of glucose had no greater effect on oxygen production than glucose alone. From this it is concluded that KH_2PO_4 and $MgSO_4$ are not directly involved in the production of the extra oxygen, whereas KNO_3 is. Microelements and iron were tested singly and together with KNO_3 and glucose to determine whether they increase the production of extra oxygen (fig. 2 B). Combined they increased oxygen production only 12.5%, whereas an increase of 48.2% was necessary to equal the amount of extra oxygen produced by cells in glucose-modified Knop's solution. Next, combinations of the major salts with a mixture containing glucose, A4 microelements, and iron were tested. Of the major salts, KNO_3 in combination with KH_2PO_4 was necessary for the complete production of extra oxygen (fig. 3 A). The combination

$KNO_3 + MgSO_4$ was no more effective than KNO_3, while the combination $MgSO_4 + KH_2PO_4$ was no more effective than glucose would be alone.

It was demonstrated that live Chlorella cells are necessary for the production of extra oxygen. No pressure change was observed when cells killed by boiling were suspended in glucose-modified Knop's solution. Also, no pressure change occurred in vessels containing glucose-modified Knop's solution without cells, during experiments lasting 5 to 10 hours. The possibility that

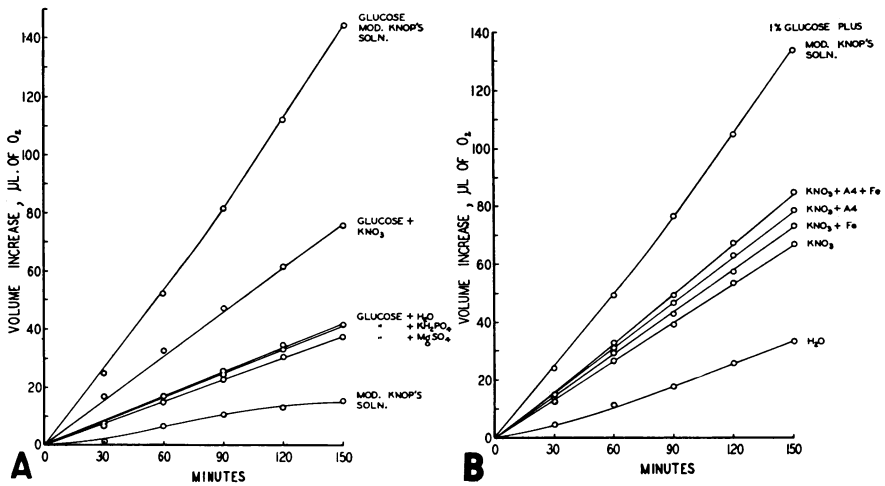

FIG. 2. A. Influence of the major salts in modified Knop's solution on the production of extra oxygen by Chlorella. Conditions: ca. 1,300 fc; KOH in side arm; plot of data after three hours of illumination. B. Influence of A4 microelements and iron on the production of extra oxygen by Chlorella supplied with glucose and KNO_3. Conditions: ca. 1,300 fc; KOH in side arm; 24 $mm.^3$ cells/vessel; plot of data after three hours of illumination.

the positive pressure changes were caused by contaminants giving off respiratory carbon dioxide which was caught by Chlorella and used for photosynthesis is thereby eliminated.

From the experiments reported it is apparent that three major constituents, glucose, nitrate, and phosphate, are necessary for the complete production of extra oxygen; and that the process ultimately responsible is nitrate reduction, for which glucose and phosphate ion are required.

If it is assumed that the process of nitrate reduction proceeds according to equation 1,

$$(1) \qquad KNO_3 + 2(CH_2O) \longrightarrow NH_3 + KOH + 2CO_2$$

$$(2) \qquad 2CO_2 + 2H_2O \xrightarrow{light} 2(CH_2O) + 2O_2$$

then for each mole of nitrate reduced, two moles of carbon dioxide would be produced and subsequently by photosynthesis (equation 2) two moles of oxygen would be liberated. To determine whether stoichiometric yields of oxygen are obtained according to these equations, cells were suspended

in media containing three concentrations of KNO_3. All of the media contained glucose, KH_2PO_4, microelements, and iron. The quantity of extra oxygen produced for a given amount of nitrate reduced was calculated as the difference between the amounts produced by cells with and without nitrate. Differences were not taken until after the slopes of the oxygen-liberation curves for the cells given nitrate became equal to the slope of the curve for the cells not given nitrate, which indicated that all of the nitrate had been reduced (fig. 3 B). The amount of extra oxygen produced for each quantity of nitrate reduced is given in table I. On the average, 109% of the theoretical yields were obtained which is added evidence that the process ultimately responsible for the production of extra oxygen is nitrate reduc-

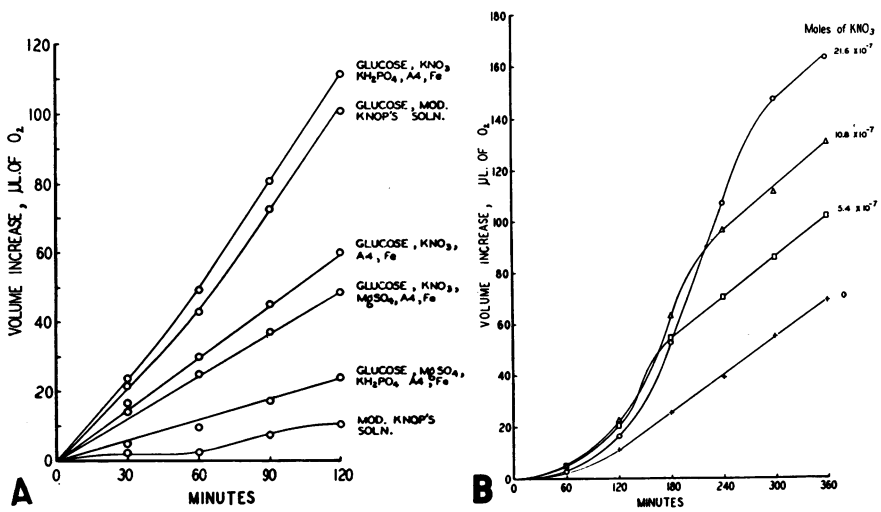

FIG. 3. A. Influence of combinations of the major salts in modified Knop's solution on the production of extra oxygen by Chlorella supplied with glucose, A4 microelements, and iron. Conditions: ca. 1,300 fc; KOH in side arm; 20 mm.^{3} cells/vessel; plot of data after three hours of illumination. B. Extra oxygen response of Chlorella to three concentrations of KNO_3. All of the media contained glucose, KH_2PO_4, A4 microelements, and iron. Conditions: ca. 1,300 fc; KOH in side arm; 25 mm.^{3} cells/vessel; plot of data from the beginning of illumination.

tion and makes the reactions of equations 1 and 2 a possible mechanism by which two moles of oxygen are produced per mole of nitrate reduced.

In light in the absence of atmospheric carbon dioxide, glucose and phosphate ion are required for the maximum rate of production of extra oxygen and concomitant nitrate reduction by Chlorella. The fact that both glucose and phosphate ion are necessary, strongly suggests that the process of nitrate reduction is coupled with the oxidation of glucose. There is little doubt that in darkness the hydrogen necessary for the reduction of nitrate is supplied by carbohydrate, but in light the problem is more complex because it is known that the rate of nitrate reduction in light is more rapid than it is in darkness. The problem of the mechanism of the light action is then presented.

TABLE I

YIELD OF EXTRA OXYGEN FOR NITRATE REDUCTION.

Moles of KNO_3 ($\times 10^{-7}$)	Extra oxygen				Per cent. theoretical yield
	With KNO_3 (1)	Without KNO_3 (2)	Yield (1 – 2)	Theoretical yield	
	$\mu l.$	$\mu l.$	$\mu l.$	$\mu l.$	
21.6	142.2	50.1	92.1	96.8	95.1
10.8	87.5	32.2	55.3	48.4	114.3
5.4	48.8	20.1	28.7	24.2	118.6
					Average 109.3

WARBURG and NEGELEIN (**8**) came to the conclusion that light accelerates the reduction by altering the absorbing mechanism so that more nitrate can penetrate the cell in a given time. The possibility nevertheless exists that light is directly involved by producing the reducing power via the photochemical reaction of photosynthesis (**7**). This photochemical reduction of nitrate can be written in the form of equation 3.

(3) $$HNO_3 + H_2O \xrightarrow{\text{light}} NH_3 + 2O_2$$

On the basis of the results reported in this paper it is not possible to assign to this reaction a substantial role in nitrate reduction, since cells in modified Knop's solution, without glucose, produced only a very slight amount of extra oxygen.

It might be argued that there would be no continuous reduction of nitrate without removal of the reduction products by assimilatory action of the organism. But the experiments of WARBURG and NEGELEIN (**8**) demonstrate that nitrate reduction by Chlorella continues even after the ammonia formed is no longer assimilated and accumulates in the medium. It has also been shown that even when carbohydrates are deficient in etiolated pea seedlings (**6**), Azotobacter (**4**), and *Aspergillus niger* and *Mucor racemosus* (**5**), these organisms continue to reduce nitrate. Recently KESSLER (**3**) was able to obtain the accumulation of nitrite in culture solutions of the green alga Ankistrodesmus by inhibiting the transformation of nitrite to ammonia by means of a low pH. These examples afford ample evidence that nitrate reduction continues even though the nitrite and ammonia formed are not assimilated.

It seems reasonable to conclude that if the photochemical reduction of nitrate, according to equation 3, plays a major role, then greater amounts of oxygen should have been produced by cells in modified Knop's solution without glucose. It is possible, of course, that the small amount of extra oxygen produced under such conditions was due to the photochemical reduction of nitrate. If this is so, then it is negligible compared with the reduction process requiring glucose. This small amount of extra oxygen, produced by cells in modified Knop's solution without glucose, may also have resulted from nitrate reduction for which carbohydrate reserves were used.

The ability of Chlorella when deprived of atmospheric carbon dioxide

and nutrient salts, but supplied with glucose, to liberate more oxygen than the amount necessary to compensate for respiration (figs. 1 B, 2 A, B and 3 B) is of particular interest. The rate of production of extra oxygen resulting from this reaction is approximately one sixth the rate of oxygen uptake during respiration with glucose as substrate. Presumably there is a mechanism, other than nitrate reduction, for the utilization of the reducing power resulting from photochemical reactions which does not rely upon the uptake of carbon dioxide. A possible mechanism is the reduction of intermediate oxidation products. This possibility is strengthened by evidence available on a photosynthetic Chlorella mutant unable to use atmospheric carbon dioxide for photosynthesis yet capable of producing oxygen in light (**2**). It is believed to accomplish this by reducing partially oxidized products of respiration.

The method of studying nitrate reduction by measuring the production of extra oxygen in light under conditions excluding carbon dioxide from the atmosphere is simple, relatively direct, and yields quantitative results. The long lag phase which was encountered is a disadvantage, but is not serious. It is possible that by working out appropriate methods this disadvantage can be overcome.

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