

NITRATE REDUCTION AND ASSIMILATION IN CHLORELLA*

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Nitrate is a common starting point for nitrogen assimilation in many microorganisms and in higher plants. The extensive literature on the reduction and assimilation of nitrate has recently been reviewed by Burström (1945). Unfortunately no comprehensive generalization of the mechanism of nitrate reduction is yet possible. The present study of nitrate reduction and assimilation in *Chlorella* has bearing both on the general features of the process and the rôle of nitrogen in algal metabolism.

Nitrate reduction in *Chlorella* has been studied by Warburg and Negelein (1920) under conditions specifically chosen to simplify certain features of the process. The cells were immersed in a "nitrate mixture" containing 0.1 M KNO_3 and 0.01 M HNO_3 and studied both at high light intensity and in darkness. In the dark the rate of oxygen uptake increased about 40 per cent when the cells were transferred from Knop's solution to the nitrate mixture; but at the same time the rate of carbon dioxide output increased still more to give a CO_2/O_2 quotient of about 1.5 and measurable amounts of ammonia were excreted. The carbon dioxide production in excess of the oxygen uptake was labelled "extra- CO_2 ." After a period of several hours the molar ratio of ammonia to extra- CO_2 approached a value of 0.5, compatible with the equation



Initially, however, and particularly in nitrogen-deficient cells, the $\text{NH}_3/\text{extra-CO}_2$ ratio was much lower than 0.5 and it was inferred that the excretion of ammonia was suppressed by attendant assimilation to cellular materials. Ammonia was the only nitrogenous excretory product although under anaerobic conditions nitrite was produced and rapidly proved toxic.

Warburg and Negelein also exposed *Chlorella* to photosynthesis-saturating illumination. Cells in the nitrate mixture then showed an excretion of oxygen and ammonia even though no carbon dioxide was provided. In light the extra- O_2 and ammonia were excreted two to three times as rapidly as the extra- CO_2 and ammonia produced in the dark. Relations of nitrate reduction to respiration and photosynthesis were studied by comparing the effects of cyanide and urethane on the three processes. The acceleration of nitrate reduction by light was explained in terms of an increased permeability to HNO_3 allowing acceleration of equation (1), the extra- CO_2 being converted photosynthetically to extra- O_2 . Alternative explanations have been suggested by Rabinowitch (1945).

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The present paper extends the study of nitrate reduction to those conditions which Warburg and Negelein chose to avoid; *i.e.*, conditions of normal growth in which the product of nitrate reduction is consumed in subsequent nitrogen assimilation. The organic materials of *Chlorella* contain approximately 10 per cent nitrogen and 50 per cent carbon. In an organism with such a high nitrogen requirement the reduction of nitrate should have observable effects on the CO_2/O_2 gas exchange ratio even during normal metabolism. Upon this expectation the present work is based.

EXPERIMENTAL

Chlorella pyrenoidosa (Emerson's strain) was grown in a continuous culture apparatus which provides uniform experimental material day after day (Myers and Clark, 1944). Knop's solution containing KNO_3 , MgSO_4 , and KH_2PO_4 with added iron and microelements was used as the culture medium as previously described (Myers, 1946). The cultures were aerated with 4 per cent carbon dioxide, maintained at a temperature of 25°C ., and illuminated at an intensity of 90 foot-candles by tungsten lumiline lamps. The population density was maintained at about 2.5 c.mm. cells/ml. At such a high population density there is considerable mutual shading of the cells. The observed growth rate is less than the maximum possible rate and corresponds to that observed at an intensity of 40 f.-c. in thin suspensions (*cf.* Myers, 1946).

Metabolic gas exchange was measured by the Warburg technique at 25°C . in Knop's solution at pH 4.5. Variation of the nitrogen source was accomplished by replacing the 0.0124 N potassium nitrate with equinormal concentrations of ammonium sulfate or potassium sulfate or by adding equinormal ammonium sulfate. For the light experiments an intensity of about 40 f.-c. was selected in order to approximate growth conditions. Illumination was provided by tungsten lamps and copper screen filters, by tungsten lamps operated at reduced voltage, or by a grid of neon discharge tubes. Voltage supply to the light source was held constant to ± 1 per cent by a voltage stabilizer.

The measurement of the CO_2/O_2 quotient at low light intensities involves considerable difficulty. One problem lies in obtaining perfectly uniform illumination of the duplicate vessels required for the indirect method. No practical means could be found to obtain this uniformity directly. The following procedure was therefore devised: at a time precisely in the middle of each experiment the positions of the two flasks and manometers of a given pair were exchanged. While this procedure tends to average out any differences in intensities between the two positions, its final effectiveness depends upon the constancy of the light source. The output of the neon grid was remarkably constant. With tungsten illumination the light intensity in any one position did show a short period variation of about ± 1 per cent which is characteristic of the voltage stabilizer; however, these variations are averaged out by allowing a 30 minute period before and after change in the flask position.

A second problem at low light intensities arises from the requirement of rather dense suspensions in order to obtain a measurable rate of gas exchange. About 12 c.mm. cells/flask were commonly used. This results in absorption of a large fraction (about 60 per cent) of the incident light and adds the requirement that the two

flasks of a given pair expose equal cross-sectional areas to the illumination. An over-all check on the precision of the method is demonstrated in the fact that the flasks could be used in various combinations and positions without effect on the observed CO_2/O_2 ratios.¹

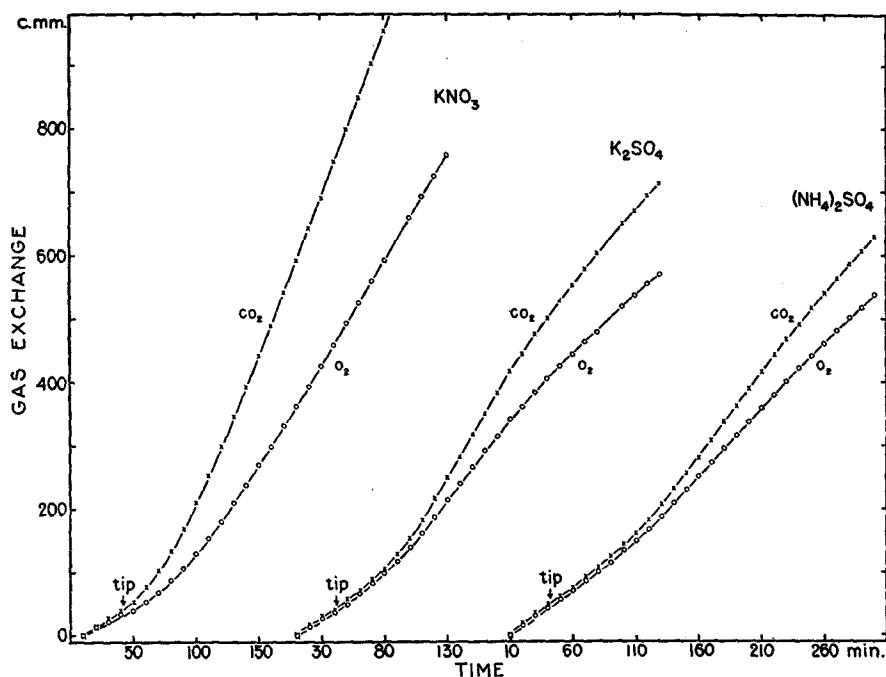


FIG. 1. Oxygen uptake and carbon dioxide production during glucose respiration of growing cells in the dark. The KNO_3 of the Knop's solution in which the cells were suspended was replaced in the second and third cases by equinormal amounts of K_2SO_4 and $(\text{NH}_4)_2\text{SO}_4$, respectively. Zero time for each set of curves represents the beginning of the manometric experiment. Glucose was added from sidearms by tipping at the times indicated.

Nitrate Reduction during Glucose Assimilation in Darkness

Chlorella grows rapidly in the dark with glucose as the only source of carbon. Fig. 1 illustrates the course of gas exchange when glucose is added to cells suspended in Knop's solution containing potassium nitrate or ammonium sulfate as the nitrogen source or without a nitrogen source (KNO_3 replaced by K_2SO_4). The cells had previously been growing photosynthetically in Knop's

¹Two of the CO_2/O_2 quotients cited in a preliminary report of this work (Myers and Cramer, 1947), now known to be in error, were obtained before the above precautions were put into operation.

solution with potassium nitrate as the nitrogen source. Examination of Fig. 1 shows that the highest and most constant rate of gas exchange is attained on nitrate, to which the metabolism of the cells had already been adapted. With ammonia as a nitrogen source the gas exchange is less rapid and shows a gradual decrease with time which may be related to a marked decrease in pH. When the cells have no supply of nitrogen a still more severe decrease in rate develops with time.

The most striking effects of the nitrogen source are seen in the r.q.² and in the changes in pH of the cell suspension presented in Table I. Uptake of nitrate is characteristically accompanied by a high r.q. and an increase in pH; uptake of ammonia, by a lower r.q. and decrease in pH. Similar changes in pH have been observed by Pratt and Fong (1940). Apparently nitrate is

TABLE I
Variation of the r.q. with the Nitrogen Source during Glucose Assimilation by Growing Cells
The r.q.'s were calculated over the time period of 200 to 300 minutes for each experiment.

Experiment	Initial pH		Nitrogen source			
			NO ₃ ⁻	No nitrogen	NH ₄ ⁺	NH ₄ ⁺ + NO ₃ ⁻
1	4.5	R.Q.	1.58	1.31	1.22	—
		Final pH	6.0	4.7	3.1	—
2	6.8	R.Q.	1.66	1.42	1.18	—
		Final pH	7.6	6.9	3.5	—
3	4.5	R.Q.	1.58	—	—	1.16
		Final pH	5.9	—	—	3.5

absorbed in exchange for a hydroxyl ion and ammonium in exchange for a hydrogen ion (or at least this is the net effect). The increase in pH on nitrate to a value of about 6.0 and attendant retention of carbon dioxide as bicarbonate must cause the calculated rate of carbon dioxide evolution (Fig. 1) and the r.q. (Experiment 1 of Table I) to be somewhat too low.

That the pH itself is not the cause of the difference in r.q.'s is demonstrated by other experiments at an initial pH of 6.8 (*cf.* Experiment 2 of Table I). Still other experiments by the indirect method, using flasks with different liquid: gas ratios yielded r.q.'s of 1.57 to 1.61 on nitrate, confirming those obtained by the direct method.

Under no conditions has an r.q. of 1.0 been observed in the respiration of glucose by growing cells. Even with no available nitrogen source the r.q. is considerably greater than unity, indicating assimilation of the glucose to

² For convenience the r.q. and other CO₂/O₂ quotients are cited without inclusion of the negative sign which obtains from the usual conventions of gas exchange.

more reduced cellular materials. Under similar conditions Gaffron (1939) has observed R.Q.'s varying from 1.2 to 2.0 in a number of different algae. Another related observation reported by Spoehr and coworkers (1946) is that *Chlorella* shows a greatly increased fat production during nitrogen deficiency.

If both nitrate and ammonia are provided the algae (Experiment 3 of Table I) the resulting R.Q. and change in pH clearly show that no nitrate reduction occurs when ammonia is present. This observation confirms the conclusion

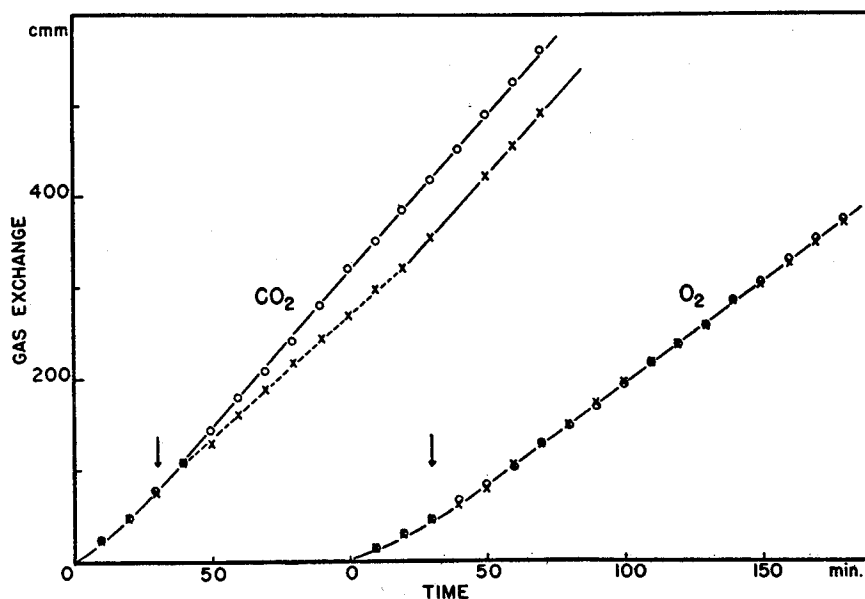


FIG. 2. Gas exchange accompanying assimilation of nitrate and ammonia during dark assimilation of glucose. Experimental details: 26 c.mm. growing cells per flask in Knop's containing nitrate and glucose; 1.0 micromol $(\text{NH}_4)_2\text{SO}_4$ (XXXX) or zero $(\text{NH}_4)_2\text{SO}_4$ (OOOO) added at the time indicated by the vertical arrows.

of Pratt and Fong (1940) that when both nitrogen sources are available *Chlorella* utilizes ammonia in preference to nitrate. Since nitrate reduction must result in products of the state of reduction of ammonia, and since no nitrate reduction occurs when ammonia is available, it follows that nitrate is reduced only in a manner intimately related to the requirements of subsequent nitrogenous synthesis.

The suppression of nitrate reduction by ammonia in growing cells submits to further experiments such as that shown in Fig. 2. If 2 micromols of ammonia are added to cells assimilating glucose and nitrate the rate of oxygen uptake is not at all affected. Carbon dioxide output, however, is reduced almost immediately to a lower constant rate which persists until the ammonia has been

utilized; the rate then returns abruptly to a value characteristic of nitrate assimilation. The R.Q. during nitrate assimilation is 1.59, during ammonia assimilation 1.22, in good agreement with the values cited in Table I. Again it is apparent that ammonia suppresses nitrate reduction. Still more striking is the evidence that the higher R.Q. during nitrate reduction results entirely from extra-CO₂ in agreement with comparable observations of Warburg and Negelein.

Nitrate Reduction during Carbon Dioxide Assimilation in Light

Many measurements of the assimilatory quotient (CO₂/O₂) for *Chlorella* in the usual nitrate-containing Knop's solution have yielded values of about 0.9 (cf. Warburg, 1948; Emerson and Lewis, 1941). Invariably the measurements

TABLE II

Variation of the CO₂/O₂ Quotient with the Nitrogen Source at Low Light Intensity

40 f.-c. illumination; 4 per cent CO₂; ~ 12 c.mm. cells/flask; Knop's at pH 4.5 with nitrogen source as indicated.

NO ₃ ⁻		NH ₄ ⁺	NO ₃ ⁻ + NH ₄ ⁺
0.76	0.68	0.91	0.94
0.68	0.65	0.96	0.93
0.67	0.66	0.91	
0.71	0.60	0.95	
0.70	0.64	0.96	
		0.97	
Average	0.68	0.94	0.94

have been made at photosynthesis-saturating light intensities. Under such conditions the cells used here have an A.Q. of 0.88. Light saturation of growth, however, occurs at an intensity considerably below that required for photosynthesis as observed in short time experiments (Myers, 1946). For the present work a light intensity of about 40 f.-c. was chosen in order to approximate the illumination under which the cells had been growing. This intensity may be described as light-limiting for both growth and photosynthesis and affords an oxygen evolution about five times as great as the endogenous oxygen uptake in the dark.

Presented in Table II are a series of measurements of the CO₂/O₂ quotient obtained by the indirect method. Since these were short experiments of about 90 minutes duration the pH (on nitrate) never rose to a value greater than 4.9 and retention of carbon dioxide could be neglected as a close approximation. The actual rates of oxygen and carbon dioxide exchange are omitted since small variations in light intensity between experiments preclude comparisons

on this basis. The quotient of gas exchange, however, is relatively insensitive to small variations in light intensity.

In light metabolism, as during glucose assimilation in the dark, nitrate reduction is apparent as a marked effect on the gas exchange quotient. The low quotient of 0.68 on nitrate has been confirmed by mass culture experiments at low light intensity which yield a quotient of 0.71 (Myers and Johnston, 1949). Again the quotients obtained with both nitrate and ammonia present are identical with those obtained with ammonia alone, indicating that nitrate reduction during photosynthesis is linked to metabolism and is not an incidental process. The important problem of the effect of light intensity on the quotient during nitrate assimilation will be treated in the following paper.

TABLE III
The Change in Gas Exchange Caused by Addition of Ammonium Sulfate after a Period of Nitrate Utilization

Neon illumination equivalent to 40 f.-c. of tungsten illumination; 4 per cent CO₂; ~ 24 c.mm. cells/flask; original solution Knop's at pH 4.5; gas exchange computed in c.mm./hour.

Experiment	O ₂ evolution			CO ₂ uptake		
	NO ₃ ⁻	NH ₄ ⁺	Change	NO ₃ ⁻	NH ₄ ⁺	Change
1	101	97	-4.0	69	98	+29
2	96.5	97	+0.5	77	104	+27
3	90	89.5	-0.5	63.5	90.5	+27
Average change.....			-1.0			+28

The low quotient on nitrate may be related to nitrate reduction. Unfortunately the experiments reported in Table II do not allow decision whether the low quotient accompanying nitrate reduction is the result of increased oxygen production or decreased carbon dioxide uptake. This problem has been attacked by addition of ammonium salt after a period of nitrate reduction.

Duplicate flasks with a circular flat bottom 10 cm.² in area and a sidearm were used with different liquid:gas ratios. About 5 mg. of dry ammonium sulfate were placed in the sidearm in lieu of a solution in order to eliminate any question of gas exchange equilibrium between cell suspension and fluid in the sidearm. A negligible volume change is introduced on addition of so small a quantity of solute. Since it is necessary to determine the gas exchange before and after addition of the ammonium salt the duplicate flasks cannot be exchanged in position in any one experiment. Two flask positions over the neon grid were selected at which the light intensities matched to within 1 per cent and in successive experiments the two flasks were used alternately in the two positions.

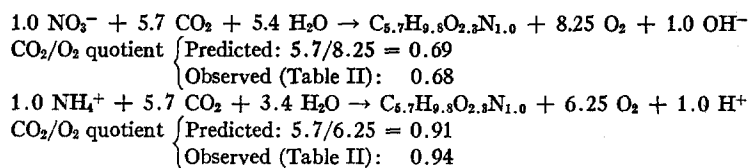
The results of three representative experiments are summarized in Table III. The quotients of gas exchange are here not very reliable since the dupli-

cate flasks required for each determination did not receive precisely the same illumination. The procedure does allow comparison of the changes in rates of oxygen and carbon dioxide exchange accompanying the transition from nitrate to ammonia utilization. The change in rate of oxygen evolution is small and perhaps negligible; the increased rate of carbon dioxide uptake is consistently large. The low quotient of nitrate reduction during photosynthetic metabolism is principally due, therefore, to a depression in carbon dioxide uptake. Such a result is to be expected on theoretical grounds. In the over-all metabolism nitrate reduction and carbon dioxide reduction are competing endergonic processes. Under light-limiting intensities the photochemical process limits the level of total metabolic energy expenditure.

Considerations from Cell Analysis

During photosynthetic growth of *Chlorella* in mass cultures about 95 per cent of the carbon dioxide and nitrate taken up can be recovered as cellular carbon and nitrogen (Myers and Johnston, 1949). As a close approximation, therefore, new cell material and oxygen are the only products of photosynthetic metabolism.

Elementary analysis of *Chlorella*, as grown for the present experiments, yields 53 per cent C, 7.5 per cent H, 28.5 per cent O, and 10.8 per cent N on an ash-free, dry-weight basis. On dividing by the appropriate atomic weights these percentages can be converted to the expression $C_{5.7}H_{9.8}O_{2.3}N_{1.0}$. By such a procedure (*cf.* Tamiya, 1932) it becomes possible to write balanced equations for over-all metabolism and thus predict the gas exchange required for the appropriate nitrogen source. Thus



If the two equations are to be compared on a rate basis, then all of the integers of the equation for nitrate utilization should be multiplied by the factor 6.25/8.25 to allow equal rates of oxygen production as demanded by the data of Table III. The equations are written, however, only with the intent of demonstrating the relative proportions of the various reactants and products of over-all metabolism. It is seen that in an organism such as *Chlorella* the over-all metabolism can be predicted equally well from cell analysis or from measurements of the gas exchange. Similar equations could be written for glucose assimilation in the dark but would require additional information on the fraction of the glucose assimilated by growing cells.

DISCUSSION

The characteristics of nitrate reduction in *Chlorella* under growth conditions may be compared with those observed by Warburg and Negelein (1920) in their highly acid nitrate mixture. Growing cells do not excrete ammonia; they reduce nitrate only at a rate compatible with further nitrogenous synthesis. This is borne out also by experiments with nitrogen-deficient cells (to be reported elsewhere) in which the rate of nitrate reduction may be very much increased. The limiting factor for nitrate reduction cannot be ascribed to the rate of entrance of nitrate into the cells. Warburg and Negelein attributed the success of their acid nitrate mixture to its high concentration of neutral HNO_3 molecules to which the cells are presumably more permeable. At the same time their acid medium, aside from its possible internal effects on the cells, also provided conditions more favorable for ammonia excretion. A change of pH from 5.0 to 2.0 decreases the external concentration of undissociated NH_4OH a thousandfold. If the internal pH of the cell remains reasonably constant, and if the only membrane-penetrating species of ammonia is the undissociated form, then at the lower pH more favorable conditions for ammonia excretion will obtain.

The observed effect of the nitrate in decreasing the carbon dioxide uptake under the light-limiting illumination used here (Table III) is entirely compatible with the extra- O_2 reported for the nitrate mixture. Warburg and Negelein did, in fact, explain their extra- O_2 as a primary extra- CO_2 produced by equation (1) and then converted photosynthetically to extra- O_2 under their conditions of light saturation and carbon dioxide limitation.

The present data offer no comparison between the actual rates of nitrate reduction in light and in darkness and therefore allow no explanation of the accelerating effect of light observed by Warburg and Negelein. The whole problem of the mechanism of nitrate reduction in light and its relation to photosynthesis will be treated in subsequent work.

Comparison of the present work on *Chlorella* may also be made to the work of Burström (1942, 1943) on nitrate assimilation in wheat leaves. Young excised wheat leaves, when illuminated, show an uptake of carbon dioxide, an increase in sugar content, and a decrease in nitrate content. Over a considerable range of light intensity only part of the carbon assimilated is recovered as sugars; the remainder is quantitatively related to the nitrate consumed. In darkness the carbon of the sugars consumed is quantitatively recovered as carbon dioxide and no nitrate is reduced. This latter characteristic, which marks an important difference between the wheat leaf and *Chlorella*, allowed Burström to postulate that nitrate reduction is intimately linked to the photosynthetic process.

Finally, it may be pointed out that a tool of considerable possible usefulness

has been developed for the study of nitrogen metabolism. The high nitrogen content of *Chlorella*, the differential effect of nitrate and ammonia on its gas exchange, and its characteristic of suppression of nitrate reduction by ammonia offer particular advantages in studies on the mechanism of nitrogen assimilation.

SUMMARY

1. Nitrate reduction and assimilation have been studied in *Chlorella pyrenoidosa* under growth conditions by observing effects on the CO₂/O₂ gas exchange quotient.

2. During assimilation of glucose in the dark, nitrate reduction is noted as an increase in the R.Q. to about 1.6 caused by an increased rate of carbon dioxide production.

3. During photosynthesis at low light intensity nitrate reduction is evidenced by a reduction in the CO₂/O₂ quotient to about 0.7 caused by a decreased rate of carbon dioxide uptake.

4. *Chlorella* will assimilate nitrogen from either nitrate or ammonia. When both sources are supplied, only ammonia is utilized and no nitrate reduction occurs. It is inferred that under the usual conditions of growth nitrate is reduced only at a rate required for subsequent cellular syntheses. The effect of nitrate reduction on the CO₂/O₂ quotient therefore provides a measure of the relative rate of nitrogen assimilation.

5. Over-all photosynthetic metabolism may be described from elementary analysis of the cells since excretory products are negligible. The gas exchange predicted in this way is in good agreement with the observed CO₂/O₂ quotients.

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