KINETIC ASPECTS OF NITRATE REDUCTION [1])

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SUMMARY

Kinetic studies were made of the photochemical reduction of nitrate and nitrite to ammonia and correlations to the photosynthetic CO_2 assimilation investigated. Two molecules of O_2 are evolved per molecule of nitrate reduced to ammonia and 1½ molecules of O_2 per molecule of nitrite.

In the rate-limiting light intensity region, the rate of oxygen production was found to be independent of the nature of the oxidant (CO_2, NO_3^- or NO_2^-); the quantum yield of nitrate reduction is equal to that of CO_2 reduction. Hence the energetic efficiency of nitrate and nitrite reduction is only about ⅔ of the efficiency of the CO_2 assimilation.

Under light saturation the rate of oxygen evolution in CO_2 containing media is not increased by a simultaneously occurring NO_3^- and NO_2^- reduction.

INTRODUCTION

In a previous article (1) we described nitrogen assimilation by cultures of green algae under various conditions. It was found that there was no conversion of inorganic nitrogen into cell nitrogen in the absence of simultaneous CO_2-assimilation, unless the N/C ratio of the algae had been disturbed during pretreatment. We found, however, that normal cells grown in a complete nutrient medium (and having a nitrogen content of 8% to 10% on a dry weight basis) if exposed to light in the absence of CO_2, excrete into the surrounding liquid an amount of ammonia which corresponds to the amount of nitrate disappearing from the nitrate-containing medium.

We considered this excretion as being due to the absence of suitable carbon skeletons which function as ammonia acceptors under normal conditions of growth. Furthermore, we tried to correlate the rate of CO_2-reduction (in the presence of CO_2) to the rate of ammonia excretion (in the absence of CO_2), both as a function of light intensity.

The conversion of nitrate into ammonia is accompanied by the production of oxygen. In the present paper, experiments are described in which the rate of NH_4^+-formation is estimated by measuring the oxygen evolution. The amount of oxygen evolved per unit of nitrate reduced or ammonia excreted is indicated by the O_2/N ratio. The respective reduction rates of CO_2 and nitrate could therefore both be followed by means of identical procedures (manometry) which allowed accurate measurements of the relative rates in weak intensities as well. Direct ammonium estimates during brief experiments in weak light are hardly feasible.

In the same way, the correlation between the rates of nitrate and CO_2-reduction is measured as a function of light intensity. Furthermore, some experiments are described which are concerned with the question as to how far the rate of oxygen output in strong "saturating" light depends upon the availability of both types of oxidants.

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Experimental methods

The algal material used, *Scenedesmus* sp., was cultivated in a modified continuous culture device (cf. Myers and Clark (2). It was made of three concentric glass tubes surrounding a fluorescent tube (daylight, 30 W nominal rating). In the inner annulus between growth chamber and lamp constant temperature water was circulated. This apparatus is shown in Fig. 1. Sedimentation of the cells was prevented by continuous aeration with a stream of 5% CO_2 in the air.

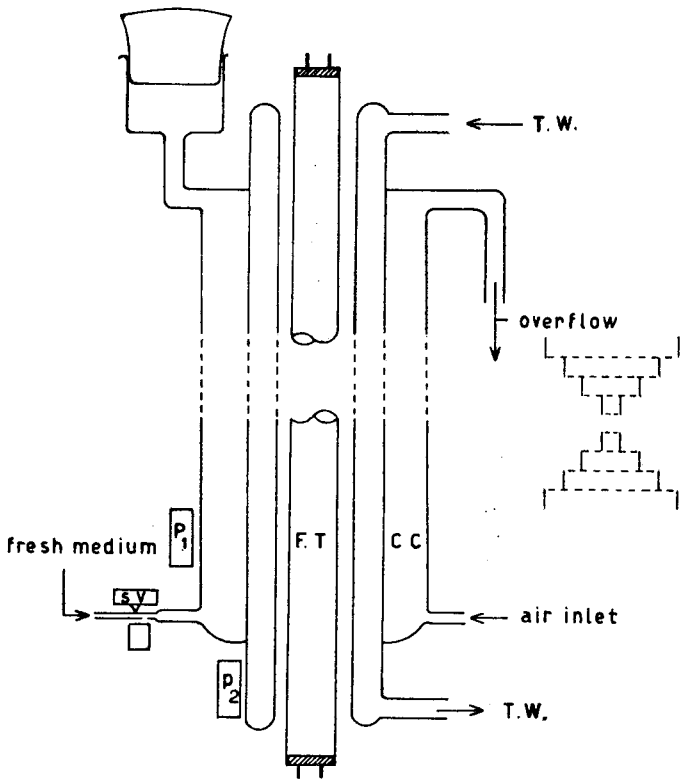

Fig. 1 Schematic drawing of the culture arrangement. C.C. : culture chamber ; volume 750 ml ; thickness culture chamber 7 mm ; F.T. : fluorescent tube, 30 Watts, daylight ; T.W. : inlet and outlet of water of constant temperature ; P_1 and P_2 : photocells ; S.V. : solenoid valve.

The density of the culture during growth was kept constant by adequate dilution with fresh nutrient solution. The supply of fresh medium was automatic and controlled, via a solenoid valve, by a system of two photo cells. One of these was exposed directly to the light source via an adjustable wire screen and the other illuminated via the algal suspension. The valve was operated by a difference between the two photocurrents. The culture density could be varied by using wire screens of different transmission.

Alternate dark and light periods were given to the algae in such a way that a complete life cycle (in accordance with observations of Tamiya et al. (3) was obtained in 24 hours at the temperature and light intensity prevailing.

At 30° C and at the end of a light period of 16 hours the culture consisted of a fairly homogeneous suspension of mature "light" cells which were about to divide. In the subsequent dark period the cells normally divided into 8 or 4 daughter cells. The pH of the KNOP culture solution was maintained at about 7.0.

For most experiments vigorously growing cells were used, harvested about 5 hours after the beginning of the light period.

Measurements of photosynthetic oxygen evolution were made with the manometric method in a 0.2 molar carbonate buffer at pH 8.4. This pH is somewhat lower than that normally used, but it was found that the oxygen production was not materially different from the rate observed in a buffer of pH 8.7. This relatively low pH was used in order to obtain comparable conditions during measurements of the reduction of either carbon dioxide, nitrate or nitrite. In the absence of CO_2, the latter two substrates are photochemically reduced to ammonia at pH values of between 7.5 and 9.0 (cf. (1), but at higher pH values toxic effects of NH_3 may interfere. In our brief experiments no such concentration of ammonia accumulated to influence the observed rates at pH 8.4.

Nitrate and nitrite reduction were determined by manometric measurements of the oxygen evolution of algae suspended in a solution of a $^1/_{15}$ M tris (hydroxymethyl) amine-methane-phosphate buffer, or by estimating the amount of ammonia excreted. After centrifuging the cells the ammonia concentration built up in the suspension medium during illumination was determined in the supernatant by means of the NESSLER technique (cf. SNELL and SNELL (4). Light absorption was measured at 410 m μ.

In our manometric experiments we used flat cylindrical vessels (bottom area $\sim$ 27 cm^2, volume $\sim$ 27 ml) each provided with 10 ml of suspension. A set of six identical vessels was used in a thermostat (30° C) and illuminated by six incandescent lamps. The highest light intensities obtainable were about 0.5 cal/cm^2 min. By using wire screens these intensities could be decreased as desired.

In several experiments a specific dose of some reagent had to be added to the suspension without disturbing the experiments. (Disturbance is hardly avoidable with the normal procedure of "tipping" from a side bulb). The arrangement used for this purpose is shown in Fig. 2. It consisted of a stopcock arrangement, the plug of which was provided with a cavity about 0.25 ml in volume.

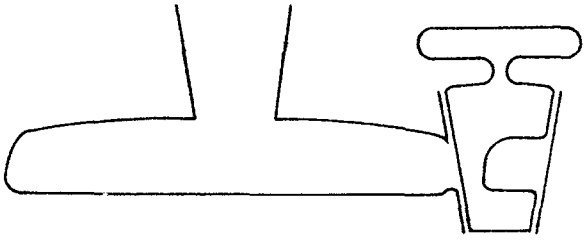

FIG. 2 ILLUSTRATION OF THE VESSEL ADAPTED TO "TIPPING" EXPERIMENTS.

Before starting the experiment this cavity had to be completely filled with the desired solution, after which the stopcock was closed and the vessel rinsed. At the desired moment during the experiment a 180° turn of the stopcock caused a fast mixing of the two liquids. Re-equilibration after this manipulation was complete in less than a minute, and an additional advantage of the arrangement was that there could be no change of the position of the vessel in the optical path.

Experimental results

O_2/NH_4 ratio

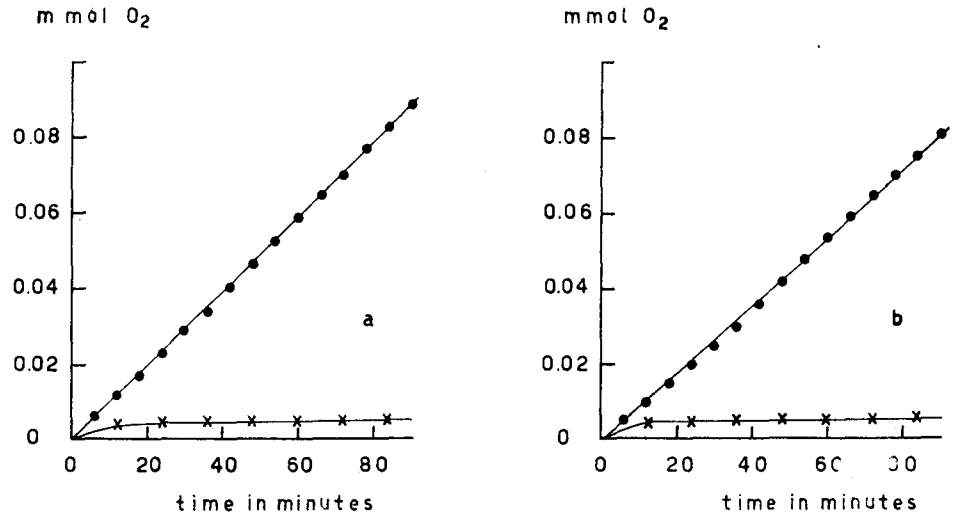

Fig. 3 Time course of oxygen evolution in light in the absence of CO_2 in media containing either KNO_3 (a), or KNO_2 (b). Crosses indicate no addition as control. Initial concentration 0.015 M nitrogen. Light intensity 0.5 cal/cm² min.

We observed that algae suspended in adequately buffered media, if exposed to light in the absence of CO_2 and in the presence of NO_3^- or NO_2^-, produced oxygen at a fairly uniform rate for a considerable time (hours). Figs 3a, b represent such experiments, and also show that oxygen evolution is negligible in a control suspension without a nitrogen and a carbon source. It was shown previously (1) that under such conditions the cells excrete ammonia in the medium at a uniform rate. A number of experiments were carried out to determine the ratio between the amount of oxygen evolved and the amount of ammonia excreted (the O_2/NH_4 quotient). The results are listed in Table 1.

Table 1 Average O_2/NH_4 quotient observed under conditions of strong and weak light in the absence of CO_2, in media containing nitrate, nitrite, or nitrate + nitrite. Suspensions buffered at pH 7.3 with a (0.07 mol.) tris-phosphate buffer.

Series	Number of experiments	N-source	Weak light	Strong light
a	5	KNO_3	1.97 ± 0.06	–
b	14	KNO_3	–	1.93 ± 0.04
c	5	KNO_2	1.45 ± 0.05	–
d	14	KNO_2	–	1.47 ± 0.03
e	5	KNO_3 + KNO_2	–	1.53 ± 0.09

There are strong indications that two molecules of oxygen are produced in both weak and strong light (series *a* and *b*) per molecule of nitrate reduced. Less oxygen is evolved per molecule of nitrite reduced (series *c* and *d*). The O_2/NH_4 quotients in this case were close to 1.5. The observation that the quotient does not differ greatly from 1.5 when both nitrate and nitrite are available probably indicates that nitrite is reduced in preference to nitrate.

Correlation between the rates of CO_2, NO_3^- and NO_2^- reduction in light

In a number of experiments we compared the rate of oxygen output as a function of light intensity in media containing NO_3^- or NO_2^- in the presence or absence of CO_2. Fig. 4 shows the rate of oxygen output in the absence of CO_2 in two parallel samples of algae, one suspended in a nitrate-containing medium (cf. Fig. 4, curve 1), the other in a nitrite-containing medium (cf. Fig. 4, curve 2). The dotted lines in this figure represent the rates of ammonia excretion (curve 3 : in the nitrate-containing medium, curve 4 : in the nitrite-containing medium), as calculated with the O_2/NH_4 quotient discussed in the preceding section.

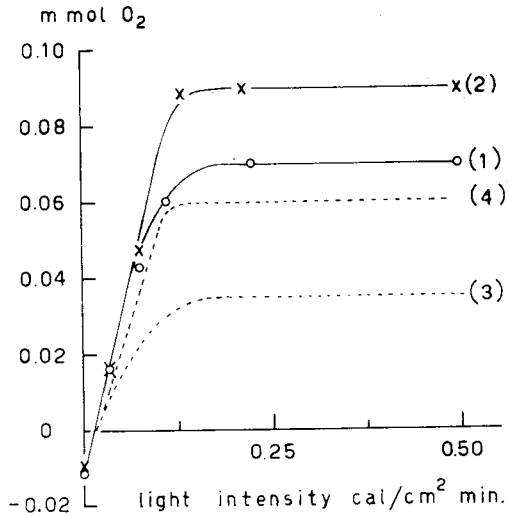

FIG. 4 RELATION BETWEEN RATE OF OXYGEN EVOLUTION AND LIGHT INTENSITY, MEASURED IN THE ABSENCE OF CO_2, IN MEDIA WITH EITHER KNO_3 (CURVE 1), OR KNO_2 (CURVE 2). DOTTED CURVES REPRESENT THE RATE OF AMMONIA EXCRETION, CALCULATED ON BASIS OF O_2/NH_4 QUOTIENTS OF 2.0 IN THE NO_3^- CONTAINING MEDIUM (CURVE 3), AND 1.5 IN THE KNO_2 MEDIUM (CURVE 4).

The rate of ammonia excretion in strong light is considerably higher in the nitrite-containing suspension than in the suspension supplied with nitrate. This phenomenon was also observed by KESSLER (5) and will be discussed in a forthcoming paper.

Fig. 5 represents the oxygen output measured at various intensities with three parallel samples of algae suspended in media containing either NO_3^- in the absence of CO_2 (open circles), or CO_2 in the absence of NO_3^- (dots), or containing both CO_2 and NO_3^- (triangles). Fig. 6 represents a similar experiment, in which, however, NO_3^- was replaced by NO_2^- . These figures

show that in weak light a linear relation exists between oxygen output and light intensity, and that with higher light intensities all processes attain saturation showing that dark reactions limit the maximum rates.

For a given amount of weak light the same amount of oxygen is evolved, irrespective of the substrate or substrates reduced. Fig. 7 again illustrates that the oxygen production in the light-limiting range is independent of the nature of the oxidant.

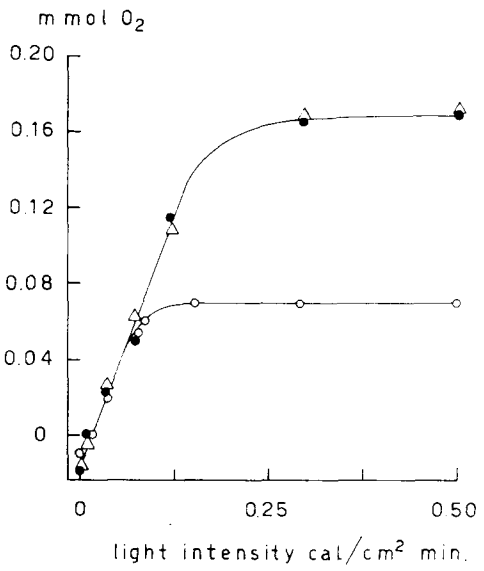

FIG. 5

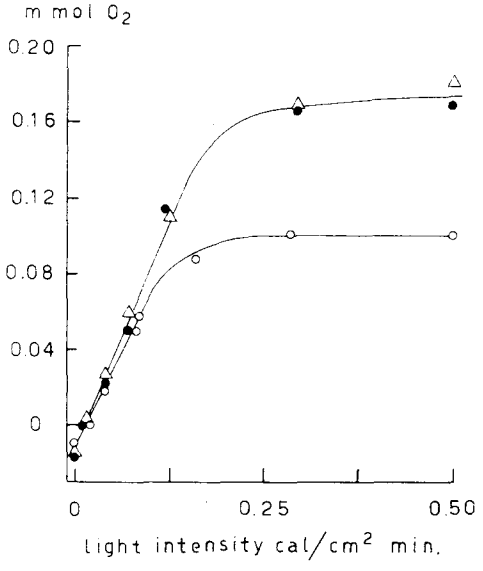

FIG. 6

FIG. 5 RELATION BETWEEN LIGHT INTENSITY AND RATE OF OXYGEN EVOLUTION IN MEDIA CONTAINING EITHER NITRATE IN THE ABSENCE OF CO_2 (OPEN CIRCLES), OR CO_2 IN THE ABSENCE OF NITRATE (CLOSED CIRCLES), OR IN THE PRESENCE OF BOTH CO_2 AND NO_3 (TRIANGLES).

FIG. 6 LEGEND AS FIG. 5. NITROGEN SOURCE KNO_2.

We may conclude that the photosynthetic quantum yield $h\mu/O_2$ is the same for nitrate, nitrite and CO_2 reduction. Since only ½ molecule of NO_3^- is reduced and $^2/_3$ molecule of NO_2^- per O_2 evolved the quantum requirement per N atom is considerably higher than that per C atom in carbon reduction.

In strong light both CO_2 and NO_3^- or NO_2^- reduction attain saturation rates. The maximum level of oxygen evolution with CO_2, $CO_2 + NO_3^-$ (cf. Fig. 5) and $CO_2 + NO_2^-$ (cf. Fig. 6) is higher than the maximum rate of oxygen evolution found with these nitrogen sources in the absence of CO_2. This approximately twofold difference in oxygen evolution between the saturation rates of nitrate and CO_2 reduction corresponds to the approximately fivefold difference between the saturation rate of oxygen output in a CO_2-containing algal suspension and the ammonia excretion in a nitrate-containing suspension in the absence of CO_2, as was described previously (1). It may be concluded from the present experiments that the same difference exists between the rates of nitrite and CO_2 reduction.

A second point of interest is that the maximum rate of oxygen output in

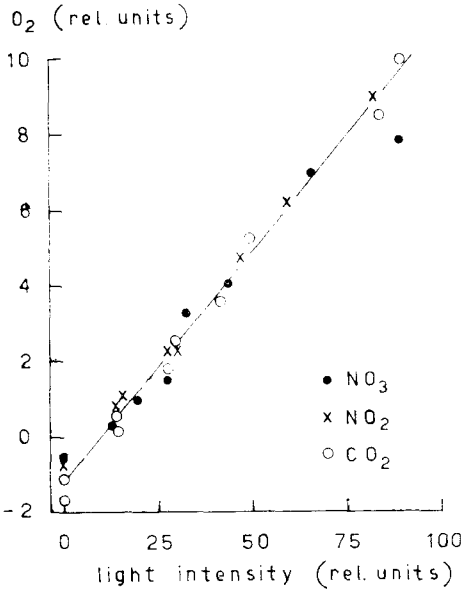

FIG. 7 RELATION BETWEEN THE RATE OF OXYGEN EVOLUTION AS A FUNCTION OF LIGHT INTENSITY, IN MEDIA CONTAINING EITHER NITRATE (CLOSED CIRCLES), NITRITE (CROSSES) BOTH IN TRIS-PHOSPHATE BUFFER (pH 8.4), OR CO_2 (OPEN CIRCLES) IN A 0.2 M CARBONATE BUFFER (pH 8.4).

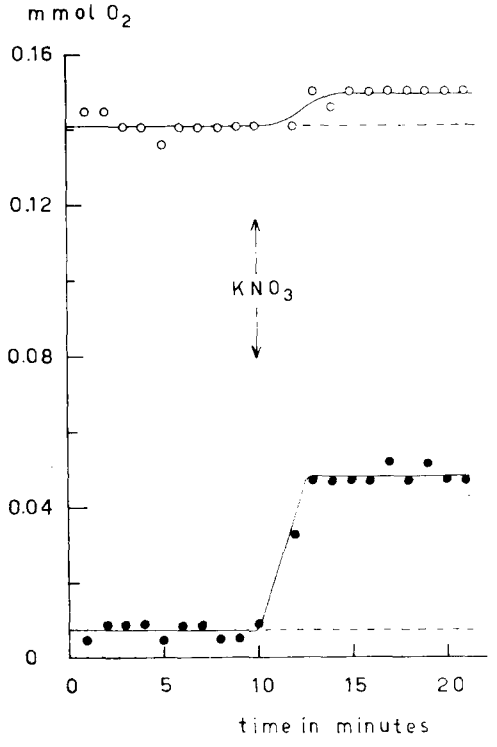

FIG. 8

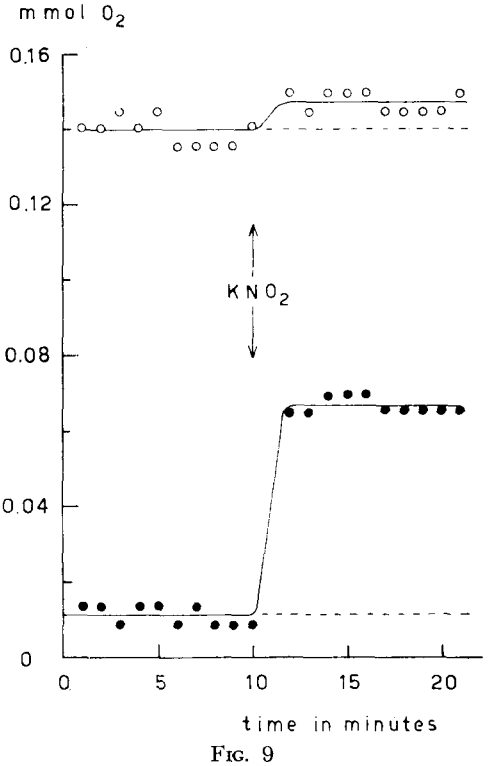

FIG. 9

FIG. 8 TIME COURSE OF OXYGEN OUTPUT IN STRONG LIGHT (0.5 CAL/CM^2 MIN.). ARROW INDICATING KNO_3 ADDITION VIA THE STOPCOCK ARRANGEMENT. TOP: 0.2 MOLAR CARBONATE BUFFER (pH 8.4). BELOW: 0.07 MOLAR TRIS-PHOSPHATE BUFFER (pH 8.4).

FIG. 9 LEGEND AS FIG 8. NITROGEN SOURCE KNO_2.

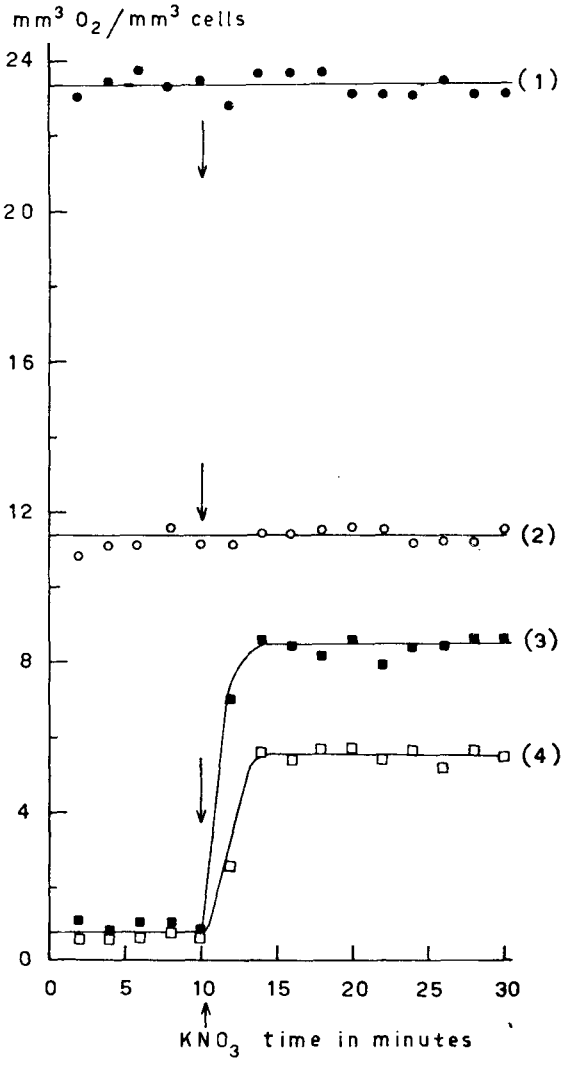

FIG. 10 TIME COURSE OF OXYGEN EVOLUTION IN STRONG LIGHT (0.5 CAL/CM² MIN.). ARROW INDICATES THE ADDITION OF KNO_3 VIA STOPCOCK ARRANGEMENT.
Normal cells : curve 1, 0.2 M carbonate buffer (pH 8.4) ;
curve 3, 0.07 M tris-phosphate buffer (pH 8.4).
N-starved cells : curve 2, 0.2 M carbonate buffer (pH 8.4) ;
curve 4, 0.07 M tris-phosphate buffer (pH 8.4).

a carbon dioxide-containing suspension is hardly if at all influenced by the presence or absence of nitrate or nitrite. The saturation rates are equal within experimental error, independent of a possible NO_3^- or NO_2^- reduction in addition to CO_2 reduction. These observations were confirmed by a number of "tipping" experiments. In these experiments, shown in Figs 8 and 9, the oxygen evolution was measured versus time in a 0.2 M carbonate buffer in saturating light. At a given moment (indicated by arrows) nitrate or nitrite was introduced with the stopcock arrangement shown in Fig. 2. This method enabled us to measure more accurately a possible increase of oxygen evolution

due to the onset of nitrate reduction. With a parallel sample of algae, and with the same technique, the oxygen evolution was measured before and after the addition of nitrate or nitrite in the absence of CO_2. These experiments show that in the absence of CO_2 a rapid increase of oxygen evolution occurs upon the addition of NO_3^- or NO_2^-. But in the presence of CO_2 this increase is very limited, amounting to some 5% or less of the prevailing rate.

We also measured the enhancing effect of nitrate on photosynthetic oxygen evolution with cell material of a decreased photosynthetic activity. The algal cells were exposed to strong light for 3 hours in nitrogen-deficient media (1). As may be seen in the experiment illustrated in Fig. 10, (came out in a similar way to those of Figs. 8 and 9), no increased rate of oxygen evolution could be observed after addition of nitrate, although the saturation rates of CO_2 reduction differed by a factor two.

Discussion

Quantitative relationships between oxygen evolution and nitrate or nitrite reduction have been observed by many workers. Davis (7) found an "extra" O_2 output in light as a consequence of nitrate reduction in glucose-containing algal suspensions, and it can be concluded from his results that two molecules of oxygen are evolved for each molecule of nitrate. Syrett (8, 9) using nitrogen-starved *Chlorella* cells, observed that in the reduction of nitrate and nitrite in darkness about 2.0 respectively 1.5 molecules of "extra" CO_2 were evolved per molecule of reduced substrate. Kessler (5) demonstrated a reduction of nitrite in light by *Ankistrodesmus* and observed a quotient (O_2/NO_2) of 1.5. A similar relationship was also found by Vanecko et al. (10) with wheat leaves. With hydrogenase containing algae (*Ankistrodesmus braunii* and *Scenedesmus obliques* "D_3"), incubated under an atmosphere of hydrogen in darkness, Kessler (11) recorded a rapid uptake of hydrogen upon addition of nitrite. He interprets his experimental results by the following equation: $HNO_2 + 3\ H_2 \longrightarrow NH_3 + 2\ H_2O$. This was in good agreement with the earlier observation of Woods (12) with *Clostridium welchii*, who observed a consumption of 4 molecules of hydrogen per molecule of nitrate and 3 molecules of hydrogen per molecule of nitrite reduced.

The experimental results all clearly indicate that the following general stoichiometric equation may be written for nitrate and nitrite reduction:

$$HNO_3 + 8\ H \longrightarrow NH_3 + 3\ H_2O \qquad (1)$$

and

$$HNO_2 + 6\ H \longrightarrow NH_3 + 2\ H_2O \qquad (2)$$

In darkness the hydrogen will be generated by oxidation of added or previously formed reduced chemical substances. In light the reducing capacity will be ultimately generated by the photolysis of water.

In the endothermic conversion of 1 mole of CO_2 to carbohydrate, expressed as (CH_2O), 112 kcal are fixed. The conversion of nitrate and nitrite to ammonia involves energy fixations of about 73 and 54 kcal per mole respectively. Since per mole O_2 evolved in the reduction of nitrate and nitrite about 36 kcal are stored (73 kcal per mole NO_3^- and 54 kcal per mole NO_2^-) the energetic efficiency of these processes is quite low compared to the efficiency of the photosynthetic CO_2 reduction (about 33%).

The above-mentioned observations fit in the general pattern of quantum conversion in photosynthesis such as was observed with chloroplast reactions and purple sulphur bacteria. They appear to confirm the generalisation that the quantum requirement of a process is stoichiometrically related to the number of hydrogen transfers, and not to the energy relations.

In our experiments we did not confirm the results of VAN NIEL and Coworkers (6) who report that *Chlorella* suspensions at high light intensities, supplied with non-limiting CO_2 concentrations, produce oxygen at a greater rate when nitrate is simultaneously present. In their experiments the increasing effect of nitrate yielded results varying from 9% to 60% of the amount of oxygen produced in the absence of nitrate. This increase is related to the photochemical reduction of nitrate. Their findings led them to the conclusion that in the presence of both nitrate and CO_2 an additional hydrogen acceptor (nitrate reducing system) is in operation, resulting in an additional amount of oxygen. In the absence of CO_2 we found a substantial rate of oxygen output in nitrate- and nitrite-containing algal suspensions, viz. from 40% to 50% of the rate of oxygen output found in the presence of CO_2 only; in the presence of both CO_2 and nitrate, however, the rate of oxygen output was only slightly increased, viz. from 0% to about 8% of the rate found in the presence of CO_2.

The said discrepancy can be understood by considering the location of the limiting factor in the photochemical CO_2 reduction. According to VAN NIEL's opinion, at high light intensities the rate of photosynthesis is limited by the concentration of the hydrogen acceptor (CO_2 or phosphoglyceric acid). If the supply of hydrogen-enzyme-complex is more than can be handled by the hydrogen acceptor mentioned, the excess reductants could be utilized by the enzymes involved in nitrate reduction, yielding an additional amount of oxygen. In our experiments such an additional amount of oxygen was not recorded. Consequently we are inclined to conclude that under conditions of strong light, even far beyond "light saturation", the supply of photosynthetic reductant does not exceed the amount which can be handled by the enzymes involved in the reduction of CO_2. This implies an earlier location in the chain of photosynthetic dark reactions of the rate-limiting factor than suggested by VAN NIEL et al., a fact supported by kinetic studies of photo-inhibition and photosynthesis in flashing light (cf. KOK (13)).

REFERENCES

1 BONGERS, L. H. J.: Thesis Wageningen (1956).
2 MYERS, J. and L. B. CLARK: *J. Gen. Physiol.* 28 (1944) 103.
3 TAMIYA, H., T. IWAMURA, K. SHIBATA, E. HASE and T. NIHEI: *Bioch. et Bioph. Acta* 12 (1953) 23.
4 SNELL, F. D. and C. T. SNELL: Colorimetric methods of analysis. London, vol. II (1949) 802.
5 KESSLER, E.: *Nature* 176 (1955) 1069.
6 NIEL, C. B. VAN, M. B. ALLAN and B. E. WRIGHT: *Bioch. et Bioph. Acta* 12 (1953) 67.
7 DAVIS, E. A.: *Plant Physiol.* 28 (1953) 539.
8 SYRETT, P. J.: *Physiol. Plant* 8 (1955) 924.
9 — —: *Physiol. Plant* 9 (1956) 28.
10 VANECKO, S. and J. E. VARNER: *Plant Physiol.* 30 (1955) 388.
11 KESSLER, E.: *Archives of Bioch. and Bioph.* 62 (1956) 241.
12 WOODS, D. D.: *Bioch. J.* 32 (1938) 2000.
13 KOK, B.: *Bioch. et Bioph. Acta* 21 (1956) 245.