ASPECTS OF NITROGEN ASSIMILATION BY CULTURES OF GREEN ALGAE

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWKUNDE
OP GEZAG VAN DE RECTOR MAGNIFICUS IR. W. DE JONG,
HOOGLERAAR IN DE VEETEELTWETENSCHAP,
TE VERDEDIGEN TEGEN DE BEDENKINGEN
VAN EEN COMMISSIE UIT DE SENAAT
DER LANDBOUWHOGESCHOOL TE WAGENINGEN
OP VRIJDAG 21 DECEMBER 1956 TE 16 UUR

DOOR

L. H. J. BONGERS

H. VEENMAN & ZONEN – WAGENINGEN – 1956

Dit proefschrift met stellingen van

LEONARD HUBERT JOANNES BONGERS

landbouwkundig ingenieur, geboren te Weert, 5 februari 1925, is goedgekeurd door de promotor, Dr. E. C. WASSINK, hoogleraar in het Plantenphysiologisch Onderzoek en de Physiologie der planten.

De Rector Magnificus der Landbouwhogeschool,
IR. W. DE JONG

Wageningen, 19 november 1956

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VOORWOORD

De voltooiing van dit proefschrift biedt mij de gelegenheid mijn dank te richten tot allen, die hebben meegewerkt aan mijn wetenschappelijke vorming en aan de bewerking van deze dissertatie.

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CHAPTER I

GENERAL OUTLINE AND METHODS

§ 1. INTRODUCTION

The primary object of this investigation was the study of nitrate assimilation by algae in the presence and absence of light. Though it is evident from the literature on this subject that nitrate reduction takes place in light as well as in darkness, only little is known about the mechanism. An attractive hypothesis assumes that the photosynthetic appàratus interferes directly in the process of reduction, but until now this has not been demonstrated conclusively. This supposition implies the existence of two mechanisms, viz. a "dark" system, in which the energy necessary for the reduction is supplied by reserve materials, and a photochemical system.

The assumption of two processes is based especially on the observation that in light in the absence of carbon dioxide the rate of nitrate reduction surpasses that in the dark. This observation, however, can also be explained in another way. During illumination the supply of oxidisable substrates might be kept constant by resynthesis from the carbon dioxide formed by respiration. In dark periods, reserve substances may be rapidly exhausted and nitrate reduction could then stop by lack of energy supply.

To obtain more insight into the capacity of the nitrate reducing systems in the presence or absence of light, it is therefore necessary to take care that also during prolonged dark periods the supply of oxidisable substrates does not limit the rate of reaction, a circumstance that has led to investigating a special type of "nitrogen-starved", or "carbon-enriched" cells.

It seemed interesting, furthermore, to try to follow the path of nitrate reduction, and to compare the rate of nitrogen assimilation in light and darkness with the rate of assimilation of more reduced intermediates, e.g. nitrite and ammonium. In experiments of such type one may expect to obtain an insight into the question in how far energy providing processes are limiting nitrogen assimilation, or in how far limitations in the process of nitrogen assimilation are introduced, e.g., by limited availability of certain necessary carbon-"skeletons".

§ 2. REVIEW OF THE LITERATURE

It would be impracticable to discuss exhaustively the accumulated knowledge on nitrogen assimilation and nitrate reduction. Therefore, the author re-

stricts himself to mention a few published reviews and to a brief discussion of those investigations, closely related to his own work.

In 1937, NIGHTINGALE has given an extensive survey of earlier literature and, in 1948, presented an additional survey covering the literature between 1937 and 1948. STREET (1949) reviewed further literature especially based on work published since 1939, and WOOD (1953) has dealt particularly with the nitrogen metabolism in higher plants. Recently, WEBSTER (1955) discussed nitrogen metabolism in a more general way. An excellent book on protein metabolism was published by CHIBNALL in 1939.

Interest in NO_3-reduction as carried out by green cells was aroused by the classical work of WARBURG and NEGELEIN (1920) with *Chlorella*. Though not working under generally physiological circumstances (pH 2.0), they observed the reduction of nitrate to ammonium by green algal cells. They found furthermore that during illumination in the absence of carbon dioxide, oxygen evolution could be obtained by the addition of nitrate. On the other hand, the presence of nitrate in the dark caused an extra carbon dioxide production. Since the "extra O_2" production in light surpassed the "extra CO_2" production in the dark, the authors supposed that under the influence of light the penetration of nitrate ions into the cells was stimulated. The replacement of "extra CO_2" by "extra O_2" can be explained from the fact that in light the carbon dioxide is again reduced to oxygen. However, it is important to notice that the observed extra O_2 production in light was more than three times as large as the extra CO_2 production in the dark.

Many investigators, using *Chlorella* cells, have confirmed the results of WARBURG and NEGELEIN also under more physiological conditions, e.g. MYERS (1948), CRAMER (1948), KOK (1951), and KROLLPFEIFFER (1951).

DAVIS (1953) demonstrated with algae that the process of nitrate reduction in light was more complicated. He found a very limited net oxygen production in light with nitrate and without carbon dioxide. However, only after the addition of an external hydrogen donor (glucose), he measured a rapid nitrate reduction with concomitant oxygen evolution. Nitrate reduction under these conditions can most easily be explained by the direct action of glucose or one of its decomposition products as a hydrogen donor. At all events these results suggest that also in irradiated cultures the nitrate reduction is coupled with the oxidation of an organic substrate. The hydrogen, originating from the "photodecomposition of water" as such would not seem to be a suitable reductant.

VAN NIEL *et al.* (1953) supposed nitrate reduction to be directly coupled with the photosynthetic mechanism. In *strong* light, the authors found a CO_2-uptake not affected by the presence or absence of nitrate. The oxygen output was, however, considerably larger when nitrate was available. In *weak* light, the CO_2-uptake was diminished by the presence of nitrate, whereas the oxygen output remained constant, independent of the presence of nitrate. The same results are given by KOK (1952) and by VAN OORSCHOT (1955). From their observations they concluded that the decreased CO_2-uptake in the presence of nitrate resulted from a competition between CO_2 and NO_3^- for the photosynthetic reductant. Under conditions of light saturation, the supply of photosynthetic reductant is more than can be handled by the enzyme systems involved in the reduction of CO_2. If now the enzyme systems involved in the nitrate reduction utilize this excess reductant, an additional amount of oxygen can be produced.

In accordance with these findings, nitrate is thus considered as hydrogen

acceptor in the same sense as carbon dioxide. Then the reduction of nitrate can be considered as a dark reaction in the same way as indicated for CO_2 (e.g. WASSINK, 1951).

In the line of thought of these investigators, the mechanism of nitrate reduction in the presence or absence of light may be the same, except for the ultimate energy source used.

MENDEL *et al.* (1951), however, suggest that two different mechanisms are responsible for the nitrate reduction in tomato leaf tissue, viz., one occurring in the light, the rate of which is about 50% greater than that in the dark. They gathered these indications for a „two way course" from the observation that the nitrate reduction in leaf discs incubated in darkness is markedly inhibited by iodo-acetate, which inhibitor had no effect on the light process.

BURSTRÖM (1943) is of the opinion that the nitrate reduction by wheat leaves in light can only occur in the presence of carbon dioxide, in close connection with photosynthesis. According to the same author (1943b) young wheat leaves are unable to reduce significant quantities of nitrate in the dark, while this would not be due to lack of carbohydrate. On the other hand, BURSTRÖM (1939) also found nitrogen assimilation by wheat roots. From these facts he concluded that the assimilation of nitrate must follow different courses in roots and leaves.

The *biochemical pathway* of nitrate reduction has been studied by EVANS and NASON, using cell-free extracts. They (EVANS and NASON 1952, 1953; NASON and EVANS 1953, and EVANS 1954) have isolated, purified, and characterized an enzyme from *Neurospora* and soybean leaves and nodules, which reduces nitrate under simultaneous oxidation of the reduced form of tri- and diphosphopyridine nucleotide. The enzyme proved to be a flavoprotein with flavine adenine dinucleotide as a prosthetic group.

NASON *et al.* (1954) furthermore isolated an enzyme from *Neurospora* and from soybean leaves which catalyzed the conversion of nitrite to ammonia.

TANIGUCHI *et al.* (1953) discovered three reductases in cell-free extracts of *Bacillus pumilus* which reduced nitrate, nitrite and hydroxylamine. Reduced methylene blue could be used as an electron donor.

NICHOLAS *et al.* (1954, 1955) have found that the addition of molybdenum to the nutrient medium increased the nitrate reductase in two *Neurospora* strains and in *Aspergillus niger*. From experimental work with isolated nitrate reductase from soybean leaves, the role of molybdenum became evident. These authors found a proportionality between the molybdenum content and the activity of the enzyme.

As is shown by EVANS (1953), reductase activity was found in the tissue of six higher plants, e.g. in potato and in barley roots and leaves. His results demonstrate that addition of TPNH and DPNH stimulates the reduction of nitrate.

These findings are not in agreement with such as found by WARBURG *et al.* (1920), and BURSTRÖM (1939, 1943b). The relatively low reduction rates found during prolonged dark periods by these authors are perhaps caused by the exhaustion of oxidisable substrate. In connection herewith the investigations of PIRSON (1937), VAN HILLE (1938) and SYRETT (1953a) are interesting. They found that the assimilation of nitrate and ammonium in nitrogen starved algae is largely influenced by the nitrogen content of the starting material. Also ROINE (1947) found a stimulated nitrogen intake in nitrogen starved *Torula* yeast.

About the formation of *intermediates* in nitrate reduction only little is known. There are some suggestions in the literature for the occurrence of nitrite. WARBURG *et al.* (1920) found this compound in a suspension of green algae, using nitrate as a nitrogen source. Recently KESSLER (1952, 1953a, b) has demonstrated excretion of nitrite with algae; the accumulation of this intermediate was highly dependent on the pH on the suspension. It is his opinion that in the range of pH 3 to pH 5 the reduction of nitrate to nitrite is stimulated while the further reduction of nitrite is limited. The occurrence of intermediates on the reduction level of hyponitrous acid and hydroxylamine has often been assumed. The consideration of these two compounds in the course of the reduction of nitrate is attractive as far as thus in each reduction step two hydrogen ions or electrons are used. The existence of these intermediates, however, has not yet been demonstrated in green cells. Only LEMOIGNE *et al.* (1937a, b) found hydroxylamine in various leaves, but LUDWIG (1938) has demonstrated that *Chlorella* does not utilize hydroxylamine as a nitrogen source. ZUCKER and NASON (1954), however, isolated an enzyme from *Neurospora* that catalyzes the reduction of hydroxylamine to ammonia. An accumulation of hydroxylamine by some *Neurospora* mutants was found by SILVER *et al.* (1954) while VERHOEVEN (1952) demonstrated the occurrence of hydroxylamine during nitrate reduction in *Denitrobacillus licheniformis.*

Nitrate has to be reduced to the ammonia level before being incorporated in the carbon skeleton for the formation of amino acids and cell protein. Many investigators accept the formation of ammonia as an intermediate in nitrate reduction. An accumulation of this compound has been demonstrated by WARBURG *et al.* (1920) with *Chlorella* while PRIANISCHNIKOV *et al.* (1931) have found it in pea seedlings. Using the tracer technique, MENDEL *et al.* (1951) found labeled ammonia nitrogen in higher plants fed with labeled nitrate.

§ 3. OUTLINE OF THE INVESTIGATION

In this section some of the ideas briefly touched upon in the introduction, will be lined up somewhat more definitely.

Our investigation started with a confirmation and extension of some of VAN OORSCHOT's findings concerning the effect of nitrogen supply on the growth of algal cultures, the composition of the cells, and the observation rates of photosynthesis in various stages of nitrogen exhaustion (*cf.* VAN OORSCHOT, 1955). These experiments are discussed in Chapter II.

Questions as exposed in § 1 have been experimentally approached in Chapters III to V included.

Chapter III builds forth upon the foundation laid in Chapter II, and the rate of nitrogen metabolism has been investigated under various external conditions, and using various nitrogen sources. Experiments are described in this Chapter, comparing rates of nitrogen assimilation in light and in darkness, with cells previously exposed to nitrogen-starvation (and carbon-enrichment) conditions, and with normal cells.[1]) Such experiments point to a requirement of nitrogen free cell compounds for nitrogen assimilation while, moreover, they led to

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[1]) In this paper we will understand by "normal cells" those obtained in a medium which does not show any obvious deficiency and which generally leads to rapid growth and multiplication. If any nutrient has been withdrawn from the culture medium for a sufficiently long period, cells thus obtained will be designed as "starved" with respect to the nutrient in question.

In the simplest type of experiment, known quantities of KNO_3 were added to cultures of nitrogen deficient cells. An added amount of nitrate was such that it would be completely taken up from the medium during the experiment. This was checked by means of the diphenyl amine sulphuric acid reagent.

From Table 1 it appears that no losses were observed.

TABLE 1. Nitrate assimilation by N-starved algae. Each culture supplied with a definite amount of nitrate. Cultures flushed with air in darkness. Temperature 30 °C

Time hrs.	Cell nitrogen mg per 5 ml susp.	NO_3-nitrogen mg per 5 ml susp.	Recovery %
0	0.63	0.50	100
22	1.13	0	100
0	0.63	0.50	100
22	1.11	0	98
0	0.39	0.98	100
22	1.40	0	103
0	0.39	0	100
22	0.37	0	95

Table 2 demonstrates that the quantities of reduced nitrogen in the suspension liquid could be neglected. Nitrite can be found in the medium, but the maximum quantities observed were very small (about 0.05 mg nitrogen per 5 ml).

TABLE 2. The distribution of the reduced nitrogen (NO_2^--N + NH_4^+-N). Nitrogen deficient cells, with abundant supply of KNO_3. Culture flushed with air, at a temperature of 30 °C in darkness

Time hrs.	Cell nitrogen mg/5 ml	Reduced nitrogen in medium mg/5 ml	Cell nitrogen %
0	0.61	0.02	100
1½	0.70	0.04	115
3	0.84	0.01	138
5½	0.96	0.05	157
7	1.10	0.04	180
8½	1.19	0.03	195

From both experiments it appears that the increase in cell nitrogen almost quantitatively accounts for the quantity of nitrate nitrogen disappeared. This means that in order to measure the velocity of nitrate reduction by the algal suspension it is sufficient to estimate the increase in cell nitrogen per unit of time.[1])

[1]) These findings are in accordance with the results of MYERS *et al.* (1949) who report a recovery of 95 % in the cells. Excretion of ammonia by the cells in their experiments also was negligible.

CHAPTER II

THE INFLUENCE OF NITROGEN SUPPLY ON GROWTH, COMPOSITION, AND PHOTOSYNTHESIS OF GREEN ALGAE

§ 1. THE INFLUENCE OF NITROGEN SUPPLY ON THE RELATIVE AMOUNTS OF PROTEIN, CARBOHYDRATE, AND LIPIDS IN THE CELL MATERIAL

a) Determination of the composition of the cell material

The relative amounts of protein, carbohydrate, and lipids were calculated from the elementary composition of the cell material according to a method indicated by SPOEHR and MILNER (1949). In comparison with CO_2, H_2O and HNO_3, cell material formed during photosynthesis is reduced as a consequence of the action of light. This material approximately has the following composition: 50% C, 7% H, 10% N, 26% O and 7% ash. The relative degree of reduction of this material can be expressed by the so-called R-value.

Considering carbohydrates, proteins and lipids as components of algal material, the R-values of these products are respectively: 28, 42 and 67.5. Using these R-values, it is possible to calculate the contents of these products in algal material of known elementary composition, assuming that no other classes of compounds are present in appreciable quantities. According to SPOEHR and MILNER this R-value is directly related to the heat of combustion per gram of the cell material. The R-value is, therefore, also a measure of the amount of energy fixed by the cells during growth.

The procedure for calculating the composition of the cell material was adopted in the experiments described in this Chapter.

b) The effect of the nitrogen content on the composition of the material

By using a nutrient medium, relatively poor in nitrogen, it was possible to obtain cell material with a large variation in composition regarding its contents of proteins, lipids and carbohydrates.

In this experiment *Chlorella vulgaris* "strain A" was used. The inoculum was pretreated as indicated by WASSINK *et al.*, (1938); 5 ml of a heavy algal suspension were inoculated into 500 ml ERLENMEYER flasks, containing 200 ml of culture medium. The nutrient solution used contained per litre 0.250 g KNO_3, 0.139 g KH_2PO_4, 0.500 g $MgSO_4 . 7H_2O$, and 2 ml of a solution containing H_3BO_3, $ZnSO_4$, $ZnCl_2$, $CuSO_4$, $FeSO_4$ and $MnSO_4$. The pH was adjusted to about 6.0. At definite time intervals samples were taken in order to estimate the dry weight and the elementary composition.

The arrow in fig. 1 at about 30 hours after the start of the illumination indicates the moment, the nitrate in the medium was completely consumed by the algae. This was checked by a diphenyl amine test in the supernatant of a small quantity of centrifuged suspension. However, the production of dry matter continued at virtually the same rate[1]) until approximately the 60th hour of illumination, which demonstrates the possibility of the production of dry matter without increase in the total amount of protein. After 60 hours of illumination the content of nitrogen in the dry matter was about 4%. When the ex-

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[1]) A more complete discussion on the rate of production of dry matter is given in the next section.

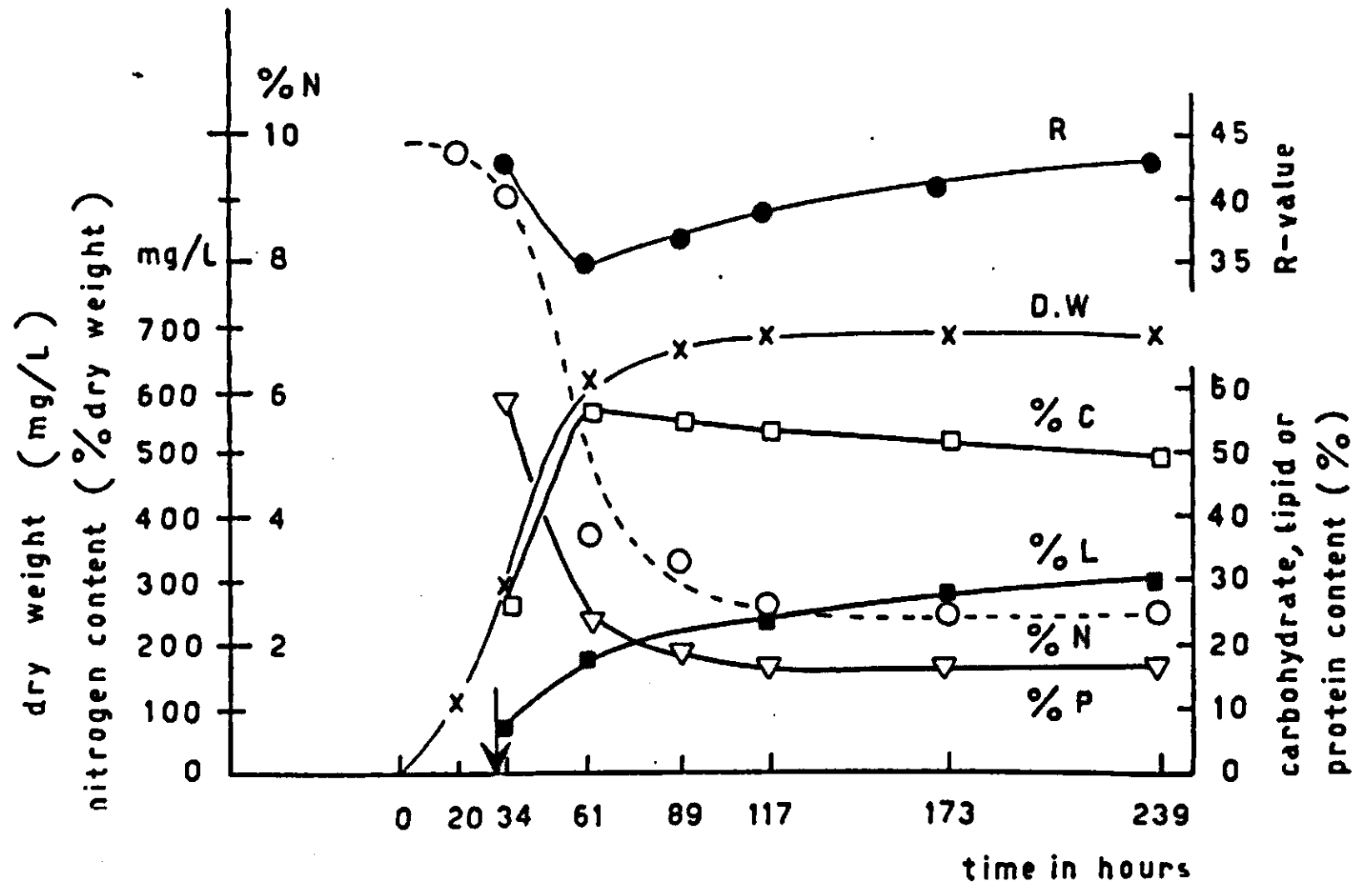


FIG. 1. Growth and composition of *Chlorella* in a medium with a limited amount of nitrate (0.25 g/l) during prolonged growth under continuous illumination from fluorescent tubes (4000 lux). The cultures were flushed with 5% CO_2 in air, and continuously shaken at a temperature of 25 °C. The arrow indicates the time at which all the nitrate in the medium is consumed. Ordinate to the left: dry weight (mg/l); nitrogen content in dry weight (%). Ordinate to the right: carbohydrate (C), lipid (L) and protein (P) content in ash free dry matter (%); R-value

posure was still more prolonged, the production of dry matter gradually decreased. After ± 100 hours of illumination the dry weight increased no more.

As a consequence of the increasing carbohydrate content during the period from 30 to 60 hours of growth and the concurrently decreasing protein content, the R-value of the material dropped from 44 to 36. This is easily understood because the R-value of carbohydrates is lower than that of proteins (28 and 42 respectively). Table 3 shows that the carbohydrate content after 60 hours of exposure has become about twice the carbohydrate content after 30 hours of exposure.

TABLE 3. Composition of cell material during growth
(data from fig. 1)

Illumination (hours)	30	60	239
Protein	60%	25%	18%
Carbohydrate	30%	56%	50%
Lipids	10%	19%	32%

During this period, the protein content has been reduced to less than half its original value and an increase of the content of lipids can already be observed. A marked drop in carbohydrate content and an increase of the lipid content was mainly found between the 60th and 239th hour of illumination. During this interval the R-value of the cell material regains its original level, mainly owing to the high R-value of lipids (namely 67.5). The consequent gain in

energy content of the cell material during this period may be attributed directly or indirectly to the reducing action of the light energy.

Up to now it is still impossible to give a definite answer to the question, whether light energy directly contributes to the turnover of carbohydrates into lipids. HAEHN *et al.* (1925) have studied fat formation in *Endomyces vernalis* and expressed the view that fatty acids are formed from pyruvic acid. LYNEN (1953) and MAHLER (1953) studied the role of coenzyme A in fatty acid metabolism. (For general discussion see these authors and BREUSCH 1952).

§ 2. SOME CHARACTERISTICS OF ALGAE WITH VARIOUS PROTEIN CONTENTS

As was stated in the preceding section, formation of dry matter also takes place in the light in the absence of nitrogen in the culture medium. It appeared, however, that the pale green cell suspension thus obtained ceased to produce dry matter as soon as the nitrogen content fell below 3–4%.

In this connection it seemed worth while to check the correlation between the nitrogen content and the rate of photosynthesis (as measured by determining the oxygen production), the chlorophyll content and the number of cells.

a) Nitrogen starvation of cells

Methods

Since the low light intensity, applied in the experiment previously described, caused a rather slow growth rate, in this and the following experiments a light intensity of about 30 klux was used. Rapidly growing cultures were obtained in this way.

A relatively heavy algal cell suspension (about 1.5 mg dry weight/ml) of *Scenedesmus spec.* was inoculated into a 300 ml KOLLE dish, containing 260 ml of a nitrogen deficient nutrient solution. The composition of the medium was the same as that used in the preceding experiment, except that KNO_3 was replaced by an equivalent quantity of K_2SO_4. The cultures were grown under continuous illumination of 30 klux, flushed with air containing 5% CO_2, at a temperature of 30°C. During the exposure, samples were taken at definite time intervals in order to determine dry weight, chlorophyll and nitrogen contents and the number of cells.

For the measurement of the light intensity rate curves of photosynthesis at different stages during the starvation period, 10 ml samples were taken, centrifuged, washed with 30 ml of distilled water and transferred to a bicarbonate buffer at pH 8.7.

Underneath the thermostat a 24 Volt, 150 Watt incandescent lamp with internal reflector (Philips Attralux, light intensity 0.60 $cal.cm^{-2}.min.^{-1}$) was mounted as a light source. Different light intensities could be obtained by using wire screen filters. The temperature during the measurements was 30°C.

Results

As shown in Table 4 and fig. 2, a considerable (4 fold) increase in dry matter was observed, the rate of which declined after 12 hours of exposure. The total quantity of nitrogen remained constant, so that the nitrogen content of the cells decreased markedly. As already stated, the rate of increase in dry weight per unit volume per hour, decreased considerably after 9–12 hours of starvation.

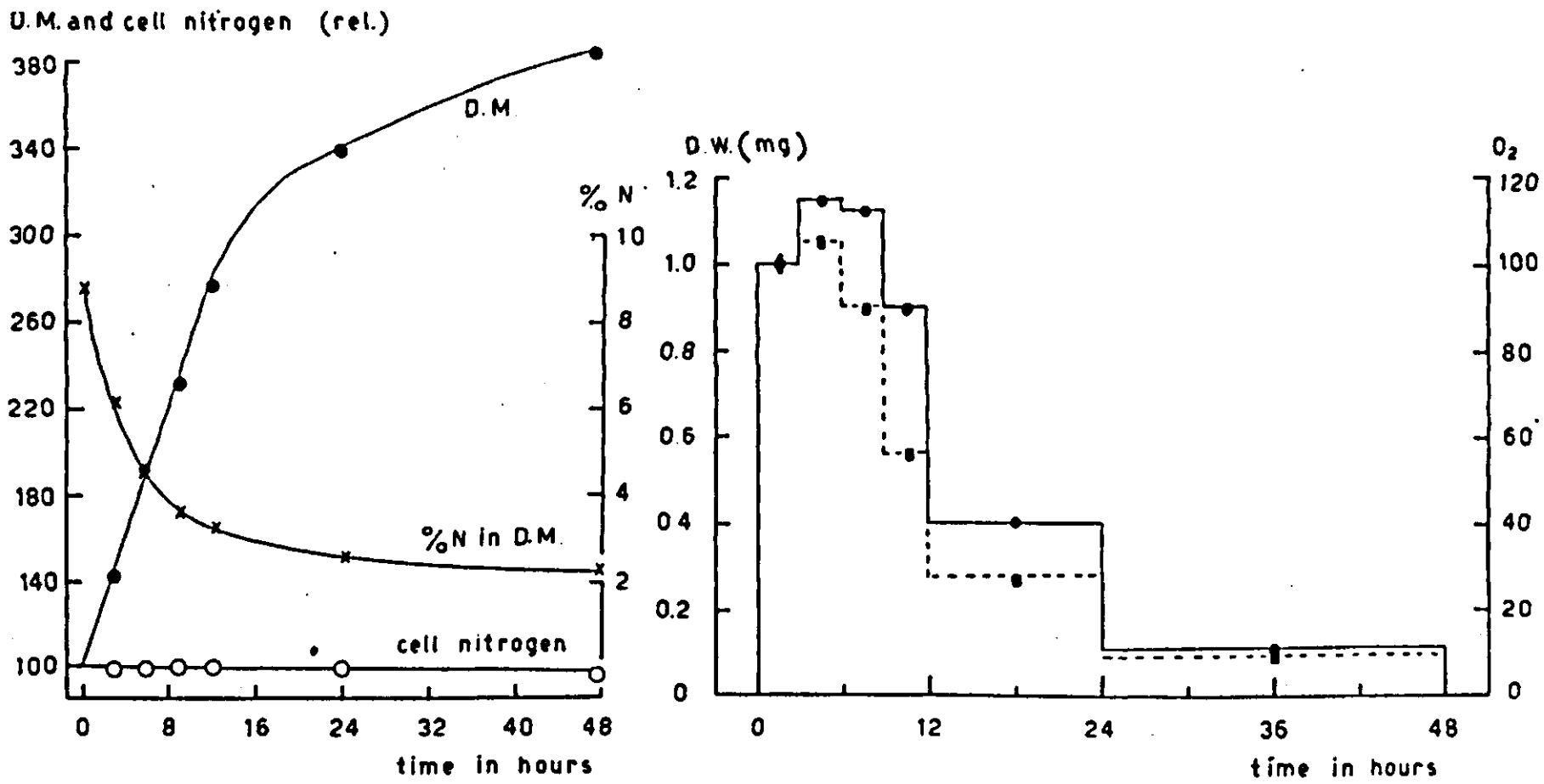


FIG. 2. Increase in dry matter (D.M.) during N-starvation in a culture medium, deficient in nitrogen. Light intensity 0.25 cal/cm² min., temperature 30 °C. Left ordinate: dry weight and cell nitrogen in relative measure; initial value = 100. Right ordinate: percentage of nitrogen in dry weight

FIG. 3. Production rate of dry matter (left ordinate) in mg per hour (solid line, data from fig. 2), and the production rate of oxygen in relative units (right ordinate) under conditions of light saturation (dotted line, data from fig. 4), measured at different moments during starvation

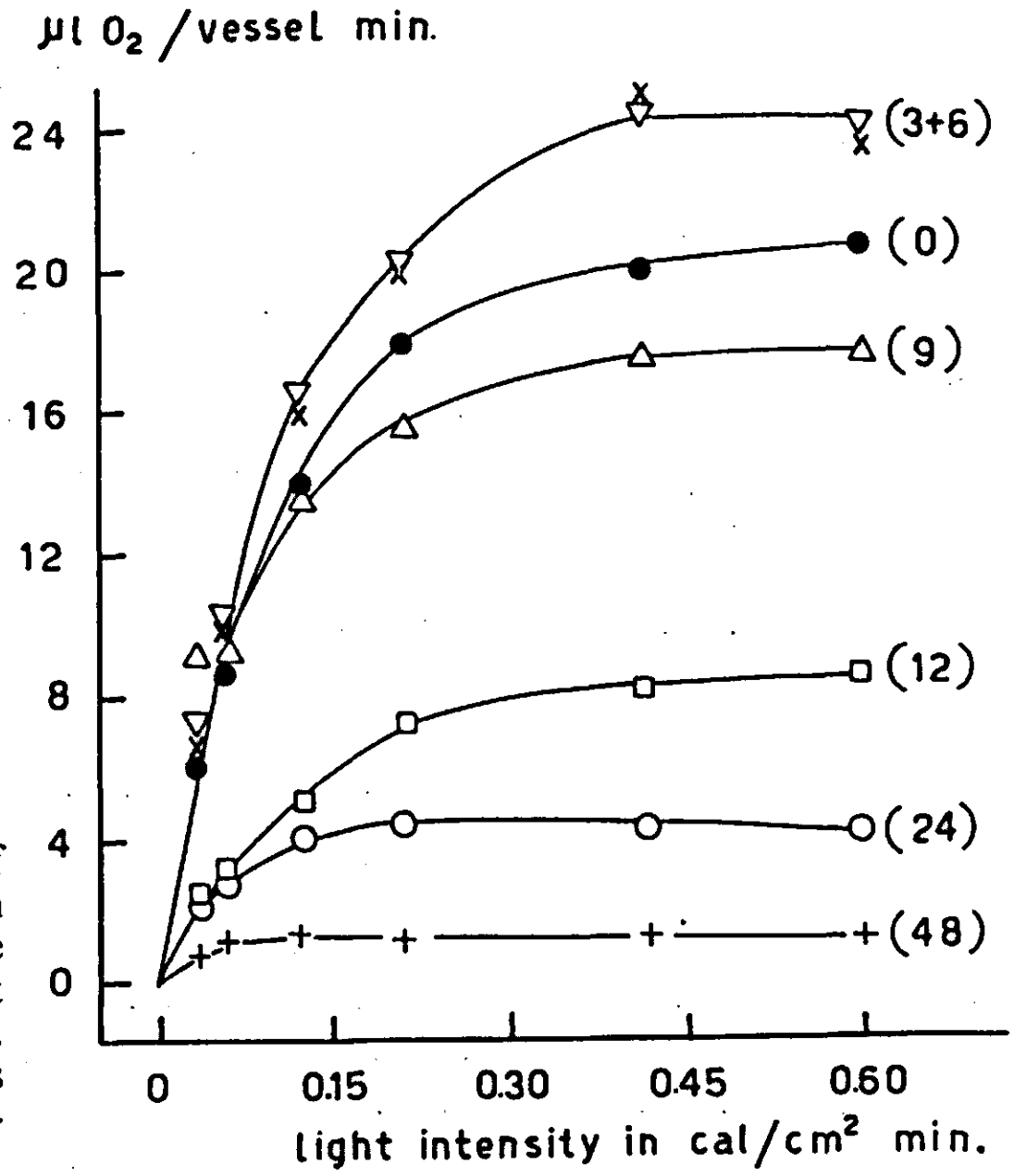


FIG. 4. Light intensity rate curves of photosynthesis measured in a 0.2 molar bicarbonate buffer (pH: 8.7) at different times during N-starvation. Figures between brackets indicate the time of starvation in hours

After 24 hours the dry matter production amounted to only 10% of its original value (fig. 3: solid line).

The light intensity rate curves of photosynthesis measured at different time intervals during the starvation period are shown in fig. 4. With the exception of the curves for 3 and 6 hours of starvation, a gradual decrease of the light saturation level is observed. After 12 and more hours of starvation such a decrease is also found in the region of limiting light intensities. After 48 hours the light saturation rate of photosynthesis has decreased to 5% of its original value, a very low rate indeed. The increase of the light saturation level (see also fig. 3: dotted line) after 3 and 6 hours of starvation is significant and may be attributed to the formation of new cells (Table 4).

TABLE 4. Photosynthesis and growth of algae *(Scenedesmus sp.)* in a nitrogen free nutrient medium. Dry weight, cell nitrogen, and chlorophyll are given in mg per 5 ml suspension. Photosynthesis is measured as oxygen output in a 0.2 molar sodium bicarbonate buffer. Light intensity 0.60 cal/cm².min.
Initial culture density 7.0 mg dry matter per 5 ml suspension. Temperature 30 °C; light intensity during starvation 30 klux

Time of exposure (hours)	Dry weight		Nitrogen		Chlorophyll		On a dry weight basis		Relative number of cells
	mg/5 ml susp.	%	mg/5 ml susp.	%	mg/5 ml susp.	%	% nitrogen	% chlorophyll	
0	7.0	100	0.61	100	0.197	100	8.80	3.82	100
3	10.0	143	0.61	100	0.225	115	6.10	2.25	115
6	13.5	193	0.61	100	0.230	117	4.50	1.70	151
9	16.9	232	0.61	100	0.225	115	3.60	1.33	153
12	19.4	277	0.63	103	0.204	104	3.25	1.05	142
24	24.3	338	0.61	100	0.148	75	2.50	0.61	145
48	26.9	384	0.59	97	0.077	39	2.30	0.29	157

Time of exposure (hours)	Dry weight production per hour (mg)	O_2-production at light saturation (µl)	O_2-production at light saturation			Dry weight per vessel (mg)
			per mg dry weight		per 0.1 mg chlorophyll	
0	–	21.7	1.55	100%	5.5	14.0
3	1.00	24.8	1.24	80%	5.5	20.0
6	1.16	24.5	0.91	59%	5.3	27.0
9	1.13	17.5	0.52	34%	3.9	33.8
12	0.83	8.55	0.22	14%	2.1	38.8
24	0.41	4.15	0.09	5.8%	1.4	48.6
48	0.11	1.35	0.05	3.2%	0.9	53.8

In order to obtain a clear picture it is preferable to recalculate the photosynthesis saturation rate values on a dry weight basis. These figures are plotted in figs 5 and 5a. A considerable decrease in the rate of photosynthesis immediately after stopping the N-supply is then observed. This decrease is linear with time during the first 14 hours of starvation (fig. 5: part A). This linear relation is also found in dry matter production as shown in fig. 2. The nitrogen content of the cells has dropped to 2 or 3% after about 12 hours (Table 4). So a close relationship seems to exist between the limiting factor responsible for the decrease of the light saturation level, and the nitrogen content. Fig. 6 represents the relation between photosynthesis saturation rate values on a dry weight basis and the nitrogen content for three similar experiments.

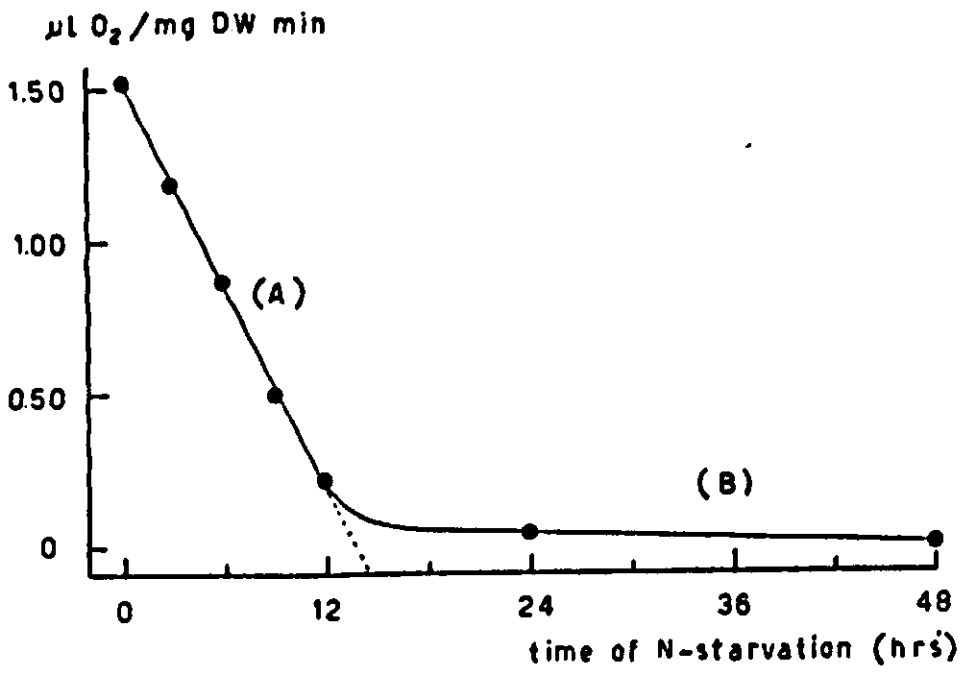


FIG. 5. Course of oxygen production rate at light saturation during nitrogen starvation, recalculated on dry weight basis from the data of fig. 4 and Table 4

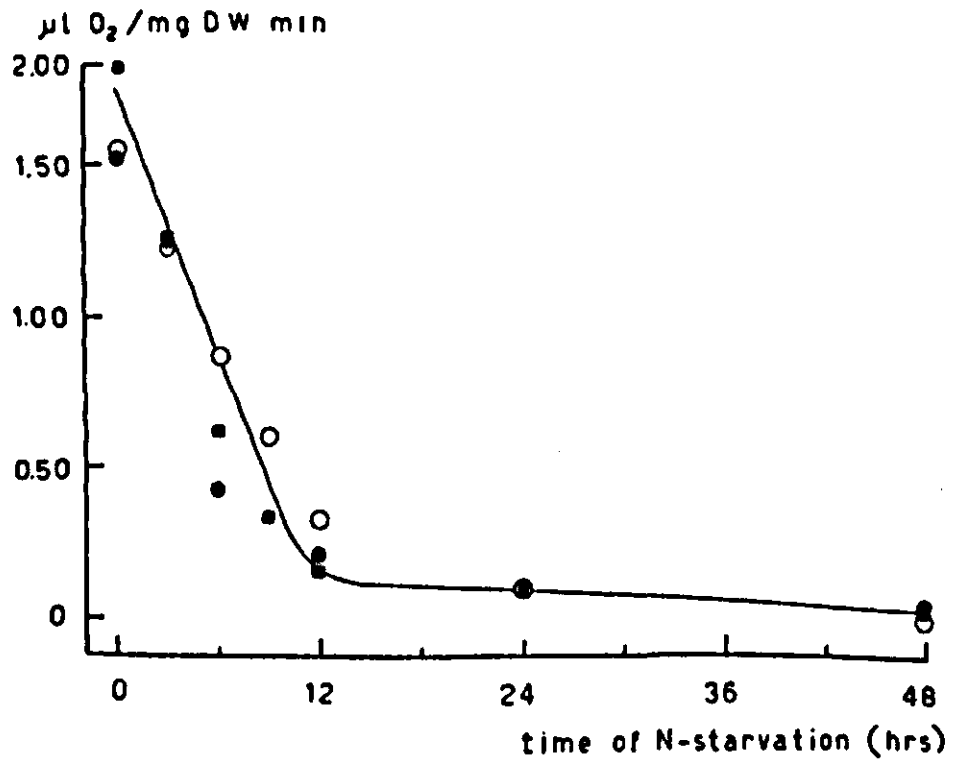


FIG. 5a. As fig. 5, for three similar experiments

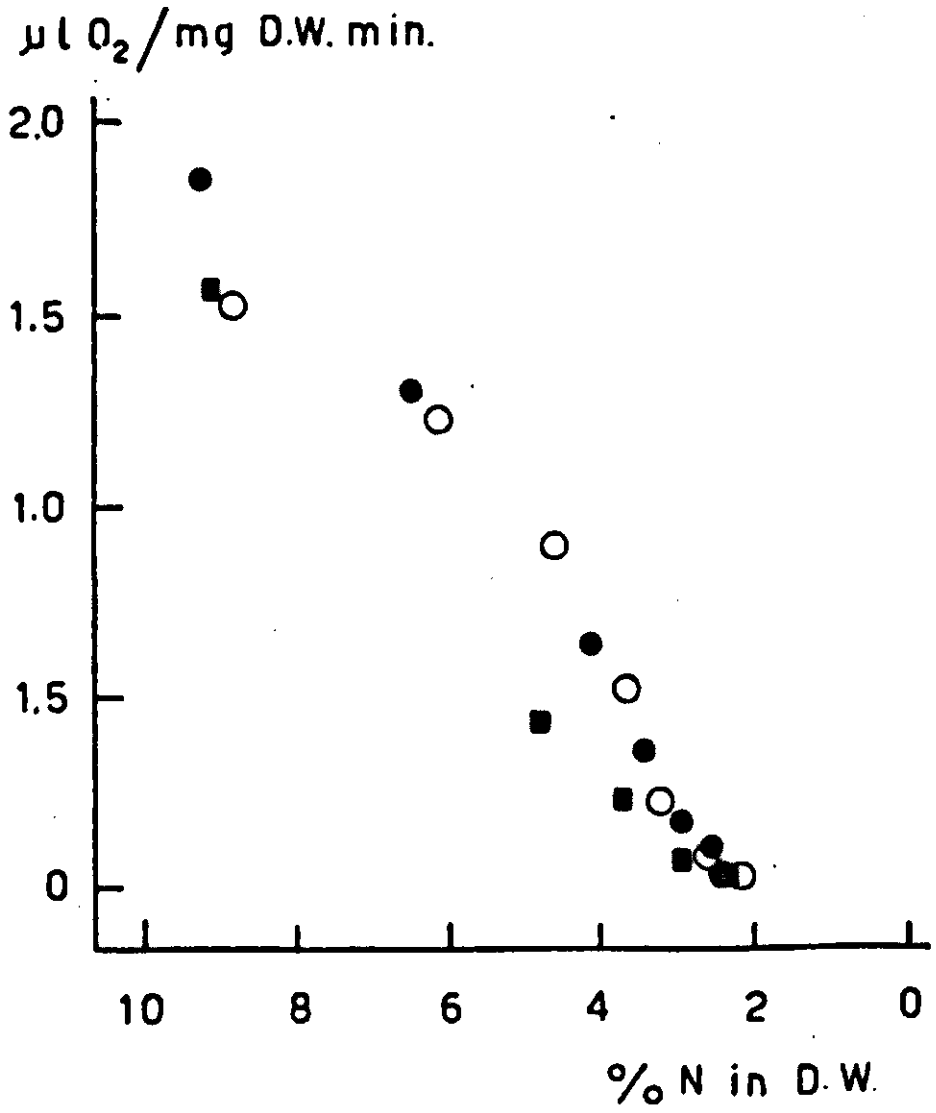


FIG. 6. Relation between the rate of photosynthesis at light saturation on dry weight basis, and the nitrogen content for three similar experiments

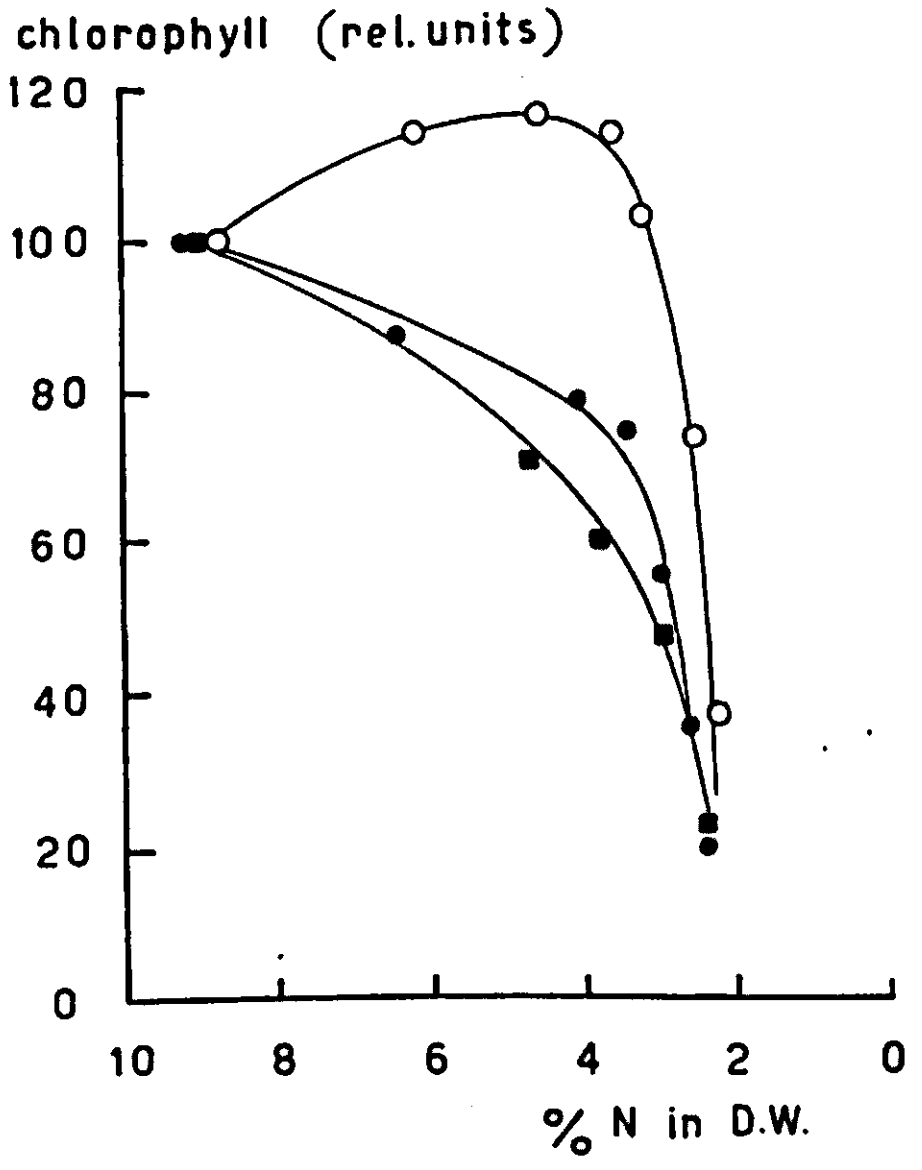


FIG. 7. Relationship between the amount of chlorophyll per unit volume of suspension and the nitrogen content of the dry matter (see also Table 4). Three similar experiments

Fig. 7 gives the relation between the quantity of chlorophyll per unit volume of the suspension, and the nitrogen content. From these results it is obvious that a critical situation is reached with a nitrogen content of 2 to 3 % on dry weight basis. At this point the activity of the cellular material is very low. The chlorophyll content has decreased to 0.29 % on dry weight basis, a very low value, so that light absorption is very low.

Thus, a remarkable coincidence exists between the moment at which the cells reach a nitrogen content of about 3%, that at which photosynthesis had decreased to a very low rate, and that at which parts A and B of the curves of fig. 5 intersect. Moreover, the approach of the minimum value of photosynthesis was accompanied by a marked chlorophyll decomposition.

In the region of 3–4% cellular nitrogen, the colour of the suspension changed from dark to pale green and finally to pale yellow.

b) Nitrogen enrichment of starved cells

Methods

By supplying soluble nitrogen to cell material, as obtained in the preceding experiments which was rich in carbohydrate but poor in nitrogen, a fast increase in nitrogen content of the cells could be achieved. During nitrogen starvation the total quantity of nitrogen per unit volume of cell suspension remained constant, and the variation of the nitrogen content per unit of dry matter was the result of the production of essentially nitrogen free material, mainly of carbohydrate nature.

Cells extremely poor in cellular nitrogen were transferred to a medium of the usual composition, containing KNO_3. The cultures were then flushed with air freed from CO_2 by inserting a soda-lime tube, and illuminated by a 500 Watt lamp. In order to estimate dry matter, cell nitrogen, chlorophyll, number of cells, and photosynthetic activity, samples were taken at different intervals during the period of the experiment (24 hours).

TABLE 5. Nitrate assimilation by nitrogen starved algae, in light (30 klux). Cultures flushed with air freed from CO_2. Increase in cell nitrogen given in mg N per 5 ml suspension. Photosynthesis measured as oxygen output in a 0.2 molar sodium bicarbonate buffer. Light intensity 0.60 cal/cm².min.; vessels containing 10 ml suspension.
Initial density 16.3 mg dry matter per 5 ml suspension. Temperature 30 °C

Time of enrichment (hours)	Dry weight		Nitrogen		Chlorophyll		On a dry weight basis		Relative number of cells
	mg/5 ml susp.	%	mg/5 ml susp.	%	mg/5 ml susp.	%	% nitrogen	% chlorophyll	
0	16.3	100	0.34	100	0.044	100	2.08	0.27	100
3	15.4	95	0.38	112	0.044	100	2.46	0.29	96
6	14.8	91	0.54	159	0.057	129	3.65	0.38	102
9	14.4	89	0.66	195	0.080	182	4.60	0.56	112
12	14.0	86	0.74	218	0.119	269	5.28	0.81	126
24	13.9	85	0.84	247	0.197	448	6.03	1.42	120

Time of enrichment (hours)	O_2-production at light saturation per 0.1 mg cell nitrogen		O_2-production at light saturation (μl)	O_2-production at light saturation		Dry weight per vessel (mg)
				per mg dry weight	per 0.1 mg chlorophyll	
0	0.53	25%	1.80	0.05 9%	2.05	32.6
3	0.47	22%	1.80	0.06 11%	2.05	30.8
6	1.02	48%	5.50	0.19 32%	4.80	29.6
9	1.91	89%	12.60	0.44 77%	7.85	28.8
12	2.14	100%	15.85	0.57 100%	6.65	28.0
24	1.85	87%	15.50	0.56 98%	3.95	27.8

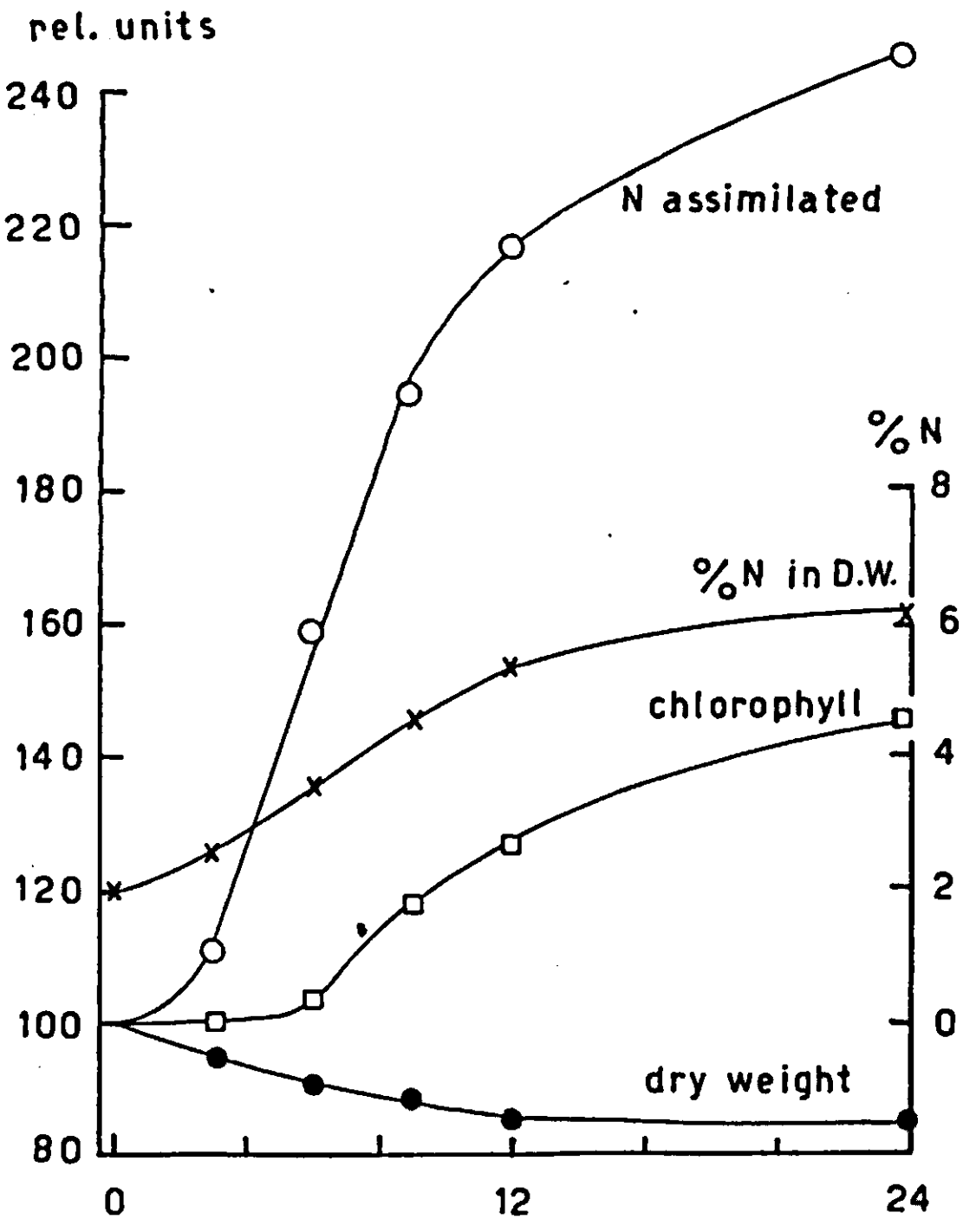
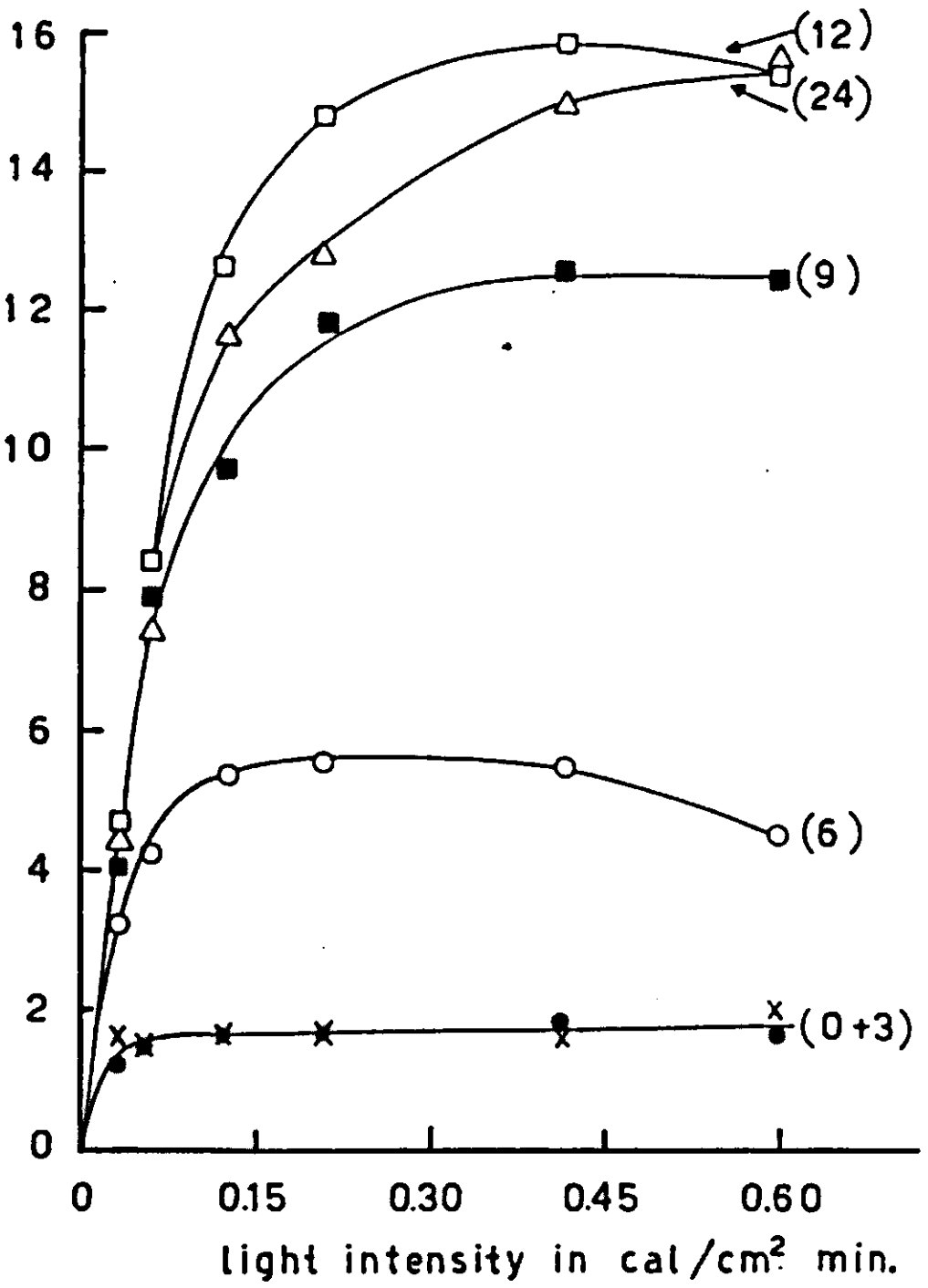


FIG. 8.
Increase in cell nitrogen, in nitrogen content, and in chlorophyll in a suspension of N-starved algae at various times after addition of 1.5 g KNO_3 per litre. Culture flushed with air freed from CO_2, and illuminated with 30 klux. Previous nitrogen starvation time 24 hours at a light intensity of 30 klux, in air with 5 % CO_2



Results

As may be seen in fig. 8, a significant increase in the amount of cell nitrogen results after NO_3-addition, while a slight decrease (15 %) in the quantity of dry matter occurs. In connection herewith, the nitrogen content in the dry matter increased from 2 to 6 %. Table 5, referring to this experiment, also shows some increase in the number of cells. The originally greenish yellow suspension again turned green by production of chlorophyll.

Fig. 9 gives the photosynthetic activity at different time intervals after the addition of KNO_3. After 6 hours a considerably increased rate of oxygen production appeared, and a maximum value was reached after 12 hours. At this time a slight

FIG. 9.
Light intensity rate curves of photosynthesis measured in a 0.2 molar bicarbonate buffer (pH: 8.7) at different times during nitrogen enrichment. Figures between brackets indicate the time of enrichment in hours. Conditions of N-starvation and enrichment as in fig. 8

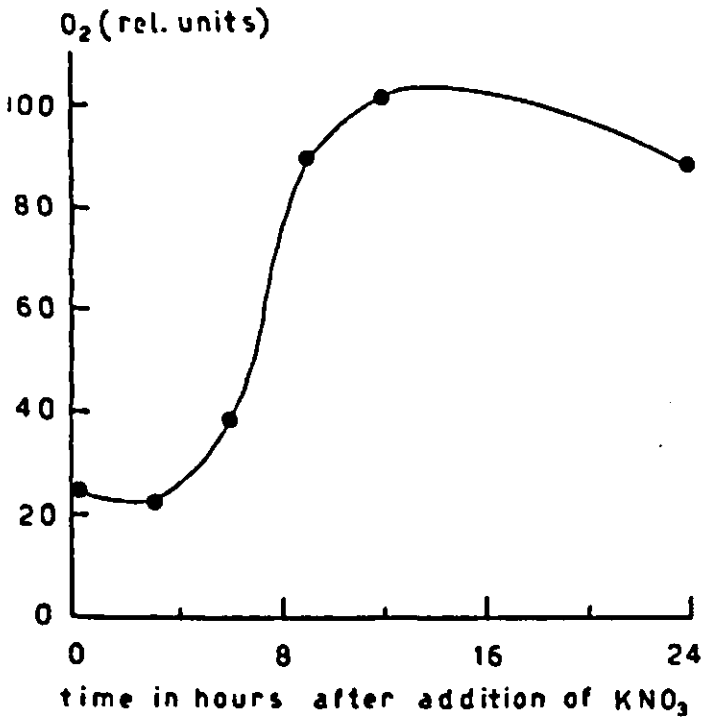


FIG. 10. Oxygen production rate in relative units at light saturation, recalculated per 0.1 mg cell nitrogen (data from fig. 9 and Table 5). Pretreatment as in fig. 8

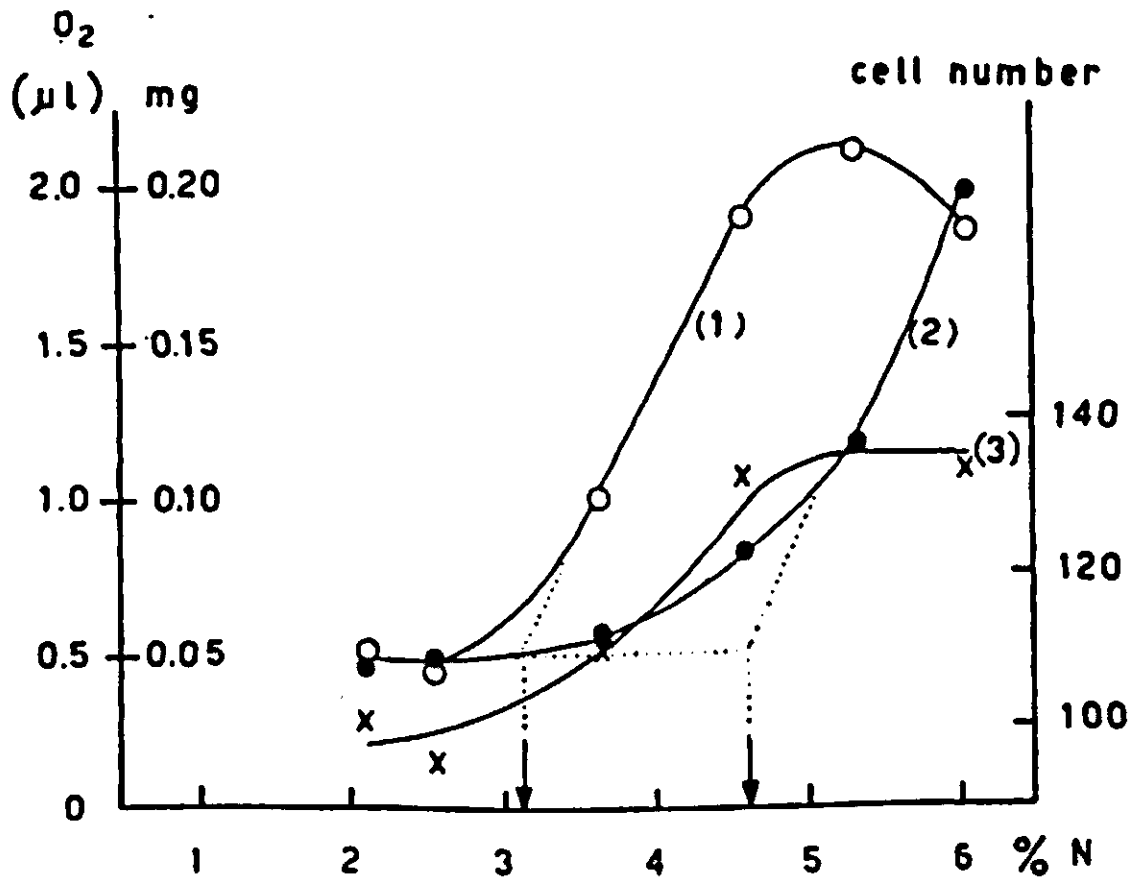


FIG. 11. Relation between nitrogen content on dry weight basis (abscissa) and oxygen output (per 0.1 mg cell nitrogen, curve 1), chlorophyll (per unit volume of suspension curve 2), and cell number (curve 3). Data from Table 5. Ordinates to the left: O_2 per 0.1 mg N, per min. (µl); chlorophyll (mg). Ordinate to the right: cell number (rel. units)

decrease in the rate of oxygen production could be observed. Probably, this must be attributed to the unfavourable action of strong light in the absence of carbon dioxide (photo-oxidation).

In this experiment the quantity of dry matter per unit volume remained almost constant (fig. 8), and the oxygen production at light saturation can be directly correlated with the amount of nitrogen per unit suspension. The results are shown in fig. 10. Similarly to fig. 5, in which the photosynthesis is found to be a linear function of the starvation time during the first 12 hours, we now find a nearly linear relation between the photosynthetic rate at light saturation and the time of nitrogen enrichment during 3–12 hours. After 12 hours of nitrogen enrichment, a constant level of oxygen production was reached. This can be explained as a result of the decrease of the production of cell nitrogen and/or the unfavourable action of strong light in the absence of carbon dioxide.

From the fact that the photosynthetic activity is gradually restored upon addition of nitrogen, it may be concluded that the reduced growth rate is not due to lack of any element other than nitrogen. The colour of the cells changes from pale yellow-green into deep green. The lag period found in fig. 10 (and also in fig. 11) probably is due to the fact that during a period of about 3 hours the photosynthetic apparatus is reorganized. After this induction period, however, we again observe a direct relationship between photosynthetic rate and nitrogen content, as was also evident in fig. 6.

Fig. 11 shows the relation between the nitrogen content and the number of cells, the chlorophyll production, and photosynthesis. The photosynthetic rate and the chlorophyll content are very low at a low nitrogen content. The critical limit of 2 to 3% nitrogen is obvious. An increasing nitrogen content from 3% upwards restores the photosynthetic activity, until a normal value is reached.

Chlorophyll production is not very quick until a nitrogen content of about 4.5 to 5% is reached. Below this nitrogen content a quick disappearance of chlorophyll was found during starvation (fig. 7).

§ 3. Discussion

The composition of algae is influenced to a high degree by the availability of nitrogen in the nutrient medium. This is easily understood since normally about half of the cell material consists of protein. When the nitrogen in the medium is exhausted, protein production will stop and, as photosynthesis continues, a shift towards exclusive production of nitrogen free compounds has to be expected, and is indeed observed. Experiments of Spoehr *et al.* (1949), Aach (1952), Fogg *et al.* (1953), and Collyer *et al.* (1954) provided evidence that the accumulation of these components in algae depends primarily on the nitrogen concentration of the nutrient medium. Fogg *et al.* (1953) suggest that in case of nitrogen deficiency the photosynthetic activity may deviate towards the production of reserve material, for instance lipids which are compounds of high energy content. Aach also holds the opinion that the reducing action of light is stored in lipids. The growth rate of material with a low nitrogen content has decreased to a very low level. This can be seen from figs 2 and 6.

The mentioned results are in good agreement with the findings reported by Kok (1952) and Aach (1952). Similar results have also been mentioned by Fogg *et al.* (1953) and Thomas *et al.* (1955). The reduced growth rate is intimately connected with the decrease of the nitrogen content of the cell-material. Since at the same time decomposition of chlorophyll occurs, the amount of absorbed light will also diminish. This is not the only cause of a reduced growth rate as was proved by the experiments of Kok (1952) and Aach (1952). They made probable that also the utilization of the absorbed light was decreased in nitrogen starved cell material.

Fig. 11, however, demonstrates that the increase in photosynthetic rate does not only depend on the increase in chlorophyll. No rapid chlorophyll production is observed until the nitrogen content has reached 4.5%. Then, however, the rate of photosynthesis has already reached more than twice its original value. Moreover, fig. 7 shows that a quick chlorophyll disappearance starts at a nitrogen content of about 4 to 5%. Table 4 shows a decrease of the photosynthetic rate to 14% of the original value after 12 hours of exposure to light in the absence of nitrogen, whereas the chlorophyll content of the suspension was still undiminished. So it seems as if the decreased growth rate is effected to a greater extent by lack of nitrogen than by the chlorophyll decomposition. The results of Pirson (1937), Aach (1952) and Thomas *et al.* (1955), are in good agreement with these findings. The influence of nitrogen starvation and subsequent nitrogen enrichment upon the photosynthetic rate was studied by Van Hille (1938). The growth rate in his experiments appeared to be lower than in our cultures, while the minimum photosynthetic value was reached only after a number of days. Notwithstanding these differences, his results are in agreement with ours. Our experiments confirm his conclusion that the recovery of the photosynthetic activity during nitrogen enrichment was not due to chlorophyll synthesis.

Restoration of photosynthetic activity by nitrogen addition has not been found by Hamner (1936), who studied the effect of nitrogen supply on nitrogen

deficient young tomato and wheat plants. In his experiments the respiration rate increased and chlorophyll production occurred after addition of nitrate, but the carbon dioxide assimilation per unit leaf area was only slightly influenced. The conditions of these experiments and the plants used differ so much of those used in the experiments discussed above, that a comparison seems hardly feasible.

From the experiments described in this Chapter it is obvious that rapid growth and a high lipid content of the material hardly occur simultaneously. Increased photosynthetic activity in starved cells after re-addition of nitrogen is evident from figs 9 and 10. The relationship between nitrogen content and photosynthetic rate is clear.

CHAPTER III

THE INFLUENCE OF SOME METABOLIC FACTORS ON NITROGEN ASSIMILATION

As has been demonstrated in Chapter II, § 2*b*, nitrogen starved cells are able to quickly restore their nitrogen content to its normal level upon addition of nitrate. It seemed, therefore, of interest to study the relation between the degree of nitrogen starvation during the pretreatment and the time course of the assimilation after renewed nitrogen supply. By adding either NO_3^- or NH_4^+, this procedure also allows a study of the nitrate reducing reaction.

§ 1. The influence of carbohydrate reserves on nitrogen assimilation

As was shown in the preceding Chapter, algae cultivated in nitrogen free media usually are relatively rich in carbohydrates. On the contrary, algae grown in complete media are relatively poor in carbohydrates. The rate of nitrogen assimilation in both types of cultures under different conditions will now be discussed.

For a preliminary experiment, the treatment of the algae before and afterwards was as follows:

Two parallel batches of algae of 150 ml each were used; the first (A) was kept in darkness, while the other (B) was exposed to light in an N-free suspension medium. During 24 hours both samples were flushed with air enriched with 5 % CO_2. Thereafter, 1.5 g KNO_3 was given to both samples, after which portions of 50 ml were taken from each sample and treated as indicated in Table 6, p. 20.

The reason why suspension A was kept in darkness was to maintain the quantity of carbohydrate at an approximately constant level during the pretreatment. Owing to respiration, a slight decrease of dry weight per unit of cell suspension occurred. This procedure was followed because of its simplicity; "normal cells" can just as well be obtained by giving light and a nitrogen source during the pretreatment. In that case, however, it would be necessary to dilute the culture of "normal cells" to the original density, since during the experiment equal quantities of cell nitrogen per unit volume of cell suspension are to be considered. The above procedure avoids this difficulty.

Table 6, and fig. 12, a and b, give the results of this experiment. Only small quantities of nitrate are assimilated by suspension A if kept in darkness (curve I).

TABLE 6. Dry matter and cell nitrogen production by algae pretreated as A and B (see text). Conditions of cultivation of samples A: I dark, air minus CO_2; II light, air minus CO_2; III light, air with 5% CO_2.
Conditions of cultivation of samples B: IV light, air with 5% CO_2; V light, air minus CO_2; VI dark, air minus CO_2. Light intensity: 30 klux; temperature 30 °C. Data in mg per 5 ml suspension.

Time in hours	Number of culture (see text)											
	I		II		III		IV		V		VI	
	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%
	Cell nitrogen											
0	0.68	100	0.68	100	0.67	100	0.68	100	0.69	100	0.68	100
1½	0.69	102	0.69	102	0.77	115	0.80	117	0.82	119	0.81	119
3½	0.69	102	0.73	107	0.90	134	1.06	156	1.04	151	1.05	154
5½	0.70	103	0.75	111	1.01	151	1.30	191	1.30	188	1.26	185
7½	0.73	108	0.75	111	1.16	173	1.48	218	1.48	215	1.39	205
9½	0.74	109	0.74	109	1.30	194	1.62	238	1.56	226	1.47	216
11½	0.74	109	0.75	111	1.50	224	1.70	250	1.60	232	1.48	218
	Dry weight											
0	6.9	100	7.2	100	7.1	100	27.1	100	26.9	100	27.2	100
1½	6.8	99	7.3	101	12.1	171	27.3	101	26.6	99	25.9	95
3½	5.9	86	6.9	96	12.9	182	26.7	98	26.8	100	23.8	88
5½	6.3	91	7.4	103	14.9	210	27.3	101	27.1	101	21.7	80
7½	6.1	88	7.9	110	16.6	234	27.8	103	26.5	99	19.8	73
9½	6.2	90	7.1	99	17.6	248	28.2	104	26.2	97	19.0	70
11½	5.8	84	7.3	101	18.6	262	29.1	107	26.3	98	18.1	67
	Nitrogen content in % of dry weight											
0	9.9		9.5		9.4		2.5		2.6		2.5	
1½	10.1		9.5		6.4		2.9		3.1		3.1	
3½	11.7		10.6		7.0		4.0		3.9		4.4	
5½	11.1		10.1		7.8		4.8		4.8		5.8	
7½	12.0		9.5		7.0		5.3		5.6		7.0	
9½	12.1		10.4		7.4		5.7		6.0		7.7	
11½	12.7		10.3		8.1		5.8		6.1		8.2	

Exposed to light in the absence of CO_2 the rate of nitrate assimilation of these cells was also negligible (curve II). However, exposure to light and flushing with air containing CO_2 caused an intense nitrate assimilation (curve III). Under the latter conditions production of dry matter occurs (Table 6) and thus the increase in cell nitrogen is to be considered as a normal growth phenomenon.

Cells originating from suspension B exhibited a rapid nitrate assimilation under all three experimental conditions. In darkness (curve VI) no production of dry matter occurred; on the contrary, a decrease in dry weight was observed. The cell suspension in light in the absence of CO_2 (curve V) shows no significant change in the quantity of dry matter during the experiment. The fraction of suspension B supplied with both light and CO_2 showed a small increase in dry matter, which however, did not appear until the experiment had lasted 7½ hours. The nitrogen content reached at that moment was nearly equal to that of normal cells. Therefore, after this moment curve IV can be compared with curve III.

We may conclude from the comparison of curves IV and VI that the rate of nitrate assimilation of nitrogen starved algae is neither influenced by light

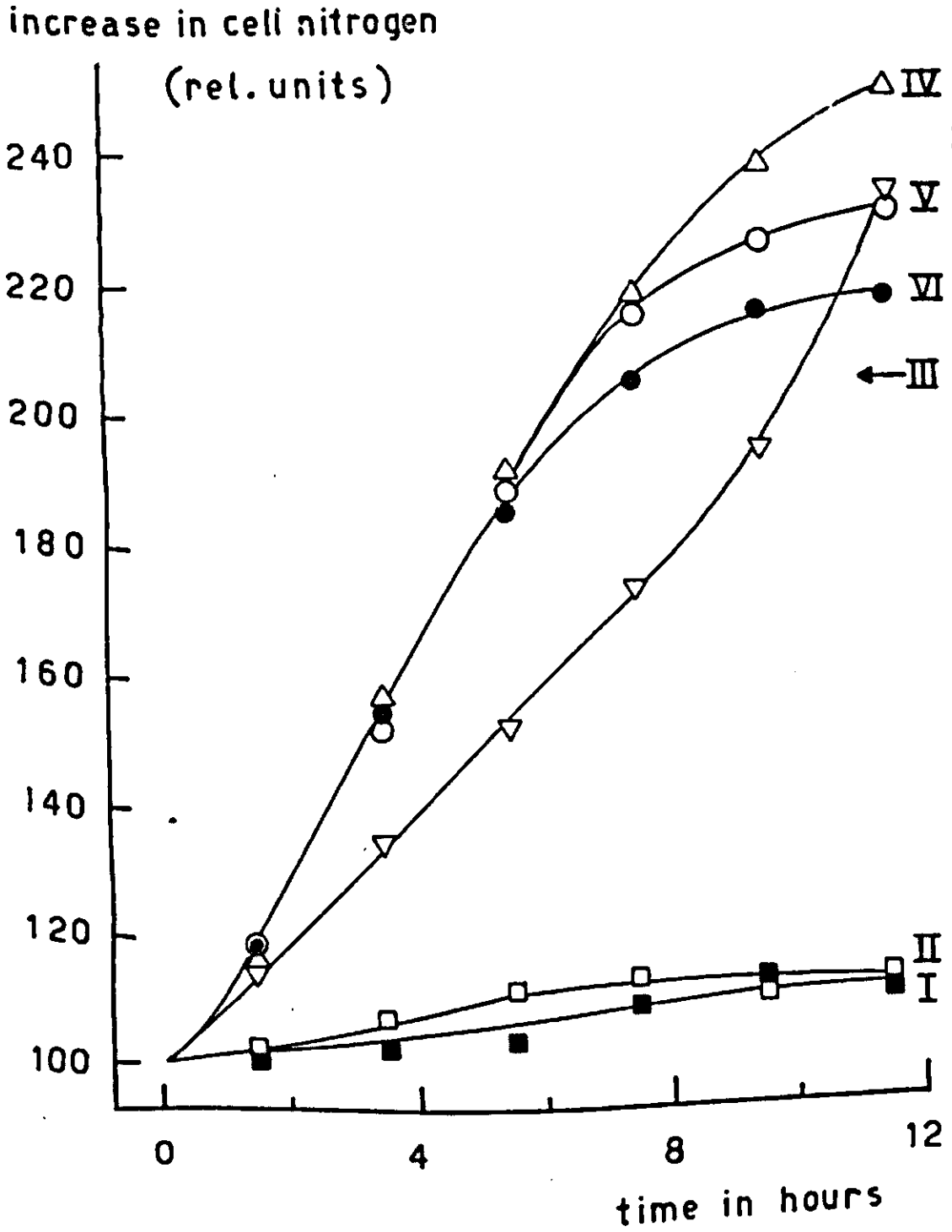


FIG. 12a

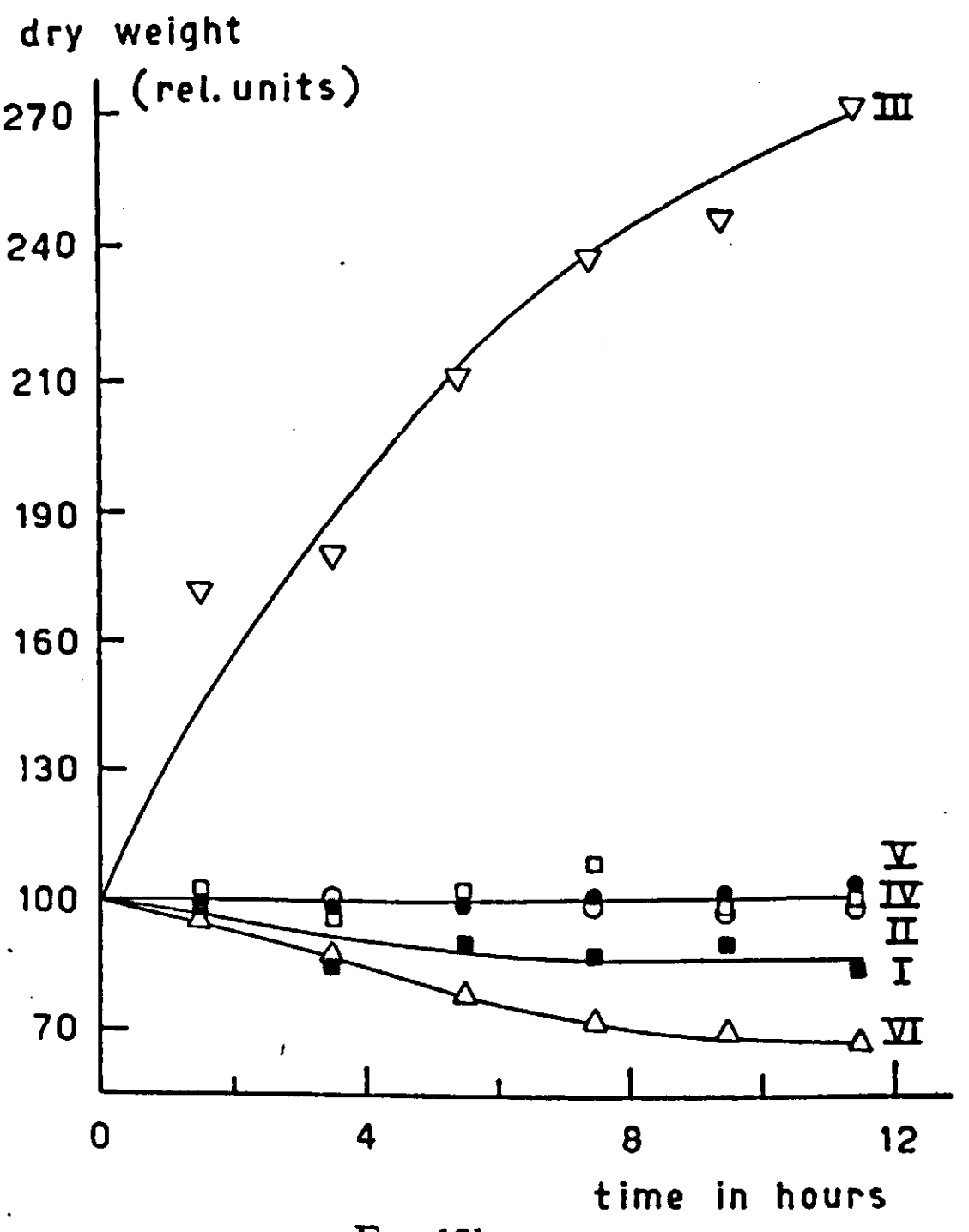


FIG. 12b

FIG. 12a. Time course of N-assimilation by algae pretreated in different ways in a nitrate containing suspension medium, under different conditions. Pretreatment A: in darkness during 24 hours, in a nitrogen free medium; B: illuminated at 30 klux in a nitrogen suspension medium during 24 hours. Both cultures flushed with air + 5% CO_2

Curve I – N-assimilation rate in dark, air minus CO_2 pretreatment A
" II – " " " light air " CO_2 " A
" III – " " " " air + 5% CO_2 " A
" IV – " " " " air + 5% CO_2 " B
" V – " " " " air minus CO_2 " B
" VI – " " " dark, air " CO_2 " B

FIG. 12b. Time course of dry matter production. Legend as in fig. 12a

nor by CO_2, during the first 6 hours after re-addition of nitrogen. Internal reserves appear necessary for nitrogen assimilation without growth. Such reserves were obtained by treating the cells with light (suspension B) in N-free media. It seems, therefore, of interest to investigate the relation between the degree of N-starvation (or carbohydrate enrichment) in light and the nitrate assimilation these cells show afterwards in darkness.

Variations in carbohydrate content can be obtained in two ways. First, by varying the duration of the exposure at constant light intensity and secondly, by applying a series of light intensities during a definite time of exposure. The latter method has the advantage of simultaneous treatment of all series and was therefore mostly used.

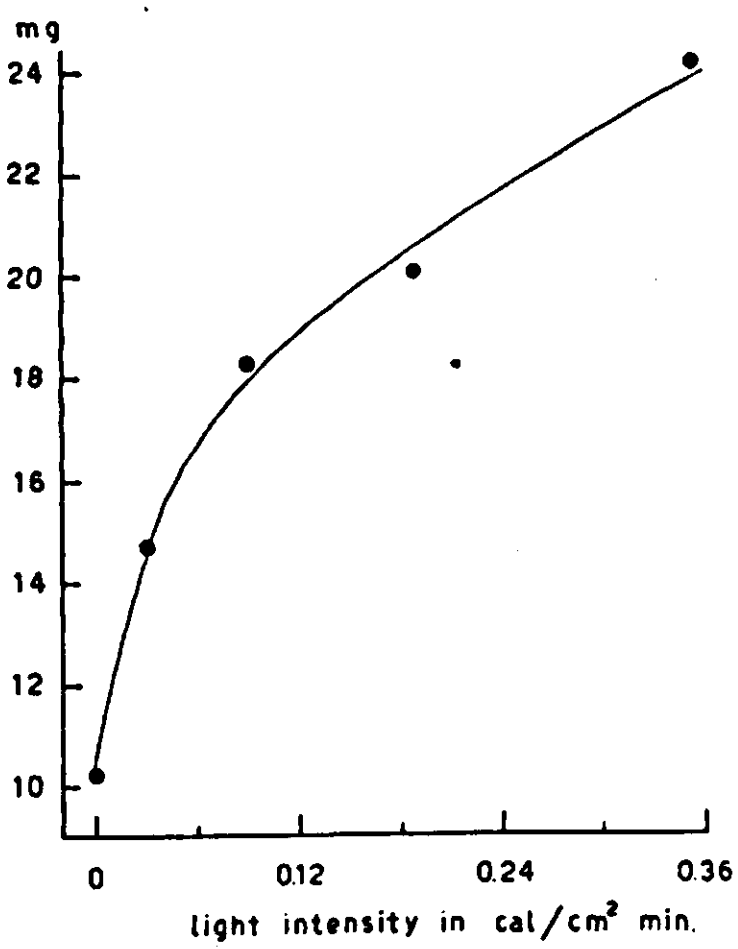


FIG. 13.
Dry weight production of algae, in mg/5 ml suspension, in an N-free medium, during 10 hours, at various light intensities, at 30 °C. Experiments with 35 ml cultures; initial culture density 2 mg dry matter per ml. Cultures flushed air with + 5 % CO_2

The experiments were carried out in a thermostat at a temperature of 30 °C, in which 5 identical 150 ml ERLENMEYER flasks – each containing 35 ml suspension – were placed and exposed to white light of different intensities which were obtained by using wire screen filters. The cultures were continuously shaken and flushed with air containing 5 % CO_2 (see also KOK *et al.*, 1954).

Fig. 13 shows the increase of dry matter during carbohydrate enrichment in a medium without nitrogen at different light intensities including darkness. No light saturation is obvious, which may be attributed to mutual shading of the cells, since dense cultures were used (2 mg dry matter per ml). After 10 hours the cells from the cultures were transferred to fresh media containing 1.5 g KNO_3

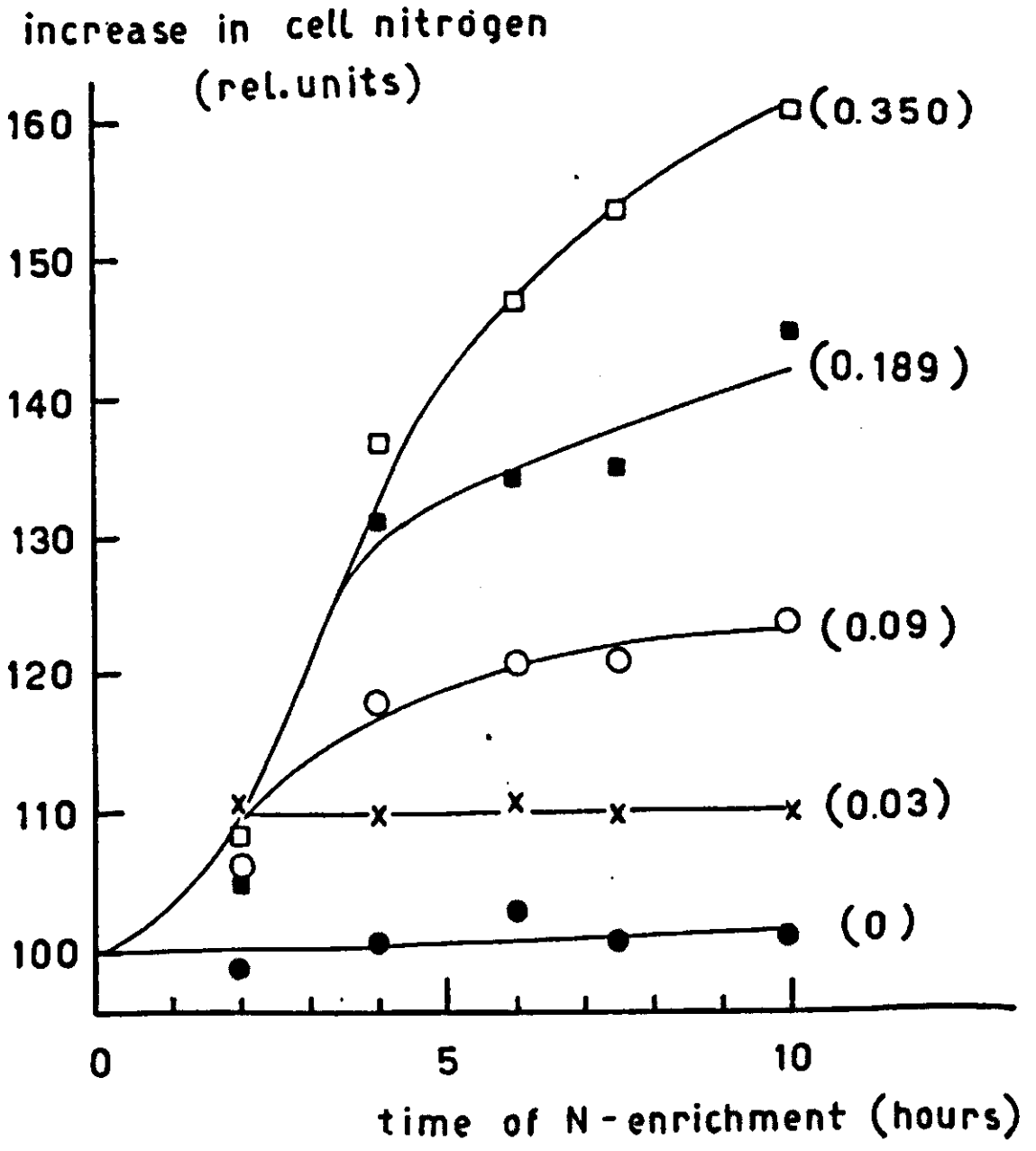
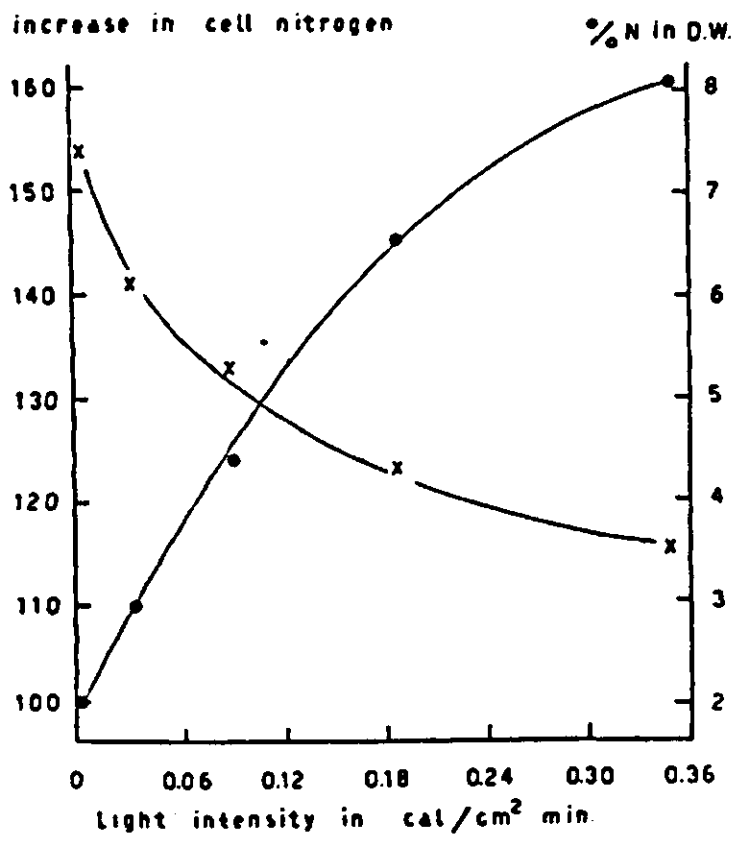


FIG. 14.
Time course of nitrogen assimilation in darkness by N-starved cells after re-addition of 1.5 g KNO_3 per litre. Figures between brackets indicate light intensities (cal/cm²min.) at which nitrogen starvation took place

FIG. 15.
Relation between the amount of nitrogen assimilated during 10 hours in darkness after re-addition of nitrate, and the light intensity during the preceding nitrogen starvation period (data from fig. 14). Crosses indicate the nitrogen content on dry weight basis of the material at the end of the starvation period (see text)



per litre and brought into darkness. The rate of N-incorporation of the 5 cultures was then followed by determining cell nitrogen, with the KJELDAHL method at definite time intervals.

Fig. 14 gives the results of an experiment of this type. As already shown in the preliminary experiment (fig. 12), the cells kept in darkness during the pretreatment did not show any nitrogen uptake. However, even the lowest light intensity used in this experiment (0.030 cal.cm^{-2}.min.$^{-1}$) already caused a small nitrogen assimilation in the subsequent dark period. The total uptake of this sample was already reached after 2 hours. Cells pretreated at higher light intensities showed concomitantly larger uptake of nitrogen. This is understandable: at low light intensities, the degree of N-starvation was small (6% N in dry weight, fig. 15), since only a small amount of dry matter was formed (fig. 13); at high light intensities, however, the nitrogen content has dropped to a lower value (3.5%, fig. 15) since much more dry matter is formed (fig. 13). It is obvious that the total amount of nitrate assimilated is greater, the lower the nitrogen content had dropped during the preceding starvation. The initial rate of NO_3-assimilation is, however, independent of the degree of pre-starvation. An obvious "light saturation" could not be observed at the highest light intensity. Indeed, this could not be expected after the comments already made on fig. 13. However, the relation of nitrate assimilation to carbohydrate reserves is evident.

§ 2. NITROGEN ASSIMILATION RATES OF NITROGEN STARVED CELLS AND OF NORMAL CELLS

We now want to compare the capacity of the nitrogen assimilating enzyme system of starved cells (tested in the dark as described) and of normal cells growing under optimal conditions. The procedure in this experiment was as follows: Two identical quantities of cells were transferred into a nitrogen deficient nutrient solution. One of the samples was exposed to a saturating light intensity (0.350 cal.cm^{-2}.min.$^{-1}$), the other was kept in darkness. To prevent possible injury to the cells at the high light intensity, the duration of the starvation was only brief, viz., 10 hours. Then both samples were supplied with KNO_3, and subsequently the suspension first kept in darkness was now exposed to light (0.350 cal.cm^{-2}.min.$^{-1}$), and, reversely, the suspension which had been exposed to light during the pretreatment, was now put in darkness. During the 20

hours the experiment lasted, both cultures were flushed with air containing 5% CO_2. The experiment was carried out at 30°C. At regular time intervals samples of both cultures were taken for determining dry weight and cell nitrogen by the KJELDAHL method.

TABLE 7. Nitrate assimilation and dry matter production by algae treated as follows:
Culture I: 0 to 10 hours: light, NO_3-free medium;
10 to 20 hours: dark, NO_3-containing medium.
Culture II: 0 to 10 hours: dark, NO_3-free medium;
10 to 20 hours: light, NO_3-containing medium.
Cultures flushed with 5% CO_2 in air during the time of experiment. Data in mg per 5 ml suspension.

Time (hrs.)	0	10	12	14	16	17½	20
			Dry matter				
Culture I	13.0	24.2	21.8	21.0	18.1	17.4	17.1
Culture II	13.0	12.3	14.4	16.4	20.3	22.6	30.1
			Cell nitrogen				
Culture I	0.87	0.87	0.94	1.10	1.28	1.33	1.40
Culture II	0.88	0.90	0.95	1.16	1.30	1.48	1.76
			Nitrogen content in % of dry weight				
Culture I	6.7	3.6	4.3	5.7	7.1	7.6	8.2
Culture II	6.7	7.2	6.6	7.1	6.4	6.5	5.9

Table 7 shows the schedule of experimentation and the results, while fig. 16 presents the nitrate assimilation rates for both cultures. During the pretreatment (0–10 hours) the quantity of nitrogen did not change, because there was no nitrogen available. After 10–20 hours, however, a fast nitrogen assimilation came through. The algae which had been irradiated in the absence of nitrogen during the pretreatment had a nitrogen content of 3.6% in dry weight and exhibited a rapid nitrate assimilation in darkness until a nitrogen content of about 7 to 8% was reached. The cells cultivated in the presence of a sufficient amount of nitrogen showed a fast production of dry matter under optimal growth circumstances, and, as a consequence, a fast nitrate assimilation too. The relative nitrogen content in these cells remained at a constant level. Fig. 16 gives the nitrate assimilation rate for both cultures. It is evident that this rate for the starved cells is equal to that of growing cells during the first 5 hours of assimilation, after which the assimilation rate of starved cells decreases.

From this experiment we conclude that the capacities of the "dark system" and of the "light system" are equal.

Using ammonium instead of nitrate as a nitrogen source, a different picture is obtained, as represented in fig. 16a. Now the assimilation rate for nitrogen in cultures of nitrogen starved cells is much faster than is found with normal cells under conditions of optimal growth. In normal cells the growth rate must be the limiting factor for the incorporation of nitrogen. We may assume that in nitrogen starved cells, the initial assimilation rate of nitrogen is determined by the maximum capacity of the enzyme system, involved in the incorporation of nitrogen. One might even consider the possibility that the mentioned rate is still not attained because of diffusion limitations. At any rate it seems clear that the maximum rate for the incorporation of nitrogen surpasses the requirement of normal algae, growing even in saturating light.

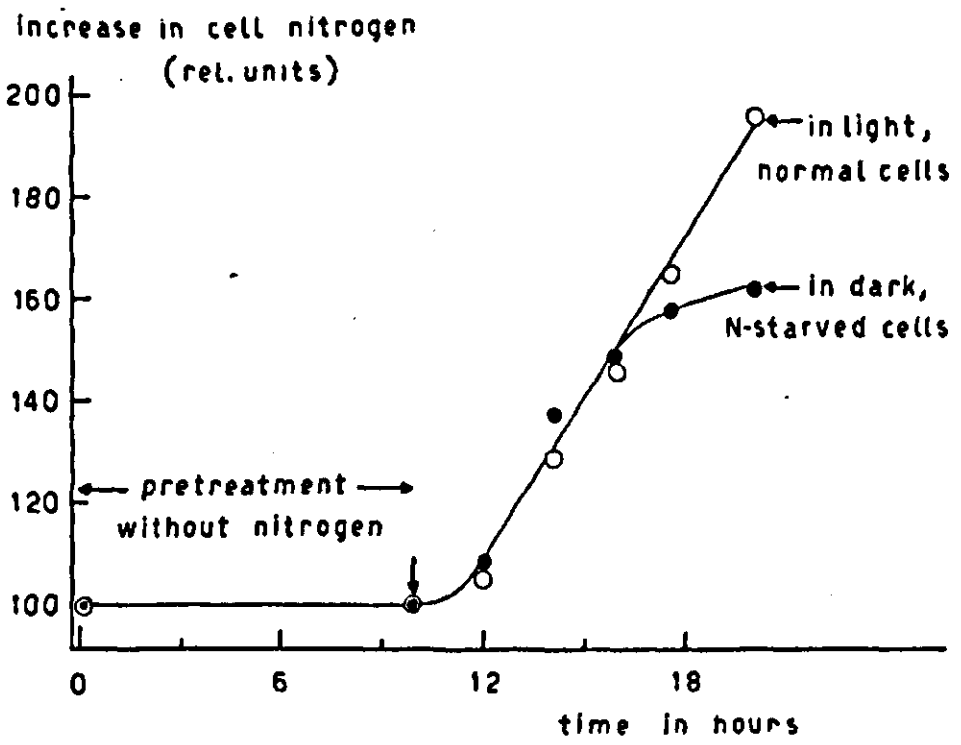


FIG. 16

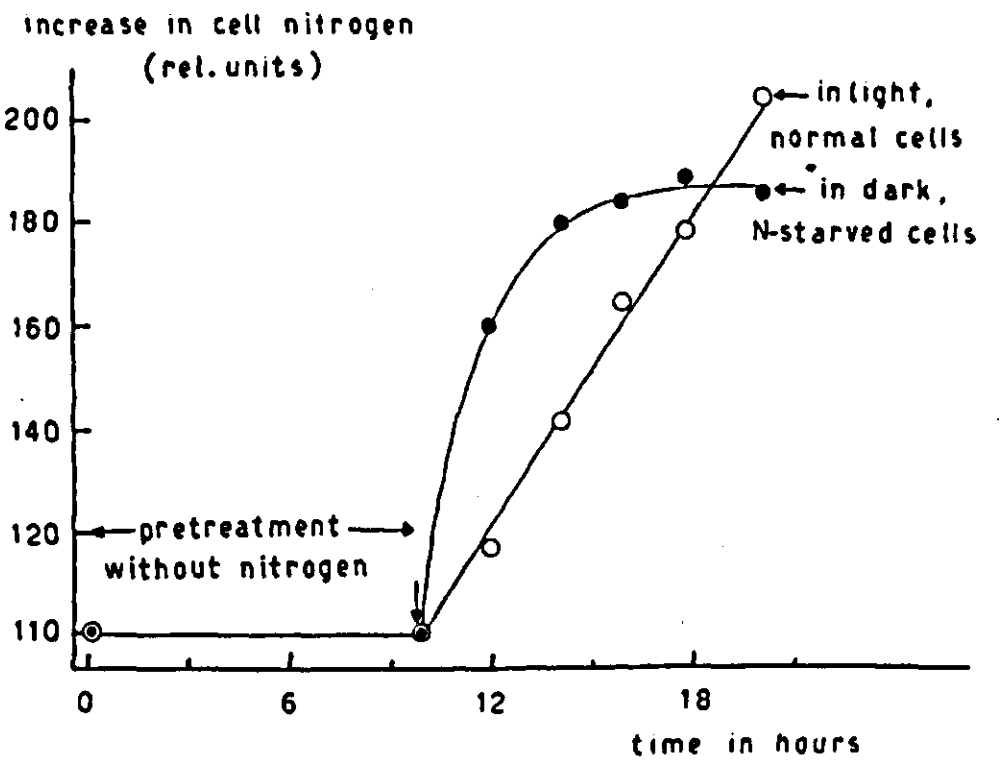


FIG. 16a

FIG. 16. Nitrogen assimilation rate of normal cells in light (0.35 cal/cm²min.) and of N-starved cells in darkness. During the pretreatment (0–10 hours in an N-free medium) one sample was kept in darkness and the other illuminated (0.35 cal/cm²min.). After 10 hours both cultures were supplied with 0.21 g/l nitrate-N. The dark culture (normal cells) was then illuminated (0.35 cal/cm²min.) and the light culture (N-starved cells) was given darkness. Both cultures flushed with air + 5% CO_2. ↓ KNO_3 added

FIG. 16a. Data as fig. 16, but instead of KNO_3, 0.21 g/l ammonium-N was given. ↓ $(NH_4)_2SO_4$ added

§ 3. NITROGEN ASSIMILATION AS INFLUENCED BY PH

For the strain used (*Scenedesmus spec.*), growth was optimal about pH 7. We maintained the pH at 7.0 ± 0.5 during periods of normal growth and during those of nitrogen starvation. During nitrogen assimilation by nitrogen starved cells light was usually given. Under these conditions and in case KNO_3 or KNO_2 is supplied nitrogen assimilation will cause the formation of hydroxyl ions which will tend to raise the pH of the suspension. If, on the other hand, ammonium salts are used as nitrogen source, the pH will shift towards lower values as a consequence of the formation of hydrogen ions. The suspensions therefore were buffered in order to avoid changes in pH.

Citrate-phosphate buffers were used for the range of pH 3 to 5, phosphate buffers for pH 5.5 to 7.5 and trisbuffer[1]) for the range pH 8.0–11.5. The buffer concentration was 1/15 molar in all cases. The nitrogen sources KNO_3, KNO_2 and $(NH_4)_2,SO_4$ were added in a concentration of 0.21 g nitrogen per litre. During the experiments the pH was regularly controlled and, if necessary, readjusted to the correct value by the addition of small quantities of either NaOH or H_2SO_4.

The results obtained with KNO_3 as an N-source are represented in fig. 17, those with KNO_2 in fig. 18, and those with $(NH_4)_2SO_4$ in fig. 19. The figures between brackets indicate the pH values of the suspensions. Fig. 20 demonstrates the relation between pH and maximum nitrogen assimilation. Obviously, pH 7

[1]) As this buffer mixture (trishydroxymethylamino methane from the Sigma Chemical Company, St. Louis, Miss., U.S.A.), contains nitrogen, it had to be investigated whether this nitrogen would be used by the cells instead of the nitrogen source intended to be checked. By adding trisbuffer to nitrogen starved cells, in the absence of other N-sources, it could be proved that such a utilization did not occur.

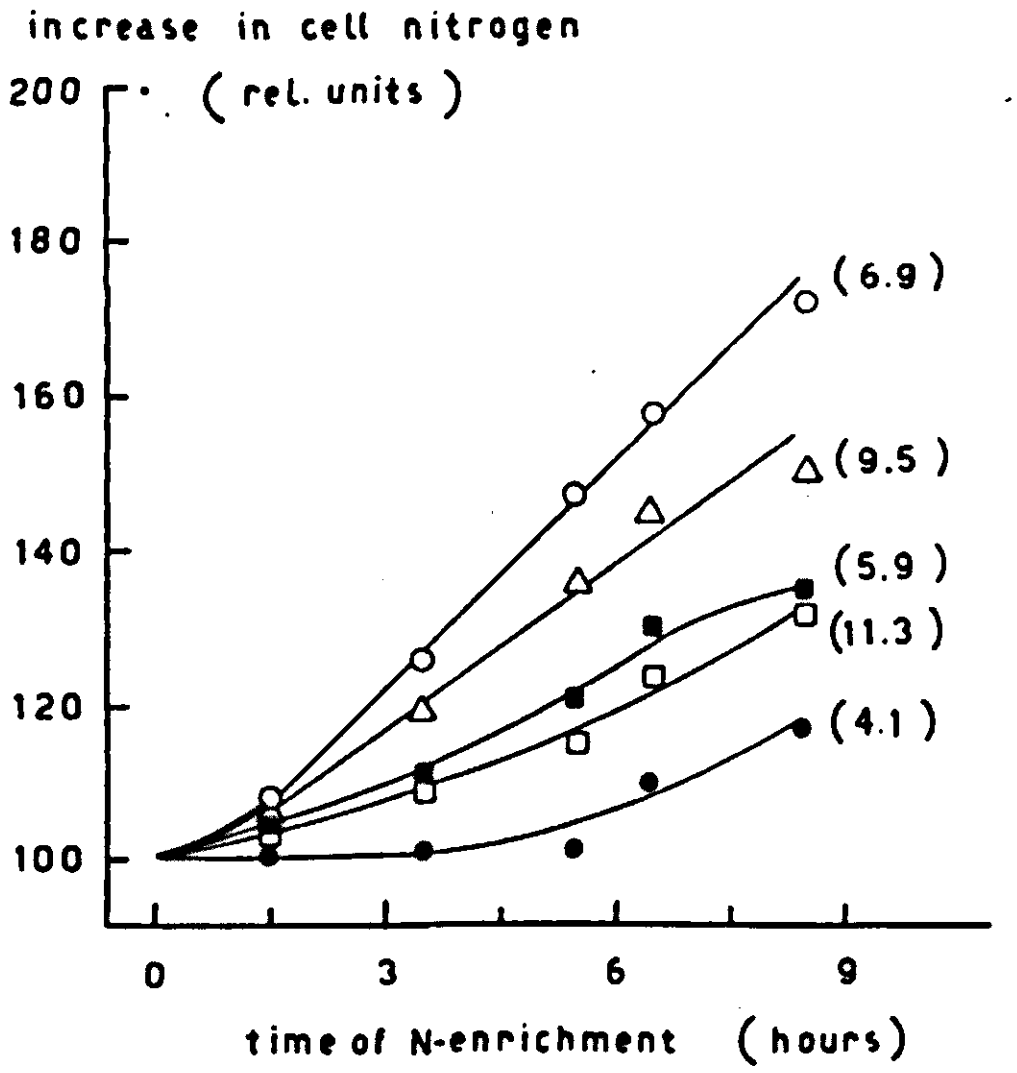


FIG. 17. Time course of nitrogen assimilation by nitrogen starved algae, at different pH values (figures between brackets) in nitrate containing media

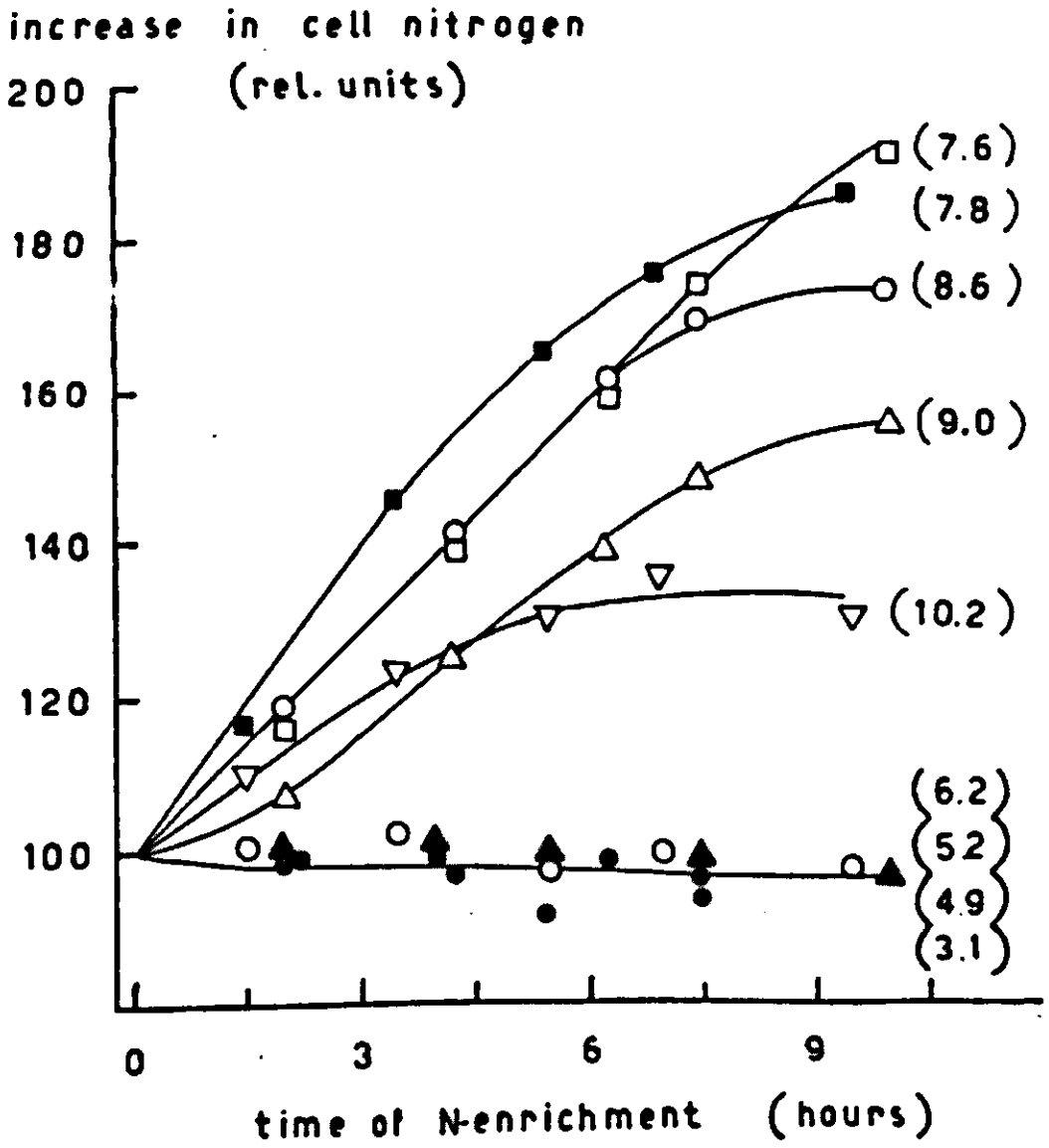


FIG. 18. Same as fig. 17, in nitrite containing media

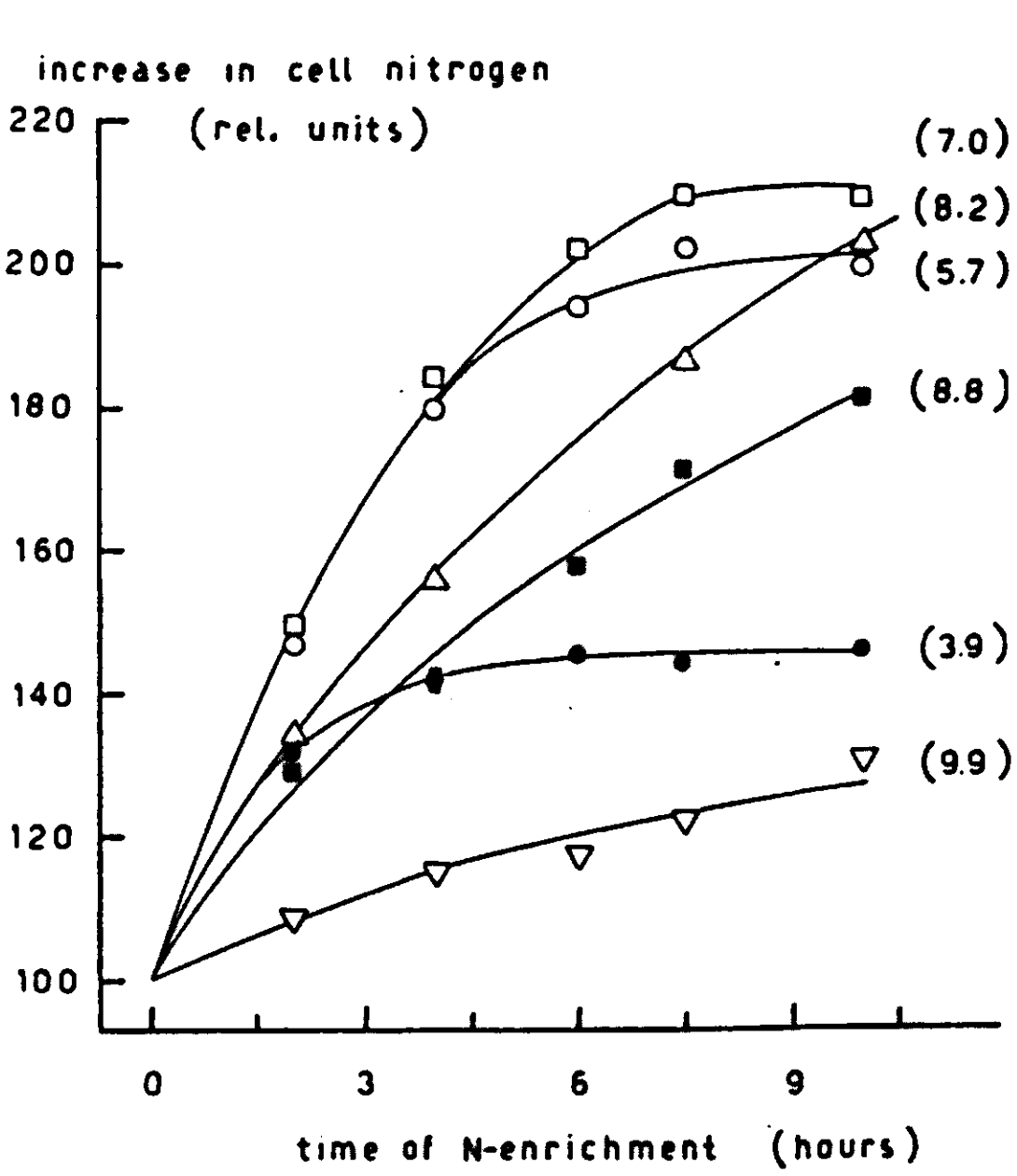


FIG. 19. Same as fig. 17, in ammonium containing media

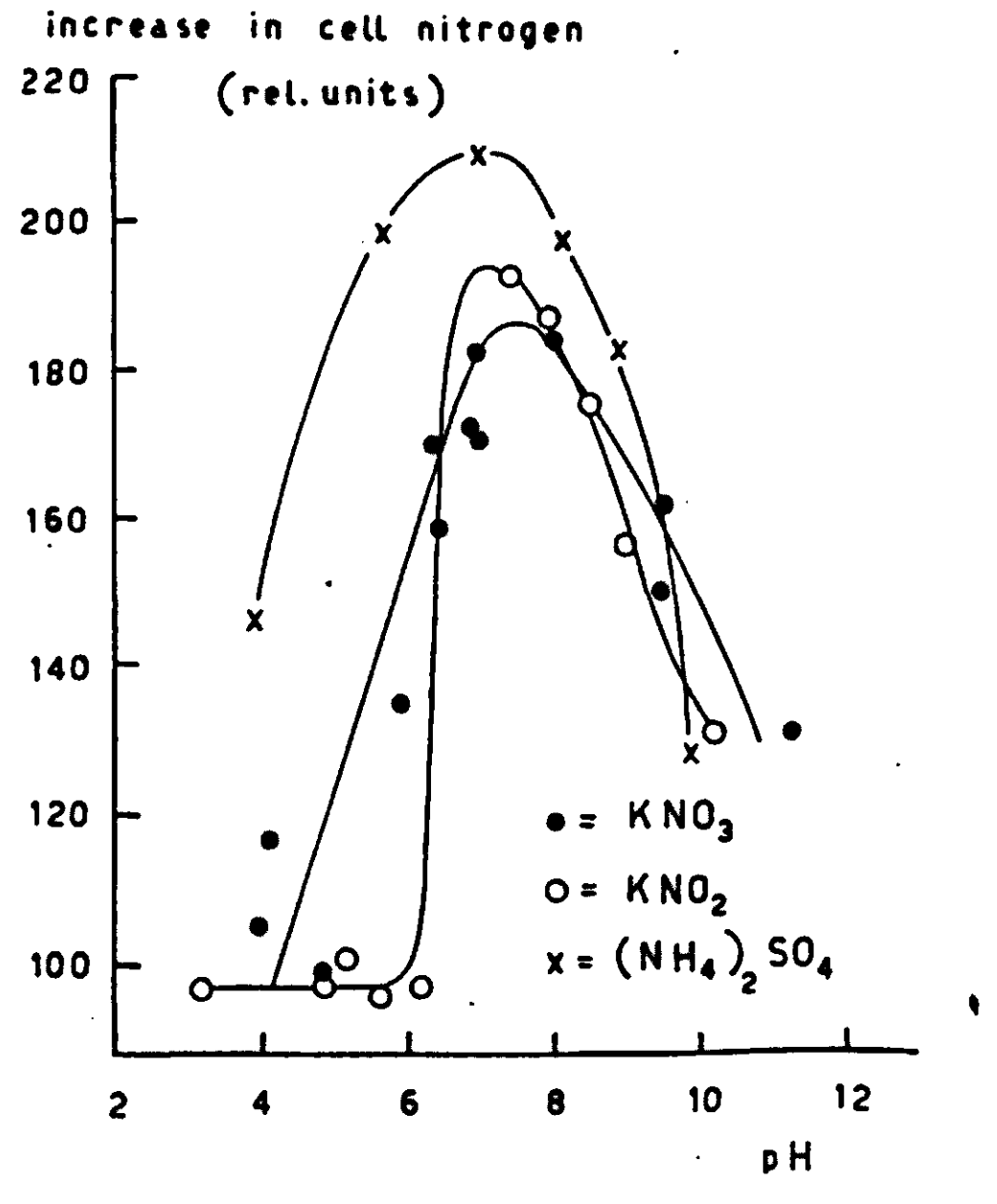


FIG. 20. Relation between pH and the amount of N assimilated at the end of the experiment

to 8 establishes the most favourable conditions with each of the three nitrogen sources used. In the case of KNO_2 the cells become very sensitive to a pH decrease below pH 7.5 to 7. Thus at pH 6.2 no nitrogen assimilation was observed at all and at pH 5 to 3 the colour of the cells rapidly turned to a dark brown.

The fact that the cells showed about the same pH optimum of the assimilation rate for all three nitrogen sources compared was fortunate since this enabled us to compare the use of these nitrogen sources at the same pH value. In further work a concentration of 1/15 molar trisbuffer was used for stabilization of the pH.

§ 4. NITROGEN ASSIMILATION AS INFLUENCED BY LIGHT AND DARKNESS

WARBURG *et al.* (1920) with green algae found that nitrate reduction was much faster in light than in darkness. MENDEL *et al.* (1951) demonstrated with tomato leaf disks that also in this case nitrate reduction was accelerated by light.

In these experiments, however, it is not taken into consideration that there must be a certain need for nitrogen, and an energy source for the reduction in case nitrate serves as a nitrogen source. Therefore, in our experiments we have used cultures of N-starved cells for the study of the effects of light and darkness in nitrogen assimilation.

The nitrogen starved cells were obtained from cultures which were cultivated for 24 hours in nitrogen free media. During the starvation period the cultures were supplied with air + 5% CO_2, and exposed to an incandescent lamp of 500 Watt with a light output of 0.25 $cal.cm^{-2}.min.^{-1}$. Under these conditions the nitrogen content dropped from about 8% to 2 or 3% N in the dry weight. Samples of this cell material were exposed to light (of the same intensity as used during the starvation period) or incubated in darkness after re-addition of nitrogen. The cultures were now flushed with air freed from CO_2 to stop growth. During the about 8 hours the enrichment in nitrogen lasted, 5 ml samples of both cultures were taken at suitable time intervals and the cell nitrogen was determined by the KJELDAHL method.

In this way we obtained an idea of the rates at which the different nitrogen sources were assimilated by the cells. The influence of light and darkness may be seen for KNO_3 in fig. 21, for KNO_2 in fig. 22, and for $(NH_4)_2SO_4$ in fig. 23.

The experiments with KNO_3, represented in fig. 21, were made with different samples of cell material but carried out under identical conditions; Table 8 also

TABLE 8. Slopes of curves of different experiments in light and dark. Cultures flushed with air minus CO_2. KNO_3 as a nitrogen source. Relative values

Experiment	Light	Dark
1	50	40
2	35	34
3	35	35
4	36	30
5	35	35
6	48	48
7	48	48
Average	41 ± 2.7	39 ± 2.7

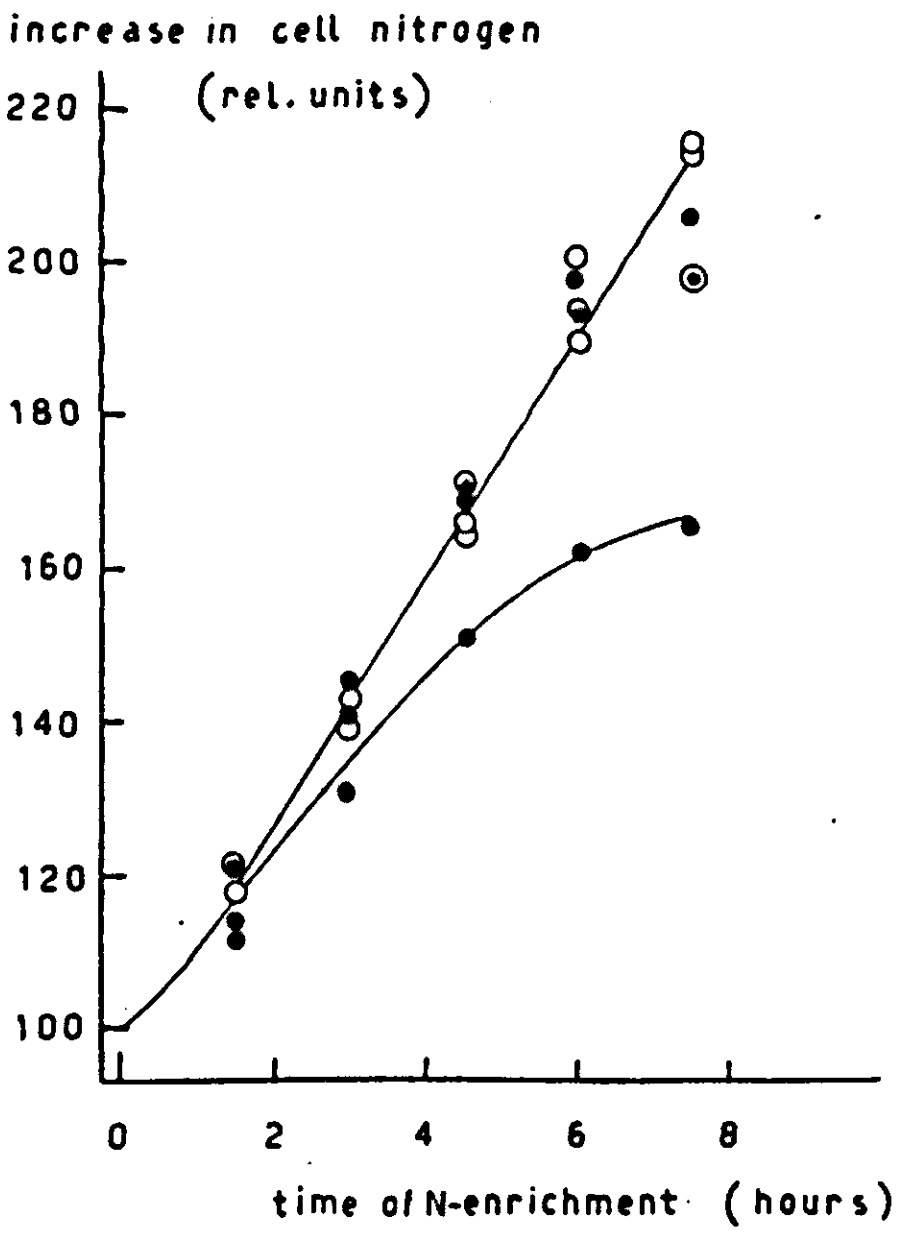


FIG. 21. Time course of N-assimilation by nitrogen starved algae in light (open symbols) and in darkness (closed symbols). Cultures supplied with 0.21 g nitrate-N per litre; flushed with air minus CO_2, pH 7.5, temperature 30 °C, light intensity 0.25 cal/cm²min.

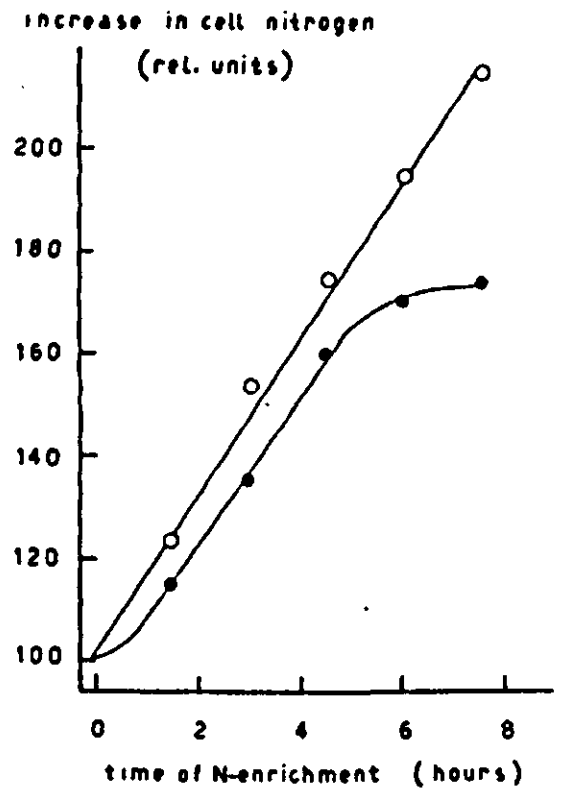


FIG. 22. Legend as fig. 21. Nitrogen source: KNO_2

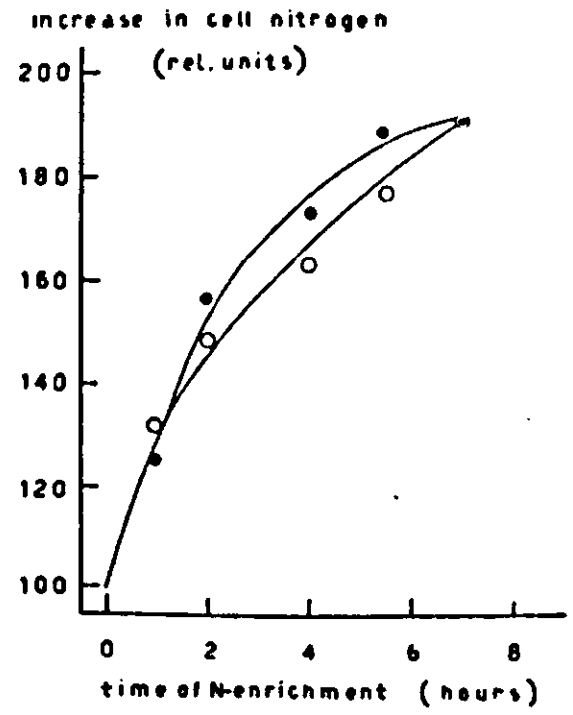


FIG. 23. Legend as fig. 21. Nitrogen source $(NH_4)_2SO_4$

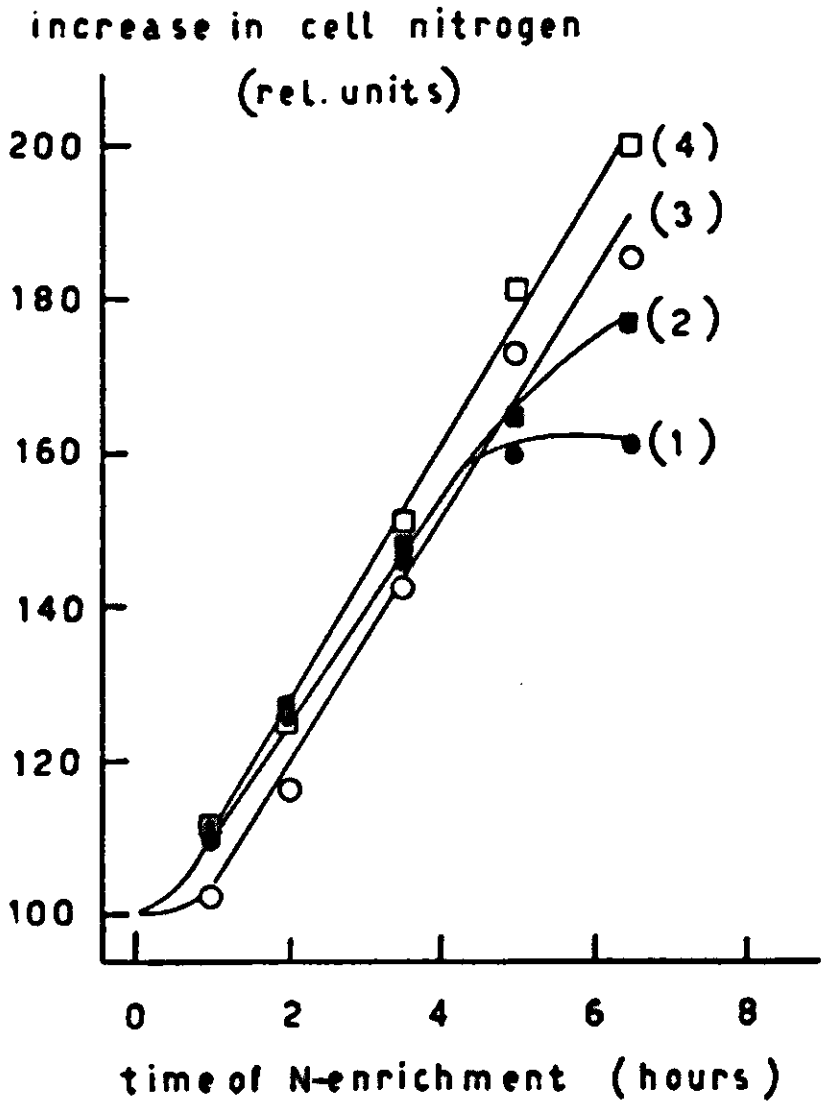


FIG. 24.
N-assimilation by N-starved algae as influenced by light and glucose. 1.5 g KNO_3 per litre
Curve 1 N-assimilation rate in darkness minus glucose
" 2 " " " " +1% "
" 3 " " " light minus "
" 4 " " " " +1% "
Cultures flushed with air minus CO_2, pH 7.5, temperature 30 °C, light intensity 0.25 cal/cm²min.

shows the standard deviation for the slopes found. This demonstrates that nitrate assimilation, in darkness is as fast as in light. In some experiments the assimilation rate in darkness was somewhat lower, up to about 20%, but during the first few hours no significant differences were observed in the assimilation rates in light or darkness. The same holds for KNO_2 and $(NH_4)_2SO_4$.

In experiments in which KNO_3 and KNO_2 have been used, an induction phase was found during the first half hour of N-uptake. This induction phase always is absent in the case of $(NH_4)_2SO_4$ and sometimes in light, as in the case of KNO_2 in fig. 22. Therefore, it should be determined whether the N-uptake in experiments with $(NH_4)_2SO_4$ really represents assimilation also during the first hour, or perhaps only accumulation of NH_4-ions inside the cells.

In order to investigate this, 5 ml samples of cell suspension were centrifuged; the precipitate was washed in 5 ml distilled water and extracted subsequently with a 5% trichloroacetic acid solution during 5 minutes. The amount of ammonium in the supernatant was then determined.

Table 9 gives the results of two experiments in which KNO_3 and $(NH_4)_2SO_4$ served as nitrogen sources. It appeared that in both cases the same quantity of nitrogen was extracted by trichloroacetic acid. This justifies the conclusion that the very rapid assimilation in using $(NH_4)_2SO_4$ is not due to an accumulation of NH_4^+ in the cells, but in reality to a nitrogen incorporation.

TABLE 9. Cells extracted with 5% trichloroacetic acid. Data in mg N per 5 ml suspension (see text)

Time hours	Total cell nitrogen		TCA extract	
	KNO_3	$(NH_4)_2SO_4$	KNO_3	$(NH_4)_2SO_4$
0	0.69	0.73	0.04	0.05
1	0.73	0.95	0.03	0.06
2	0.82	1.16	0.05	0.03
3½	0.94	1.40	0.04	0.05
5	1.01	1.46	0.05	0.04
7	1.08	1.50	0.03	0.05

§ 5. THE INFLUENCE OF GLUCOSE ON THE NITROGEN ASSIMILATION

It was shown earlier in this paper that, in experiments with nitrogen starved cells the supply of energy in the form of light does not accelerate the assimilation rate of nitrogen; it was the same in light and darkness irrespective of the nitrogen compound used (§ 4).

External supply of energy can also be accomplished by addition of glucose. As illustrated in fig. 24, this did not significantly affect the assimilation rate, neither in darkness nor in light. The assimilation was not more rapid in light with additional supply of glucose than in darkness in the absence of glucose. In dark, the presence of glucose was expressed only in the total quantity of assimilated nitrogen, which is easy to explain since the quantity of oxidisable substrate is larger so that more nitrogen can be assimilated. The initial assimilation rate, however, is not influenced.

§ 6. DISCUSSION

The study of nitrogen assimilation under different conditions reveals that this assimilation is most obvious when the nitrogen content of the cells drops below a certain value. The process of nitrogen enrichment in nitrogen starved cells can be seen as a "recovery" process, a rebalancing to the normal N-content, restoring the initial N/C quotient. By inhibiting the nitrogen intake, this quotient declines; the relative and in light also the absolute amount of carbohydrates increases. If, on the other hand, the carbon assimilation is inhibited, the N/C quotient was never found to exceed the value observed with material obtained under "normal" conditions. Such cells contain 8 to 10% nitrogen and about 50% carbon in relation to total dry matter, the N/C quotient thus being 0.16–0.20. The fact that there is an upper limit of the N-content of about 10% indicates that under conditions of C-deficiency, nitrogen containing reserve substances are not built up to any appreciable degree. The upper limit of the N-content may be deemed to be close to that in proteins as the major nitrogen containing substances, viz. 1/6.25 *i.e.* about 16%, yielding an N/C quotient of about 0.30. Since, however, certain non-proteinaceous compounds are invariably incorporated into cell structures (*e.g.* in the cell-wall, as well as in certain rather constant cell constituents, as carotenoids, phospholipids, etc.) it is understandable that the mentioned value is never fully reached. We may well accept the experimentally established value of 10% nitrogen (N/C $\sim$ 0.20) as an empirical maximum.

A further indication in this direction is provided by the experimentally established fact that nitrogen assimilation by cells having an N/C quotient of about 0.20 only takes place when a useful carbon source is available at the same time or is provided simultaneously by photosynthesis. However, this does not hold for cells with an N/C quotient lower than 0.16 (or a nitrogen content lower than 8% of the dry matter). Such cells tend to incorporate nitrogen into cell material even without simultaneous administering of a useful carbon source. The amount of nitrogen assimilated by such cells depends on the degree of nitrogen depletion. If the nitrogen content of the cells is high, only small quantities of nitrogen are assimilated; cell material with a low nitrogen content is capable of forming large quantities of cellular nitrogen under such conditions. It should be observed, however, that the initial rate of nitrogen assimilation is independent of the degree of depletion (figs 14 and 15).

Under conditions of nitrogen depletion, nitrogen assimilation takes place in light and in darkness at the same rate. This holds both for nitrogen supplied as ammonium (fig. 23) and as nitrate (fig. 24). We may conclude that the rate of nitrogen assimilation as observed in the presence of ammonium is determined by the rate at which nitrogen is coupled to some carbon skeleton (fig. 16a). This carbon skeleton no doubt is derived from the excess carbon compounds present in the nitrogen starved cells.

Some further interesting conclusions may be drawn from a more close comparison of figs 16 and 16a. In these figures the assimilation of nitrogen is compared in light (under light saturation) with normal, growing cells, and in darkness with nitrogen starved cells. Normal cells in strong light (in the presence of CO_2) assimilate ammonium and nitrate at much the same rate. This has been verified in many other experiments. For nitrate, the same rate is also observed in darkness, using nitrogen starved (carbon enriched) cells. For ammonium,

however, the rate in darkness in carbon enriched cells is definitely higher than the one obtained in light with "normal" cells. It follows that nitrogen starved cells, in the case of ammonium, are capable of a higher rate of nitrogen incorporation than cells under optimum conditions of photosynthesis. The most obvious reason for this difference seems to be the presence of a stock of nitrogen free carbon compounds in the nitrogen starved cells. We may, therefore, conclude that the rate of nitrogen assimilation observed in this case is that at which nitrogen is coupled to some carbon skeleton, which is present in or derived from the carbon compounds in the nitrogen starved cells. It would seem that, in principle, four rate determining reactions are feasible viz.:

1. a reaction converting reserve carbohydrates into some "nitrogen acceptor",
2. a reaction coupling nitrogen to the "acceptor" postulated under 1,
3. diffusion of the nitrogen source into or inside the cells,
4. a reaction converting the nitrogen source into a specific nitrogen compound or group coupling with the carbon skeleton.

Provisionally, no definite choice between these possibilities seems feasible. We are forced to conclude that in the light even under optimal conditions of photosynthesis, the rate of ammonium assimilation is determined by the rate at which carbon compounds are made available by photosynthesis.

The situation in the light by supplying nitrate instead of ammonium does, so far, not call for a different explanation. However, the situation in dark does, in which the rate of nitrate assimilation, contrary to that of ammonium assimilation, does not exceed that of normal cells in light. Comparing thus the rates of nitrate and ammonium assimilation in darkness (for which many more direct examples will be presented in Chapter IV) leads to the conclusion that nitrate assimilation, as compared with ammonium assimilation is governed by a limiting step of lower capacity (which may well be enzymic in nature) then in the case of ammonium assimilation. For this reason, of the possibilities summed up for limitation of the rate of ammonium assimilation, the number 1. appears to be ruled out. Experiments to be discussed in Chapter V indicate that also the possibility 2. is improbable since these experiments render it likely that nitrate is reduced to a compound near to or identical with ammonium. Observations to be presented in Chapter IV indicate that a rate limiting, probably enzymatic step is intercalated in the reduction process of nitrate preceding nitrogen assimilation.

We may briefly touch the question whether the equal rates of nitrate assimilation observed in darkness (with nitrogen starved cells) and in light (with "normal" cells) also represent equal capacities for nitrate reduction, and thus equal capacities of the rate limiting enzyme system(s) under both conditions. The following two possibilities may be distinguished in this respect:

1. The capacity of the "dark" and "light" systems are the same. In other words: The highest assimilation rate observed in saturating light is the maximum attainable (equal to the maximum rate observed in the dark with nitrogen deficient cells), the rate is enzymatically limited.
2. The capacity of the "light" system surpasses that of the "dark" system, and even under conditions of light saturation of photosynthesis the measured rate of the "light" system is lower than the maximum value.

As will be discussed in Chapter V, the last mentioned assumption is improbable.

CHAPTER IV

ASSIMILATION RATES WITH DIFFERENT NITROGEN SOURCES IN NITROGEN STARVED CELLS

The experiments presented in the foregoing Chapter led to the hypothesis that the maximum rate of nitrate assimilation is controlled by an enzymatic step. The reduction of nitrate to ammonium requires several steps and it is likely that several enzyme systems are involved. It seems possible to get an impression of the relative velocities of various steps by comparing the assimilation rates of nitrogen compounds of different degrees of reduction.

§ 1. EXPERIMENTS

A proper selection of nitrogen compounds, however, is difficult. Naturally NO_3^- must be regarded as the most oxidized form. Since it was possible to demonstrate excretion of NO_2^- in the suspension media of cultures in which KNO_3 was the nitrogen source (to be discussed in Chapter V), it seems justified to consider this compound as the first reduction product of KNO_3. The present knowledge concerning hydrogen transfer in living systems leads to the supposition that hyponitrite is formed in the next step (*cf.* WEBSTER 1955). By further hydrogenation hydroxylamine and ammonium may be obtained. This reaction course can be represented as follows:

$$HNO_3 \xrightarrow{2H} HNO_2 \xrightarrow{2H} HNO \xrightarrow{2H} NH_2OH \xrightarrow{2H} NH_4OH$$

This way of representation is attractive in so far as in every further reduction step two hydrogen ions or electrons are used. However, the existence of intermediates at the reduction level of hydroxylamine has not yet been demonstrated in green cells. It is difficult, at the one hand, to test hyponitrite inasmuch as this compound is very unstable. On the other hand, hydroxylamine – even in low concentrations – is poisonous, and for this reason could hardly be considered in the scope of this investigation. Consequently, only NO_3^-, NO_2^- and NH_4^+ were left to be considered. In extremely low concentrations hydroxylamine has been tested as a possible nitrogen source, but no assimilation could be measured.

The assimilation rates observed for the three nitrogen sources are illustrated in figs 25a (in light) and 25b (in darkness). The assimilation rate for NH_4^+ distinctly surpasses those for NO_3^- and NO_2^-. The slopes indicating the assimilation rates are 81 for NH_4^+ and 35 for NO_3^- and NO_2^-. Light does not have any influence on the assimilation rate. Table 10 gives some further data of similar observations with their standard deviations.

TABLE 10. Assimilation rates of starved cells with different nitrogen sources (relative values)

Experiment	KNO_3	KNO_2	$(NH_4)_2SO_4$
1	35	35	81
2	35	35	82
3	38	39	85
4	34	34	83
5	43	45	81
Average	37 ± 1.7	38 ± 2.0	82 ± 0.8

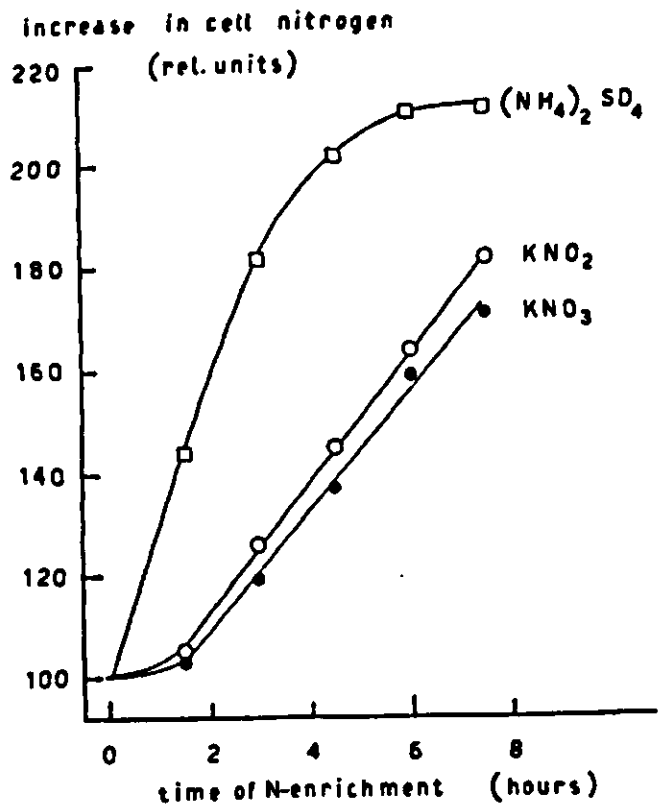


FIG. 25a. N-assimilation rates by N-starved algae in light (0.25 cal/cm²min.) in the absence of CO_2; 0.21 g N per litre given as KNO_3, KNO_2 or $(NH_4)_2SO_4$. Culture flushed with air minus CO_2, pH 7.5, temperature 30 °C

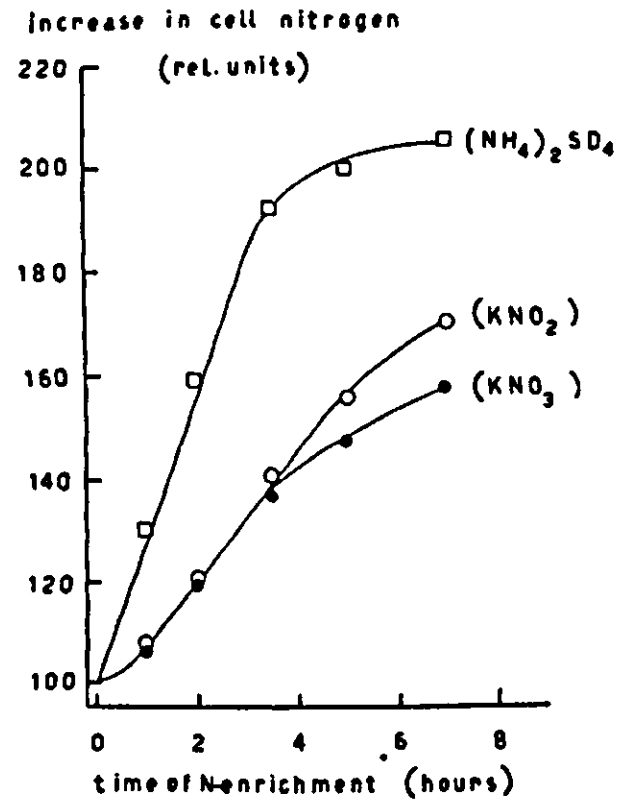


FIG. 25b. As fig. 25a, but in darkness

According to this Table the assimilation rate with NH_4^+ is more than twice that with NO_3^- or NO_2^-, which means that the rate of operation of the enzyme system S_4 (Scheme 1) is higher than that of the systems S_1, S_2 and S_3. When starting with NO_3^- instead of NH_4^+, the NH_4 produced may be assimilated with a velocity greater than its production rate. If this would not be so, the maximum assimilation rate for NO_3^- should be equal to that of NH_4^+.

SCHEME 1

$$NO_3 \xrightarrow{S_1} NO_2 \xrightarrow{S_2} \text{unknown intermediates} \xrightarrow{S_3} NH_4 \xrightarrow{S_4} \text{cell nitrogen}$$

The assimilation rates for nitrate and nitrite are equal, which probably means that the first reduction step from NO_3^- is "NO_2^-" which is not assimilated with a greater velocity than nitrate. In the following Chapter we will demonstrate a nitrite excretion in the suspension medium, with nitrate as a nitrogen source. Both facts appear to imply that the production of nitrite takes place more rapidly than its reduction. Consequently, $S_1 > S_2$ and $S_4 > S_{1,2,3}$. The slowest reaction or reactions are then involved in the reduction of $NO_2^- \rightarrow NH_4^+$. As the velocity of the overall reaction is determined by the slowest reaction, one of the enzymes controlling the reduction of $NO_2^- \rightarrow NH_4^+$ can be considered as the bottleneck in the overall reaction.

According to a personal communication, Professor C. B. VAN NIEL had been able to demonstrate a consumption by algae of hydroxylamine supplied in very low concentrations. He based his procedure on the influence of hydroxylamine on oxygen production. In this relation it seemed useful to study the assimilation of hydroxylamine also in our experiments.

Since our set-up was such that an increase of 0.1–0.2 mg N could be correctly estimated, and since addition at once of the total quantity of hydroxylamine required would certainly have a toxic influence, an amount of 0.11 mg hydroxyl-

amine nitrogen was added in 10 portions with intervals of half an hour, beginning 30 minutes after the start of the experiments. If we suppose now that each quantity of hydroxylamine added has been assimilated before addition of the next one, the concentration of toxic matter will remain low; the maximum concentration will amount to only 3.1×10^{-5} molar.

At the beginning, and after 6 hours, *i.e.* one hour after the addition of the last quantity of hydroxylamine, the amount of cell nitrogen was determined by the KJELDAHL method, which proved that no hydroxylamine was assimilated (Table 11). If the hydroxylamine supplied would have been assimilated, the amount of cell nitrogen would have increased from 0.58 mg to 0.69 mg per 5 ml suspension, which certainly would have been clearly detectable. It seems, therefore, that the inhibiting action of hydroxylamine prevents its consumption so that hydroxylamine was not utilizable as a nitrogen source in our experiments.

TABLE 11. Nitrogen starved cells supplied with 0.11 mg hydroxylamine nitrogen per 5 ml suspension; control suspension with 0 mg nitrogen. pH of the suspension 7.0 (phosphate buffer). Temperature 30 °C.

Time (hours)	Cell nitrogen mg/5 ml suspension			
	0.11 mg hydroxylamine add.		Control cultures	
	I	II	I	II
0	0.57	0.59	0.58	0.57
6	0.58	0.59	0.60	0.60

§ 2. DISCUSSION

It may be concluded from the preceding that, in nitrate assimilation one of the steps in the reduction of NO_2^- to NH_4^+ must be considered as the limiting one. More evidences herefor is found in Chapter V, where the excretion of nitrite is demonstrated. It is also supported by the fact that the rates of assimilation of nitrate and nitrite are the same. The end product of the nitrate reduction, NH_4^+, can be assimilated with greater velocity than it is formed with.

That nitrate and ammonium should be equivalent nitrogen sources as found by PIRSON *et al.* (1952) is not in accordance with our results. Under the experimental conditions used by these workers a more rapid assimilation of ammonium cannot be observed, because the nitrogen assimilation depends on the rate of growth and, as demonstrated in Chapter III, the capacity of the nitrate assimilating system is sufficient to meet the requirements. Moreover, we demonstrated in fig. 16a, that the assimilation rate of NH_4^+ in nitrogen starved cells is much faster than it is in growing cells. This means that the assimilation rate in normal cells might also be much faster if photosynthesis was faster. The photosynthetic rate, however, obviously limits the rate at which NH_4^+ is incorporated.

KRATZ *et al.* (1955) found a higher growth rate on ammonium than on nitrate for *Anacystis nidulans*. Also the observations of VIRTANEN *et al.* (1949) with low-nitrogen *Torula* yeast and those of SYRETT (1953a,b) with nitrogen starved *Chlorella* cells demonstrate that the increase of cell nitrogen in an ammonium medium is much higher than in a nitrate solution.

Many investigators accept the formation of ammonium as an intermediate in nitrate reduction. WARBURG *et al.* (1920) found an ammonium accumulation in the medium and, as will be shown in Chapter V, the rate of accumulation depends on light intensity. PRIANISCHNIKOV *et al.* (1931) found an accumulation of ammonium with pea seedlings and also in the experiments of MENDEL (1951) there is some evidence for ammonium as an intermediate in the reduction of nitrate.

Ammonium, the most reduced form of nitrogen, can be considered as the end product of nitrate reduction. The further assimilation of this product can be seen as a reaction of keto-dicarboxylic acid with ammonium from which the primary amino acids originate (*cf.* ROINE 1947). The central position of ammonium between nitrate reduction and protein synthesis is evident.

In considering ammonium as the end product of nitrate reduction we wonder about its precursor. Positive evidence as to the occurrence of hydroxylamine as an intermediate in nitrate reduction and precursor of ammonium is reported by LEMOIGNE *et al.* (1937a,b), who found it in various leaves. Furthermore, recently ZUCKER & NASON (1955) isolated an enzyme from *Neurospora* that catalyzes the reduction of hydroxylamine to ammonium. These authors suggest that reduced DPN acts as an H-donor. Also TANIGUCHI *et al.* (1953) found evidence for the reduction of hydroxylamine, using cell free extracts of *Bacillus pumilis*. Moreover, VERHOEVEN (1952) demonstrated the occurrence of free hydroxylamine in the process of nitrate reduction by *Denitrobacillus licheniformus*. SILVER & MCELROY (1954), however, with some *Neurospora* mutants found an accumulation of hydroxylamine, but free hydroxylamine was not utilized as a nitrogen source. BURSTRÖM (1939a) assumes that in the course of nitrate reduction the consideration of hydroxylamine as an intermediate is improbable, while LUDWIG (1938) has found that *Chlorella* utilized hydroxylamine as a nitrogen source neither in acid nor in alkaline media. Moreover, PETHICA (1954) demonstrated that hydroxylamine is not utilized by *Azotobacter*. As was also proved in our experiments, hydroxylamine – even in very low concentrations – is not assimilated. Furthermore it is also doubtful whether a compound as toxic as hydroxylamine will accumulate in quantities suitable for analysis.

Nevertheless, hydroxylamine may act as an intermediate, but the rate of its formation may well be smaller than that of its removal. In such a case the intermediate cannot be demonstrated. The reduction rates for nitrate are in accordance with a conversion of 0.1 mg nitrate nitrogen into protein nitrogen per hour for about 5 mg dry weight per ml. Consequently, an equal quantity of nitrogen in intermediates, *e.g.*, hyponitrous acid and hydroxylamine will be converted per hour. This quantity of hydroxylamine, applied at once would correspond to 1.4×10^{-3} molar. According to RABINOWITCH (1945), such a concentration causes a complete inhibition of photosynthesis. So it must be assumed that this concentration exerts a poisonous influence. Since, however, it has been demonstrated that the rate limiting steps are just in the region of hydroxylamine formation, it is quite unlikely that a poisonous concentration of this intermediate would ever be built up. Therefore, experiments to test the possible occurrence of hydroxylamine as an intermediate are to be considered as invalid when they start with a poisonous concentration. In our experiments, using hydroxylamine, we therefore took precautions never to allow any poisonous concentration. Nevertheless, as reported, no indication for the assimilation of externally applied hydroxylamine could be found.

As a precursor of hydroxylamine, hyponitrous acid $(NOH)_2$ is of a certain theoretical interest. AUBEL (1938) believes to have demonstrated its occurrence in *Escherichia coli* fed with nitrate during anaerobiosis, while VIRTANEN & RAUTANEN (1952) hold that *Torula* yeast has a mechanism for assimilation of the group = C-NOH. MOZEN & BURRIS (1954) observed the assimilation of labeled nitrous oxide, N_2O, a compound at the reduction level of hyponitrous acid, in various nitrogen fixing organisms. CHADHARY *et al.* (1954), however, demonstrated that neither nitrous oxide nor hyponitrous acid were assimilated by *Azotobacter*. Therefore, so far there seems no sufficient unambiguous experimental evidence for both nitrogen compounds, hyponitrous acid and hydroxylamine, as intermediates in nitrate reduction.

Owing to their volatile and poisonous character, their application as substrates for nitrogen assimilation appears to meet with considerable difficulties.

CHAPTER V

INTERDEPENDENCIES OF CARBON AND NITROGEN ASSIMILATION AS INDICATED BY EXCRETION OF INTERMEDIATES OF NITRATE REDUCTION INTO THE MEDIUM

As was discussed in Chapters III and IV, under certain conditions nitrite can be utilized as a source of nitrogen. It is generally assumed that this component is the first reduction step of nitrate. This assumption is mainly based on the observation made by a number of workers (BURSTRÖM 1945, DITTRICH 1930, EGGLETON 1935, SOMMER 1936, and KESSLER 1952, 1953a,b), who found excretion of nitrite in a nitrate containing suspension medium. Dependent upon the conditions, also the ammonium ion may be taken up from the suspension medium or excreted into it.

In general, the concentration of such intermediates in the medium depends upon their relative rates of formation and removal. Only as long as the rate of formation surpasses the rate of uptake, there is a possibilibity for accumulation in the medium.

We studied the excretion of intermediates by suspending algae, pretreated in various ways, in media supplied with 1.50 g KNO_3/litre. In either light or dark-

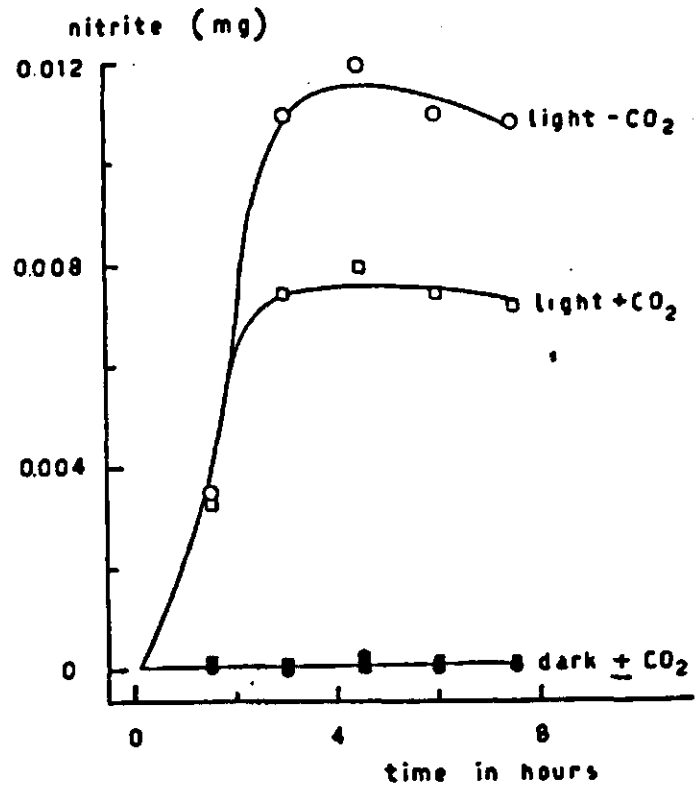


FIG. 26.
Excretion of nitrite (mg NO_2-N per 5 ml suspension) in nitrate containing media, in light (0.25 cal/cm² min.) or in darkness. Cultures flushed with air, either enriched with 5% CO_2 or freed from CO_2. Temperature 30 °C, pH 7.5; 5 µl normal cells per ml suspension

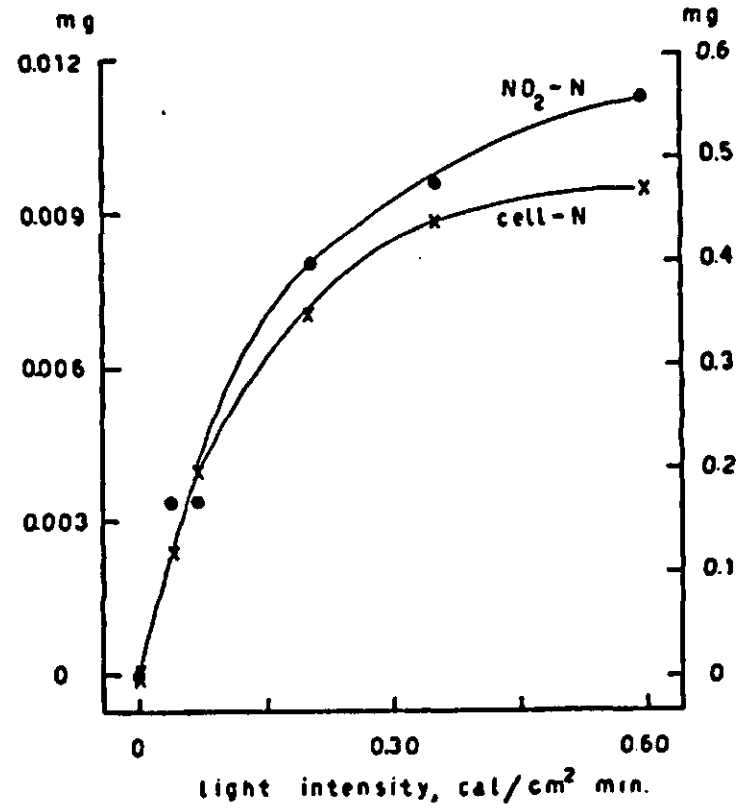


FIG. 27.
Relation between nitrite excretion and the production of cell nitrogen at different light intensities. Media containing 1.5 g KNO_3 per litre. Cultures flushed with air + 5% CO_2, temperature 30°C, pH 7.5; 5 µl normal cells per ml. Time of exposure 6 hours. Ordinate to the left: mg nitrite-N per 5 ml suspension. Ordinate to the right: cell-N per 5 ml suspension

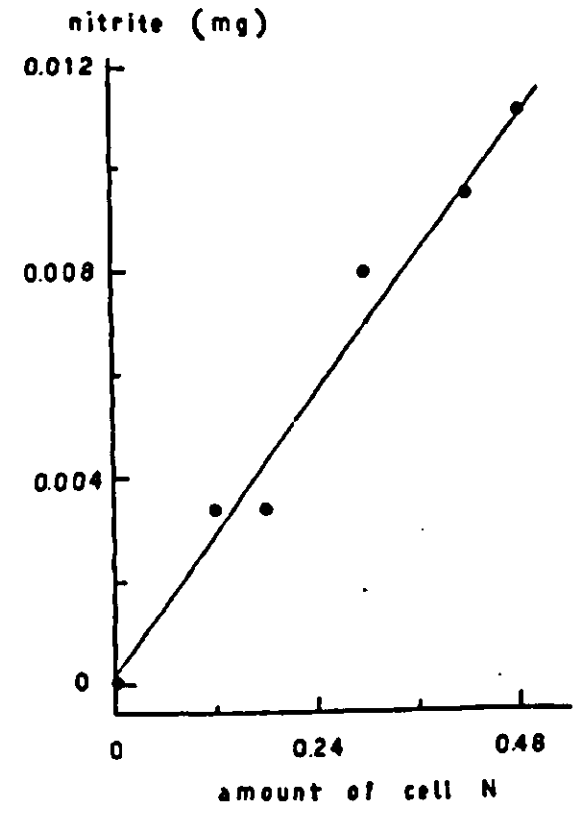


FIG. 27a.
Relation between the concentration of nitrite (mg NO_2-N per 5 ml suspension) built up in the medium, and the production of cell nitrogen in 6 hours (data fr om fig. 27)

ness and in the presence or absence of CO_2, 1 ml samples were taken at definite time intervals during each experiment. The cells were centrifugated, and the nitrite and ammonium concentrations determined in the supernatant.

§ 1. THE EXCRETION OF NITRITE INTO THE MEDIUM

a. Nitrite excretion by algae of normal N-content

As long as algae are grown in an NO_3^- medium of suitable pH, one always finds detectable amounts of nitrite in the suspension medium. If algae are resuspended in fresh medium, a steady state concentration of NO_2^- is built up after a few hours of growth.

This is shown in the experiment of fig. 26, which furthermore illustrates an influence of both light and CO_2 on the final concentration. The factor light was studied in more detail, and representative experiments are given in figs 27 and 28.

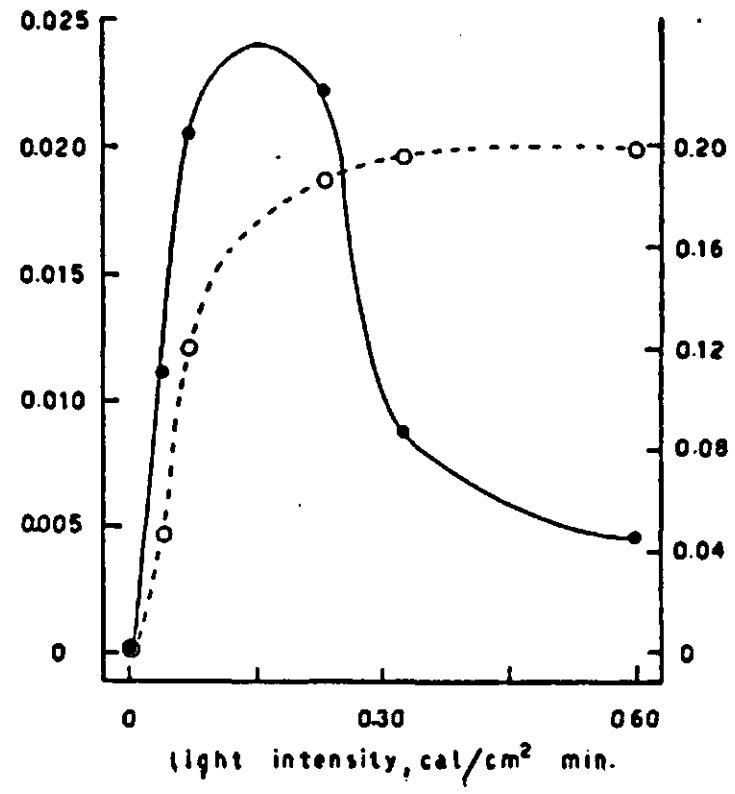


FIG. 28.
Nitrite excretion (closed circles) and ammonium excretion (open circles) by normal cells in nitrate containing media as influenced by different light intensities. Cultures supplied with air minus CO_2. Temperature 30°C, pH 7.5; 5 µl normal cells per ml. Data as mg N per 5 ml suspension. Ordinate to the left: nitrite excretion. Ordinate to the right: ammonium excretion

In fig. 27 we plotted the amount of nitrogen fixed in the cells and the amount of NO_2^- excreted into the medium during an exposure of 6 hours to various light intensities (this duration of exposure is long enough to establish final levels of NO_2^- concentration, *cf.* fig. 26). After 6 hours, free nitrite amounts to 2.5 % of total cell nitrogen formed. There is a linear relation between the final nitrite concentrations at different light intensities and the formation of cell nitrogen at these intensities. This is given in fig. 27a. In this figure we used the data of fig. 27, but plotted the final NO_2^- concentrations as a function of the formation of cell nitrogen. This relation could indicate that a given nitrate assimilation is always correlated with a specific final concentration of free nitrite.

Fig. 28 gives an experiment in which the algae were deprived of CO_2. No nitrogen is fixed in the cells under these conditions but ammonium is excreted instead, a fact further discussed in the next section. The production of NH_4^+ during the six hours duration of the experiment is plotted as a dotted line in fig. 28. It shows that nitrate reduction occurs to a considerable extent, its rate increasing with the light intensity.

Only in weak and medium light intensities nitrite formation is found to occur at a rate comparable to that observed in the presence of CO_2. The decreased nitrite excretion at high light intensities could be a consequence of

1. re-oxidation of nitrite to nitrate,
2. accelerated reduction of nitrite to ammonia,
3. decreased production of nitrite.

The second and third possibilities are improbable: an accelerated rate of reduction must be considered improbable since, as will be shown later, NH_4^+-excretion did not increase at high light intensities. Consequently, it seems most likely to postulate a process in which nitrite is re-oxidized to nitrate, probably by photo-oxidation. In view of the chemical properties of nitrite such an oxidation could well occur.

b. Nitrite excretion by N-starved cells

Table 12 shows an experiment in which nitrate (0.21 g per litre) was added to N-starved algae, either kept in light or darkness.

TABLE 12. Formation of cell nitrogen and excretion of nitrite in the culture medium by N-starved cells, in light and darkness with KNO_3 as a nitrogen source. Light intensity 0.25 cal.cm^{-2}.min.$^{-1}$. Concentrations are expressed in mg N per 5 ml suspension. Temperature 30 °C

Time (hours)	Dark			Light		
	Cell-N	NO_2^--N	$100\frac{NO_2^-\text{-N}}{\text{cell-N}}$	Cell-N	NO_2^--N	$100\frac{NO_2^-\text{-N}}{\text{cell-N}}$
0	0	0	0	0	0	0
1½	0.11	0.004	3.6	0.11	0.005	4.5
3	0.22	0.009	4.1	0.21	0.016	7.6
4½	0.37	0.016	4.3	0.35	0.018	5.1
6	0.52	0.015	2.9	0.53	0.017	3.2
7½	0.52	0.015	2.9	0.59	0.016	2.7

It may be seen that initially no nitrite was present in the medium. This was to be expected since the cells had assimilated all available nitrogen during the previous starvation period. However, after re-addition of nitrate, nitrite rapidly

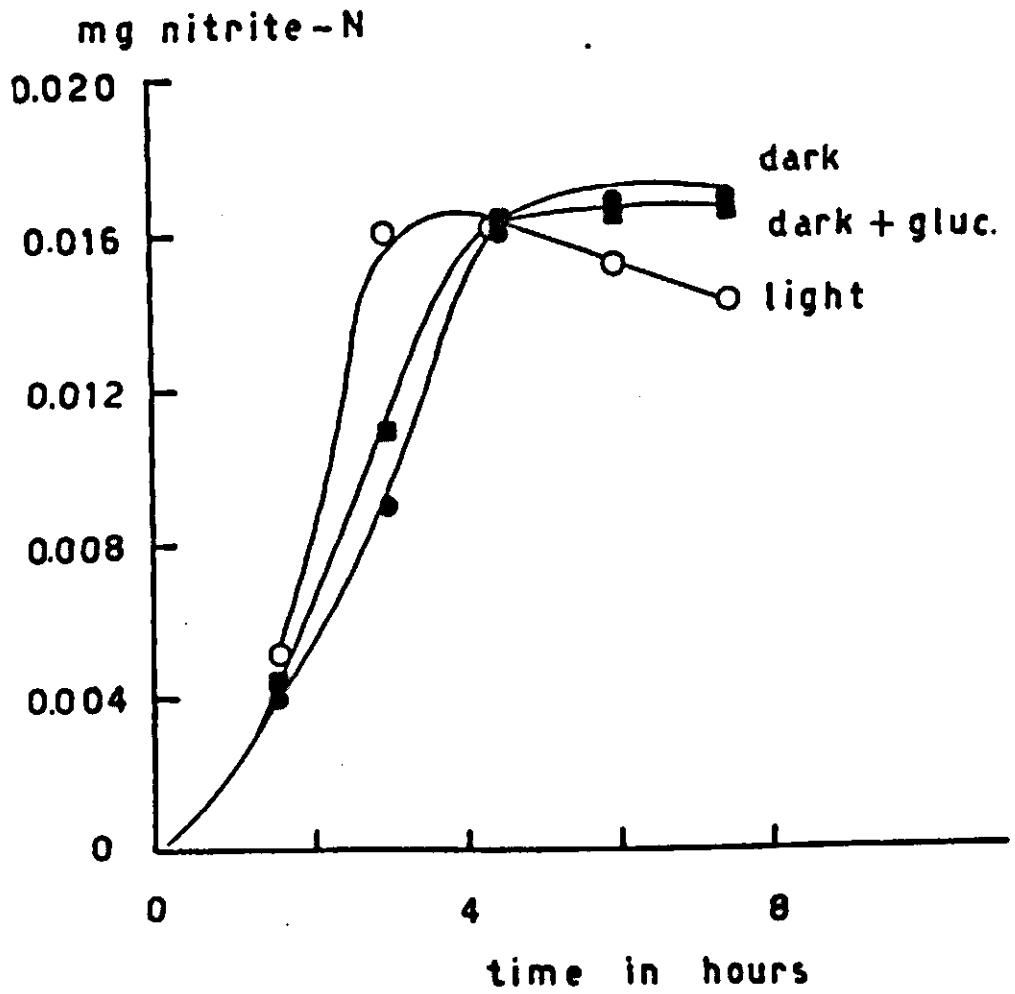


FIG. 29.
Time course of nitrate excretion (mg NO_2-N per 5 ml suspension) into the medium by N-starved cells in dark, in light (0.25 cal/cm²min.), and in dark with 1 % glucose. Cultures supplied with air freed from CO_2, temperature 30 °C, pH 7.5; 15 µl N-starved cells per ml, in a medium containing 1.5 g KNO_3/l

occurred in the suspension and after 3 to 4 hours a maximum concentration was attained. In most cases no further increase was observed. Thereafter, a slight decrease, amounting to 10–20 %, was found sometimes. As may be seen from Table 12, the production of cell nitrogen proved to be constant up to 6 to 7 hours after re-addition of nitrate. The fact that the rate of nitrite excretion declines after about 4 hours might indicate that a steady state equilibrium between production and uptake is then attained.

Experiments presented in the foregoing led to the hypothesis that the slowest enzymatic step involved in the reduction of nitrate had to be located somewhere in the reaction chain between NO_2^- and NH_4^+. As far as the reduction of nitrate to nitrite is concerned, two possibilities exist, namely, it may be either enzyme-limited or controlled by the supply of energy. In order to check this, we have studied the influence of the external energy sources: light and glucose. Fig. 29 shows the amount of nitrite excreted in the course of N-rebalancing as influenced by light (0.25 cal.cm^{-2}.min.$^{-1}$) and glucose (1 %); a parallel sample of algae was kept in darkness as a control. The curves show a time lag of about an hour. Whereas the final nitrite concentration in the three experiments is about equal (0.016 mg N/5 ml), this concentration is attained faster in the suspension to which light or glucose was supplied. This indicates an acceleration of NO_3-reduction by additional energy supply, the effect of light surpassing that of glucose.

c. The influence of the pH

As has been shown in Chapter III (page 25), the nitrite assimilation depends strongly on the pH-value of the suspension medium and shows a pronounced optimum around pH 7 to 8. It seemed interesting to check to what extent the excretion of nitrite is influenced by pH. For this reason, N-starved algae were supplied with 1.50 KNO_3 g/1 in media with different pH-values, ranging from 5 to 10.5. The steady state nitrite concentrations found in this way are given in fig. 30. As can be seen in this figure, the highest concentrations were found in the pH range of 7 to 8. We found (*cf.* Chapter III, fig. 20) the same pH optimum for

the process of nitrite assimilation. KESSLER (1952, 1953a,b) studied nitrite excretion by various strains of algae, and observed a wide variation in regard to pH optimum. This might indicate that the pronounced optimum we observed is only specific for our strain and our conditions of cultivation. However, no observations at pH-values beyond 7 were made by KESSLER, and this point may require further study.

§ 2. THE EXCRETION OF AMMONIUM INTO THE MEDIUM

We observed that algae of a normal N-content, if exposed to light in the presence of NO_3^- in the absence of CO_2 excrete ammonium into the medium at a uniform rate during a considerable time. This is illustrated in fig. 31 which also shows that no detectable amounts of NH_4^+ are excreted if the cells are kept in darkness or illuminated in the presence of CO_2. The ammonium excretion increases linearly with the time of exposure, but after 6 to 8 hours, the process usually declines, especially in bright light, which tends to damage the cells in the absence of CO_2.

The amounts of NH_4^+ excreted are substantial and exactly equal to the amount of cell nitrogen formed in case CO_2 had been given under otherwise comparable conditions. This also is illustrated in fig. 31: the crosses indicate the increase in cell nitrogen observed in a comparable sample of algae exposed to the same light intensity in the presence of CO_2. Fig. 31 also shows that in the absence of CO_2 no N-compounds are incorporated into the cells.

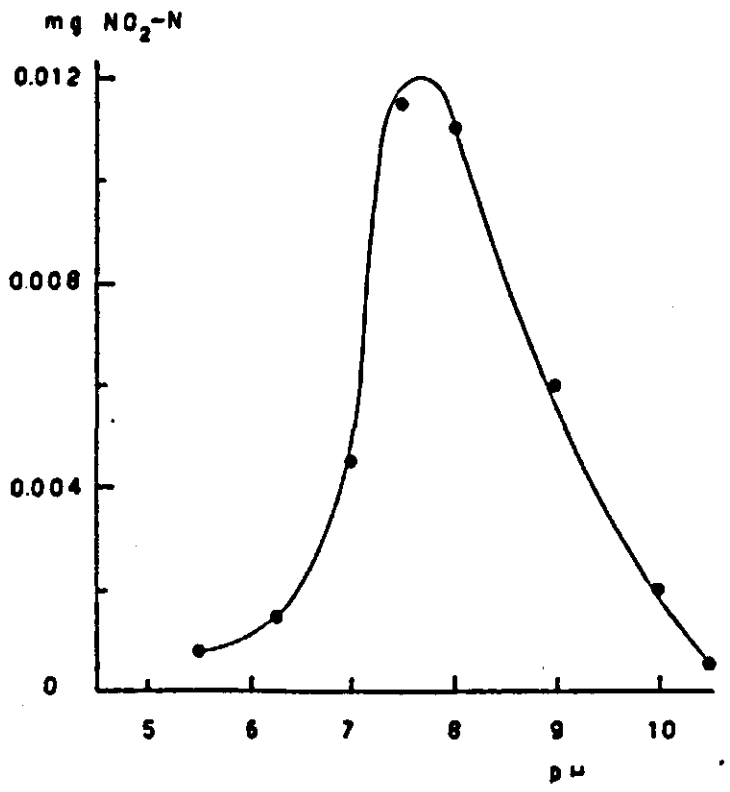


FIG. 30.
Influence of pH on nitrite excretion (mg NO_2-N per 5 ml suspension) by nitrogen starved cells after re-addition of nitrate. 15 µl N-starved cells per ml, temperature 30 °C, darkness, experiment duration of 7½ hours

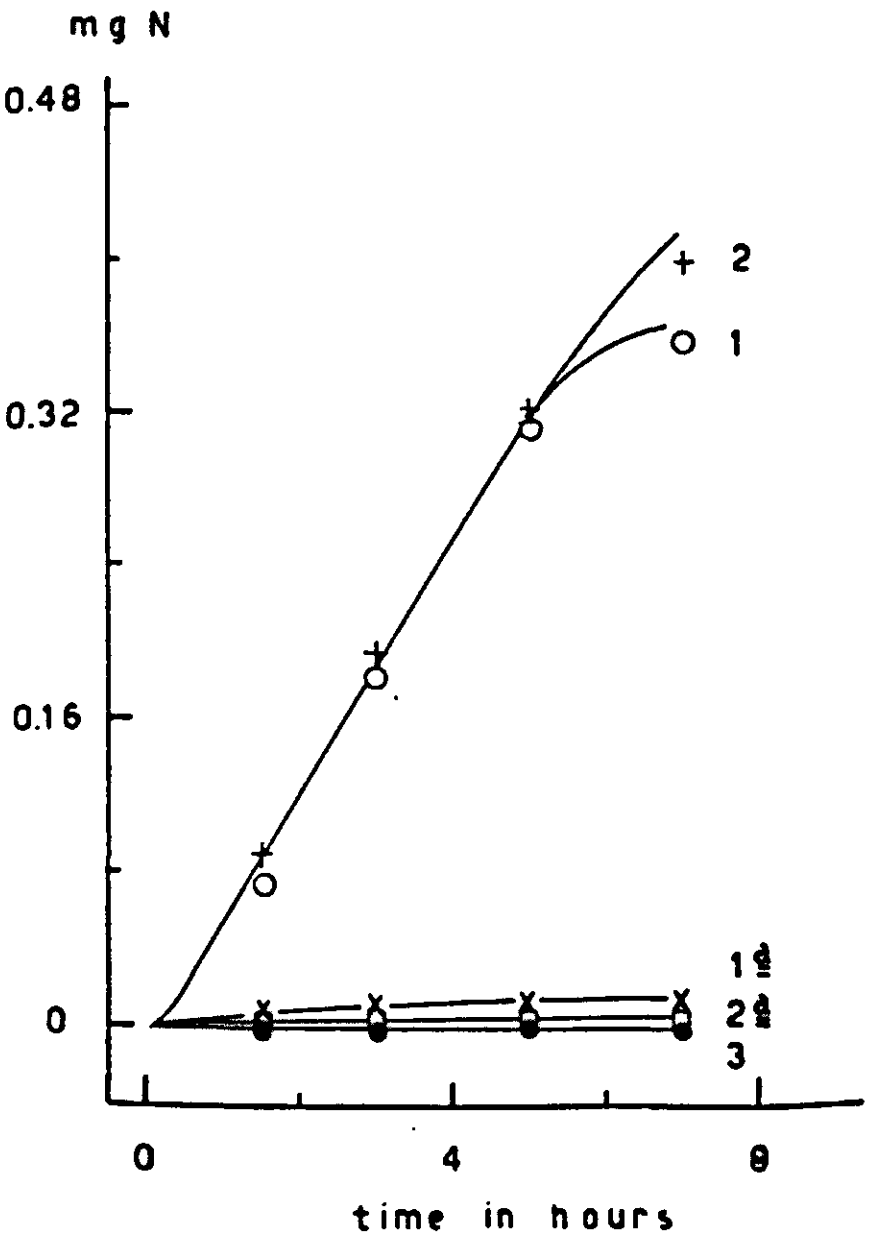


FIG. 31. Excretion of ammonium and formation of cell nitrogen in nitrate containing media.
a. In light, in the absence of CO_2. Curve 1: excretion of ammonium, curve 1a: formation of cell nitrogen
b. In light, in the presence of CO_2. Curve 2: formation of cell nitrogen, curve 2a: excretion of ammonium
c. In darkness. Curve 3: formation of cell nitrogen and excretion of ammonium
Data in mg N 5 per ml suspension. Light intensity 0.25 cal/cm²min.; 6 µl normal cells per ml suspension, pH 7.5, temperature 30 °C

Obviously, these experiments lead to the conclusion that (at least in strong light) nitrate reduction runs independently of CO_2-reduction, regardless whether the ultimate product is cell nitrogen or ammonium nitrogen. We may also conclude that under these conditions the reduction of nitrate is driven by the light.

a. The correlation between CO_2- and NO_3-reduction

The relation between the light intensity and the rate of nitrate reduction, measured as the rate of NH_4^+-excretion in the absence of CO_2 is shown in fig. 32 (closed circles). It appears that this relation has the character of a saturation curve. A dark process limits the maximum rate attainable in strong light.

It is interesting to analyse further this rate curve of NO_3-reduction and to correlate it with the rate curve of CO_2-reduction, both measured against light intensity with parallel samples of algae. The open circles of fig. 32 represent such measurements of the rate of O_2-exchange with algae suspended in a 0.2 molar bicarbonate buffer of pH 8.7. We may accept that during the O_2 measurements the photosynthetic quotient was close to unity. Each mole O_2 evolved then represents the reduction of one mole of CO_2.

In very weak light, below the compensation point of photosynthesis, no NH_4-production is observed. This can be easily understood since the photosynthetic system still has respiratory CO_2 available. At low and medium light intensities, algae deprived of CO_2 show nitrate reduction (measured as NH_4^+-production) at a rate comparable to that of CO_2-reduction (measured as O_2-production) in normal cells. Table 13 gives N/C ratio's from data obtained as indicated above in the light limiting range. It looks as if at low light intensities this ratio is about 1, but experimental accuracy is small at these light intensities. If the reduction of 1 molecule of nitrate yields 2 molecules of oxygen (which we actually found in

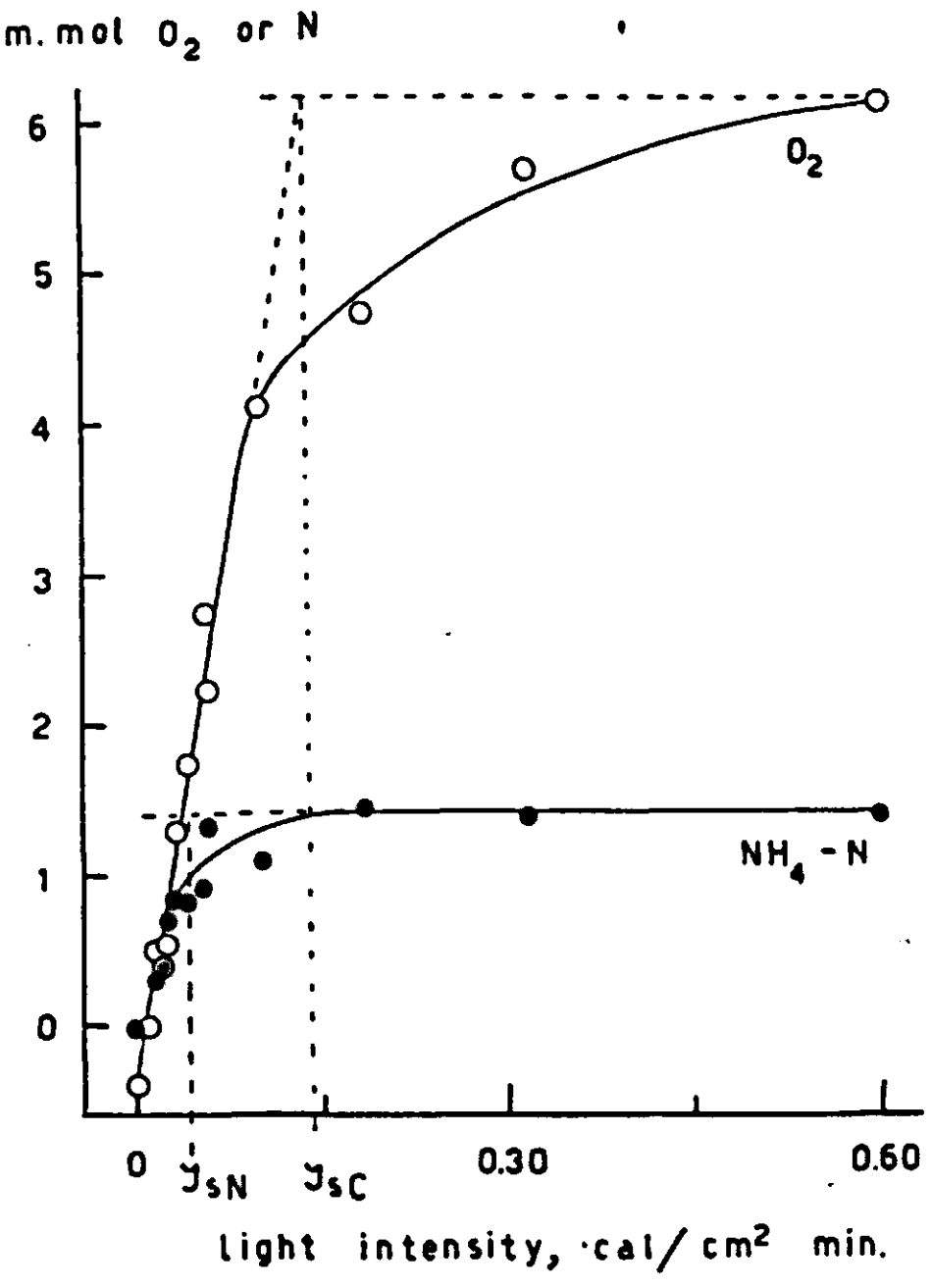


FIG. 32.
Relation between light intensity and rate of ammonium excretion in a nitrate containing medium in the absence of CO_2, pH 7.5 (closed circles) and rate of oxygen output of a parallel sample of algae in a 0.2 molar bicarbonate buffer at pH 8.7 in the absence of NO_3 (open circles). Both experiments: 8 µl of normal cells per ml suspension, temperature 30 °C

some preliminary experiments) an NO_3/CO_2 quotient = 1 would mean the production of 2 molecules of oxygen in nitrate reduction against 1 molecule in carbon dioxide reduction for equal numbers of light quanta. In the light limiting range the production of "reducing agent" may be assumed to limit both NO_3 reduction and CO_2-reduction. If we accept comparable general pathways for both types of reduction, the relative amounts of nitrate and CO_2 reduced at a given light intensity will be proportional to the respective numbers of reducing agent units involved in both reductions. Roughly these numbers may be represented by the theoretical number of hydrogen atoms required in the balance equations. These numbers are 4 for CO_2-reduction, and 8 for nitrate reduction, according to, respectively:

$$CO_2 + 4H_2O \longrightarrow CH_2O + O_2 + 3H_2O, \text{ and}$$

$$4H \quad 4OH$$

$$\tfrac{1}{2}N_2O_5 + 8H_2O \longrightarrow NH_3 + 2O_2 + 6\tfrac{1}{2}H_2O$$

$$8H \quad 8OH$$

It thus would follow that, for a given amount of light in this light intensity region, the NO_3/CO_2 ratio in experiments of the type described theoretically would be 0.5, and not 1. The "molar" NO_3/CO_2 ratio = 0.5 equals an N/C ratio (on mg basis) of 0.58. As follows from Table 13 this value has not been generally found, but is approached quite satisfactorily by the most reliable data at the somewhat higher light intensities.

It should, however, not be forgotten that a too close comparison of the NO_3 and CO_2 data does not seem warranted since the compared experiments had to be performed in different media. More experimental data will be required for a full discussion of the situation; such experiments are now being set up.

TABLE 13. Average N/C ratio's observed in the light limiting range. CO_2- and NO_3-reduction measured separately. Culture density 0.31 mg chlorophyll per 10 ml

Light intensity cal/cm².min.	Ratio NO_3/CO_2	Number of experiments	Standard deviation
0.015	0.76	4	0.25
0.020	1.10	3	0.36
0.034	0.65	4	0.14
0.042	0.74	4	0.26
0.057	0.55	4	0.10
0.060	0.55	2	0.10

This type of experiment, of course, presupposes that in the absence of CO_2 the absorbed light is consumed in the nitrate reduction process with the same efficiency as otherwise in CO_2-reduction.

In strong light both carbon and nitrogen assimilation attain saturation rates. Fig. 32 shows that the maximum level of CO_2-reduction is several fold higher than the maximum rate of NO_3-reduction in the absence of CO_2. In fact the ratio between these two observed levels usually corresponds quite well with the normal N/C ratio observed by analysis of the elementary composition of

Chlorella (*cf.* KOK, 1952, VAN OORSCHOT, 1955). This is illustrated by the results of experiments given in Table 14. The average N/C ratio observed in these experiments is 0.22 which corresponds to an N-content of 11 % of the dry weight of the algae.

TABLE 14. CO_2- and NO_3-reduction measured separately (see text) at saturating light intensities. Data in mg C and mg N per hour. Density 0.31 mg chlorophyll per 10 ml suspension

Experiment	mg N	mg C	N/C	Computed % N
1	0.13	0.62	0.21	10.5
2	0.24	0.75	0.32	16.0
3	0.23	0.81	0.28	14.0
4	0.20	0.92	0.22	11.0
5a*	0.20	0.73	0.27	13.5
5b**	0.13	0.97	0.14	7.0
6a*	0.12	0.52	0.23	11.5
6b**	0.11	0.92	0.12	6.0
Average			0.22	11.0

* Suspension containing about 90 % "light" cells (see text)
** Suspension containing about 90 % "dark" cells (see text)

The first four experiments of Table 14 show relatively high values. The conditions probably have been such that either CO_2-assimilation was lower or NO_3-reduction was higher than normal. TAMIYA *et al.* (1953), NIHEI (1954), and IWAMURA (1955) showed that during the life cycle of *Chlorella* large variations of the photosynthesis rate may occur, especially "light" cells showing a low rate of CO_2-incorporation. Therefore, in the last four experiments of Table 14 we have used parallel samples of algae in different stages of development. "Light" cells (*cf.* observations 5a, 6a, Table 14) *i.e.* large cells which are about to divide, show a relatively much higher rate of NO_3-reduction, resulting in a high calculated N/C ratio. Small, "dark" cells (*cf.* observations 5b, 6b, Table 14) on the other hand show a low N/C ratio. This difference is mainly due to a decrease of the CO_2-assimilation characterizing the maturity of the algae. The rate of NO_3-reduction if calculated either per unit chlorophyll or per unit dry weight remains rather constant.

In fig. 33, we compared the rate of NO_3-assimilation (in the presence of CO_2, measured as formation of cell nitrogen) and the rate of NO_3-reduction (in the absence of CO_2, measured as NH_4^+-excretion) in weak and strong light. Whereas in strong light the rates of both processes are equal, in weak light the assimilation of nitrate appears to be suppressed by the simultaneously occurring CO_2-reduction. This suggests a competition between the CO_2- and NO_3-reducing systems for some sort of reducing material supplied by light. This holds only in weak light, because the supply of reducing material is then limited. In strong light, the supply of the reducing material is such that also in the presence of CO_2, the NO_3- and CO_2-reducing systems attain saturation.

The ratio of the two rates of nitrate reduction observed in weak light in the presence and absence of CO_2, is about 1 : 5. This means that in the presence of

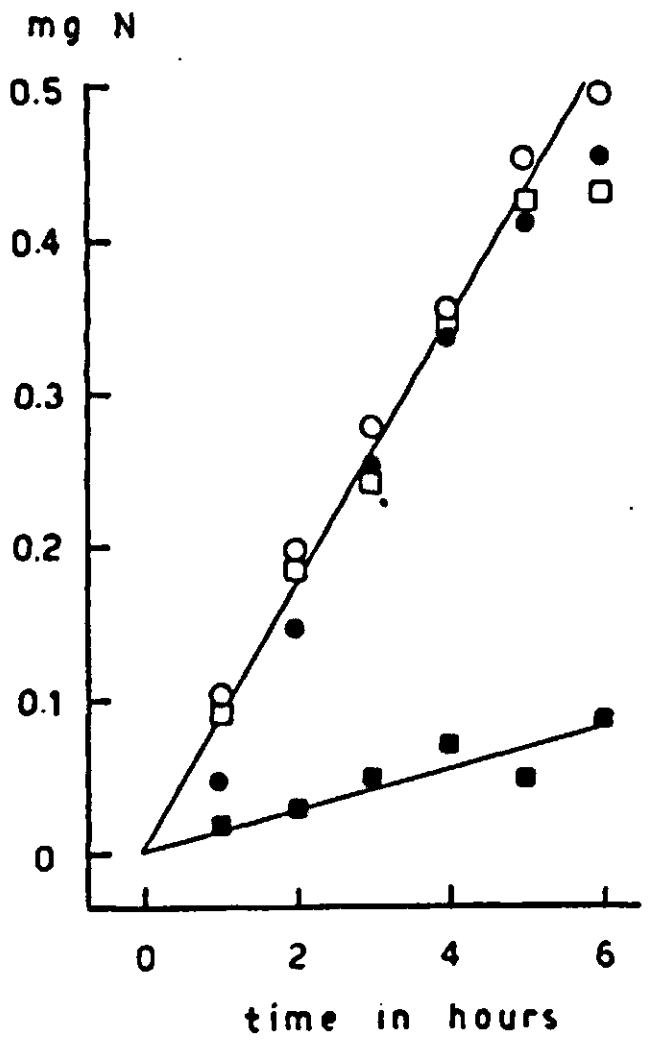


FIG. 33.
Time course of formation of cell nitrogen in the presence of CO_2 and excretion of ammonium in the absence of CO_2. Light intensities 0.60 cal/cm²min. and 0.10 cal/cm²min. Excretion of ammonium in weak light (□) and in strong light (○).; formation of cell nitrogen in weak light (■) and in strong light (●) Temperature 30°C, pH 7.5; 8 µl of normal cells per ml suspension. Data in mg N per 5 ml suspension

CO_2, algae are produced which are characterized by a normal N/C ratio. On the other hand, it indicates that in the absence of CO_2, a deviation of all the reducing material towards NH_4-production occurs.

§ 3. DISCUSSION

Accumulation of nitrite can be observed with nitrogen starved algae in light as well as in darkness after re-addition of nitrate. Normal cells show excretion of nitrite only when illuminated in nitrate containing media.

If we neglect photo-oxidative effects under conditions of strong light and lack of CO_2, the excretion of nitrite can generally be considered as a process accompanying nitrate reduction. In cultures of normal algae kept in darkness, no nitrate reduction and nitrite excretion is found. After re-addition of nitrate to cultures of nitrogen starved algae, however, rapid nitrate assimilation and nitrite excretion occur.

We can accept that one of the early intermediate steps in nitrate reduction can freely exchange nitrite with the medium surrounding the algae, and that the concentration of the excreted nitrite is correlated with the reduction rate of nitrate.

Accumulation of ammonium is shown by normal algae only when illuminated in the absence of CO_2. The excretion of ammonium under these conditions may be explained as a side reaction of the normal nitrate assimilation. This side reaction, however, will occur only if the incorporation of nitrogen is blocked by lack of carbon compounds so that no amino acids can be synthesized. As a consequence of this, accumulation of ammonium inside the cell will occur, resulting in excretion into the medium.

The excretion rate of ammonium will depend upon the rate at which nitrate is reduced. In weak light the reduction rate of nitrate is low, and the excretion rate of ammonium will also be low. In strong light in the absence of CO_2, high rates of ammonium excretion were found; the excretion rate shows light saturation. The maximum excretion rate is exactly equal to the amount of cell nitrogen

formed in case CO_2 had been given under otherwise comparable conditions (fig. 33).

Also for carbon dioxide reduction a maximum reduction rate can be established. The maximum reduction rates of nitrate and carbon dioxide have a ratio of about 1 : 5. This means that by simultaneous CO_2- and NO_3-reduction, algal material will be formed with a nitrogen content of about 10%, on dry weight basis.

The observation that in medium light intensities the reduction of nitrate is suppressed by the simultaneously occurring CO_2-reduction (fig. 33), suggests a competition between the NO_3- and CO_2-reducing systems for some sort of reducing material generated by light energy. In strong light, the reducing material is generated in excess, and both systems will be saturated. Under these conditions in the presence of CO_2, an amount of cell nitrogen is built up, equal to the amount of ammonium excreted into the medium in the absence of CO_2.

We thus have seen that in case no nitrate is assimilated, nitrate reduction in light nevertheless may occur and is then manifest by excretion of NH_4^+ into the medium. Thus nitrate reduction in light is not strictly dependent on simultaneous CO_2-reduction.

CHAPTER VI

GENERAL DISCUSSION

From the experiments described in Chapter II, it is obvious that the photosynthetic capacity is correlated to the nitrogen content of the material. The question to what extent the decline of the photosynthetic rate has to be ascribed either to the accumulation of photosynthetic products or to the decreased protein content could hardly be answered. During growth in nitrogen free cultures a chlorophyll decomposition was recorded, but the decrease in the photosynthetic rate could not be explained by a diminished absorption of light, a conclusion also drawn by VAN HILLE (1938), AACH (1952), and KOK (1952). In studying recovery processes, we found that the addition of nitrate to nitrogen starved algae raised the photosynthetic activity, confirming the findings of VAN HILLE (1938). So it seems as if the production of proteins restores the photosynthesizing apparatus.

VAN HILLE (1938) did not observe a decreasing effect of carbohydrate accumulation on the photosynthetic rate. He found no decrease in the photosynthetic rate in a *Chlorella* culture with bicarbonate and without nutrient salts, but instead an increase of about 70% after an illumination of 25 hours. The rate, however, is expressed in terms of volumes of oxygen per unit volume of suspension. With this method, an increase in dry matter may well be responsible for the increase in photosynthetic rate. In nitrogen deprived cultures, however, VAN HILLE observed a decreased rate, and this rate could only be increased by the addition of nitrate to the medium. From this it is evident that also in his cultures the photosynthetic rate was correlated with the nitrogen content of the medium, and thus with the protein content of the material formed. Such decreased photosynthetic rates were also found by TAMIYA and coworkers (TAMIYA *et al.*, 1953, NIHEI, 1954, and IWAMURA, 1955). They showed that during the life cycle of *Chlorella* large variations of the photosynthetic rate may occur. Two distinct forms in the course of growth were observed by these authors; cells,

small in size, called "dark cells" and larger "light cells". The photosynthetic rate found with dark cells was about six fold faster than the rate observed with cultures of light cells. The difference in nitrogen content between the two types of cells is remarkable: the nitrogen content of the dark cells was 7.0–9.5%, that of the light cells was much lower, viz. 5.2–5.7% (*cf.* TAMIYA 1953). The chlorophyll content in the light cells was decreased to about 30%. Thus, also in the daily cycle, photosynthetic activity and nitrogen content of the cells seem closely related.

The way in which living cells adjust their protein to carbohydrate ratio will become somewhat understandable by experiments described in Chapters III and IV. In nitrogen starved algae we found the assimilation rate of ammonium twice that of nitrate. This indicates that the ammonium ion can enter cell metabolism, avoiding a bottleneck reaction in the nitrate reduction. However, during growth in an ammonium containing nutrient solution, the nitrogen assimilation rate was about equal to that found in nitrate containing media. Therefore, a regulatory mechanism must be supposed at some final state of the formation of cellular nitrogen compounds. The fact that the faster rate is only found with nitrogen starved (= carbon enriched) algae (*cf.* fig. 16a), has led us to the conclusion that the availability of a carbon skeleton, an N-acceptor, determines the assimilation rate, in algae cultivated in ammonium containing nutrient solutions. Using nitrogen starved algae, *i.e.* cells relatively rich in carbohydrate reserves, such acceptors will be present, and appear to be available in larger amounts than in quickly growing cells, which condition accelerates ammonium incorporation. The formation of such acceptors thus seems to be a decisive factor in the incorporation of nitrogen into cell constituents. This supposition is supported by the observations of Chapter V that in the presence of nitrate under conditions of CO_2-absence in the light NH_4^+ is excreted into the medium. Under such conditions the production of nitrogen acceptors will be inhibited, and amino acid formation will be limited by lack of suitable carbon skeletons. For a more detailed discussion, see Chapter III, § 6.

The observations discussed above, lead to the conclusion that the NO_3^--reducing system comprises a specific rate limiting step. We concluded that this step must be located between the oxidation levels of nitrite and ammonium. The special reasons for this conclusion were that in nitrogen starved cells ammonium is assimilated more rapidly than either nitrate or nitrite and that the same assimilation rates are found for nitrate and nitrite (Chapter IV). Moreover, the excretion of nitrite under certain conditions points to the same conclusion (Chapter V). These facts together suggest that the reduction of nitrate into ammonium proceeds as follows:

$$\begin{array}{ccccccccc} (NO_3) & \longrightarrow & (NO_2) & \longrightarrow & ? & \longrightarrow & NH_2OH\ ? & \longrightarrow & (NH_4) \longrightarrow \text{cell nitrogen} \\ \downarrow\uparrow & & \downarrow\uparrow & & & & & & \downarrow\uparrow \\ NO_3^- & & NO_2^- & & & & & & NH_4^+ \\ \text{(External)} & & & & & & & & \text{(External)} \end{array}$$

( ) indicates: enzyme bound compound, in equilibrium with internal and external concentrations.

The picture given in this scheme of reaction is in good agreement with experimental results obtained with cell-free plant extracts. ECKERSON (1924) found a

rapid reduction of nitrate into nitrite in juice of tomato plants. More recently, an enzymatic reduction of nitrate into nitrite was observed by EVANS *et al.* (1953); a reduction of nitrite into ammonium by NASON *et al.* (1954), while ZUCKER *et al.* (1955) found a reduction of hydroxylamine into ammonium.

Based on theoretical considerations, WEBSTER (1955) arrived at the conclusion that the existence of intermediates as given in the above scheme, including hyponitrite and hydroxylamine, has to be accepted. This consideration is based on the assumption that in the course of the reduction of nitrate every further reduction step requires two hydrogen ions or electrons.

Nitrate reduction in algae generally takes place in light as well as in darkness. The assimilation in darkness, also in the case of nitrate, depends on the availability of oxidisable reserve materials. Provided sufficient assimilates are stored in the cells, the nitrate assimilation rate is independent of illumination.

The nitrate assimilation rate observed under optimal conditions of growth (saturating light intensity and excess carbon dioxide) does not surpass that occurring in darkness in an equivalent amount of nitrogen starved algae. These facts lead to the conclusion, that the energy supply of the nitrate assimilating system does not depend directly on light.

On the other hand, the results of EVANS *et al.* (1953) present strong evidence for the possibility of a direct, photochemical nitrate reduction. With nitrate reductase preparations of some higher plants they found a reduction of nitrate into nitrite in the light in the presence of reduced triphosphopyridine nucleotide (TPNH) and active grana. Also in darkness in the presence of TPNH a substantial nitrate reduction was found, the total amount of which was, however, smaller than in light. They suggest that the greater yield in light could be a consequence of the regeneration of reduced TPNH from the oxidized form by the light. NASON *et al.* (1954) demonstrated enzymatic reduction of nitrite into ammonium by DPNH, using enzyme extracts from *Neurospora* and soybean leaves.

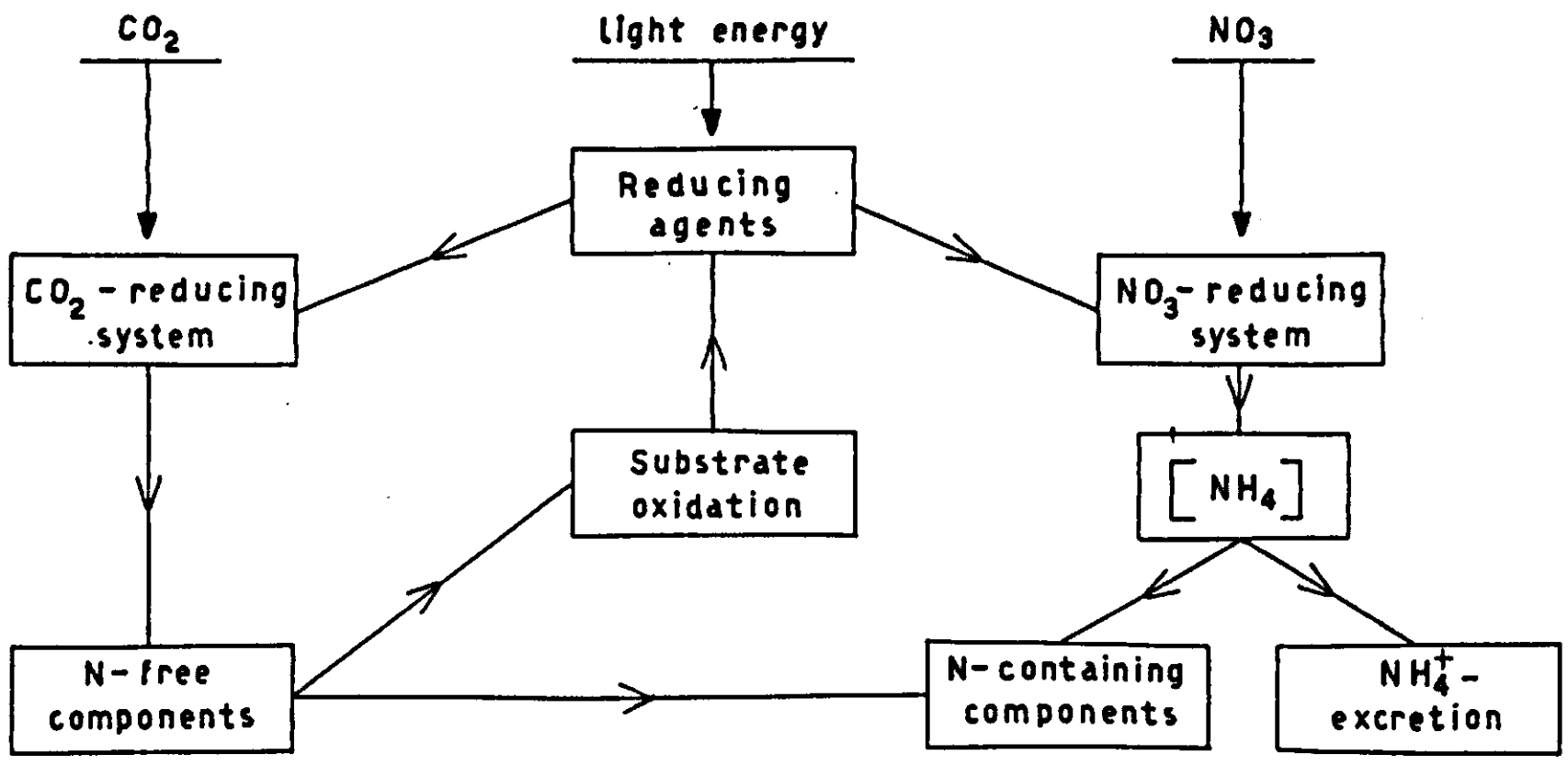
It thus appears that nitrate reduction only depends upon the availability of reduced co-enzymes, which can be supplied either by light or through the respiratory breakdown of carbohydrates in darkness. In our experiments with nitrogen starved algae which have a surplus of assimilates, it is likely that such a generation of reducing power can proceed during a prolonged dark period.

Evidently, nitrate reduction and protein formation are dark processes, analogous to the reduction of carbon dioxide. This can be symbolized in the following scheme, (see p. 48), in which energy rich phosphates and reduced co-enzymes may act as reducing agents.

This scheme assumes that the action of the nitrate reducing system is quite independent of that of the carbon dioxide reducing system, while both systems compete for the available reducing material. The independence is supported by the data of Chapter V, which demonstrate an ammonium excretion during a number of hours in illuminated algal cultures in the absence of CO_2. The competition for the reducing material is demonstrated by the experiments of fig. 33. In these experiments we observed that in weak light the rate of NH_4^+-excretion was suppressed by the simultaneously occurring CO_2-reduction.

In Chapter V the capacities of both systems have been measured separately. These experiments demonstrated a roughly five fold greater capacity of the carbon dioxide reducing system as compared with the nitrate reducing system. The nitrate reduction rates have been measured as an excretion of ammonium.

SCHEME 2



Under ample provision with CO_2, no ammonium excretion is found, but a formation of nitrogen containing cell components. The elementary composition of this material is approximately 50% C and 10% N, indicating a ratio of 5 to 1.

As was shown by WARBURG *et al.* (1920) in light an extra amount of O_2 will be produced as a consequence of nitrate reduction. Therefore, under conditions of simultaneously occurring CO_2- and NO_3-reduction the CO_2/O_2 quotient will be influenced and may deviate significantly from 1. This quotient, therefore, can be considered as a measure for nitrate reduction. We have discussed in Chapter V (p. 41) that the reduction of 1 molecule of nitrate probably leads to the production of 2 molecules of oxygen. A CO_2/O_2 quotient of 0.70 means the reduction of 70 molecules of CO_2 and a production of 100 molecules of oxygen. If 70 molecules of CO_2 give an output of 70 molecules O_2, the "extra" amount of 30 molecules of O_2 then represents the reduction of 15 molecules of NO_3. The NO_3/CO_2 ratio is then 15/70 or about 1/5.

Data on nitrate reduction in literature reveal great differences in this respect. MYERS and CRAMER (1948) with nitrogen starved algae found a CO_2/O_2 quotient of 0.74 in strong light, and 0.17 in weak light. These quotients correspond to NO_3/O_2 ratio's of 1/5 and 2/1 respectively. If normal cells were used, the CO_2/O_2 quotient also deviated appreciably from unity, the value being about 0.70 in weak light and about 0.90 in strong light, nearly equal to the quotient found if ammonium was used instead of nitrate. This would indicate that by N-starved algae in weak light relatively more nitrate is reduced (with respect to the volume of CO_2 assimilated) than in strong light. In strong light the relative amount of nitrate reduced, would be very small, indicating the formation of material with a lower nitrogen content than the material cultivated at low light intensities. Such low nitrogen contents are reported by MYERS (1946) and KOK (1952). The statements of AACH (1952), however, show the reverse: at high light intensity he found higher nitrogen contents than in the case of low light intensities. The results of VAN OORSCHOT (1955) on the other hand, do not show any difference in N-content at all between material cultivated at high or low light intensities.

According to KROLLPFEIFFER (1951), a significant deviation of the CO_2/O_2 quotient from unity was only found in weak light, if the exposure to weak light

was immediately preceded by an exposure to a high light intensity. This suggests that, according to conditions of pretreatment, the NO_3/CO_2 ratio may be influenced.

Also from the experiments of VAN NIEL *et al.* (1953), KOK (1952), and VAN OORSCHOT (1955), a rough estimation of the NO_3/CO_2 ratio's can be made. In strong light, they found CO_2/O_2-quotients varying from 0.68 to 0.85, indicating NO_3/CO_2 ratio's of 1/4 to 1/12. The data discussed show that the results, as measured in our experiments, closely agree with NO_3/CO_2 ratio's as observed by various authors under more generally physiological conditions.

SUMMARY

Normal algae are characterized by an N-content of 8% to 10% and a C-content of about 50% (N/C ratio 0.18). After removal of nitrogen from the culture medium the protein and chlorophyll contents decrease, until a final composition is reached with a nitrogen content of 2% to 3% (on a dry weight basis). Correlated herewith, the rate of photosynthesis decreases, and the carbohydrate content of the cells increases. We also followed the time course of the reverse process: the re-balancing of N-starved cells to normal composition and N/C ratio, after re-addition of nitrogen to the culture medium. During this process, the rate of photosynthesis increases (Chapter II).

We made quantitative studies of the uptake of NO_3^-, NO_2^-, and NH_4^+ under various conditions. Provided pH is well controlled (the pH optimum for the consumption of all three sources investigated was found to be between 7 and 8) nitrite is metabolized by the algae at exactly the same rate as nitrate.

The conversion of inorganic nitrogen into cell nitrogen does not occur in the absence of simultaneous CO_2-assimilation unless the N/C ratio of the algae has been disturbed during pretreatment (Chapter III).

The total amount of nitrogen assimilated is greater, the lower the N-content had dropped during the previous starvation. The initial rate of N-assimilation, however, is independent of the degree of pre-starvation, in case a nitrogen source (nitrate or ammonium) is re-added to the algae (Chapter III).

It appeared that the rates of nitrate assimilation both in light in the absence of CO_2 and in darkness (i.e. in both cases under exclusion of growth) in N-starved cells was equal to the maximum rate of nitrogen assimilation in normal cells under conditions of optimal growth (Chapter III).

The conclusion is that external energy supply could be excluded as a rate determining factor of the process of nitrogen rebalancing. This was emphasized, furthermore, by the fact that addition of glucose did not increase the rate of nitrate assimilation. Therefore, the capacity of the NO_3^--assimilating system must be equal in light and darkness.

In the absence of CO_2, either in darkness or in light, nitrogen starved algae show a much faster assimilation of NH_4^+ than under conditions of full growth. Therefore, in actively growing cells the limiting factor for nitrogen assimilation is the availability of suitable carbon "skeletons".

In nitrogen starved cells, nitrate and nitrite are assimilated at equal rates, which are much lower than that of ammonium assimilation. Therefore, the limiting step in the assimilation of nitrate has to be located somewhere between the reduction levels of NO_2^- and NH_4^+ (Chapter IV).

In accordance herewith was the observation of a relatively strong nitrite excretion during the period of N/C-rebalancing in case NO_3^- was used as a

source of nitrogen. This excretion had a temporary character, and during the first 4–5 hours amounted to 5–8 % of the total amount of incorporated nitrogen. It showed a pronounced optimum at pH 7–8, and was not influenced by light.

A nitrite excretion of quite similar a character is shown by cells with a normal N/C ratio in case they are exposed to light in the absence of CO_2. Both the rate and the amount of the excretion are optimal at a relatively low light intensity. In the presence of CO_2, the NO_2^--excretion is suppressed.

In addition to the nitrite excretion, normal cells, if exposed to light in the absence of CO_2, show a much more conspicuous excretion of ammonium. At medium light intensities, all absorbed light energy can be used for this conversion. In this conversion, per molecule of NO_3^- reduced, a number of quanta was required comparable to the number required per molecule of CO_2 reduced in normal photosynthesis. In strong light, the NH_4^+ excretion proceeds for a number of hours at the same speed as the rate of N-incorporation found if CO_2 were present. In the latter case, the end products of NO_3^--reduction are organic compounds and no detectable amounts of NH_4^+ are excreted.

The correlation between the rate of CO_2- and NO_3^--reduction and the light intensity was estimated with normal algae. It appeared that the two processes could be shown to run rather independently of each other and to compete for the reducing power generated by the light. The ratio of the capacities of the two systems appeared to agree closely with the N/C ratio's observed in algae of normal composition (Chapter V).

SAMENVATTING

Het stikstof- en het koolstofgehalte van algenmateriaal, dat in een volledig voedingsmedium verkregen wordt, is tamelijk constant. Het stikstofgehalte bedraagt 8 à 10 % en het koolstofgehalte ongeveer 50 % (berekend op droge stof). Bij groei in een voedingsmedium, zonder bruikbare stikstofbron, kan het koolstofgehalte aanmerkelijk stijgen; het chlorophylgehalte daalt dan en ook het stikstofgehalte, totdat een minimale waarde bereikt wordt van 2 à 3 % N. Bij het bereiken van dit stikstofgehalte is de photosynthesesnelheid zeer laag, maar de oorspronkelijke snelheid en ook de samenstelling kan weer verkregen worden door aan deze culturen een bruikbare stikstofbron te verstrekken (Hoofdstuk II).

De omzetting van anorganische stikstofverbindingen in organische celcomponenten heeft alleen plaats indien gelijktijdig koolzuur gereduceerd wordt, tenzij de normale N/C-verhouding verstoord is, tengevolge van een voorafgaande stikstofhongering. De totale hoeveelheid geassimileerde stikstof is dan afhankelijk van de hongeringsgraad. Het bleek verder, dat de snelheid waarmede dit materiaal stikstof assimileert, onafhankelijk is van de hongeringsgraad, de lichtenergie en toevoeging van substraten, en dat die snelheid gelijk is aan de maximale snelheid van nitraat- en ammoniumassimilatie onder omstandigheden van groei (Hoofdstuk III). Ammoniumstikstof wordt echter door dit voorgehongerd materiaal sneller geassimileerd. Daarom moet de langzaamste en snelheidbepalende stap in het proces van de nitraatreductie gelocaliseerd worden vóór ammonia. Daar nitriet niet sneller geassimileerd wordt dan nitraat en onder bepaalde omstandigheden (afhankelijk van de pH) zelfs door de cellen in het medium uitgescheiden kan worden, lijkt het waarschijnlijk, dat deze langzaamste stap na nitriet verondersteld moet worden (Hoofdstuk IV).

Indien normale algen belicht worden in media, die nitraat bevatten, bij afwezigheid van CO_2, dan wordt behalve nitriet, ook een beduidende hoeveelheid

ammonia (afhankelijk van de lichtintensiteit) in het medium afgescheiden. Het aantal afgescheiden moleculen ammonia is in zwak licht ongeveer gelijk aan het aantal moleculen koolzuur dat geassimileerd wordt als nitraat afwezig is. Per molecuul gereduceerd CO_2 en nitraat worden dus ongeveer evenveel lichtquanta gebruikt. Bij gelijktijdige aanwezigheid van nitraat en koolzuur kan een onderlinge concurrentie worden aangetoond om het reducerende agens. In sterk licht vindt, eveneens bij afwezigheid van koolzuur, ammonia-uitscheiding plaats. De snelheid hiervan is echter 5 maal zo laag als de snelheid waarmee koolzuur wordt gereduceerd (in overeenstemming met de N/C-verhoudingen in normale cellen). Indien nitraatassimilatie en nitraatreductie (bij afwezigheid van koolzuur) vergeleken worden, dan blijkt, dat de snelheden van deze processen in sterk licht gelijk zijn. Hieruit volgt, dat het nitraatreducerend proces niet afhankelijk is van producten van de photosynthese (Hoofdstuk V).

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STELLINGEN

I

De photosynthese snelheid van stikstofarm algenmateriaal is niet gecorreleerd met de chlorophylconcentratie.

(Dit proefschrift)

II

In algenculturen kan de reductie van nitraat in donker en in licht met gelijke snelheid geschieden.

(Dit proefschrift)

III

De reductie van nitraat in een biologisch systeem kan beschouwd worden als een donkerproces.

IV

Het begrip „groei" wordt door VAN HILLE op een onjuiste wijze gehanteerd.

(VAN HILLE. Diss. Utrecht 1938)

V

Het gebruik van algen bij de biologische reiniging van afvalwater, zal de zuurstofvoorziening in het afvloeiende water kunnen bevorderen.

VI

Het waarde verminderende karakter van onkruidzaden komt in de formule ter berekening van de gebruikswaarde voor zaaizaden op een onvoldoende wijze tot uitdrukking.

VII

Bij de verklaring van de z.g. minnend- en mijdendheid van graslandplanten speelt de concurrentie een zeer belangrijke rol.

VIII

Tegen het door TURNER geconstateerde verband tussen daglengte en optimale N/K-bemesting zijn ernstige bezwaren in te brengen.

(W. I. TURNER and V. M. HENRY. "Growing plant, in nutrient solutions". John Wiley & Sons, Inc. New York 1939)

IX

De betekenis van practische werkzaamheid in een laboratorium voor plantenphysiologisch onderzoek voor de aanstaande landbouwkundig ingenieur wordt, in het kader van een algemene oriëntering, nog niet voldoende in aanmerking genomen.

oefschrift L. H. J. Bongers