THE QUANTITATIVE RELATION BETWEEN RATE OF PHOTOSYNTHESIS AND CHLOROPHYLL CONTENT IN CHLORELLA PYRENOIDOSA

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Contents.

CHAPTER I.

Introduction.

Ever since the discovery of the principles of plant metabolism the photosynthesis of the green plants has been the object of a great number of investigations. The results of the most important research work have been summarized by STILES (1925) and by SPOEHR (1926) in two monographs on photosynthesis and surveys on the latest investigations have been given in the papers of GAFFRON and WOHL (1936) and of EMERSON (1936 and 1937), to which is referred for a review of the evolution of photosynthetic research.

The photosynthesis in the green plants actually is a reduction of carbon dioxide by means of light energy. The light energy is taken up by the chlorophyll. The carbon dioxide reduction, however, can only be achieved in the living plant tissue; photosynthesis by chlorophyll in vitro could never be established. Which factor in the plant, besides the chlorophyll, is concerned in the photosynthesis cannot be said. It is likely that in the living chloroplast a special structure of the chlorophyll molecules is present, which is necessary for the photosynthesis and which is disturbed by chlorophyll extraction.

If no external factor is limiting, it might be expected that the rate of photosynthesis is proportional to the chlorophyll content unless another factor in the plant limits the assimilation process.

It has been tried by several authors to discover a quantitative relation between the rate of photosynthesis and the chlorophyll content.

PLESTER (1912) studied the assimilation activity of the leaves of green, yellow and variegate varieties of different plants. Though he observed that the photosynthesis of all leaves with lower chlorophyll content is less than that of normal leaves, the lower assimilation rate was not proportionate to the chlorophyll concentration. PLESTER's methods of photosynthesis determination were rather crude, so his results are not conclusive.

In the well known experiments of WILLSTÄTTER and STOLL (1918) the assimilation activity is given as the assimilation number (= „Assimilations-Zahl") which is the photosynthesis in grams of carbon dioxide absorbed per hour per gram of chlorophyll. In a number of different objects widely divergent assimilation numbers were found. Most striking are the high assimilation numbers in *aurea*-varieties of *Sambucus nigra* and *Ulmus*. Also the leaves of etiolated plants gave high assimilation numbers.

WILLSTÄTTER and STOLL explained the differences in assimilation numbers by assuming that an enzyme is playing a part in the process and that this enzyme determines the rate of photosynthesis. As the enzyme reaction is the rate determining process, this theory was in accordance with the fact that the photosynthesis has a temperature sensivity that is much higher than in photochemical processes. Therefore it is a highly interesting point in the work of these authors that in leaves with a high assimilation number the temperature sensitivity decreases. This points to a determination — in this case — of the rate of photosynthesis by the photochemical reaction.

EMERSON (1929) repeated the experiments of WILLSTÄTTER and STOLL with the unicellular alga *Chlorella* measuring photosynthesis manometrically by the method of WARBURG (1919, 1920). For controlling the chlorophyll content, cells were cultivated in nutrient media containing different iron concentrations. EMERSON found the assimilation number for *Chlorella* about constant within the cultures of one set and concludes that the photosynthesis is a function of the amount of chlorophyll just as it is of external factors too. The fact that the curves of different series are not superimposed is considered by EMERSON a matter of minor importance. However, this is an indication that there must be another factor in the cells limiting the photosynthesis, or limiting the chlorophyll activity. Moreover, in EMERSON's „shorter" curve (his fig. 3) the assimilation numbers are not constant as the curve does not pass through the origin.

Once more the ratio between photosynthesis and chlorophyll content in *Chlorella* was investigated by FLEISCHER (1934). This author supposed that the irregularities in EMERSON's results might have been caused by the fact that EMERSON did not regularly measure respiration but applied a uniform correction for it. FLEISCHER found an about constant assimilation number in cultures with normal nutrient solution and in media with graded amounts of iron or nitrogen. So it seems indeed that the amount of chlorophyll is a rate determining factor in photosynthesis. In cells grown in media with a low magnesium concentration very inconstant assimilation numbers were found.

In the experiments of EMERSON and ARNOLD (1933) on photosynthesis of *Chlorella* exposed to flashes of light was demonstrated that the light reaction and so the chlorophyll, as far as its rôle in the light reaction is concerned, is not the rate determining factor, as the dark reaction proved to require much more time and therefore always limits the rate of photosynthesis. The

authors hold the view that the results indicating a constant ratio between chlorophyll content and rate of photosynthesis must be attributed to chance.

The experiments described in this paper have been undertaken to investigate whether a constant ratio between the rate of photosynthesis and the chlorophyll content really exists. If this could be stated it would be an indication that the chlorophyll plays a part in the limiting process of photosynthesis being the BLACKMAN reaction.

Furthermore, it was investigated by which factors this ratio could be affected. The effect of low concentrations of magnesium, found by FLEISCHER, seemed an interesting point. The results obtained with cells cultivated in media with graded amounts of magnesium have been published in a preliminary note (VAN HILLE 1937) and moreover in Chapter IV. It was supposed that the external conditions during the growth of the cells might have an influence, as the results obtained in the sets of cultures not simultaneously cultivated use to yield divergent results.

CHAPTER II.

Experimental Methods.

Material.

Experiments were carried out with the well known experimental object *Chlorella pyrenoidosa*. A pure strain was secured from the collection of Prof. Dr. E. G. PRINGSHEIM at Prague. This strain had been cultived in a test-tube on a solid substrate of unknown composition.

For all experiments *Chlorella* cells grown in fluid culture-medium were used.

The algae were cultivated in Erlenmeyer flasks of 100 cm^3, covered with cotton-wool stoppers. The flasks were filled with 50 cm^3 of the nutrient solution. The composition of the nutrient solutions is always given in the concerned chapter. The nutrient compounds were dissolved in distilled water. It proved not to be necessary to distil the water from glass to glass. Cultures provided with distilled water from the brass still or from the glass still do not show any difference.

The flasks filled with the nutrient medium were sterilized for half an hour at 100° at a pressure of 1 atmosphere, because most of the cultures contained 1½ per cent of glucose. This way of sterilizing always proved to be sufficient. Bacterial infections

appeared only incidentally after inoculation of the medium with algae or after the cultures had been used for experiments. The bacteria show a quick development by the high pH and the glucose and so an infected culture was easily recognized by the bacterial film formed on the surface of the nutrient solution within a few days.

Culture media, containing no glucose were sterilized at 120° at a pressure of 2½ atmospheres for 20 minutes.

The cultures were inoculated by means of a metal needle, the end of which had been bent into an eye with a diameter of 6 mm. This needle, after being heated in a BUNSEN flame, was put in the test-tube or in the culture in which the *Chlorella* cells grew. The eye then bears a film of the nutrient solution containing a number of cells. These were brought into the sterile nutrient solution. In this way the inoculation could generally be carried out without causing infections.

Cultivation.

Cultures were grown in diffuse daylight in a greenhouse. The temperature was not constant. In winter the temperature fluctuated about 21°. On warm days in summer the temperature could rise appreciably and sometimes amounted for a short time to over 30°. No injury was effected by these high temperatures lasting for such a short time. The greater part of the experiments, moreover, were carried out in winter.

Chlorella, when cultivated with glucose, supplies thick suspensions in a short time. The cells, especially in young cultures are rather heavy, so they settle on the bottom of the culture

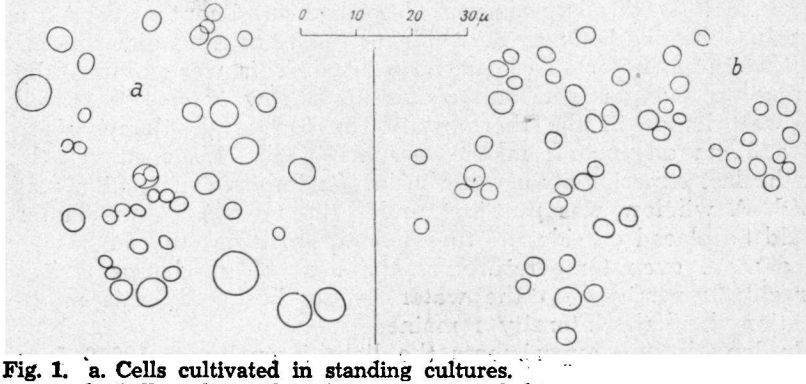


Fig. 1. a. Cells cultivated in standing cultures.
b. Cells cultivated under continuous shaking.

flasks. Experimental cultures were stirred daily in order to distribute the cells and the nutrient compounds evenly in the culture. Part of the cultures were cultivated under continuous shaking. Growth was not accelerated by the shaking but the cells showed a greater mutual conformity (see figure 1). This had no influence on the cell metabolism, so the shaking has no essential effect on later experiments.

The pH determination.

The pH of the nutrient solution was determined colorimetrically by means of „HELLIGE's Microcomparator". The method has the advantage that only little test solution is needed for the pH determination. This was necessary because the same culture was used for estimating photosynthesis several times. The method is not accurate. In the pH range, within the reach of two different indicators not always the same pH values are found. The relations of the quantities of test solution and some indicators as given in the manual proved not to be satisfactory.

Determination of Photosynthesis.

Photosynthesis was determined by the manometric method of WARBURG (1919). The water-basin was heated electrically and fitted with two stirring apparatus driven bij an electromotor. Temperature was kept constant by a toluene regulator at 25,5° C.

The light of a series of incandescent lamps, placed behind the window in the backside of the thermostat, was reflected by a mirror in the thermostat on the bottoms of the vessels containing the cell suspensions. In this way an illumination with 4000 Lux was obtained. This illumination has been used only in a part of the experiments described in Chapter V. As a surplus illumination was desirable in the remaining experiments a light intensity of 12000 Lux was used. This was obtained by fixing up a watertight tank containing a row of closely spaced 56 watt lamps in the thermostat. The top of the lamps were at a 3 cm distance from the bottoms of the WARBURG vessels. In the last experiments a waterbasin of another construction was used. A window was put just under the vessels, so the lamps could be placed outside the thermostat, which has the advantage of a more even temperature, as the heat of the lamps is less directly transmitted to the water in the basin. By this modification the light intensity remained the same.

In long lasting experiments the light intensity of 12000 Lux

has an injurious effect on the cells. Especially in aged cells and in cells cultivated at a low light intensity the chlorophyll is destructed and the protoplasm is damaged. (HARDER 1933). Most experiments were not extended over a longer period than half an hour, so the injurious light effect could be neglected. Only in the experiments of Chapter VII, carried out with old cells which were exposed to the light for a long time, and in the experiments described in Chapter VIII with cells cultivated in darkness the injury was perceptible.

In the experiments the conical vessels of WARBURG with one side-bulb and without central well were used. The capacity of the vessels was about 20 cm^3.

Before the experiment the culture in question was thoroughly shaken to get an even distribution of algae in the culture medium. A quantity of the cell suspension was poured sterilely in a calibrated centrifugal tube. This quantity ran from 20 cm^3 for the young cultures to 0,5 cm^3 for cultures forming dense suspensions. This suspension was centrifuged till the supernatant culture liquid was quite clear. Of this liquid the pH was determined.

As a suspending fluid 7 cm^3 of WARBURG's carbonate mixture no. 9 was employed. This mixture is composed of 85 parts M/10 sodium bicarbonate plus 15 parts M/10 sodium carbonate. The WARBURG vessels were filled with 7 cm^3 of the cell suspension by means of pipettes.

Because the ratio between the rate of photosynthesis and chlorophyll was investigated, always thin cell suspensions were used, as otherwise the cells would overshade each other so the amount of chlorophyll might not show its maximal achievement. This concentration of cells had been determined previously by examining the photosynthesis of various quantities of algae from one culture and by determining up to which concentration the assimilation was proportional to the quantity of algae.

When the algae in the vessels had been submerged in the thermostat they were illuminated and shaken for an adjustment period of 15 minutes. During this period the stop-cocks were closed to allow the water vapor in the gas space to get into equilibrium with the carbonate mixture. The cell suspension obtained the temperature of the waterbasin and during this time the induction (SMITH (1937)) took place. At the end of the adjustment period the stop-cocks were opened momentarily to level the BRODIE fluid in the manometer; after closing the stop-cocks the first reading was done. The oxygen production was

followed manometrically at 5 or 10 minute intervals, for half an hour at least; from which, according to the formula given by WARBURG, the exact amount of liberated oxygen was computed.

Determination of respiration.

The computing of the respiration in all the investigations of photosynthesis is a source of uncertainty (v. d. PAAUW 1932). When the cells contain a great quantity of assimilation products the rate of respiration is higher than when the cells have not been able to photosynthesize for a long time (FRENCH, KOHN, TANG 1934).

It could be demonstrated that the respiration is less before the determination of the photosynthesis than afterwards. It is probable that during the photosynthesis the respiration increases. This increase, however, does not appear from a decreased rate of photosynthesis (cf. Chapter V). In all the experiments I determined the respiration for half an hour immediately after the photosynthesis.

From the moment on which the light is switched off, the development of oxygen goes on for a short time. Five minutes sufficed for the establisment of the equilibrum. A decrease of the respiration during the first half hour after the photosynthesis was not stated. In young cultures the respiration amounts to one tenth of the photosynthesis; in older cultures the respiration increases in proportion to the photosynthesis. When cultivating cells without glucose the respiration is $\frac{1}{30}$ and less of the photosynthesis in young cells.

The rate of respiration thus obtained in half an hour was applied as a correction on the rate of photosynthesis.

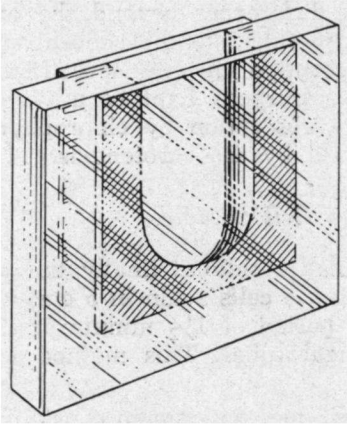
Determination of the chlorophyll content.

After the determination of the respiration the cell suspension used in the experiment was poured out in centrifugal tubes of pyrex glass. The algae were centrifuged, the carbonate mixture was decanted, the cells were boiled in methanol for a few seconds and centrifuged again. The young cells after having been boiled once are completely decolourized; the cell residuum is white. In older cells, even by repeated boiling, part of the chlorophyll cannot be extracted. This is, however, easily done by suspending the cells in one or two drops of water. At the second extraction with methanol also the chlorophyll of the old

cells was then completely taken up. The extracts were poured together and methanol was added up to 10 cm³.

Of this chlorophyll solution the absorption was determined at the wavelength of 6600 Å by means of the spectral pyrometric method (ORNSTEIN 1935).

The cuvettes were made of pieces of 5 mm thick plateglass that had been cut in a U-shape (figure 2). At both sides coverglasses of planeparallel glass were pasted with a special coverglass lac (Deckglaslack 1 a Dr. G. GRÜBLER and Co Leipzig). For low chlorophyll concentrations cuvettes of 1 cm gauge were



made by pasting two pieces of U-shaped plateglass together. The cuvettes have a content of 3 and 6 cm³ respectively.

It has often been stated that a chlorophyll solution follows the absorption laws of Beer and Lambert unless the concentration is too high (HUBERT 1935).

This was proved also in my experiments. According to these laws for clear solutions of pigments in uncoloured media

$$\log \frac{Io}{I} = kcd$$

in which:

Io = light intensity after passing through the uncoloured medium,
I = light intensity after passing through the pigment solution,
k = absorption coefficient,
c = concentration of the pigment,
d = thickness of the layer.

At the use of cuvettes of constant gauge, the concentration is proportional to $\log \frac{Io}{I}$.

The absorption spectrum of diverse chlorophyll extracts was determined. By plotting the $\log \log \frac{Io}{I}$ against the wave length the shape of the absorption curve is independent of the concentration of the chlorophyll; so the measured absorption curves are directly comparable (WEIGERT 1916).

The chlorophyll extracts of cells of different assimilatory activity, of different age, or cultivated in different nutrient media do not show differences beyond the experimental error. Hence it is allowed to calculate the concentration of the chlorophyll from the absorption of one special wave length. It is evident that for this purpose light of a wave-length has been chosen of which the absorption by chlorophyll is maximal and which is not absorbed by the carotenoids.

Determination of the Cell volume.

Of the extracted cell residuum finally the volume was determined by suspending the cells again in 5 cm^3 water and centrifuging them during a period of 1½ minute at a uniform speed in TROMSDORFF centrifugal tubes. This method involves many objections.

a. The cell volume does not remain constant during the photosynthetic experiment, but increases in almost all cases.

b. The cell volume that is read after centrifuging is dependent on the rate of settling, consequently of the specific gravity of the cells. Cells having a high specific gravity will be quickly centrifuged out, forming a compact centrifugate, while specifically light cells settle slowly into a loose and voluminous sediment. The volume of the cells determined by the TROMSDORFF method therefore is to a certain extent a reverse measure of the specific weight of the cells. The only significance of the volume of the cells is to have a measure for the amount of substance participating in the metabolism. To this purpose the cell weight is hardly a better measure than the cell volume as the quantity of cell-wall substance and store-starch has a far greater influence on the weight than the living protoplasm.

The cell volumes are recorded only for the sake of completeness; in my opinion they do not have much value in the calculation of the results.

CHAPTER III.

Growth and Photosynthesis of Chlorella pyrenoidosa.

Introduction.

The experiments described in this chapter were done with pure cultures of *Chlorella pyrenoidosa*. The nutrient solution had the following composition:

$FeSO_4$	0,03 g.
Na citrate	1,— g.
KNO_3	1,26 g.
$MgSO_4$	2,46 g.
KH_2PO_4	1,22 g.
Glucose	15,— g.
Aq. dest.	1 l.

This medium was used in the experiments of FLEISCHER (1934) as normal nutrient solution.

The cultures were cultivated in 100 cm^3 Erlenmeyer flasks, which had been sterilized and inoculated as described in Chapter II. Cultures that had been contaminated by bacterial infections were left out of consideration. The Erlenmeyer flasks were placed in a greenhouse. The light intensity was low, because these experiments were carried out in the months November and December 1937. In this time the sky was almost heavily clouded throughout. The temperature in the greenhouse was about 21°. On a few days when during a short time the sun was shining it could rise to 28°. Under these circumstances all cultures showed a rapid and regular growth.

Although the cultures are cultivated under equal conditions, they often show individual differences, but these are not essential. The results recorded in one table or graph always relate to one culture.

Photosynthesis, chlorophyll content, cell volume, cm^3 nutrient solution, pH, were determined by the methods, reported in Chapter II.

The results of these determinations are represented as follows:

Time : days after inoculation of the culture.

Photosynthesis : mm^3 oxygen evolved in the light during half an hour + mm^3 oxygen absorbed in the dark during an equal following period of time.

$\frac{Photosynthesis}{cm^3\ nutr.\ sol.}$: the photosynthesis of the algae per 1 cm^3 nutrient solution.

Chlorophyll : the relative concentration of chlorophyll ($\frac{Io}{I}$).

$\frac{Chlorophyll}{cm^3\ nutr.\ sol.}$: the relative amount of chlorophyll in the algae per 1 cm^3 nutrient solution.

$\frac{Cell\ volume}{cm^3\ nutr.\ sol.}$: the cell quantity in mm^3, determined by the TROMSDORFF method, present in 1 cm^3 nutrient solution.

$\frac{Photosynthesis}{Chlorophyll}$: the photosynthesis of a sample of cells divided by its relative chlorophyll content, or in other words, the photosynthesis of the cells per unit of chlorophyll. This value consequently is proportionate to the assimilation numbers ("Assimilations-Zahl") according to WILLSTÄTTER and STOLL (1918).

If in my work is referred to assimilation numbers, the photosynthesis per unit of chlorophyll is meant. The absolute value of the ratio $\frac{\text{photosynthesis}}{\text{chlorophyll}}$, however, is not comparable with the assimilation number of WILLSTÄTTER and STOLL, but must be multiplied by an unknown constant.

After the inoculation, the nutrient solution was still quite clear. The first observation was made when the first green colouring was visible.

Results.

A perusal of table 1, column 3 and figure 3a shows that the photosynthesis makes a great progress in the beginning, reaches a maximum and then decreases.

TABLE 1.

Time	pH	Photosynthesis	Chlorophyll	Cell volume	Photosynthesis
		cm^3 nutr. sol.	cm^3 nutr. sol.	cm^3 nutr. sol.	Chlorophyll
6	6,9	8,356	0,012	0,4	6,90
10	7,1	62,2	0,137	3,2	4,55
12	7	108,2	0,267	4,1	4,06
18	6,8	131,2	0,392	6,5	3,35
26	6,7	79,0	0,391	11	2,02
31	6,8	56,4	0,383	11,2	1,47

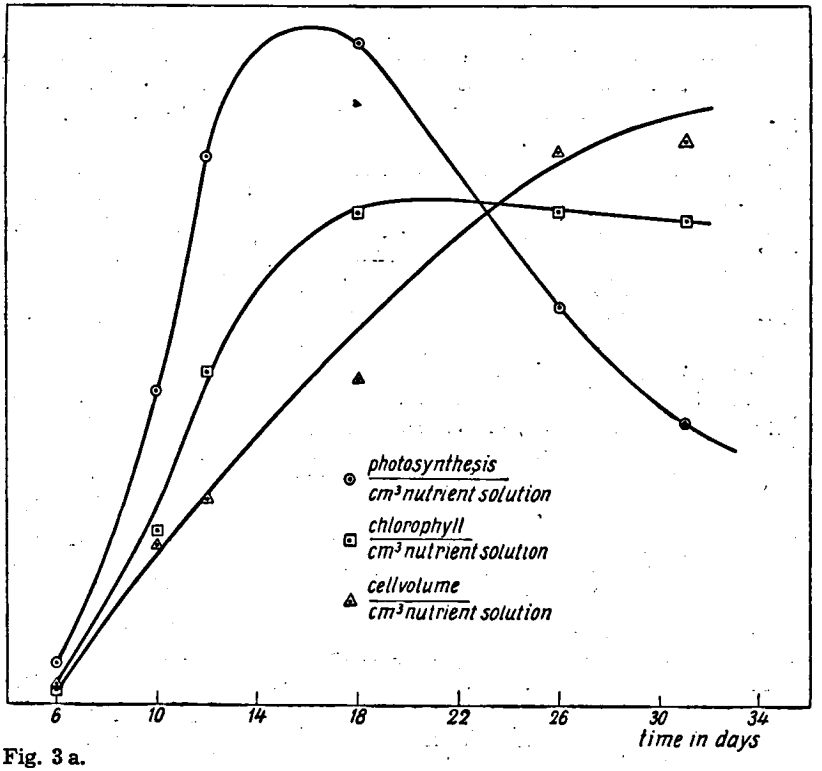


Fig. 3 a.

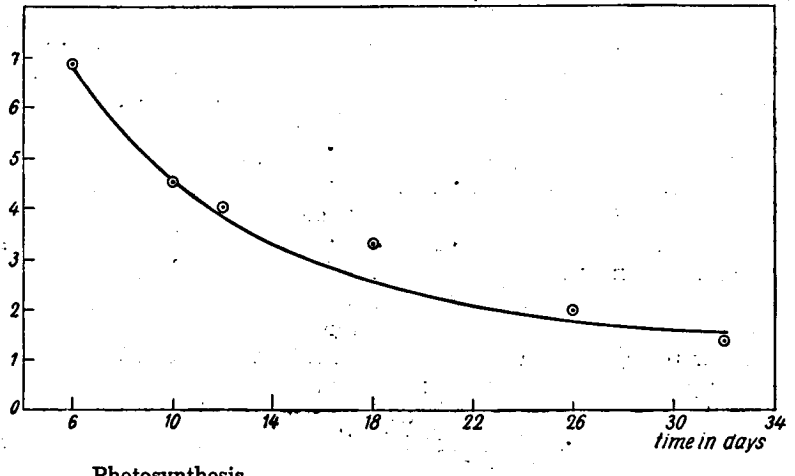


Fig. 3 b. $\frac{\text{Photosynthesis}}{\text{Chlorophyll}}$ in course of time of culture with normal nutrient solution, cultivated in diffuse daylight.

The chlorophyll content (table 1 column 4) increases first, reaches a maximum some days later than the photosynthetic maximum and then decreases too (for discussion see Chapter VIII, page 748).

The cellvolume increases continuously during the time of observation; the increase is greatest in the beginning.

As table 1, column 6 and figure 3b show, the assimilation numbers are not constant, but continuously decrease. This means that the chlorophyll content increases faster than the photosynthesis. Even during the time of unchecked development in the culture, the assimilation numbers become lower.

As in the first experiment the highest assimilation number is found, it is possible that a still higher assimilation number might have been found if an experiment could have been done earlier. From the shape of the curve it cannot be concluded where the maximal value of the assimilation number lies.

It was examined which factors in the nutrient solution might unfavourably affect the photosynthesis in order to explain the decrease of the assimilation numbers.

I. The pH is not constant.

It must be born in mind that the photosynthesis of the cells was not determined in the nutrient medium but in the carbonate mixture, at a pH of 8,5. If the pH in the nutrient solution should have an injurious influence on the photosynthesis a recovery of photosynthesis in the carbonate mixture must be expected. The photosynthesis, however, does not recover in the carbonate mixture, but remains constant during many hours.

EMERSON and GREEN (1938) observed that the photosynthesis of *Chlorella pyrenoidosa* was not influenced by media of a pH between 5 and 8,5. It is, therefore, improbable that the pH would have affected the ratio $\frac{\text{photosynthesis}}{\text{chlorophyll}}$.

II. During the growth of the culture, the inorganic substances and glucose are consumed, so the concentration of the nutrient solution decreases. Because rapid growth takes place in the beginning, the concentration of the nutrient compounds must also decrease sharply in the beginning. This is in accordance with the fact that during the first experiments the assimilation numbers show the greatest decline. If the decrease of the assimilation numbers should be a direct reaction on the concentration of the nutrient compounds, higher assimilation numbers ought to be found in a more concentrated nutrient solution, respectively lower assimilation numbers in a less concentrated nutrient solution.

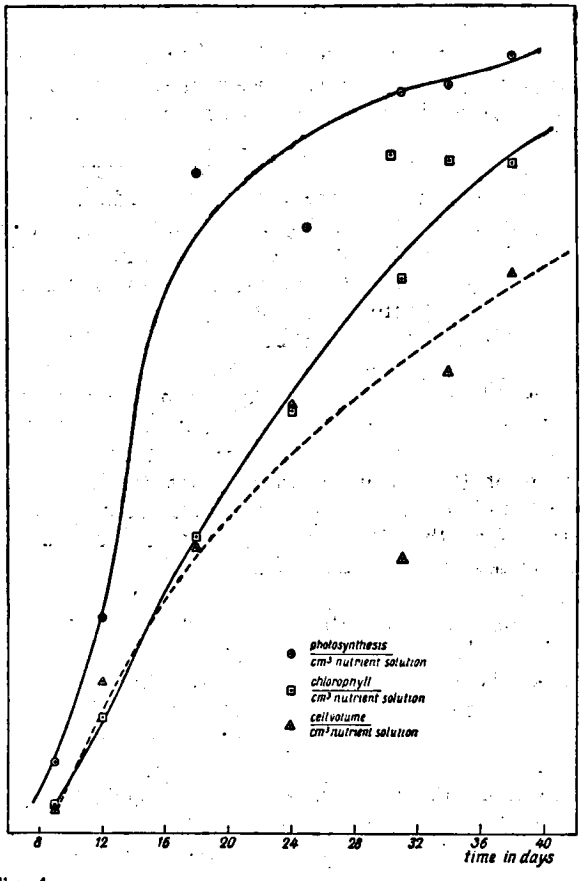


Fig. 4 a.

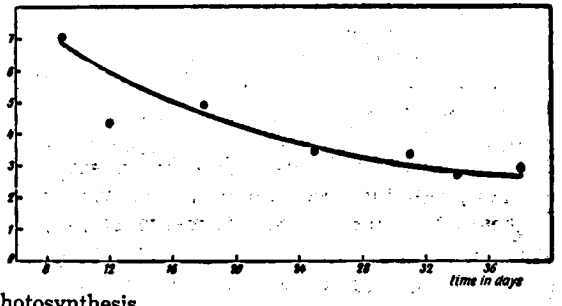


Fig. 4 b. $\frac{\text{Photosynthesis}}{\text{Chlorophyll}}$ in course of time of culture with 2 × concentration of the nutrient solution, cultivated in diffuse daylight.

(In Fig. 4 a $\frac{\text{Cellvolume}}{\text{cm}^3 \text{ nutr. sol.}}$ is indicated by a dotted line, as the values are very irregular).

This was tested as follows: the nutrient components were given in the same mutual proportion, but to twice and to half the concentration respectively. The results of the measurements of two cultures that were provided with these modified nutrient solutions are reproduced in table 2 and 3, moreover in figure 4a and 4b, and figure 5a and 5b.

These cultures have been inoculated on the same day as the culture of table 1 and have grown under the same conditions of light intensity and temperature.

From the graphs it is evident that the photosynthesis and the chlorophyll content are appreciably influenced by the concentration of the nutrient solution. The maximal values attained are proportionate to the concentration of the nutrient solution.

The maximal values of the assimilation numbers (figure 4b and 5b), however, are not affected by the concentration of the nutrient solution; the curves of figure 3b, 4b and 5b are almost identical. The maximal values of all three cultures are almost on the same level.

The curves drop off most rapidly in the beginning.

TABLE 2.

2 × nutrient solution.

Time	pH	Photosynthesis	Chlorophyll	Cell volume	Photosynthesis
		cm^3 nutr. sol.	cm^3 nutr. sol.	cm^3 nutr. sol.	Chlorophyll
9	7,1	22,3	0,036	0,77	7,05
12	6,9	68,7	0,146	4,8	4,38
18	7	208,0	0,374	9	4,92
25	7	191,5	0,547	13,5	3,50
31	—	234,0	0,700	8,7	3,35
34	7	236,6	0,847	14,6	2,72
38	6,4	245,8	0,845	17,7	2,91

TABLE 3.

$^1/_2$ × nutrient solution.

Time	pH	Photosynthesis	Chlorophyll	Cell volume	Photosynthesis
		cm^3 nutr. sol.	cm^3 nutr. sol.	cm^3 nutr. sol.	Chlorophyll
6	7	11,2	0,015	0,43	7,70
8	7,3	35,0	0,067	1,4	5,35
11	7,1	78,4	0,155	3,5	5,08
18	6,6	32,8	0,133	6,0	2,46
26	6'9	25,7	0,130	9,4	1,98
31	6,9	20,9	0,149	—	1,40
34	6,9	20,3	0,115	14,3	1,77

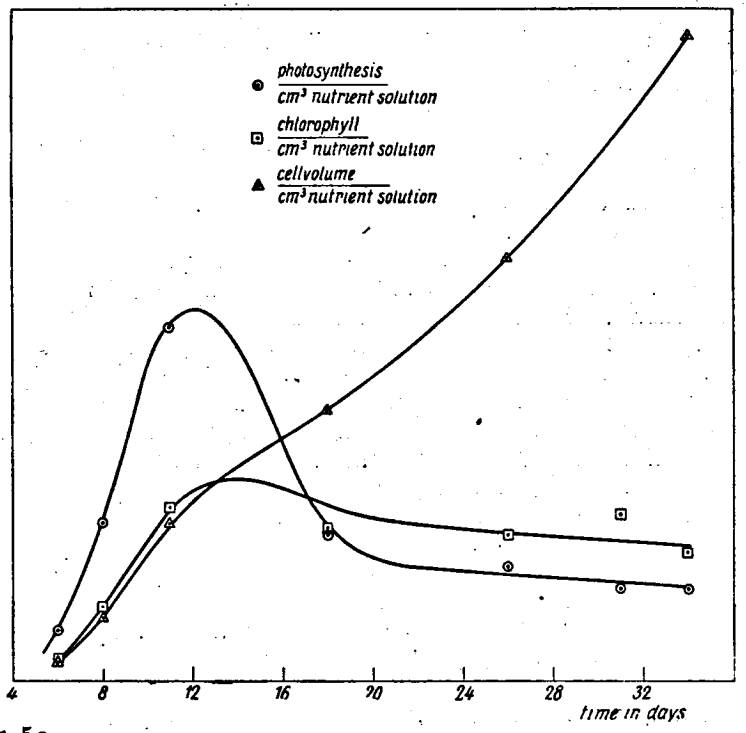


Fig. 5 a.

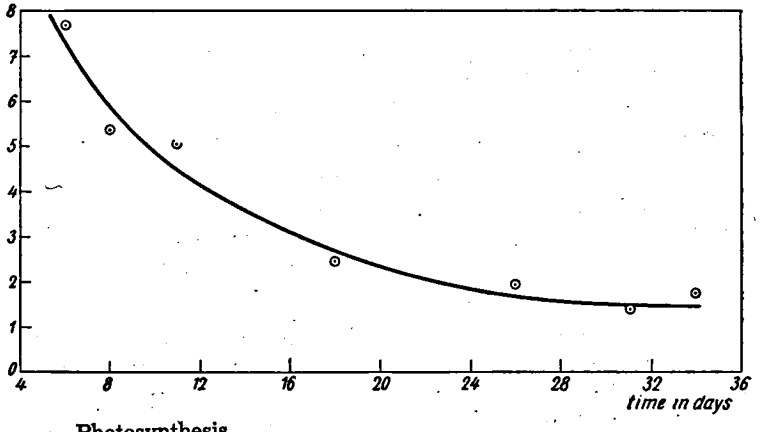


Fig. 5 b. $\frac{\text{Photosynthesis}}{\text{Chlorophyll}}$ in course of time of culture with $^1/_2$ × concentration of the nutrient solution, cultivated in diffuse daylight.

So, the level of the assimilation numbers is not directly influenced by the concentration of the nutrient medium. Otherwise, the highest assimilation number of the culture of table 3 should agree with the assimilation number of the culture from table 2, when this one had exhausted $^3/_4$ of its nutrient solution.

In figure 6 the curves of $\frac{\text{photosynthesis}}{\text{cm}^3 \text{ nutr. sol.}}$ of figure 3a, 4a and 5a are given together.

From this, it appears that the rising part of the curves coincide; consequently the photosynthesis in the first period of growth of the cells in the culture is independent of the concentration of the nutrient solution. PEARSALL and LOOSE (1937) demonstrate, in their ample investigations on the growth of *Chlorella vulgaris* in pure culture, that in the first period after inoculation the growth gets on according to an exponential curve.

This is also the case for the rapid growth of *Chlorella pyrenoidosa*, if photosynthesis is taken as a standard for growth.

From figure 6 appears that the exponent is not influenced by the concentration of the nutrient solution, until the nutrient solution (or one of its components) becomes limiting factor.

During the exponential growth, the chlorophyll curves too almost coincide. Therefore, as the concentration of the nutrient solution proves not to affect the rate of the chlorophyll formation, nor the rate of photosynthesis, but since in course of time, it appears as limiting factor, it is not to be expected, that the assimilation numbers, being the quotient of photosynthesis and chlorophyll content, will be influenced by the concentration of the nutrient solution, during the exponential growth.

Because the nutrient solution does affect the duration of the period of the exponential growth, the curve of the assimilation numbers must drop off less sharply in the higher concentrations than in the lower concentrations. This is apparent from comparison of figure 3b, 4b and 5b.

As the highest assimilation numbers were found in the experiments with the youngest cultures, I tried to accelerate the growth, in order to be able to carry out the first experiment within fewer days after inoculation.

In order to secure faster growing cultures, the cells were cultivated at a temperature of 27° and in the constant light of a PHILIPS 500 Watt bulb, at a distance of 90 cm from the cultures. To obtain a regular growth the cultures were continuously shaken. The frequency of shaking amounted to 90 to the minute with a amplitude of 1,5 cm.

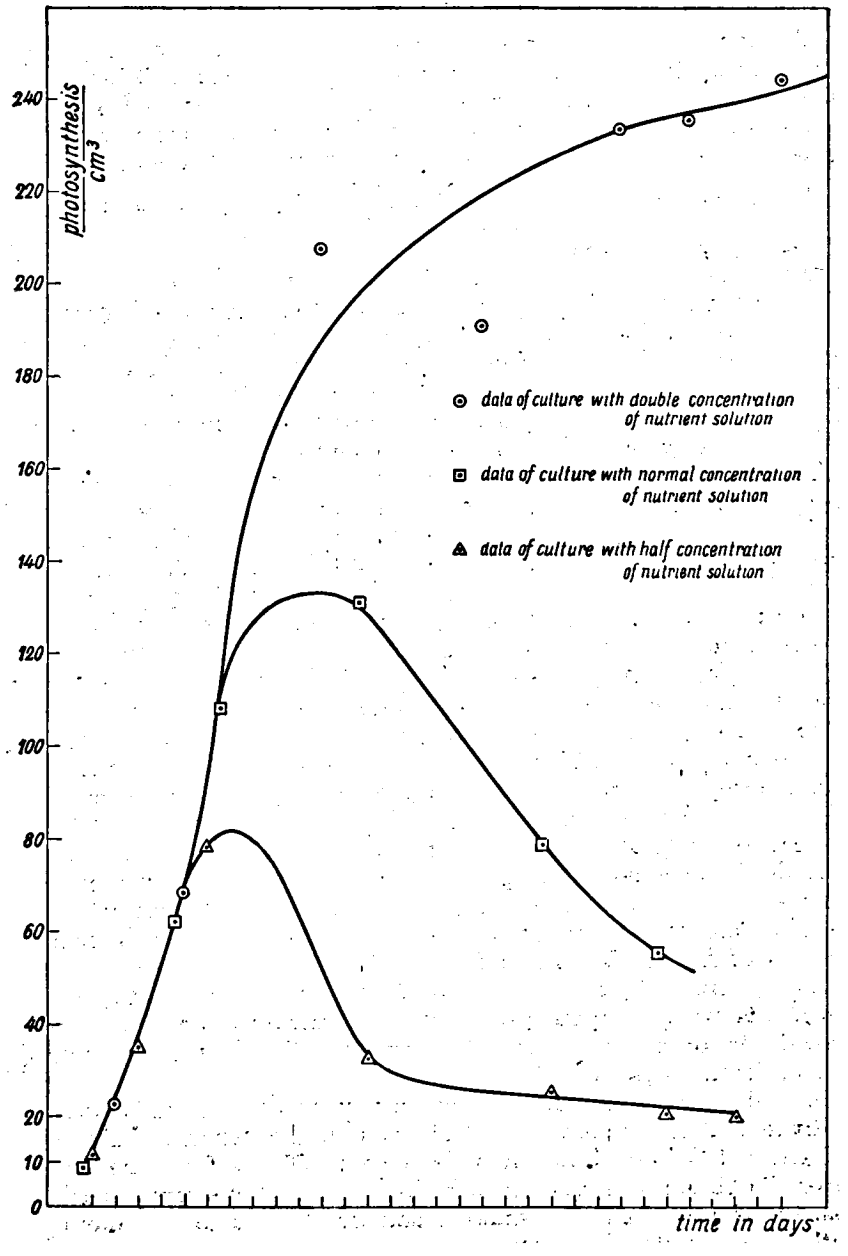

photosynthesis
cm³
240
220
200
180
160
140
120
100
80
60
40
20
10
0
data of culture with double concentration of nutrient solution
data of culture with normal concentration of nutrient solution
data of culture with half concentration of nutrient solution
time in days

Fig. 6.

The results obtained with three cultures provided with three different concentrations of nutrient solution are recorded in table 4, 5 and 6, moreover illustrated by the graphs in figure 7, 8 and 9.

Under these different conditions, however, it is impossible to carry out the first experiment earlier after the inoculation.

TABLE 4.

Normal nutrient solution.

Time	pH	Photosynthesis cm³ nutr. sol.	Chlorophyll cm³ nutr. sol.	Cell volume cm³ nutr. sol.	Photosynthesis / Chlorophyll
6	7	18,4	0,066	1,17	2,80
10	8,2	94,0	0,508	6	1,85
12	8,2	79,1	0,468	7	1,69
16	7,4	44,6	0,386	8,5	1,15
19	7,6	29,5	0,374	10	0,82
25	7,8	16,3	0,370	13,1	0,44
34	7,4	21,3	0,360	11,5	0,59

TABLE 5.

Normal nutrient solution 2 × concentration.

Time	pH	Photosynthesis cm³ nutr. sol.	Chlorophyll cm³ nutr. sol.	Cell volume cm³ nutr. sol.	Photosynthesis / Chlorophyll
8	7,6	26,5	0,075	3,9	3,530
10	7,6	161,0	0,753	17,6	2,130
12	6	157,6	0,850	14,7	1,850
16	7	134,6	0,798	14,9	1,825
19	5,4	39,2	0,756	19,5	0,518
25	7,8	39,6	0,574	25,7	0,691
31	—	42,8	0,706	34,3	0,607

TABLE 6.

Normal nutrient solution $^1/_2$ concentration.

Time	pH	Photosynthesis cm³ · nutr. sol.	Chlorophyll cm³ nutr. sol.	Cell volume cm³ nutr. sol.	Photosynthesis / Chlorophyll
5	6,7	4,35	0,016	0,27	2.664
9	8,2	24,6	0,173	3,03	1,423
11	8,2	17,5	0,218	3,4	1,208
16	8	15,5	0.247	3,48	0,435
19	8,2	7,6	0,179	4,7	0,492
31	6,8	3,5	0,055	5,2	0,637

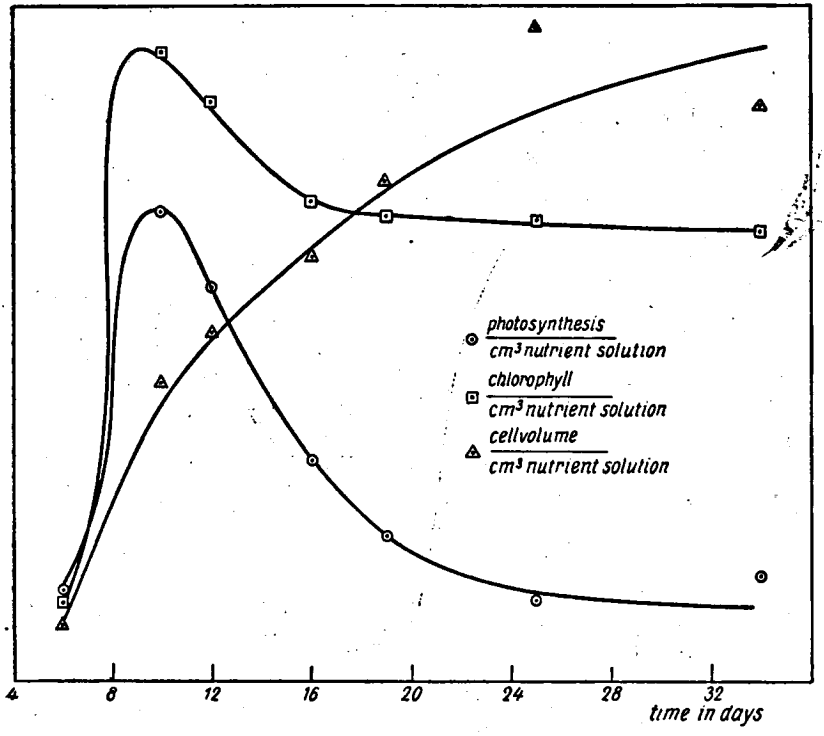


Fig. 7 a.

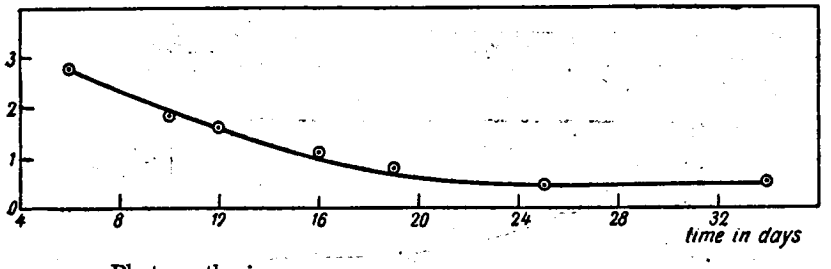


Fig. 7 b. $\frac{\text{Photosynthesis}}{\text{Chlorophyll}}$ in course of time of culture with normal nutrient solution, cultivated in continuous light.

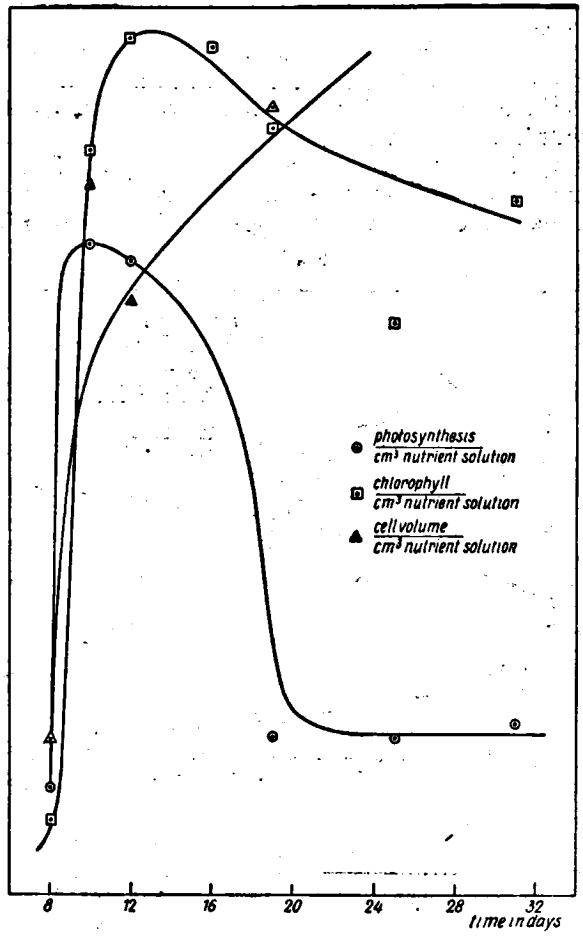


Fig. 8 a.

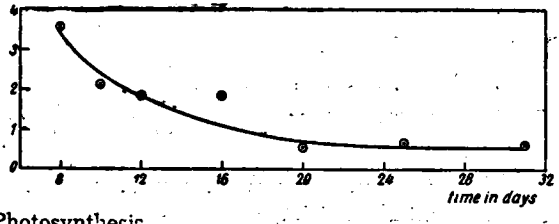


Fig. 8 b. $\frac{\text{Photosynthesis}}{\text{Chlorophyll}}$ in course of time of culture with 2 × concentration of the nutrient solution, cultivated in continuous light.

Also in these cultures the first green colouring was observed at the sixth day.

When the culture had reached this stage of development the growth was more rapid than in the cultures in the greenhouse, so the maximal values of chlorophyll and photosynthesis were attained within fewer days.

Comparing the data of figure 3 and figure 7, respectively table 1 and table 4 it appears that the constant hight and the higher temperature do not have an unfavourable influence on

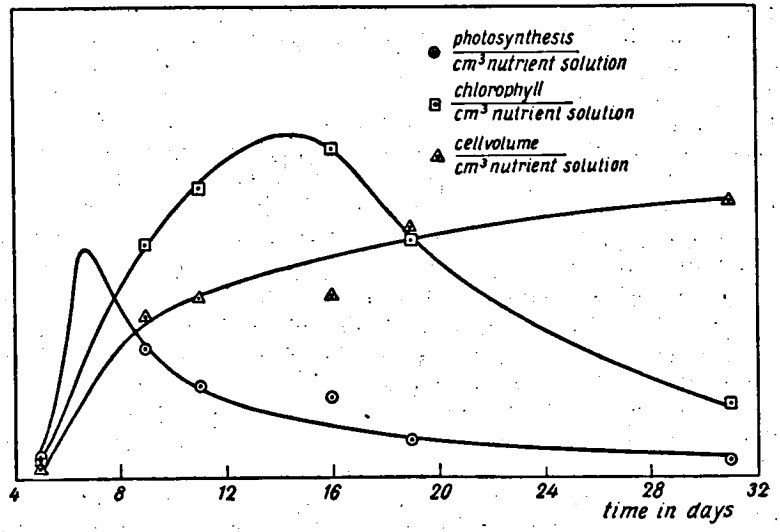


Fig. 9 a.

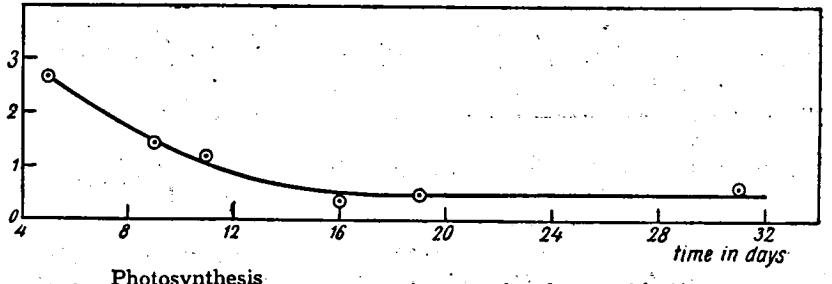


Fig. 9 b. $\frac{\text{Photosynthesis}}{\text{Chlorophyll}}$ in course of time of culture with $^1/_2$ × concentration of the nutrient solution, cultivated in continuous light.

the formation of the chlorophyll, as the maximal chlorophyll content of the culture is even higher under these circumstances. The photosynthesis, however, is unfavourably affected. The maximal photosynthetic value is lower and the decrease is sharper than in the cultures in the greenhouse.

Also the assimilation numbers are lower. The maximal values of the assimilation numbers, found in the first experiment with each culture, are ± 50 per cent of the maximal assimilation numbers of the cultures in the greenhouse.

Further it is found again that the maximal values of the assimilation numbers cannot be influenced by the concentration of the nutrient solution.

Since the assimilation numbers decrease from the moment that the first greening is visible, it can be understood why the authors, who investigated the relation between photosynthesis and chlorophyll content, always found irregular values. WILLSTÄTTER and STOLL (1918) too found the highest assimilation numbers with leaves of higher plants when these were just unfolded.

EMERSON (1929) found smaller assimilation numbers with higher chlorophyll concentrations in *Chlorella vulgaris* and moreover a great difference between the assimilation numbers of two different series. This is understandable, when the different series were not cultivated under the same circumstances or when cultures of different age were used.

Also FLEISCHER (1934) found that the assimilation numbers of the cultures of one series gave a closer agreement than those of different series. The irregularities in the magnesium series in the experiments of FLEISCHER will be discussed in Chapter IV.

So, the highest photosynthetic activity both in higher plants and in *Chlorella* is found with very young organisms. Therefore, if *Chlorella* cultures are used for experiments when they have formed dark green and thick cell suspensions, the assimilation numbers have already decreased to the half or less!

Theoretical:

The process of photosynthesis can be devided into

a. a light sensitive or photochemical process.

b. a dark chemical or BLACKMAN process.

It may be that each of these processes must be devided into part processes. Since, the various theories on photosynthesis do not agree on the character of these processes, and the ex-

periments are not conclusive, I will stick to the general division, mentioned above.

The observed decrease of the assimilation numbers can be explained in two ways.

A. The light reaction falls off, so an amount of the chlorophyll becomes inactive.

B. The dark chemical or BLACKMAN reaction declines, so during the growth of the culture an ever smaller part of the radiant energy absorbed in the light reaction is converted further in the BLACKMAN reaction.

ad A. Several theories exist on the function of the chlorophyll in the photosynthetic process. These theories can be classified in two groups.

1. Chlorophyll is photosentisizer.

The chlorophyll absorbs the energy of the light and transfers it upon some other substance.

2. Chlorophyll is photochemical agent and enters into reaction with carbon dioxide or an hydrated form of carbon dioxide.

If the decline of the assimilation numbers should be ascribed to the function of chlorophyll, so it must be assumed that in the first case, chlorophyll is unable to transfer the absorbed energy. If chlorophyll is photo-inactive, there is no fluorescence, for instance in colloidal solution. Photo-active chlorophyll ever yields a part of its energy as fluorescence. Though no quantitative fluorescence measurements were done, the fluorescence was still clearly visible in aged cultures. This appears to me to be an argument that it is improbable, that chlorophyll would not be able to transfer the absorbed light energy.

In the second case it might be assumed that chlorpohyll turns inactive by a chemical reaction. I tried to demonstrate a chemical transformation of the chlorophyll by determination of the absorption spectra of chlorophyll extracted from algae of very divergent photosynthetic activity. This failed. The spectra all agree within the experimental error.

Of some cultures of different photosynthetic activity the temperature sensitivity was determined. As appears from the following data the temperature sensitivity remains fairly constant in spite of differences in cultivation and age. The temperature sensitivity is given as the Q_5 (20,5° C.—25,5° C.) being the quotient of the rates of photosynthesis at 25,5° C. and 20,5° C.

From these data it is obvious that the photosynthesis is not limited in older cells by:

1: the carbon dioxide diffusion (which might be thought

Light during growth	Temperature during growth	Age of culture	Photosynthesis / Chlorophyll	Q_s
diffuse daylight	± 21°	35 days	1,14	1,5
" "	± 21°	17 days	3,82	1,4
" "	± 21°	20 days	2,13	1,6
continuous artificial light	± 26°	20 days	1,13	1,5

as the cell walls of the older cells become thicker (cf. PEARSALL and LOOSE 1937),

2: by the photochemical process (which would be the case if the chlorophyll became inactive and so the quantity of photoactive chlorophyll would limit the rate of photosynthesis),

for the temperature sensitivity of diffusion is considerably lower and that of photochemical processes = 1.

Moreover, it will be proved in the experiments described in Chapter VIII that the opinion that chlorophyll is inactivated by photosynthesis itself cannot be sustained.

Consequently there is no reason to suppose that chlorophyll is the cause of the decline of the assimilation numbers.

ad B. The decline of the assimilation numbers, therefore, must originate from limiting of the photosynthesis by the BLACKMAN reaction.

Usually, the BLACKMAN reaction is considered as an enzymatic reaction, on account of its temperature sensitivity.

So the decrease of the assimilation numbers could be explained by presuming that the formation of the BLACKMAN enzyme is a process, that is more sensitive to the circumstances in the culture during the growth of the algae than the chlorophyll formation. Assuming this theory there must exist, besides the enzyme formation, an inactivation of the enzyme. This is apparent from the decline of the ratio $\frac{\text{photosynthesis}}{\text{cm}^3 \text{ nutr. sol.}}$ after the attainment of the photosynthetic optimum. Hence it is also possible that the enzyme is formed in a constant ratio to the chlorophyll but that during the exponential growth of the algae it already turns inactive.

As a consequence the assimilation numbers must not be considered as a measure for the chlorophyll activity, but as a measure for the BLACKMAN reaction, for the assimilation number indicates which part of the absorbed light energy is converted further into chemical energy in the BLACKMAN reaction.

To my mind the assimilation number therefore is the best item

by which the photosynthetic activity can be measured, to prefer to the photosynthesis per cell volume, per cell number, per dry weight or per fresh weight.

How the decline of the assimilation numbers must be explained will be reported in the subsequent chapters.

CHAPTER IV.

Influence of Magnesium on the relation between Chlorophyll content and rate of Photosynthesis. The Experiments of Fleischer.

Introduction.

FLEISCHER (1934) cultivated chlorotic *Chlorella* cells by deficiency of iron, nitrogen or magnesium, in order to investigate the ratio photosynthesis-chlorophyll. With cells chlorotic by deficiency of iron or nitrogen, FLEISCHER found the rate of photosynthesis proportional to the chlorophyll content.

The behaviour of the *Chlorella* cells in nutrient solution with graded quantities of magnesium, however, was quite different. No definite proportionality between the chlorophyll amount and rate of photosynthesis was found. FLEISCHER describes his results:

"At low concentrations of magnesium the rate of photosynthesis is relatively independent of the chlorophyll content. As the magnesium concentration is increased, the rate of photosynthesis rises rapidly and during the rise is relatively independent of the chlorophyll content. Eventually the rate of photosynthesis reaches the value indicated by full nutrient determinations and at this point the relation between the rate of photosynthesis and chlorophyll content is comparable to the relation existing in iron and nitrogen graphs for similar values."

FLEISCHER explains the magnesium effect by assuming that magnesium plays a part in the process of photosynthesis in addition to its effect upon the chlorophyll content.

Though this may be true, it is not a sufficient explanation of the phenomenon, because the same may be said of nitrogen. It would be more probable that lack of iron, which is no constituent part of the chlorophyll molecule, would disturb the proportionality of photosynthesis and chlorophyll.

FLEISCHER found cells being highly chlorotic but having an almost normal assimilation number. Also he found cells with

a normal chlorophyll content having an abnormal low assimilation number. However, FLEISCHER gives no data of the magnesium concentrations which gave rise to these phenomena.

Methods.

In my experiments I followed the method of FLEISCHER.

In the nutrient solution, given in Chapter III, 1,42 g sodium sulfate was substituted for 2,46 g magnesium sulfate.

The magnesium standard solution contained 1 mg magnesium per cm^3. I used magnesium sulfate in the standard solution, because magnesium chloride, used by FLEISCHER, on account of its hygroscopical quality is less suited for quantitative work.

Varying amounts of the magnesium standard solution were added to Erlenmeyer flasks of 100 cm^3, containing 50 cm^3 nutrient solution. FLEISCHER added varying amounts of the standard solution to the cultures so as to give concentrations ranging from 0,02 to 2,0 parts per million, being in correspondence with 0,1 cm^3 — 0,001 cm^3 of the standard solution to 50 cm^3 of the nutrient solution.

The normal nutrient solution (cf. page 691) contains 2,46 g magnesium sulfate per litre, or 25 mg magnesium per 50 cm^3.

As in FLEISCHER's experiments the cultures were grown in constant light. Growth must have been a little more rapid in FLEISCHER's experiments than in mine. With his cultures it was possible to carry out experiments three days after inoculation already. My first series were inoculated as described in Chapter II. With these series experiments could not be done before the sixth day. In order to acquire a more rapid growth, I inoculated new series with greater quantities of algae. For that purpose, a mature culture was no longer shaken but put aside. So a thick layer of cells was formed on the bottom. The nutrient solution was decanted and replaced by sterile water. With 2 cm^3 of this cellsuspension the cultures were inoculated by means of sterile pipettes. On this way too, it was impossible to carry out experiments earlier than after 4 or 5 days. FLEISCHER does his experiments 3 to 6 days after inoculation.

From the experiments of Chapter III, it is apparent that within 3 days the ratio photosynthesis-chlorophyll of one and the same culture can shift appreciably. This difference will be the greater in different cultures which moreover are supplied with a various composition of nutrient components.

That is why, as much as possible, I carried out my experiments within one series on the same day.

Results.

It appeared that *Chlorella* needs only little magnesium. Even if no magnesium at all was added to the nutrient solution there was some development of the algae, though distilled water and pure salts were used.

From the results tabulated in table 7, it is evident that after 6 or 7 days the assimilation numbers of the different cultures are fairly constant.

TABLE 7.

Amount of magnesium in cm^3 of the nutrient solution	Time	Photosynthesis / Chlorophyll	Chlorophyll / Cell volume
0,004	6	2,52	37
0,006	6	2,87	27
0,01	7	2,63	43
0,03	6	2,46	36,7
0,05	6	2,34	26
0,07	7	2,76	38
0,1	7	2,68	45
1	6	3,00	39
2	7	3,37	35
5	6	2,64	35

Cultures with less magnesium than 0,004 of the magnesium standard solution did not supply enough cells to carry out accurate photosynthetic determinations.

Because the ratio chlorophyll — cell volume too was rather constant, there cannot be question of chlorose here, though the lack of magnesium appeared clearly from the slight development of the cultures. It is, however, possible that this disagreement with FLEISCHER's results was caused by the different way of determining the cell volume.

FLEISCHER notes that the cells chlorotic by magnesium deficiency were larger than cells which were green and abundantly supplied with magnesium. In his experiments the cell volume was determined by measuring and counting of cells. The cell volume in my experiments has been determined by centrifuging the cells after the chlorophyll had been extracted. If the cells from the cultures with magnesium deficiency contain more water than the normal cells, it is possible that the difference in chlorophyll amount per cell volume is lost by extraction of the cells with methanol.

TABLE 8.

Mg content in cm³ of Mg standard sol.	Time	Photosynthesis / Chlorophyll	Chlorophyll / cm³ nutr. sol.	Chlorophyll / Cell volume	Cell volume / cm³ nutr. sol.
0	4	1,31	0,032	38	0,85
	6	0,92	0,035	30	1,17
	9	1,02	0,039	18	2,14
	12	0,88	0,033	12	2,72
	16	0,97	0,055	14	4,00
	17	0,76	0,040	—	—
0,001	4	1,44	0,026	35	0,75
	6	1,49	0,044	28	1,14
	9	1,12	0,043	12	3,59
	12	0,85	0,045	12	3,74
	16	1,31	0,044	10	4,40
	17	1,16	0,057	—	—
0,003	4	1,54	0,032	37	0,88
	7	1,12	0,068	29	2,31
	9	0,98	0,044	14	3,12
	12	0,97	0,057	12	4,94
	16	0,47	0,041	6	6,50
0,007	4	1,16	0,020	31	0,62
	7	1,95	0,106	37	2,80
	9	1,03	0,106	31	3,40
	12	0,65	0,095	18	5,30
	16	1,17	0,073	10	7,10
	17	1,14	0,073	—	—
0,02	4	1,33	0,012	24	0,52
	7	1,87	0,064	34	1,88
	9	1,58	0,170	38	4,40
	12	0,70	0,256	20	12,80
	16	0,58	0,146	19	7,50
0,03	4	1,23	0,018	34	0,52
	7	1,98	0,086	35	2,4
	9	1,31	0,246	36	6,8
	12	0,73	0,246	20	12,4
	16	0,55	0,142	14	10,3
0,04	4	1,21	0,018	28	0,63
	6	2,19	0,103	35	3,00
	9	1,37	0,220	34	6,46
	12	0,78	0,181	23	7,90
	16	0,46	0,187	23	7,70
0,05	4	1,59	0,035	31	1,1
	6	1,99	0,256	34	7,7
	9	0,65	0,217	21	10,3
	12	0,36	0,200	17	11,6

By the other way of inoculating it was possible with another series to carry out the first experiments after four days. Though it is not with certainty to discriminate, it does not seem probable that this way of inoculating with great quantities agrees with the inoculation as done by FLEISCHER. The results of this series are recorded in table 8.

By this way of inoculating, the culture contains already in the first period of growth so many cells that they form a light green suspension. These cells are dividing slowly during the first days after inoculation. So the aged cells are still appreciably influencing the results when an experiment is done four days after inoculation, because within this time only few new cells have been formed. The culture, from which was inoculated, had on the day of inoculation an assimilation number of 0,450.

In this way too, FLEISCHER's experiments could not be affirmed. After four days the assimilation numbers of all cultures were constant and about three times as high as in the original culture. Difference became visible in the cultures by continuing the experiments. In the cultures with little magnesium no further growth appeared and the assimilation numbers became lower. In the cultures with more magnesium the suspension of algae became denser and in the first days the assimilation numbers increased. The maximum of the assimilation number was never reached later than on the seventh day and then declined too in the same way as in the culture provided with normal nutrient solution.

No more the results of FLEISCHER could be confirmed with cultures of *Chlorella vulgaris*. These too give results completely corresponding with those of *Chlorella pyrenoidosa*, only the photosynthetic activity is somewhat lower.

CHAPTER V.

Influence of the products of Photosynthesis on the Photosynthetic rate.

Introduction.

In Chapter III has been demonstrated that during the growth of the cultures the photosynthetic activity (expressed as the assimilation number) becomes ever lower.

In the cultures cultivated in continuous light, the photosynthesis can go on unremittently. In these cultures more

photosynthetic products can be formed than in cultures grown in diffuse daylight.

So, it might be possible, that the photosynthesis would be inhibited by accumulation of photosynthetic products and that for this reason the first mentioned cultures show lower assimilation numbers than the latter.

It is often cited in literature that the accumulation of photosynthetic products causes a decreased rate of photosynthesis. The first notice of this theory has been given by BOUSSINGAULT (1868) on account of decreased photosynthesis of leaves removed from the plant. Further evidence has been given by investigations of SAPOSCHNIKOFF (1893).

In many experiments, as well with detached leaves as with leaves attached to the plant a decline of photosynthesis was found after a prolonged illumination. It should be expected that this effect, if it must be attributed to the accumulation of the photosynthetic products, should make its appearance earlier in detached leaves than in leaves attached to the entire plant, as the latter are capable to translocate synthesized carbohydrates to other parts of the plant.

EMERSON (1937), however, remarks that a more constant rate of photosynthesis is obtained in experiments with excised leaves. So it seems probable that the unaccountably fluctuating rates must be ascribed to the methods of determination of photosynthesis, making higher demands upon keeping constant the external factors in the case of experimental work with entire plants.

HARDER (1933) in his experiments with *Fontinalis* found that definite fluctuations in prolonged experiments on photosynthesis arise when the light intensity during the growth of the experimental plants varies from the light intensity used during the photosynthetic determination. If these light intensities correspond within certain limits there is no decrease of photosynthetic rate. After a dark period of twelve hours *Fontinalis* when illuminated shows a slowly increasing photosynthesis, which reaches its maximal activity in the course of hours.

KOSTYTSCHEW, BAZYRINA and TSCHESNOKOV (1928) working with plants in their natural stand found great fluctuations of the assimilation rate. In their experiments with leaves of several higher plants an assimilation was found being high in the morning, declining and even stopping at the midday. In many cases it was observed that carbon dioxide was evolved in much greater quantities than during the determinations of

respiration. In the afternoon several plants showed a second assimilation maximum. However, the curves of the photosynthetic activity of different plants on different days are so divergent that a definite conclusion cannot be given.

The authors emphasize that the stopping of the assimilation cannot be ascribed to an exposure to too intense light or too high temperature because also on rainy and cloudy days this phenomenon was observed. Therefore it was supposed that these irregularities were due to the accumulation of carbohydrates. After the criticism by MONTFORT (1929) KOSTYTSCHEW (1931) recants this idea. In a further investigation BAZYRINA and TSCHESNOKOV (1930) found a very regular day-assimilation curve, independent from the fact whether the assimilation products during the photosynthesis are translocated (*Pisum*) or accumulated (*Solanum*).

A number of other authors working with plants in different climates (HENRICI (1921), GUTTENBERG and BUHR (1935), MÖNCH (1937)) ascribe the decline of the rate of photosynthesis wholly or partly to the accumulation of photosynthetic products. GUTTENBERG and BUHR especially marked an inhibitory effect of starch. KURSSANOW (1933) found with several higher plants a day-assimilation curve with two maxima (cf. KOSTYTSCHEW) and remarked that the photosynthesis was decreased the more when the translocation of carbohydrates was inhibited. In his experiments with *Cladophora* he obtained the same results. This is important because by working with a waterplant he could eliminate the influence of air humidity and the behaviour of the stomates. Besides he found that the photosynthetic activity decreased by cultivating the algae in a 1 per cent invertsugar solution. This too is imputed to the accumulation of carbohydrates, as the invertsugar is taken up and assimilated.

Investigations carried out in the laboratory in artificial light in several cases proved to give a constant rate of photosynthesis during a long time.

BOYSEN JENSEN and MULLER (1928) could not find an influence of the photosynthetic products in *Fraxinus excelsior*. Neither could KJÄR (1937) with seedlings of *Sinapis alba*. This author, however, stated that the assimilation products were rapidly transported, so an intense staining with iodine could never be found.

Very constant results were obtained in the experiments by MITCHELL (1936). For seven hours at a stretch an intensive and constant rate of photosynthesis was found. Even the air humidity

and the condition of the stomates proved to have little influence.

As in a unicellular organism a translocation of assimilation products is impossible it was to be expected, that the accumulation of the photosynthetic products in *Chlorella* would have a greater influence on the photosynthesis than in higher plants.

Methods.

For these experiments *Chlorella* cells were used which at the start of the experiments were free of photosynthetic products. To that purpose cultures were grown in the normal nutrient solution (cf. page 691) but glucose was omitted, for *Chlorella,* like higher plants, is able to assimilate glucose in the dark and to convert it into starch (BÖHM (1883), GENEVOIS (1928, 1929)).

Growth without glucose supply is much slower (cf. Chapter VIII). The cells contain more chlorophyll, but the rate of photosynthesis, calculated per unit of chlorophyll (= assimilation number), is not greater than in cultures cultivated in an organic medium.

Before the experiment was carried out the culture was put in the dark for twelve hours. After chlorophyll extraction no staining with iodine was produced than.

As under conditions of continued exposure the light intensity of 12000 Lux used in the other experiments has an injurious effect on the chlorophyll, a light intensity of 4000 Lux was used in these experiments. In this illumination the photosynthesis of the cells amounts to 80 per cent of the assimilation in the light intensity of 12000 Lux.

In these experiments dilute cell suspensions were used in order that the prolonged photosynthesis would have little influence on the composition of the carbonate mixture.

Results.

The results of an experiment extended over 67,5 hours are tabulated in table 9.

Only little growth took place during this time because the cells were deprived from all nutrient salts.

Table 9 gives the intervals during which the cells were illuminated and the observed photosynthesis and respiration per half hour. A perusal of the table shows that the circumstances in course of the experiment were not unfavourable for the cells, because the rate of photosynthesis is not lower at the end of the experiment than at the start.

TABLE 9.

Illumination	Intervals of observation	Photosynthesis per ½ hour	Respiration per ½ hour
	30 min.	90	
	30 min.	113	
4½ hours	30 min.	108	
Light	30 min.	96	
	30 min.	104	
	2 hours	108	
	30 min.		25
18 hours	30 min.		20
Dark	5½ hours		12
	11½ hours		12
	30 min.	25	
	2 hours	85	
	1 hour	116	
	2 hours	119	
25 hours	3 hours	115	
	2 hours	153	
Light	13 hours	?	
	30 min.	155	
	30 min.	156	
	30 min.	141	
	2 hours		36
19 hours	2½ hours		46
Dark	14 hours		30
	30 min.		31
1 hour	30 min.	143	
Light	30 min.	153	

With iodine treatment on the cells that were subjected to the experiment in the parallel vessels the depth and degree of the staining was followed. At the start of the experiment no staining by iodine was found.

After half an hour of photosynthetic activity only a very faint reaction with iodine is visible.

After 2½ hours illumination a distinct iodine reaction is found in a part of the cells. In other cells small dark points or no staining are observed.

After 25 hours of photosynthetic activity the greatest part of the cells is stained intensively with iodine. Other cells show no reaction.

The rate of photosynthesis after 25 hours continual illumination does not decrease but still increases. So nothing is perceivable of an inhibition by accumulation of synthesized carbohydrates. When the cells have been in darkness for a long time, the rate of photosynthesis is not greater than after a light period. So the disappearing of products of photosynthesis has no stimulating influence on the assimilatory process. On the contrary the rate of photosynthesis continuously increases during the illumination.

The influence of a preceding light period on the respiration is described by VAN DER PAAUW (1932) for *Hormidium* and by FRENCH, KOHN and TANG (1934) for *Chlorella pyrenoidosa*. The results of my experiments with *Chlorella* are in agreement with the results of the latter authors showing a gradual decrease after a light period to a constant value.

Of two cultures (see table 10) of the same age, containing about an equal amount of chlorophyll, one culture had been cultivated in constant light and the other in diffuse daylight (cf. Chapter III). It was thought, that the photosynthesis would have been higher in constant light so that if photosynthesis were inhibited by accumulation or carbohydrates, it should be the case here.

Before the photosynthetic experiment the amount of starch of the cells from both cultures was determined with the iodine test. It appeared that exactly the cultures, cultivated in diffuse daylight, contained more starch.

TABLE 10.

Cultivation	Photosynthesis / Chlorophyll	Chlorophyll cm³ nutr. sol.	Cell volume cm³ nutr. sol.	Photosynthesis cm³ nutr. sol.	
in constant light	1,62	366	8,1	545	little starch
in diffuse daylight	2,45	386	8,4	947	much starch

It is apparent from this that also in this case no inhibition of photosynthesis is found in cells containing more photosynthetic products.

It is often observed in more aged cultures that in a standing culture a portion of the cells does not settle but remains suspended in the nutrient solution. The greater amount of store starch makes the specific gravity of the cells increase. Cells, which show a deep starch staining with iodine can much faster

be concentrated by centrifuging than other cells. I applied this fact to make cells photosynthesize, which were centrifuged out in short time, apart from cells that were only centrifuged after a very long time.

Before the experiment was done, it was ascertained that the former contained more store products than the latter.

TABLE 11.

	Photosynthesis / Chlorophyll	Photosynthesis / Respiration
Mixture of both cells	1,465	7,8
First centrifugate	1,472	6,9
Last centrifugate	1,092	8,7

It appears from table 11 that the photosynthesis per unit of chlorophyll is highest in cells with the highest amount of reserve carbohydrates, which also in this case have no inhibiting effect on the photosynthesis.

The last column of table 11 shows that the rate of respiration is higher in the presence of reserve carbohydrates.

Conclusions.

It is obvious from the experiments of this Chapter, that an inhibition of photosynthesis by accumulation of carbohydrates never could be demonstrated. Cells containing a large amount of synthesized carbohydrates in contrary show a higher photosynthetic activity in comparison with cells containing few assimilatory products.

From the last experiment it appears that one culture may contain cells of different metabolic capacity. The results of photosynthetic experiments with *Chlorella* are always an average of the rate of photosynthesis of cells in different states present in the suspension. It is evident from this last experiment that the calculation of the rate of photosynthesis per cell volume must give appreciable irregularities. The cells which have the lowest specific gravity, will take the greatest volume with the TROMSDORFF method, while their assimilatory activity is slighter.

Nor is the respiration rate in experiments with *Chlorella* a useful standard for the quantity of organic matter concerned in metabolism, as assumed VAN DEN HONERT (1930) for *Hormidium*. When accepting this idea the assimilation activity in the last

mentioned experiment would be considered to be the highest in the cells with the less photosynthesized products while, in my opinion, it is more correct to say that in these cells the respiration is low. That the assimilation activity is lower than in the other cells (though to a less degree than the respiration) appears from the calculation of the photosynthesis per chlorophyll content (assimilation number).

That the glucose has no injurious influence on the assimilation number appears from the fact that the cultures with inorganic nutrient solution do not have higher assimilation numbers than the cultures grown in organic nutrient solution. The cells of the organic cultures, moreover, have the highest assimilation numbers in the beginning, viz. when the concentration of the glucose is still the greatest.

Though the accumulation of carbohydrates may cause a decrease of the rate of the assimilation in higher plants, in experiments with *Chlorella* no inhibitory effect could be demonstrated.

CHAPTER VI.

The Blackman reaction and the decomposition of Hydrogen peroxide.

Introduction.

In the theory of WILLSTÄTTER and STOLL (1918) the decomposition of a peroxide would be responsible for the liberation of oxygen produced in photosynthesis.

In experiments with *Chlorella*, WARBURG and UYESUGI (1924) found that the decomposition of hydrogen peroxide and the BLACKMAN reaction showed a certain conformity in sensitivity to several narcotics. YABUSOE (1924) compared the influence of temperature on the BLACKMAN reaction and on the decomposition of added hydrogen peroxide and found that the two processes were both linear functions of temperature, while the influence of temperature on other biological processes for instance on the respiration of *Chlorella* showed the characteristics of the VAN 'T HOFF and ARRHENIUS' rule.

WARBURG and his co-workers held these results to support the theory of WILLSTÄTTER and STOLL and explained such differences as were found between the two reactions by the fact that in the case of the BLACKMAN reaction it was not hydrogen peroxide which played the part of substrate of catalase activity, but some organic peroxide.

In several theories on the mechanism of photosynthesis the formation of H_2O_2 (FRANCK 1935) or some other compound (WILLSTÄTTER 1933, GAFFRON and WOHL 1936) is considered to be the reaction preceding the liberation of oxygen in photosynthesis.

Other authors have pointed out that the coinciding occurrence of catalase activity and the BLACKMAN reaction in green plants is merely casual. Catalase is a very unspecific enzyme and occurs in numerous organisms, which show no BLACKMAN reaction or even never evolve oxygen in their metabolism.

FRENCH (1934) demonstrated that the splitting of H_2O_2 could be caused by means of 1: an enzyme; 2: a surface action.

The latter is the case with yeast as FRENCH concluded from the data of SÖHNGEN and SMITH (1924) on the temperature sensitivity of the decomposition of H_2O_2, which deviates from the temperature sensitivity of the splitting activity of *Chlorella* cells, decomposing hydrogen peroxide by an enzyme.

The influence of temperature on the catalase activity as found by FRENCH does not correspond with the results of YABUSOE (1924). EMERSON and GREEN (1937) showed that though there is a remote resemblance between the two curves for *Chlorella pyrenoidosa*, they are not identical and "surely insufficient to serve as evidence for a relationship between the two processes. Between the two curves for *Chlorella vulgaris* there seems to be no resemblance whatever". These authors, moreover, were able to reduce the BLACKMAN reaction and nevertheless found an undiminished capacity to split added hydrogen peroxide.

The conclusion of their paper is that the hypothesis that the BLACKMAN reaction involves the decomposition of some peroxide by catalase is deprived of experimental support but that it cannot be said that the BLACKMAN reaction is not a peroxide decomposition.

As the decline of the assimilation described in Chapter III was attributed to a reduced capacity of the BLACKMAN reaction, I investigated the catalytic power of the catalase reaction in the *Chlorella* cells during the growth of the culture.

If the catalase activity should give a similar curve as the photosynthetic activity, this would be an experimental support for the hypothesis that the BLACKMAN reaction involves the decomposition of some peroxide by catalase.

Methods.

For the determination of the catalase activity I followed the methods of WARBURG and UYESUGI (1924).

Of a sample of cells, centrifuged from their nutrient solution and suspended in 7 cm^3 of WARBURG's carbonate mixture no. 9 the rate of assimilation and respiration was determined. Hereafter 2 cm^3 hydrogen peroxide solution was put into the side bulb and shaken for ten minutes, in order to obtain the temperature of the waterbath and subsequently mixed up with the cell suspension. The level of the manometer was read immediately.

In the first experiments I used a concentration of hydrogen peroxide yielding a concentration of $\frac{1}{300}$ N when the hydrogen peroxide had been mixed up with the cell suspension. WARBURG and UYESUGI used the same concentration. The concentration of the hydrogen peroxide was determined by titrating with a $KMnO_4$ solution of known concentration after acidifying with sulphuric acid (cf. TREADWELL 1927). The strain of *Chlorella pyrenoidosa* used in my experiments had a very high catalase activity. With readings of 5 minutes it was impossible to get constant values for the hydrogen peroxide splitting with the concentration of $\frac{1}{300}$ N. In order to obtain a more constant rate of catalytic decomposition a concentration of hydrogen peroxide of $\frac{1}{200}$ N was used in all experiments described in this chapter. Readings were made every minute: Maximal values for the rate of hydrogen peroxide decomposition were reached at the second or the third reading. The maximal peroxide splitting was computed as the average of the five successive readings at which the highest catalytic power was found.

The concentrations of algae in the WARBURG vessel in all experiments have been chosen so as to secure a constant rate of peroxide splitting for at least five minutes.

The peroxide decomposition in my experiments is higher as compared to the BLACKMAN reaction than in the experiments of WARBURG and UYESUGI (1924) and of EMERSON and GREEN (1937). As the rate of the BLACKMAN reaction matches with the values found by these authors, the catalase activity of the *Chlorella* strain, used by me, must be higher. Moreover a more concentrated peroxide solution was used.

Because the amount of oxygen liberated during the peroxide decomposition was notably higher than during the photosynthesis, the splitting of hydrogen peroxide has been calculated per ten minutes, while the assimilation has been given per half hour.

Otherwise, it would be impossible to combine the results of the rates of photosynthesis and of hydrogen peroxide decomposition in one graph.

Like WARBURG and UYESUGI (1924) I made no correction for the respiration during the peroxide decomposition.

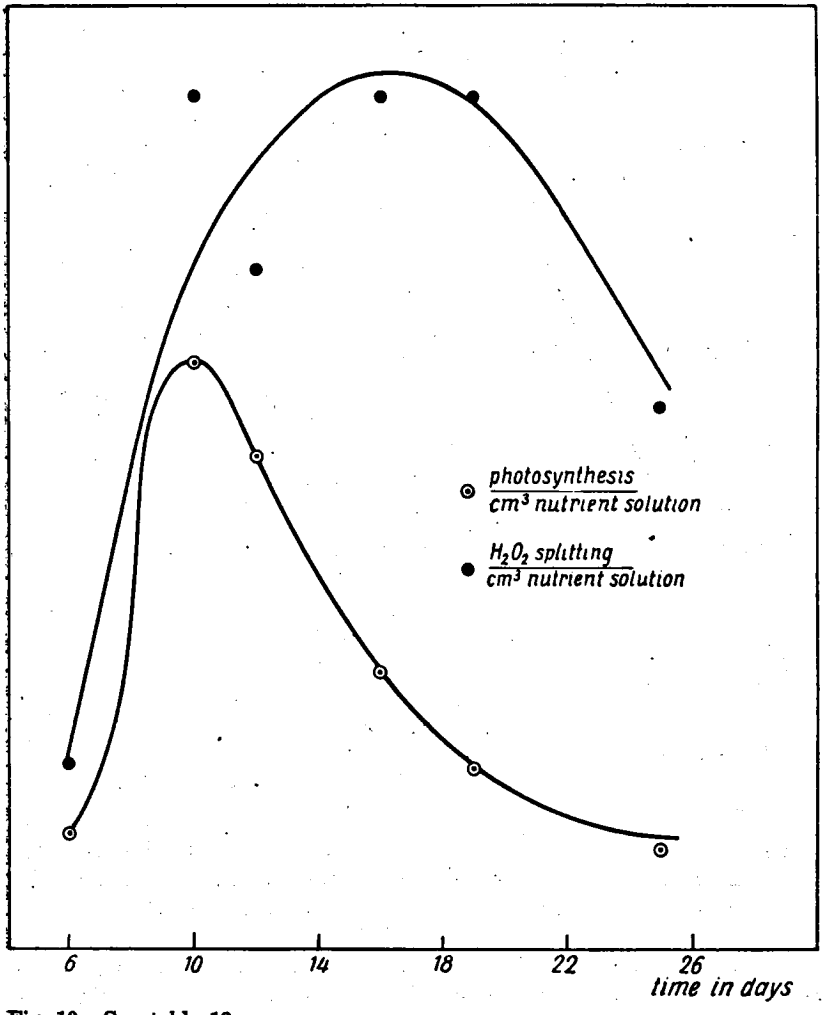


Fig. 10. See table 12.

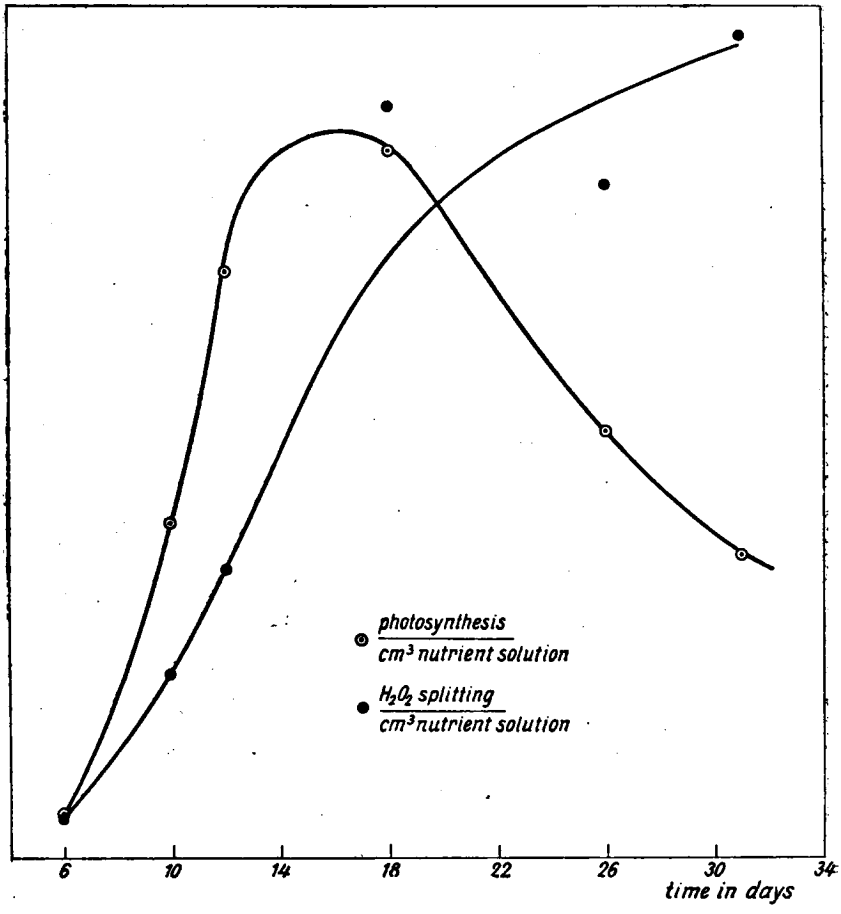


Fig. 11. See table 13.

Results.

The experiments on the rate of splitting added hydrogen peroxide have been carried out with the cultures of which the assimilatory activity has been described in Chapter III.

Of four cultures, which were differently cultivated and provided with different concentrations of the nutrient solution, the results are recorded in table 12, 13, 14, 15 and illustrated in figure 10, 11, 12, 13.

TABLE 12.
Normal nutrient solution. Cultivated in constant light.

Time	Photosynthesis per 1/2 hour cm³ nutrient solution	H_2O_2 decomposition per 10 min. cm³ nutrient solution
6	18,4	29,2
10	94,0	136,2
12	79,1	109
16	44,6	137
19	29,5	137
25	16,3	97

TABLE 13.
Normal nutrient solution. Cultivated in diffuse daylight.

Time	Photosynthesis per 1/2 hour cm³ nutrient solution	H_2O_2 decomposition per 10 min. cm³ nutrient solution
6	8,35	7,5
10	61,2	34
12	108,6	54
18	131,2	139
26	79,0	125
31	56,4	152

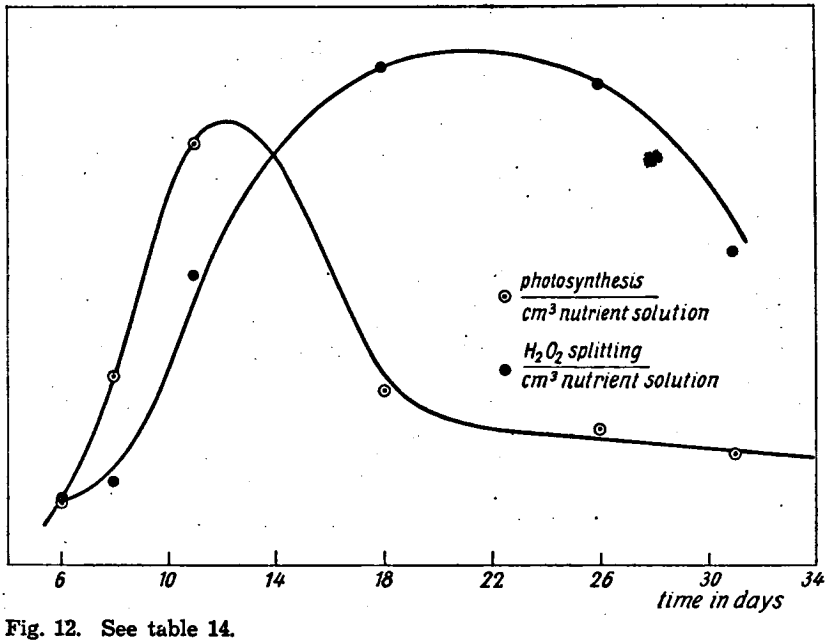


Fig. 12. See table 14.

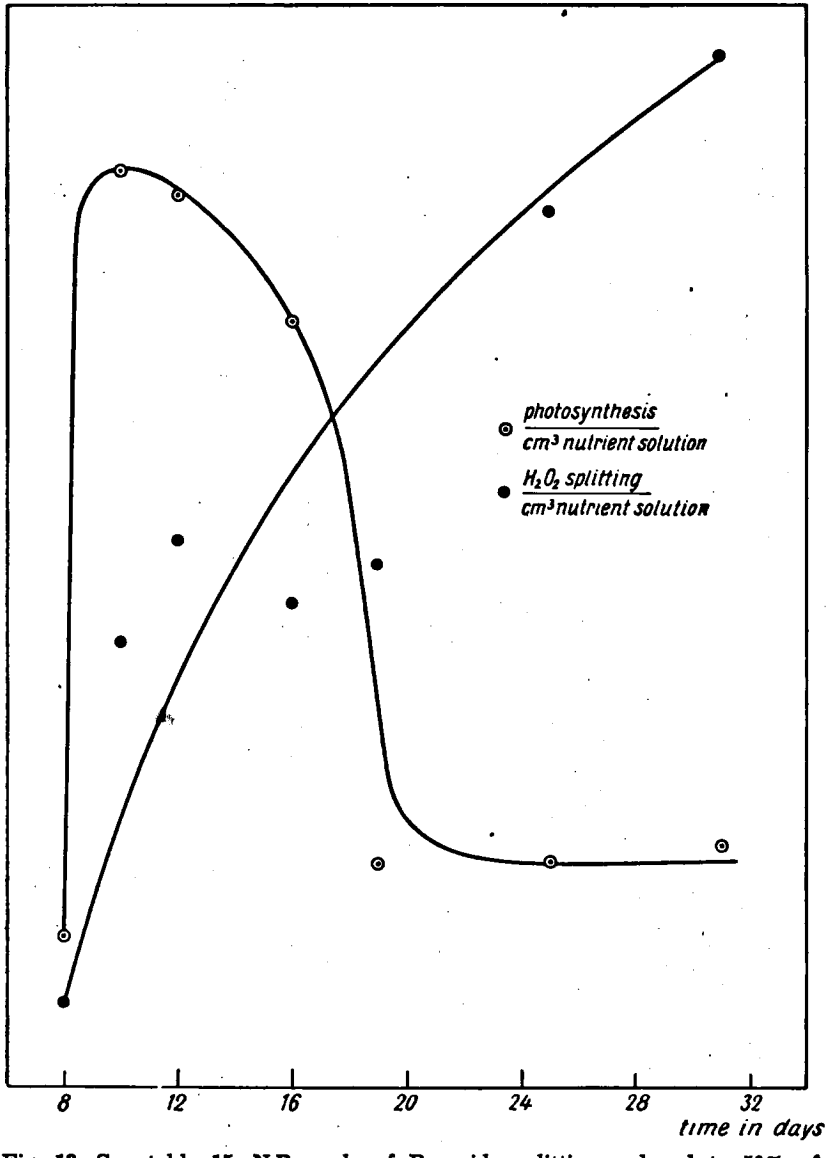


Fig. 13. See table 15. N.B. scale of Peroxide splitting reduced to 50% of that of the figures 10, 11, 12.

TABLE 14.

Normal nutrient solution 1/2 concentration. Cultivated in diffuse daylight.

Time	Photosynthesis per 1/2 hour cm³ nutrient solution	H_2O_2 decomposition per 10 min. cm³ nutrient solution
6	11,2	12,1
8	35,0	15,4
11	78,4	54
18	32,8	92
26	25,7	89
31	20,9	58

TABLE 15.

Normal nutrient solution 2 × concentration. Cultivated in constant light.

Time	Photosynthesis per 1/2 hour cm³ nutrient solution	H_2O_2 decomposition per 10 min. cm³ nutrient solution
8	26,5	29,7
10	161,0	156
12	157,6	193
16	134,6	169
19	39,2	184
25	39,6	308
31	42,8	362

Though the results of the determinations of the amounts of splitted peroxide are rather fluctuating, it is obvious that the curves of photosynthesis and hydrogen peroxide decomposition show no correlating course in any of these cases.

As has already been discussed in Chapter III the curves of photosynthetic activity all show a distinct maximum. The peroxide splitting, on the contrary, increases in the first period of growth in the culture and subsequently remains approximately constant.

It seems highly probable that the enzyme of the BLACKMAN reaction is inactivated during the growth of the culture, while the catalase still survives.

The method of cultivation has a widely different influence on the BLACKMAN reaction and on the hydrogen peroxide decomposition. This is very striking when comparing the values given in table 15 and figure 13.

The rate of photosynthesis in these cultures reaches its maximal value early and, in spite of the high concentration of the nutrient compounds, the BLACKMAN reaction decreases quickly at the constant illumination and the high temperature in the incubating room.

The peroxide decomposition, however, still increases appreciably thanks to the high concentration of the nutrient solution and is much higher in the last determination than during the time when the assimilatory activity is maximal.

That the peroxide decomposition is strongly dependent on the concentration of the nutrient solution is also apparent from the rates in the cultures of table 14 and figure 12. The catalase effect here is on the main highly decreased and is even lower in the last experiment.

Conclusions.

The capacity to split hydrogen peroxide and that of the BLACKMAN reaction do not show similarity in the course of their activity during the growth of *Chlorella pyrenoidosa* in pure culture. Both are differently affected by the external conditions prevailing in the cultivation of the cells. It is therefore unlikely that the BLACKMAN reaction — the name given to the rate determining process, when photosynthesis has been saturated both with light and with carbon dioxide — should proceed by means of the same mechanism as the peroxide decomposition.

CHAPTER VII.

Effects of the composition of the nutrient solution on the development of Chlorella. Effects of Nitrogen supply.

Introduction.

In Chapter III the experiments with cultures of *Chlorella*, supplied with various concentrations of the normal nutrient solution have been described. It appeared from the determinations of the photosynthesis that the growth of *Chlorella* proceeds at a rate, which is independent of the concentration of the nutrient solution, but that the time during which the growth continues is proportional to this concentration (cf. figure 6).

Consequently the maxima of photosynthesis, chlorophyll content and cell volume are also proportionate to the concentration of the nutrient medium. So this means that the nutrient supply limits the growth of the *Chlorella* cells in culture.

It was to be expected that not the nutrient solution as such

would limit the growth, but that one of the compounds would act as limiting factor.

In order to investigate which compound of the nutrient medium was the limiting factor, nutrient media were made, varying in such a way as to give one of the compounds in double, respectively half the concentration, whereas the other compounds were given in the normal dose.

Starting from the nutrient solution of Chapter III page 691, the nutrient media were prepared by changing in the „normal" solution the quantities of the successive elements as follows:

for Fe plus:	0,06 $FeSO_4$ and 2,- Na-citrate	gram p. litre
„ Fe minus:	0,015 $FeSO_4$ and 0,5 Na-citrate	„ „ „
„ N plus:	2,52 KNO_3	gram per litre
„ N minus:	0,63 KNO_3	„ „ „
„ Mg plus:	4,92 $MgSO_4$	„ „ „
„ Mg minus:	1,23 $MgSO_4$	„ „ „
„ P plus:	2,44 KH_2PO_4	„ „ „
„ P minus:	0,61 KH_2PO_4	„ „ „
„ Glucose plus:	30,- Glucose	„ „ „
„ Glucose minus:	7,5 Glucose	„ „ „

In each of these different nutrient solutions a set of cultures was inoculated.

In 100 cm^3 Erlenmeyer flasks 50 cm^3 nutrient solution was pipetted. Sterilization and inoculation were done as usually. All cultures were cultivated in the greenhouse.

It is not necessary to give the complete growth curves of all these cultures. In none of the cases they show a fundamental deviation from the curves given in Chapter III. Hence only some characteristic values of the growth curves are recorded in table 16. As such the following values have been chosen:

1. Maximal photosynthesis per cm^3 nutrient solution.
2. Maximal chlorophyll content per cm^3 nutrient solution.
3. Time, in number of days after inoculation, on which the maximal rate of photosynthesis is reached.

The analogous data of the cultures of which the growth is described in Chapter III and which were cultivated under about the same environmental conditions in the greenhouse are reproduced for comparison at the head of table 16.

TABLE 16.

	Max. Photosynthesis / cm³ nutr. sol.	Max. Chlorophyll / cm³ nutr. sol.	Day on which Max. Photosynthesis / cm³ nutr. sol. is reached.	
Normal nutr. sol. ½ concentration	78,4	0,180	11	(5)
Normal nutr. sol.	132	0,400	18	(12)
Normal nutr. sol. 2 × concentration	245	0,850	38	(30)
Fe plus	158	0,561	12	(7)
Fe plus	156	0,567	20	(9)
Fe minus	83,7	0,309	12	(7)
N plus	179	1,060	38	(31)
N minus	101	0,258	10	(4)
P plus	121	0,424	11	(7)
P plus	110,3	0,474	20	(10)
P minus	124	0,500	11	(7)
Mg plus	143,5	0,518	17	(10)
Mg plus	140,8	0,537	15	(9)
Mg minus	120	0,495	11	(7)
Mg minus	122	0,435	15	(8)
Glucose plus	115	0,487	20	(9)
Glucose plus	128,5	0,506	21	(10)
Glucose minus	134,2	0,505	15	(8)

There is much variability as to the time (number of days) on which the maximal rate of photosynthesis in the cultures was reached. This is partly caused by the fact that the cultures were inoculated with cells from different cultures. This, however, is necessary because the cultures used for the inoculation during these manipulations have a great chance to get contaminated as has already been alluded to in Chapter II. If inoculation from such a culture is continued then, infection may appear in all subsequently inoculated media. To avoid this, several cultures must be used for the inoculation of one series. These cultures differed in density of suspension and in age, by which the first growth of the new culture is appreciably affected. On this account it is a better standard to calculate the number of days between the first visible greening and the attainment of the maximal assimilation. These data are presented in brackets in the last column of table 16 and prove to be rather regular.

The maximal rate of photosynthesis of all cultures could not be determined with the same accuracy as it was not possible to carry out the experiments on every day with all the cultures of so large a series. For this reason it may be that the data on the maximal photosynthetic rate of the different cultures as given in the table differ more than in reality. The assimilation curves in fact show a sharp maximum, and not always the determinations will have been done at this very moment.

As the course of the curve of the chlorophyll content shows a broad top, the maximum of the chlorophyll amount is rather equal in the different cultures, except in the case of low iron supply and in the case of nitrogen plus and -minus media. The data on cultures at a low level of iron supply, however, are not reliable because the cultures deteriorated by bacterial infections before the maxima were reached.

With a high iron supply a somewhat higher photosynthetic maximum is reached. The curve, however, shows a sharp optimum (fig. 14); after reaching the maximum of $\frac{\text{photosynthesis}}{\text{cm}^3 \text{ nutr. sol.}}$ the rate of photosynthesis decreases rapidly which points to the deficiency of another factor. This factor undoubtedly is the nitrogen supply, as will be shown below.

In the cultures on a high nitrogen level (cf. figure 14) the same $\frac{\text{photosynthesis}}{\text{cm}^3 \text{ nutr. sol.}}$ curve appears as has been found in Chapter III with the cultures supplied with the double concentration of nutrient compounds. It is typical of this curve that the maximum $\frac{\text{photosynthesis}}{\text{cm}^3 \text{ nutr. sol.}}$ is maintained for a long period. This curve is found in none of the other nutrient media; in all other cultures the rate of photosynthesis declines soon after having passed its maximal value.

The curve $\frac{\text{photosynthesis}}{\text{cm}^3 \text{ nutr. sol.}}$ of the nitrogen-plus culture differs from the curve of the culture supplied with the nutrient solution in double concentration only in the lower value of the maximum of the assimilation. This must be caused by the difference in concentration of the other compounds of the nutrient solution and on account of the investigations it must be the concentration of the iron, which prevents the assimilation to surpass a certain maximum.

The chlorophyll concentrations in the different cultures prove

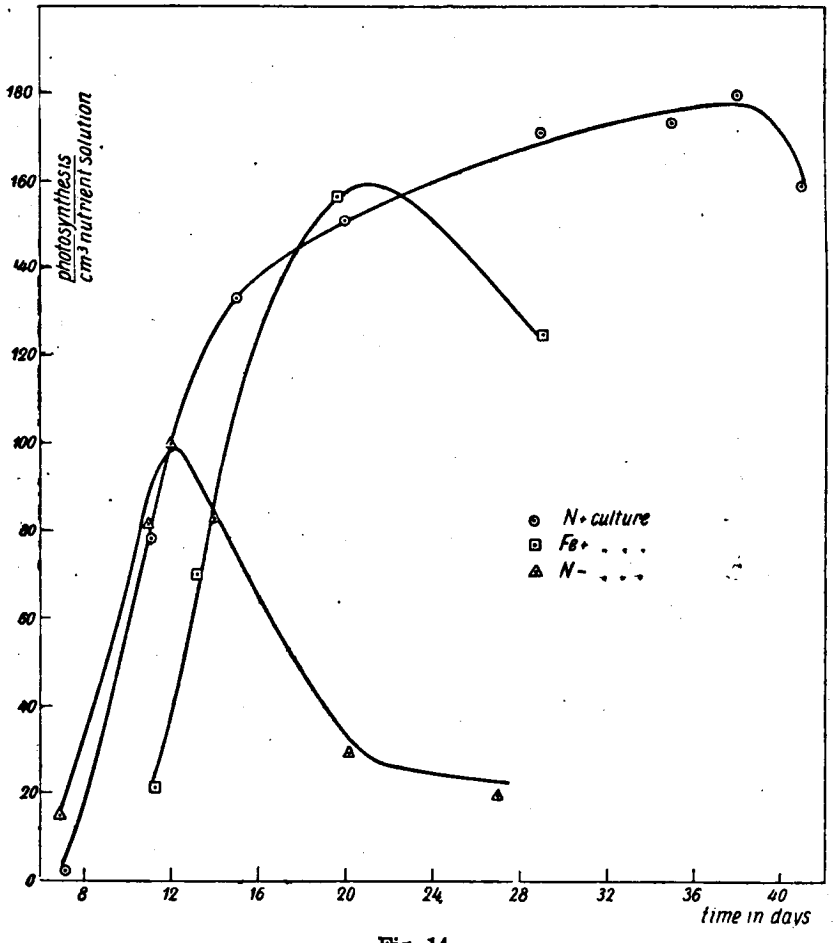


Fig. 14.

to be proportional to the nitrogen content. That the values of the chlorophyll content of the cultures described in Chapter III are lower than the values of this last series must be ascribed to the fact that the series were not cultivated simultaneously and so they were not exposed to the same external conditions during the growth.

If one of the other compounds of the nutrient solution, for instance magnesium, phosphorus, glucose are varied, no differ-

ence is found, neither in the attained maxima nor in the slope of the curves of assimilation and of chlorophyll content. So in the composition of the applied "normal" solution the concentration of these elements are actually not limiting the growth of the *Chlorella* cells in culture.

A large amount of nitrogen enables the cells to sustain the maximal assimilation for a long time. The photosynthesis in the N-minus cultures and also in the cultures supplied with the normal nitrogen amount must decline by nitrogen deficiency. During the growth the amount of nitrate decreases. The nitrate is converted into a form unable to keep the assimilatory process going on.

The rôle of the iron is of quite a different character. Though an increase of the iron concentration enables to a higher photosynthetic maximum (see Fe plus cultures) the photosynthesis in case of the normal iron amount does not decrease until another compound (in this case nitrogen) makes the rate of carbon fixation decline. If iron plays a part in the assimilatory process, which must be accepted on account of the investigations indicating that in assimilation a catalysis by a heavy metal plays a part (WURMSER 1921; WARBURG 1919, 1920, 1921), the above experiments allow to accept that the iron is not converted into an inactive form.

As far as the other elements of the nutrient solution are concerned it is not yet possible to conclude on their rôle in the assimilatory process on account of the mentioned experiments, but this would be possible by varying their concentrations to such an extent that they would limit the growth of *Chlorella* cells in the culture.

The influence of nitrogen on photosynthesis was investigated more in detail by adding potassium nitrate to cells with a low photosynthetic activity.

Methods.

In these experiments always cells were used which had passed their maximal value of $\frac{\text{photosynthesis}}{\text{cm}^3 \text{ nutr. sol.}}$ by aging. So these cultures did not show their maximal rate of photosynthesis per unity of chlorophyll (= assimilation number) since a long time, because this ratio decreases in the cultures from the very start of the growth. It was ascertained first that the cells were still alive.

After the rate of photosynthesis of these cultures had been determined in the carbonate mixture nr. 9 (WARBURG) under the normal conditions, from the side-bulb 2 cm³ of a KNO_3 solution were added in such a concentration that in the WARBURG vessels the KNO_3 concentration of the nitrogen-plus cultures was obtained. This concentration amounts to 2,52 g/l KNO_3 = 0,025 N. In the control vessel 2 cm³ of distilled water were added to the cells.

Results.

In table 17 and figure 15 the results of the photosynthetic measurements of two equal samples of cells from the same culture are recorded. In this experiment cells of a nitrogen-minus culture were used. The experiment was done on the 27th day after the inoculation. The photosynthetic maximum, stated on the tenth day, has been passed already for a considerable time.

The assimilatory activity of the cells was low:

$$\frac{\text{Photosynthesis}}{\text{cm}^3 \text{ nutr. sol.}} = 20$$

$$\frac{\text{Photosynthesis}}{\text{Chlorophyll}} = 0{,}898$$

The ratio $\frac{\text{photosynthesis}}{\text{cm}^3 \text{ nutr. sol.}}$ has been maximal 101 (cf. table 16). The ratio $\frac{\text{photosynthesis}}{\text{chlorophyll}}$ is determined at 5,54 in the first experiment done with this culture.

After having ascertained that the photosynthesis of the samples differed less than 5 per cent, the one sample was supplied with 2 cm³ of KNO_3 solution and the other with 2 cm³ of distilled water. The results of the respiratory and assimilatory measurements per half hour are given in table 17 and figure 15.

The entire experiment extended over 34 hours, of which the cells were exposed to the light for 8¼ hours.

Table 17 and the graph 15 show that by adding KNO_3 to the cells the rate of photosynthesis increases gradually in the course of the experiment. The oxygen output rises from 22,3 to 199,8 mm³ per half an hour, which means an increase to 900 per cent of the original rate. The control cells also show a slight increase in photosynthetic rate, reaching at the end of the experiment an oxygen emission amounting to 152 per cent of the initial rate per half an hour.

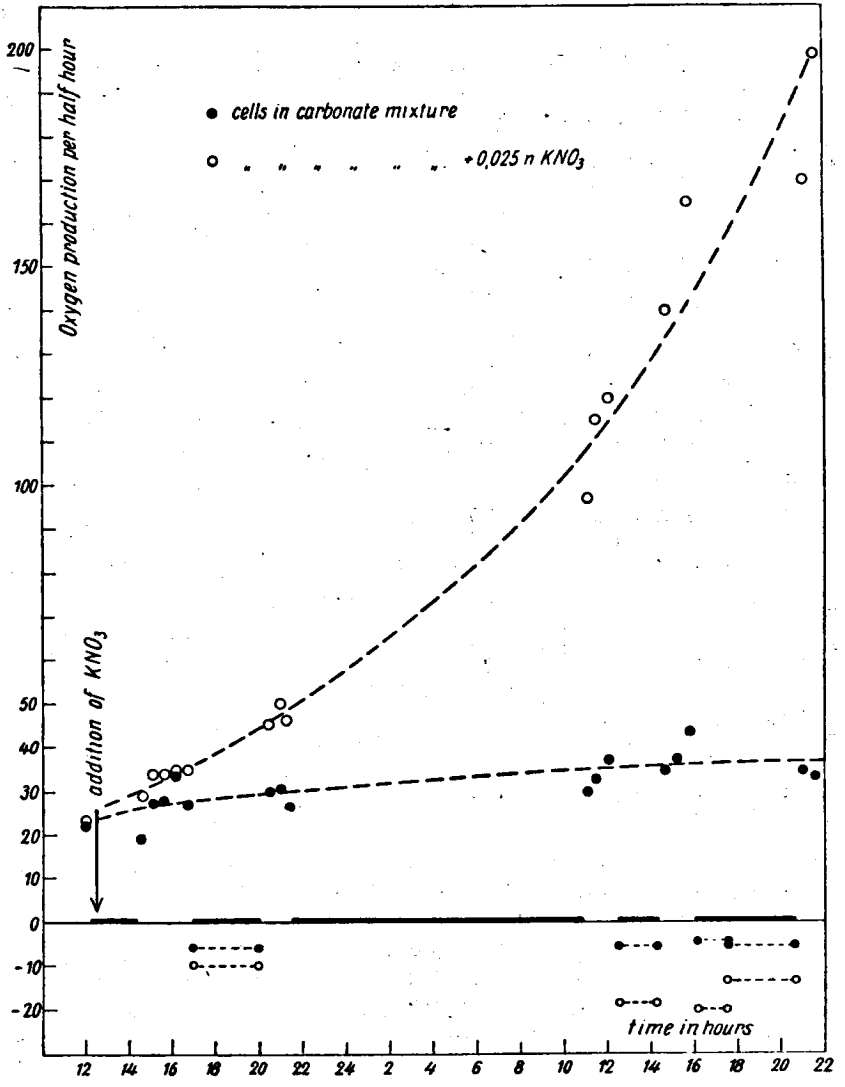

Oxygen production per half hour
cells in carbonate mixture
" " " " " " " + 0,025 n KNO3
addition of KNO3
time in hours
200
150
100
50
40
30
20
10
0
-10
-20
12
14
16
18
20
22
24
2
4
6
8
10
12
14
16
18
20
22

Fig. 15.

TABLE 17.

	Time	Rate of photo-synthesis per half hour	Rate of respiration per half hour	Rate of photo-synthesis per half hour	Rate of respiration per half hour
Light	11.45—12.15	22,3		23,3	
Dark	12.30	addition of KNO_3		addition of aq. dest.	
»	12.45—14.15		5,55		4,8
Light	14.20—14.50	28,8		19,1	
»	14.50—15.20	33,6		27,4	
»	15.20—15.50	33,6		28,0	
»	15.50—16.20	34,8		34,4	
»	16.20—16.50	34,8		27,4	
Dark	16.55—20.05		9,55		5,7
Light	20.10—20.40	45,0		30,0	
»	20.40—21.10	50,0		30,2	
»	21.00—21.30	46,8		26,7	
Dark	21.30—10.50		?		?
Light	10.55—11.25	97,0		30,4	
»	11.25—11.55	115,0		33,7	
»	11.55—12.25	120,8		38,6	
Dark	12.30—14.20		18,3		5,4
Light	14.30—15.00	140,0		35,2	
»	15.00—15.30	?		37,2	
»	15.30—16.00	165,0		44,2	
Dark	16.05—17.25		20,6		4,5
»	17.25—20.45		13,7		5,2
Light	20.50—21.20	170,5		34,8	
»	21.20—21.50	199,8		35,5	

When at the end of the experiment the chlorophyll amount of the used cells was determined, it appeared that it had not remained constant. At the beginning of the experiment an equal sample of cells as used in the experiment contained a chlorophyll concentration of 0,312. At the end of the experiment the chlorophyll content of the cells supplied with nitrate has risen to 0,494 = 159 per cent of the original concentration. It is evident from this that the increase of photosynthesis cannot be ascribed to the new formation of chlorophyll alone, as the quantity of newly formed chlorophyll is much smaller in

proportion than the photosynthetic increase. As will be seen from the following experiments the formation of new chlorophyll is not necessary for the increase of the photosynthesis. In many cases a marked increase of photosynthesis was found without any increase of the chlorophyll content. In some cases it could be stated that the chlorophyll increase appeared later than the increase of the assimilation.

Also the respiration rate of cells supplied with KNO_3 increases. This increase of the respiration rate can already be observed in the dark period, directly following the addition of KNO_3. This increased respiration is a phenomenon for itself. In the succeeding dark periods the respiration is increased not only by the application of the nitrate, but also by the photosynthetic products accumulated in the preceding periods of higher rate of carbon fixation (cf. Chapter V).

Investigations on the addition of nitrogen during the photosynthetic experiment were published in a paper of PIRSON (1937) during the course of my experimental work. PIRSON used in his investigations cells of *Chlorella vulgaris* f. *pyrenoidosa,* grown in inorganic media with nitrogen deficiency. His experiments too were carried out with the WARBURG method; the cells, however, were suspended during the photosynthetic measurements in their culture medium, not in carbonate mixture. The concentration of the added nitrogen was lower than in my experiments, viz. 0,01 N.

In my opinion it is better to work in a medium containing no other nutrient compounds than the one which must be added and of which the effect on the rate of photosynthesis has to be investigated. In the latter case the cell *growth* is inhibited by deficiency of the other nutrient compounds and so the increase of the rate of assimilation can be ascribed more safely to a *recovery* of the assimilatory mechanism.

The age of the cultures used in some of PIRSON's experiments is 12 days. It is not to discriminate whether his cultures had already reached their maximal development as to their photosynthesis and chlorophyll content. Growth in inorganic media in my experiments is a little slower than that with glucose addition, but the nitrogen concentration in the nutrient medium used by PIRSON is so low that the maximum, no doubt, must be reached within short time.

Notwithstanding these different methods the results of PIRSON are in agreement with my findings. In his experiments too the rate of photosynthesis is markedly affected in a short time by

nitrogen supply and in comparison the amount of newly formed chlorophyll is small.

As is evident from table 17 and figure 15 the rate of photosynthesis rises gradually; it requires some time before the effect of nitrate gets apparent. Whether this effect is realized in the dark or in the light is of no significance for the photosynthetic rate.

SCHIMPER (1888) held the view that nitrate could only be converted into organic substances in the light by green plants, and assumed that chlorophyll was necessary for this process. His theory, however, was refuted by GODLEWSKY (1903) who demonstrated in experiments on the chemical composition of seedlings of *Triticum* that proteins could be synthesized by green plants in the dark as well as in the light. For a protein synthesis in the dark it is, however, necessary that the plants contain a sufficient amount of carbohydrates. The favourable effect of the light on the synthesis of proteins must in the first place be the supply with carbohydrates. Besides GODLEWSKY assumes that also the energy, necessary for the nitrate reduction, can be derived directly from the light.

HAMNER (1936) examining the effect of nitrogen supply on the rate of photosynthesis and respiration of *Triticum* cultivated at N-deficiency, finds that addition of nitrogen increases the rate of photosynthesis but that the effect is dependent on the amount of carbohydrates in the plant. HAMNER's experiments with *Triticum* are not quite comparable with the experiments with *Chlorella*. The experiments extended over such a long period of time, that the plants supplied with extra nitrate showed a considerable growth. At the end of the experiment (after ± 14 days) the control-plants showed a definite injury, for the lower leaves were partly or completely dead and the other ones were yellowish green in colour. It stands to reason that the rate of photosynthesis of these plants was low and could not serve as a control for the plants supplied with nitrogen.

With *Chlorella* I have examined whether the increase of photosynthesis by nitrogen addition is dependent on the amount of reserve carbohydrates in the cells and on the possibility of forming new carbohydrates by illumination.

In the culture used in the experiment of table 17 and figure 15 it has been stated that this one contained a large amount of carbohydrates at the beginning of the experiment. This is understandable from the slight development of the culture (by the deficiency of nitrogen) in a nutrient medium abundantly contain-

ing glucose (1½ per cent). Moreover, it appears from the high respiration rate at the beginning of the experiment, amounting to 20 per cent of the rate of photosynthesis.

In the following experiments the influence of carbohydrates is clearly demonstrated.

In table 18 and figure 16 the results are recorded of an experiment also done with cells cultivated in nitrogen-minus medium.

The age of the used culture was 57 days. Consequently there exists a marked N-deficiency. The photosynthetic rate is particularly low; assimilation number = 0,422. The nitrogen in all vessels was supplied at the same time, but two of the vessels were kept in the dark for different periods of time. It now becomes obvious, that the longer the dark period, the more the photosynthetic rate has made progress. Consequently a sufficient amount of reserve materials has been present in the cells to restore the photosynthetic mechanism.

It seems rather curious that the rate of photosynthesis in all samples with N-supply is not the same at the end of the first light period. The effect of the nitrate supply on the increase of

TABLE 18.

	Time	control cells suspended in carbonate mixture O_2 liberated per ½ hour	cells in carbonate mixture + 0,025 n KNO_3 O_2 liberated per ½ hour	cells in carbonate mixture + 0,025 n KNO_3 O_2 liberated per ½ hour	cells in carbonate mixture + 0,025 n KNO_3 O_2 liberated per ½ hour
Light	12.05—12.35	86,5	86,0	Dark	Dark
»	12.35—13.55	102,5	98,0	»	»
»	13.55—14.25	115	102	»	»
»	14.25—14.55	113	120	»	»
»	15.05—15.35	130	105	107	»
»	15.35—16.05	124	106	162	»
»	16.05—17.45	123,5	124	150	»
»	17.50—19.30	128	124	155	»
»	19.30—21.30	125	133	158	»
»	21.40—22.10	115	124	146	134
»	22.10—22.40	119	142	158	212
»	22.40—23.10	120	136	166	212
»	23.10—23.40	112	137	164	208
Dark	23.43—10.43	-53	-63	-63	-65
Light	10.55—11.25	130	190	202	259
»	11.25—11.55	136	187	185	300

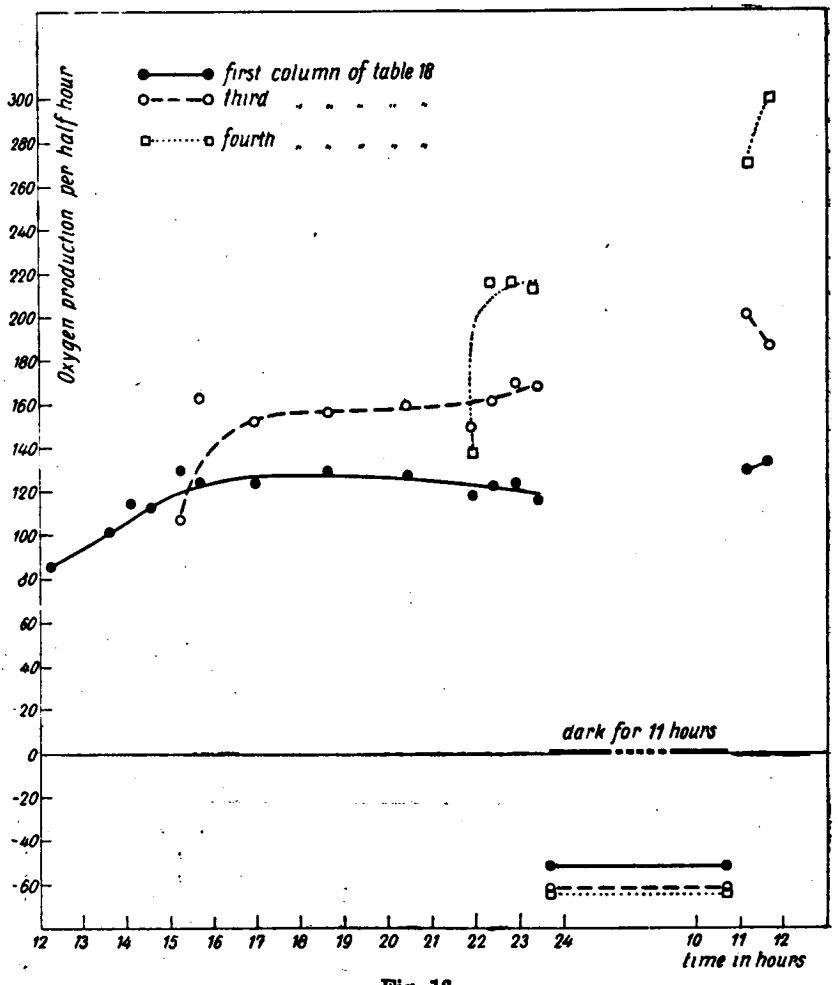


Fig. 16.

the photosynthetic rate seems less in the light than in the dark. This, however, must be ascribed to the injurious effect of the prolonged exposure to light of high intensity (see p. 686). By this the assimilatory system is injured, so the photosynthetic rate is the higher, the shorter the preceding illumination has been continued.

The respiration rate during the dark period is equally high in the three vessels supplied with nitrate. Most likely it is not allowed to conclude from this that the injurious effect of the light acts only on the assimilatory system and not on the respiration. As described before, the respiration rate is increased by a greater amount of photosynthetic products; where the illumination has been the longest, the most photosynthetic products will have been accumulated and consequently the respiration shall be mostly increased in this case. As the respiration rate is nevertheless equally high in the three cases, this points to an injurious effect of the light on the respiration as well. That, on the long run, the injury of the light is rather sweeping, is evident from the fact that after a dark period of 11 hours a recovery has not yet appeared. Though the photosynthetic rate of all three samples has become higher than on the previous day, the photosynthetic rate is highest in the culture which has been exposed to illumination for the shortest period.

Quite a different result was obtained in the following experiment (see table 19). The used culture had been cultivated in the normal nutrient solution with ½ per cent of glucose instead of the usual supply of 1½ per cent. The culture was 36 days old; the last 10 days it had stood in the dark, so the assimilatory products for the greater part had been dissimilated. This is apparent from the respiration rate, being only $\frac{1}{12}$ of the assimilatory rate.

When potassium nitrate has exerted its effect on the cells during 24 hours the following data are obtained:

First column: In the control cells, illuminated during 12 hours, the photosynthetic rate has remained constant. The chlorophyll content has been decreased by the long intensive illumination.

Second column: These cells, supplied with nitrate and illuminated during a long period, have been able to photosynthesize and consequently have produced a large amount of storage material. The photosynthetic rate has increased more than twice. Also the chlorophyll content has appreciably increased.

Third column: Cells not supplied with nitrogen and illuminated during a short period of time, have preserved their initial chlorophyll content. The photosynthetic rate is somewhat higher than in the control cells mentioned in the first column, which must be ascribed to the fact that these cells have been exposed for a shorter time to the injurious effect of the light intensity.

Fourth column: The cells supplied with nitrogen and il-

TABLE 19.

All cells suspended in the carbonate mixture.

Time		O_2 liberated per 1/2 hour	O_2 liberated per 1/2 hour	O_2 liberated per 1/2 hour	O_2 liberated per 1/2 hour
Light	16.00—16.30	47,4	45,6	Dark	Dark
			KNO_3 added	"	KNO_3 added
"	16.40—17.10	48,6	49,4	"	"
"	19.30—20.00	48,3	55,5	"	"
"	21.45—22.15	43,1	56,1	"	"
Dark	22.20—10.45	-3,0	-5,5	"	"
Light	10.55—11.05	48,9	107,5	"	"
"	11.25—11.55	51,4	112,0	"	"
"	15.40—16.10	46,4	113,3	52,0	53,0
"	16.10—16.40	46,0	108,5	56,3	54,7
Chlorophyll content before the experiment		0,187	0,187	0,187	0,187
Chlorophyll content after the experiment		0,140	0,343	0,185	0,214
mm^3 cellvolume before the experiment		12	12	12	12
mm^3 cellvolume after the experiment		9,5	14	12,6	11,1

luminated for a short period of time have formed some more chlorophyll, the assimilation rate has not increased in this case.

So these phenomena can be well explained by the theory of GODLEWSKY. The photosynthetic rate is only increased if the cells are able to form new proteins. For this both a suitable nitrogen source and carbohydrates are necessary. If one of these two is lacking the photosynthesis does not increase. The light is only needed for the formation of carbohydrates. If these are present the improvement of the photosynthesic system takes place just as well in the dark as in the light. It is not probable in my experiments that light has a promoting effect on the increase of the activity of the assimilatory process. It is not with certainty to discriminate because the assimilatory mechanism is damaged by continued illumination, which injury is dependent again on the age and the chemical composition of the cells.

As appears from the described experiments, the rate of photosynthesis is only increased when the formation of new proteins is possible and the increase is not dependent on the chlorophyll content. Once more it appears from this fact that the process of photosynthesis is limited by a protoplasmic factor.

When by an organism proteins are formed from nitrate taken up from the medium, the nitrate must be reduced. This reduction of the nitrate happens in the dark as well as in the light, as may be concluded from the fact that the photosynthetic rate is equally raised whether the nitrate has affected the cells in the dark as in the light.

In the light the photosynthetic rate is measured only by the production of oxygen. It might be thought that the extra oxygen production originated from the nitrate reduction and not from the photosynthesis, which is a carbon dioxide reduction.

By the investigations of WARBURG and NEGELEIN (1920) on the nitrate reductive power of *Chlorella* was shown that by illuminating *Chlorella* cells in a nitrate mixture great quantities of oxygen were liberated, originating from the nitrate reduction. By the differences in methods, however, the results of WARBURG and NEGELEIN are not comparable with the experiments described above. The experiments of these investigators were carried out with young cells rich in nitrogen. The cells that had been in a 0,1 N $NaNO_3$ solution before the experiment were suspended in a 0,1 N $NaNO_3$, 0,01 N HNO_3 mixture during the experiment. When illuminated the extra oxygen liberation immediately took place, *also* when the cells were deprived of carbon dioxide and so the assimilation was inhibited.

The addition of HNO_3 is an essential point in the experiments of WARBURG and NEGELEIN. When the cells are suspended in a 0,1 N $NaNO_3$ solution the nitrate reduction was not measurable with the manometric method.

In my experiments only in cells with nitrogen deficiency an increase in oxygen liberation appears and even in this case only when in the cells a sufficient amount of carbohydrates is present.

It could be decided whether the increase in oxygen production caused by addition of nitrate originated from nitrate reduction or from carbon dioxide reduction by inhibiting the photosynthesis in a natural way. WARBURG and NEGELEIN showed that, in this case, this can be done best by lowering the carbon dioxide concentration in the medium, since when using narcotics no extra oxygen is emitted in the nitrate reduction either.

In the experiment, the results of which are recorded in table 20, one sample of cells was suspended in the carbonate mixture, and an equal sample of cells of the same culture in the nutrient solution, to which no carbon dioxide was added. In the carbonate mixture the photosynthesis is much higher than in any other medium (EMERSON 1936). Both samples of cells were supplied with an equal amount of KNO_3. The cells were high in content of carbohydrates. By the higher assimilation rate in the carbonate mixture, this sample of cells could make still more, but the other sample being suspended in the nutrient solution could build up carbohydrates from the glucose.

The experiment was extended over 9 hours at constant illumination.

TABLE 20.

	O_2 liberated per $^1/_2$ hour cells in carbonate mixt. + KNO_3 0,025 N.	O_2 liberated per $^1/_2$ hour cells in nutrient medium containing KNO_3 0,025 N.
Beginning of the experiment	57	23
9 hours later	121	33

In the cells suspended in the carbonate mixture the extra oxygen output at the end of the experiment amounts to $121 - 57 = 64$ mm³ per half an hour. If this extra oxygen production would originate from the nitrate reduction, the oxygen production of the cells suspended in the nutrient solution should have raised to $23 + 64 = 87$ mm³ per half an hour. This is not the case as is seen from the table. So the increase of the oxygen production must not be ascribed to nitrate reduction but to a higher rate of carbon dioxide reduction as only the latter is affected by the lower concentration of carbon dioxide in the medium.

The form in which nitrogen is supplied to the cells is of great influence. I tried to affect the photosynthesis with NH_4Cl instead of KNO_3. The results, obtained by PIRSON with NH_4Cl are rather questionable. A distinct increase of the photosynthetic rate is not ascertained by PIRSON and in a later paper (1938) he tends to speak of an inhibition.

Like in the nitrate addition, I experimented with a higher concentration (0,025 N) than PIRSON did (0,01 N). The effect of the NH_4Cl application was such that, after a dark period of

one hour during which NH_4Cl could exert its influence on the cells, the photosynthesis was completely inhibited. This is shown by the data tabulated in table 21.

TABLE 21.

		O_2 liberated per ½ hour Control	O_2 liberated per ½ hour KNO_3 addition	O_2 liberated per ½ hour NH_4Cl addition
Dark	11.00—12.00	-34	-43	-67
Light	12.10—12.40	180	181	-22
Dark	12.45—14.15	-42	-63	-86
Light	14.20—15.20	192	214	-53
„	15.20—17.00	186	232	-73
Dark	17.15—10.45	-40,5	-71,5	-56
Light	10.50—11.20	182	489	-42,4
Chlorophyll content before the experiment		0,225	0,225	0,225
Chlorophyll content after the experiment		0,189	0,209	0,226

The respiration rate is still more stimulated by NH_4Cl than by KNO_3 supply. The chlorophyll formation appears to be able to go on in spite of the inhibition of the photosynthesis. This was established by PIRSON too (1938). The increase of the chlorophyll content at NH_4Cl addition in this experiment is not beyond the experimental error. It is, however, certain that the final chlorophyll content in the cells supplied with NH_4Cl was higher than in the other samples of cells.

Conclusions.

On the basis of the mentioned data it is evident that the decrease of the photosynthetic rate at aging of the cultures must be ascribed to a deficiency of available nitrate. By addition of a new amount of nitrate the assimilatory capacity is recovered if the *Chlorella* cells are able to form new proteins. The photosynthetic increase is independent of the chlorophyll content.

CHAPTER VIII.

Growth and assimilatory Activity of Chlorella grown in darkness and in inorganic media.

Introduction.

In one of the experiments with aged cells (cf. table 22), to which nitrogen was added in the WARBURG vessels, the first of the samples of cells was kept in the dark for 12 hours, the second one was exposed to light for 6 hours and the third one was in darkness for the entire duration of the experiment. The whole experiment extended over 24 hours.

When at the end of the experiment the chlorophyll content of the cells was determined, the chlorophyll content of the three samples of cells supplied with nitrate appeared to have equally increased, independently of the time of illumination. Only in the control sample of cells the chlorophyll content had decreased.

It must be concluded that the chlorophyll formation took place independently of the light and so *Chlorella* proves to be able to build up chlorophyll in complete darkness.

The ability to form chlorophyll in darkness belongs, as SCHIMPER (1885) reports, to all lower plants up to and including the *Bryophyta*. The *Pteridophyta* still are partly able to form chlorophyll without light, but a number of ferns requires light for greening. With the *Anthophyta* this capacity of greening in darkness is limited to the cotyledons of the *Coniferae* (SACHS 1865) (except *Larix europaea* (WIESNER 1876)) and the seeds of some Angiosperms (LOPRIORE 1904).

In the theories on the chlorophyll formation given by RUDOLPH

TABLE 22.

Rate of photosynthesis in the first half hour	31,8	Darkness	Darkness	33,0
Addition of nitrate	KNO_3	KNO_3	KNO_3	—
Period, in hours, of exposure to light	12	6	0	12
Rate of photosynthesis, 24 hours after the beginning of the experiment per half hour	93,9	91,2	—	26,4
Chlorophyll content before the experiment	0,230	0,230	0,230	0,230
Chlorophyll content at the end of the experiment	0,290	0,292	0,290	0,172

(1934) the phase in which light is required, is always set quite at the end of the process of the construction of the complicated chlorophyll molecule, because etiolated leaves, when illuminated, show a high rate of chlorophyll formation.

SCHARFNAGEL (1931) presumes that chlorophyll is formed out of protochlorophyll by means of light. RUDOLPH tends to regard protochlorophyll as photosensibilisator in the process of chlorophyll formation. According to LIRO (1908) the chlorophyll formation by light out of its precursor is a process observed also in vitro; this, however, is denied by SCHARFNAGEL.

From the fact that chlorophyll in lower plants can be formed in darkness, it is evident that these theories have no general validity, since lower plants contain a factor that makes the action of light superfluous; consequently the effect of light in this process is not specific.

The absorption spectrum of chlorophyll developed in darkness in *Nostoc punctiforme* was tested by ETARD and BOUILHAC (1898), of *Chlorella vulgaris* by RADAIS (1900). None of these authors could state differences between the absorption spectra of chlorophyll formed in darkness and of chlorophyll formed in light.

I too compared the methanol extracts of the chlorophyll formed in darkness and in light and found no discrepancy. As, according to the absorption spectra, the chlorophyll developed in the dark was identical with normal chlorophyll, it was investigated whether there exists some difference between the photosynthetic activities. This research still has another interest, because it enables to examine whether the decrease in assimilation number, described in the previous chapters, is caused by the illumination.

PANTANELLI (1903) has found that by intensive illumination the photosynthesis comes to an end. These "fatigue" effects involve a ceasing of the protoplasmic streaming and a chlorophyll destruction. It might be possible, that this phenomenon appeared to a minor extent in my experiments, for the assimilation number is the lower, the greater the amount of light the cells have been exposed to. This phenomenon can also be ascribed to other factors, and in the experiments of the foregoing chapter has been proved that the exhaustion of the nutrient solution is the principal cause of the decrease of the assimilation number.

However, there is a possibility that chlorophyll having never been exposed to light would show an abnormal behaviour when illuminated, and also it was of interest to investigate whether

the further assimilatory system was affected by the complete absence of light during the growth of the cells.

Methods.

As the increase of the chlorophyll in the experiment mentioned in the beginning of this chapter is rather slight and so the formation of chlorophyll in darkness is not quite convincing, I have grown cultures of *Chlorella* in complete darkness. The cultures were supplied with the normal nutrition or with the nitrate plus nutrient medium both containing 1.5 per cent of glucose. Immediately after the inoculation the culture flasks were placed in a light tight cupboard in a dark room. The development of the culture then was scanty. The cells poorly divided and did not form much chlorophyll. The highest measured ratio $\frac{\text{cm}^3 \text{ nutr. sol.}}{\text{chlorophyll}}$ was 0,054, while a culture with the normal nutrient solution cultivated in the light shows a maximal ratio of 0,500. If, however, during the growth the cultures were continuously shaken, the development improved noticeably and the chlorophyll formation did not vary from that in cultures grown in light. To this purpose, the culture flasks, wrapped up in black paper, were put in the shaking machine, which was boarded up with black paper. To which factor this disparity in growth of standing and shaken cultures must be ascribed is not quite conceivable. Of course the shaking does provide an even distribution of the cells and of the nutrient compounds. That the shaking would aid the ventilation and so the respiration does not look probable. The ventilation must have been rather poor by the double packing in paper (cf. also results with cultures cultivated in inorganic medium in this chapter). [1])

When the cultures were to be used in an experiment, a sample was taken out of the culture, centrifuged and suspended in the carbonate mixture at as little light as possible. The exposure to light during the determination of the photosynthesis consequently must be considered as the very first illumination of the cells.

Results.

As the temperature in the incubating room in darkness was low, the cells grew slowly in the beginning and the further

[1]) With higher plants it proved to be impossible to stimulate the chlorophyll formation in darkness by shaking. Seedlings of *Zea Mays* and *Pisum sativum* growing well in the dark at the cost of the storage material in the seed, remained fully etiolated in the shaking machine.

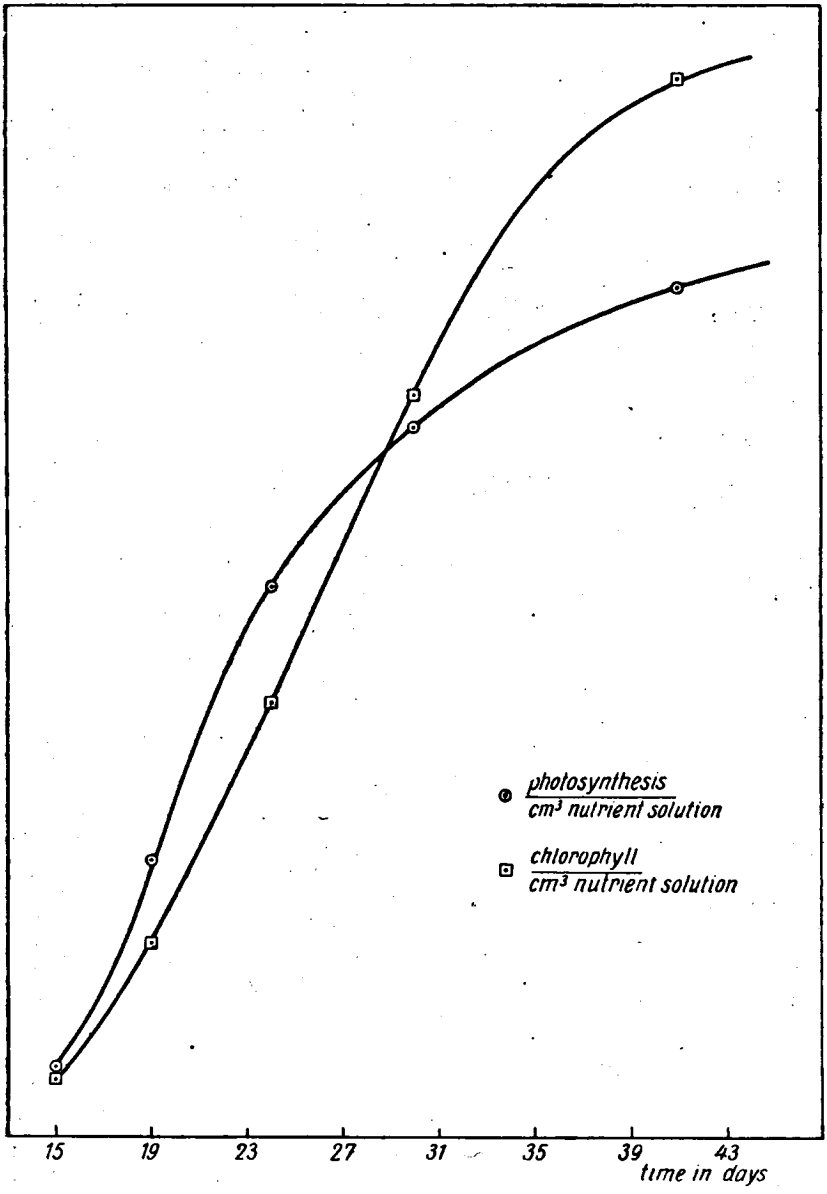

photosynthesis / cm³ nutrient solution
chlorophyll / cm³ nutrient solution
15
19
23
27
31
35
39
43
time in days

Fig. 17.

growth too was a little slower than in the preceding experiments. This appears from the length of time elapsing after the inoculation and the moment on which the first experiment could be carried out and from the smoother slope of the curves in figure 17. This figure and table 23 represent the data of assimilation per cm^3 nutrient solution per half an hour and the chlorophyll content per cm^3 nutrient solution, at an illumination of 12000 Lux

TABLE 23.

Time	Photosynthesis / cm^3 nutr. sol.	Chlorophyll / cm^3 nutr. sol.	Photosynthesis / Chlorophyll
15	4,54	0,009	5,16
19	47,8	0,122	3,81
24	105,2	0,241	4,35
30	137,8	0,576	2,99
41	166,-	0,840	1,97

It is apparent that there exists no essential difference with the cultures grown in light.

The readings per half an hour of photosynthetic measuring are constant from the beginning and show the normal time of induction (SMITH 1937). The chlorophyll, however, is highly sensitive to the intensive illumination. It appears that the chlorophyll concentration decreases if the cells are exposed half an hour to an illumination of 12000 Lux. The percentage of the chlorophyll, decomposed during the determination of photosynthesis varied between 0 and 15 per cent and is the higher, the older the cells. Young cells preserve their chlorophyll content or form even more chlorophyll. In computing the results, the average of the chlorophyll concentration before and after the experiment was taken.

The chlorophyll in aging cells became ever more photounstable. For this reason it was of no sense to extend the experiments over a longer period. Moreover, a curious phenomenon was met with: the chlorophyll in aged cultures came out of the cells and formed a colloidal chlorophyll solution in the nutrient medium. When the cells had been centrifuged out of the nutrient solution, the supernatant liquid was brightly green of colour. This chlorophyll solution did not give fluorescence and was decolourized within a few days when standing in diffuse daylight in an open vessel. The phenomenon that the chlorophyll

diffuses out of the cells gives the explanation of the decrease of the chlorophyll content of the cultures grown in light (see Chapter III). In the latter cultures the chlorophyll when diffused out is photo-oxidized in the light with the oxygen originating from the photosynthesis in the culture. In the cultures grown in the dark the chlorophyll is preserved in colloidal solution as no oxydation can occur in the medium that must be very low in oxygen tension by the respiration of the cells.

As the growth of the cultures in the dark does not show any essential deviation from that of the cultures grown in light, it seemed rather probable that the latter were growing heterotrophically too. For this reason cells were grown in the normal nutrient solution, however, not containing glucose. The growth of the cells was very slow in this medium as appears from table 24.

TABLE 24.

Time	$\frac{\text{Photosynthesis}}{\text{Chlorophyll}}$	$\frac{\text{Photosynthesis}}{\text{cm}^3 \text{ nutr. sol.}}$	$\frac{\text{Chlorophyll}}{\text{cm}^3 \text{ nutr. sol.}}$
13	5,40	2,16	0,004
34	2,06	3,16	0,015
97	1,04	12,9	0,123

On the 97th day the chlorophyll concentration amounts only to one fourth of the maximal chlorophyll concentration of the cultures provided with a nutrient solution containing glucose. By the way it may be remarked that it was investigated whether the addition of a growth promoting substance had any influence on the growth of the cells. To Dr. L. PONS I am indepted for supplying a biotine preparation of known activity. An influence of biotine could not be stated. (For the preparation of biotine cf. PONS 1938).

In spite of the slight development, the ratio $\frac{\text{photosynthesis}}{\text{chlorophyll}}$ is low in aged cells, which is not due to a deficiency of inorganic nutrient compounds. The poor development of the cells must be ascribed to a deficiency of organic substances. The conditions in the culture are apparently not of a nature to make possible a sufficient assimilation to supply the want of carbohydrates of the cells. As the photosynthetic activity of the cells in the carbonate mixture in the WARBURG vessels is sufficiently high to form a considerable amount of carbohydrates, the assimilation

in the culture flasks must be inhibited by deficiency of carbon dioxide. The cottonwool stoppers do not allow a sufficient aeration. Therefore I made a strong air current pass through cultures provided with inorganic nutrient solution. Growth is then only a little slower than in cultures provided with a nutrient solution containing 1½ per cent of glucose. The cells grown under these circumstances give an assimilation curve and a chlorophyll curve which do not deviate from the curve found in cells supplied with an organic carbon source. The maxima of chlorophyll per cm^3 nutrient solution and of photosynthesis per cm^3 nutrient solution too lie approximately on the same level.

A divergence is only found in the rate of respiration which was in the beginning of the growth of inorganically cultivated cells so small that no measurable change in pressure per half an hour was stated. This low respiration rate of the cells must be ascribed to the deficiency of carbohydrates. When the cells are still in their state of rapid division no carbohydrates are accumulated. When the cell-division in the older cultures decreases, more carbohydrates are accumulated and the rate of respiration increases.

Conclusions.

Cultures of *Chlorella* covered by cottonwool stoppers have an insufficient aeration. Supplied with glucose the growth is for the greater part heterotrophic and goes on equally well in complete darkness, if care is taken for a good distribution of the nutrient compounds by shaking of the culture. It is of no importance for the assimilatory capacity of the cells whether the cultures have build up their carbohydrates from carbon dioxide or from glucose; brought under equal external conditions the assimilatory activity is the same.

CHAPTER IX.

Discussion of the results.

In the preceding chapters the results are described of investigations on the growth and on the photosynthetic activity of *Chlorella pyrenoidosa.*

The rate of photosynthesis was determined while the cells were suspended in WARBURG's carbonate-bicarbonate mixture number 9, using the manometric method of WARBURG.

When the rate of photosynthesis of samples of cells from differ-

ent cultures must be compared, it is a problem per which entity the measured photosynthesis must be computed. As the chlorophyll is the only measurable substance in the green plants which can be said to be involved in the assimilatory process, the photosynthesis (determined per half hour at a temperature of 25,5° C.) per unity of chlorophyll was considered as an item for the assimilatory activity. This ratio is proportional to the assimilation number ("Assimilations-Zahl") according to WILLSTÄTTER and STOLL (1918); only the values in my work cannot be compared with the values of these authors as in my work merely relative chlorophyll concentrations were determined.

The ratio $\frac{\text{photosynthesis}}{\text{chlorophyll}}$ was found to be inconstant, and from experiments done with the same culture after different periods of time it appeared that this ratio is the highest in very young cells and declines from the very beginning of the growth in culture. This means that during the growth more chlorophyll is formed than corresponds with the increase of the photosynthetic capacity; so the photosynthesis per unity of chlorophyll decreases. As there was no reason to accept that the photochemical activity of the chlorophyll was decreased, it must be assumed that the factor of the BLACKMAN reaction, which may be an enzyme, increases at a slower rate than the chlorophyll. It follows from this that the amount of chlorophyll and the BLACKMAN factor increase independently of each other.

The BLACKMAN reaction limiting the rate of photosynthesis when the cells are satured with carbon dioxide at a surplus of light intensity, has a high temperature sensitivity. If the decrease of photosynthetic activity was caused by the diffusion of carbon dioxide through the cell walls that become thicker in older cells, or by inactivation of the chlorophyll, the temperature sensitivity of the assimilation process should decline in older cells, as diffusion- and photochemical processes have a lower temperature coefficient than the BLACKMAN reaction (VAN DEN HONERT 1930). However, it appeared that the temperature sensitivity did not vary with the age and the cultivating methods of the cultures, so it is allowed to assume that the BLACKMAN reaction always is the limiting process.

Different experiments were carried out to investigate which factors might affect the ratio $\frac{\text{photosynthesis}}{\text{chlorophyll}}$. An influence of the accumulation of photosynthetic products could not be stated.

The experimental results of FLEISCHER (1934), who found in young cells cultivated with magnesium deficiency considerable differences in the ratio $\frac{\text{photosynthesis}}{\text{chlorophyll}}$, could not be affirmed.

It appeared that the chlorophyll and the BLACKMAN factor were differently affected by the external circumstances during the growth of the cultures. By constant light and high temperature the chlorophyll formation was stimulated but the BLACKMAN factor was unfavourably affected and so the assimilation numbers were considerably lower than in cultures of the same age and density cultivated in diffuse daylight.

As according to the theory of WARBURG and UYESUGI (1924) the BLACKMAN reaction represents the splitting of a peroxide and the catalase activity of the *Chlorella* cells was supposed to be responsible for this reaction, it was investigated whether the power of splitting hydrogen peroxide showed the same variations in activity as had been found for the BLACKMAN reaction. This appeared not to be so. The power of splitting hydrogen peroxide is much less sensitive to unfavourable conditions in the culture during the period of growth of the cells. The decrease, found for the BLACKMAN reaction in all cultures, was never stated for the catalase activity. Therefore it seems improbable that the mechanism of the BLACKMAN reaction can be identified with the catalase reaction. If, indeed, the oxygen liberated by green plants in photosynthesis originates from the splitting of some peroxide, it must be assumed that in the dark chemical reaction another process is involved that limits the rate of photosynthesis and, as a consequence, represents the BLACKMAN reaction, or it must be assumed that this peroxide is splitted by another agent than that which is responsible for the splitting of added hydrogen peroxide.

By varying the concentration of the nutrient solution the rate of growth was not affected, but the length of the period of growth was; consequently the reached maximum of density of the cell suspension, of the chlorophyll content and of the photosynthetic capacity of the culture were proportional to the concentration of the nutrient compounds in the medium.

As the result of the determination of the growth and photosynthesis in media in which the nutrient compounds were given in different mutual proportion, it could be stated that the amount of available nitrate limits the photosynthetic capacity of the culture. Even in the cultures with a high amount of nitrate the photosynthesis decreases by nitrate deficiency in course of

time. It could be demonstrated that, when the concentration of iron limits the growth of the cells, the photosynthesis does not decrease by iron deficiency but remains constant until it is decreased by nitrate deficiency.

In cells of which the assimilatory activity was decreased by nitrate deficiency, the addition of nitrate appeared to have a highly stimulating effect on the rate of photosynthesis, while the chlorophyll content was only slightly increased. The rate of photosynthesis could, however, only be increased when the cells contained a sufficient amount of reserve carbohydrates. So it seemed probable that the added nitrate was used for the formation of new proteins, by which the photosynthesic rate was increased. This, however, does not directly require the assumption that in photosynthesis an enzyme of proteid-like nature is involved. The proteins are so closely related to the protoplasm and the actions of the protoplasm are so numerous and unapproachable that any further conclusion seems premature.

From the differences in behaviour of the rate of photosynthesis and of the chlorophyll content at nitrate addition, it was stated in several experiments that the increase of photosynthesis was never accompanied by an adequate increase but sometimes even by a decrease in chlorophyll content, while in other cases it was found that an increase of chlorophyll content did not involve a higher photosynthetic rate. This proves that the decrease of the assimilation numbers can not be explained by assuming a time effect on the photosynthetic activity of the chlorophyll and that the BLACKMAN reaction, being the rate determining process in photosynthesis, is not directly dependent on the concentration of the chlorophyll.

However, it appeared impossible to raise the assimilation numbers above a certain value. The abnormally high assimilation numbers found by WILLSTÄTTER and STOLL with leaves of etiolated higher plants could not be reproduced with *Chlorella*, for not only the synthesis of proteins took place in darkness equally well as in light, but also the chlorophyll formation and growth are possible in complete darkness if only carbohydrates are present.

The assimilation numbers of etiolated leaves, which are only surpassed by the ratios found in some *aurea*-varieties of different plants, are so much higher than the assimilation numbers found in *Chlorella* (EMERSON 1936) and *Hormidium* (VAN DEN HONERT 1930) that in these lower plants just as in normal higher plants the chlorophyll must be in excess in proportion to

the rate determining factor of photosynthesis. As this cannot be changed by cultivation in complete darkness and as also chlorotic *Chlorella* cells appeared to have constant assimilation numbers (EMERSON 1929, FLEISCHER 1934), there must exist — in spite of the independence of chlorophyll content and BLACKMAN reaction — an internal factor in the green plants which prevents the assimilation numbers to diverge widely, a fact which is not yet understood.

Summary.

1. The photosynthetic activity of *Chlorella pyrenoidosa*, expressed by the ratio $\frac{\text{photosynthesis}}{\text{chlorophyll}}$ is the highest in young cultures and declines from the beginning of the growth.

2. By cultivation under different external conditions of light and temperature, cells of different photosynthetic activity can be formed.

3. The decline of the photosynthetic activity cannot be explained by accumulation of photosynthetic products.

4. A specific effect of magnesium deficiency on the photosynthetic activity of young cells, as has been found by FLEISCHER, could not be reproduced.

5. The decline of the photosynthetic activity cannot have been caused by inactivation of the chlorophyll, but must be ascribed to the activity of the BLACKMAN reaction.

6. The changes in the rate of photosynthesis and of catalase activity do not correspond, so it seems unlikely that the BLACKMAN reaction and the splitting of added hydrogen peroxide should proceed by means of the same mechanism.

7. If the decline of the total photosynthetic capacity of an aging culture is caused by nitrate deficiency, it can be raised by nitrate addition. This can be achieved equally well in light as in darkness if only the cells contain a sufficient amount of carbohydrates. If no carbohydrates are present the rate of photosynthesis can only be increased in light when new carbohydrates are photosynthesized.

8. The increase of the photosynthetic activity by nitrate addition is established independently of the chlorophyll.

9. The growth and chlorophyll formation of *Chlorella* in glucose containing media are equally good in darkness as in light. The absence of light during the growth does not have an effect on the photosynthetic power of the cells.

10. The fact whether the cells have built up their carbohydrates of carbon dioxide or of glucose does not affect the photosynthetic activity.

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