PHOTOXIDATION PROCESSES IN NORMAL GREEN *CHLORELLA* CELLS

I. THE BLEACHING PROCESS

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INTRODUCTION

The variability of the pigment content of leaves in relation to the ecological and developmental factors has now been recognised by many authors (see SIRONVAL[1], WENDEL[2]). The work of TAMIYA *et al.*[3], with *Chlorella* cells, has also revealed such variations. The origin of these variations is still obscure and probably very complex. This emphasises the necessity for further investigations.

The present study deals with the relatively simple case of pigment photoxidation (pigment bleaching) in very intense light. The process has been studied by various authors since NOAK[4] (see *e.g.* MONTFORT[5], 1936–1953; FRANK AND FRENCH[5b], 1941; AACH[6,7], 1952–1954; KANDLER AND SCHÖTZ[8], 1956; HAGER[9], 1957). However, as pointed out by RABINOWITCH[10], its exact kinetics has never been studied in detail.

We have tried to do so using a high-pressure lamp (the same as used by KANDLER AND SCHÖTZ in their experiments on *Chlorella* mutants).

The experimental results are published in two papers. The first is concerned with the bleaching kinetics themselves; in the second several facts are co-ordinated to permit some new speculations on the causes of the bleaching in intense light.

MATERIAL AND METHODS

The experimental material consists of two different strains of normal green *Chlorella*, the strains K and P. Strain K (*Chlorella pyrenoidosa*) has been cultivated by KANDLER for several years and has often been used by this author. It is relatively resistant to the light action. Strain P (*Chlorella vulgaris*) was kindly sent to us by Prof. PIRSON. It is much more sensitive than strain K.

The two strains are cultivated following the method of KANDLER[11]. They are kept in the dark for 24 hours before the experiments.

The suspension exposed to light is made sufficiently thin to permit a good illumination of all the cells (about 0.5 mg dry weight per cm^3). By continuous shaking in a glass vessel the different cells receive approximately the same quantity of light.

The light is supplied through a water thermostat maintained at 28° C (except in the cases where the temperature effect is studied). The lamp is a Xenon high-pressure lamp XBF 6,000 from Osram. It yields about 100,000 lux at the bottom of the vessel. Lower intensities are obtained

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by the use of suitable screens. Naturally, the light arriving inside the vessel comprises only the visible part of the spectrum (for the exact emission spectrum of the lamp, see KANDLER AND SCHÖTZ[8]).

The gaseous phase inside the vessel is generally normal air. When pure oxygen, pure nitrogen, or CO_2-containing air is used, it is bubbled continuously through the vessel.

The pH of the suspension is maintained by a phosphate buffer. In some cases, however, a bicarbonate–carbonate buffer (90:10; pH 9.6) was used (see the text).

The pigments are extracted in methanol or in a mixture acetone–methanol. These solvents are more suitable than aqueous acetone in the case of *Chlorella*. The various pigments are separated by paper chromatography (method of BAUER modified by SIRONVAL[12]). The chlorophylls are eluted in ether, and their absorption is measured in a Beckman spectrophotometer. The calculations are made according to COMAR[13]. The carotenoids are extracted in chloroform. Their absorption is also measured in a Beckman spectrophotometer. The quantities are estimated by using a standard curve previously established with pure products.

RESULTS

I. The principal factors governing the bleaching of Chlorella *cells in light*

a. General kinetics of bleaching

In very intense light (about 100,000 lux), *Chlorella* cells do not immediately lose their pigments to a noticeable degree. During the first hour and a half of illumination of strain P in air (or, with the less sensitive strain K, during the first 2–5 hours), there is slight loss of pigment, amounting to scarcely 10–20 % of the initial quantity. However, after this time, the rate of bleaching grows rapidly, reaching 30–50 % per hour of the initial content, and this rapid rate remains approximately unchanged until the colour has completely disappeared.

We may consider the first phase of very slow decrease in pigment content as *an induction phase* introducing the following rapid bleaching. The two phases appear clearly from Fig. 1 (curves 2) which gives three examples of bleaching in air in the case of the very sensitive strain P.

b. The light-intensity factor

As is well known, the pigment destruction by light occurs only at high intensities. On Fig. 2, we have plotted the rate of bleaching against a scale of intensities from 0 to 100,000 lux (sensitive strain P). It can be seen that the bleaching becomes only perceptible at 50,000 lux, and that the destruction is greatly accelerated above 70,000 lux. Between 70,000 and 100,000 lux the rate increases approximately 7-fold, so that the 70,000 lux value appears as a limit between the low intensities incapable of causing considerable bleaching, and the high intensities leading to a rapid destruction.

c. The composition of the gaseous phase

The presence of oxygen in the gaseous phase is also very important for the bleaching process. In pure oxygen, the total photodestruction of the pigments is reached many hours before that in air. This is mainly due to the reduction of the induction phase (from 90 min in air to 30 min in oxygen), whereas the bleaching phase is little activated (Fig. 1, curves 1). In contrast, an atmosphere of pure nitrogen almost totally inhibits the bleaching, even if a high light intensity is continuously supplied for several hours (Fig. 1, curves 3).

The photoxidative character of the process is therefore evident. There are two

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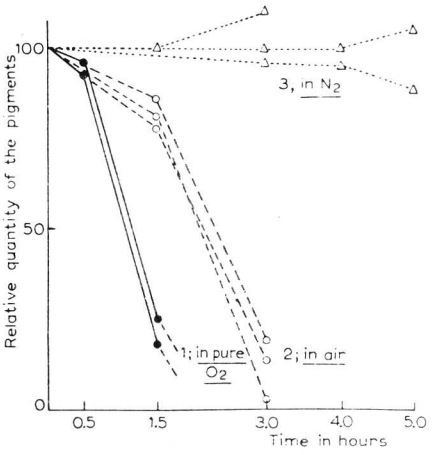

Fig. 1. The kinetics of bleaching in high intensity light (about 100,000 lux). It is evident that the presence of O_2 is necessary for bleaching. The induction and bleaching phases are clearly distinct. (Initial quantity of the total pigments = 100.)

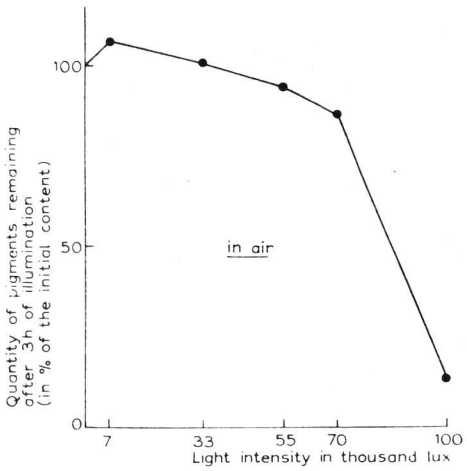

Fig. 2. Relation between light intensity and bleaching in air.

factors necessary: sufficient light energy supply and the availability of a certain quantity of oxygen.

The increase of the CO_2 content of the gaseous phase (up to 1 %) or the addition of bicarbonate–carbonate buffer (90:10) to the suspension medium does not modify greatly the rate of photodestruction. Table I shows that in the presence of CO_2, the bleaching is always a little retarded. The result is the same if the gaseous phase consists of air + CO_2 or of oxygen + CO_2. The influence of CO_2 is not at all sufficient to prevent a complete disappearance of the pigmentation; the only effect is a certain delay in the destruction.

TABLE I

THE INFLUENCE OF CO_2 ON THE PHOTODESTRUCTION OF THE TOTAL PIGMENTS

Light intensity: about 100,000 lux

Nature of the gaseous phase	*Period of illumination*	*% of total pigments remaining at the end of the illumination time*	
		in the presence of CO_2	*in the absence of CO_2*
(1) air*	3 h	21	13
(2) air*	3 h	36	16
(3) air	3 h	7	4
(4) air	3 h	13	6
(5) O_2	1 h 30 min	19	17
(6) O_2	1 h 30 min	39	23

* The CO_2 is supplied by addition of bicarbonate–carbonate buffer (90:10). In the other cases, the CO_2 is added to the gaseous phase (up to 1 %).

d. The pH and the temperature of the suspension medium

It is difficult to notice a difference in the rate of bleaching in relation to the pH

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of the *Chlorella* suspensions. We used systematically different pH values between 5 and 9 (the gaseous phase being air) without finding significant variations.

Three temperatures were also tested (18, 28 and 38° C). The kinetics found for the bleaching in air at these temperatures for the *Chlorella* strain P is shown in Fig. 3. It is clear that the temperature effect—if it exists—is negligible, when compared with the effect of the presence of oxygen in the gaseous phase.

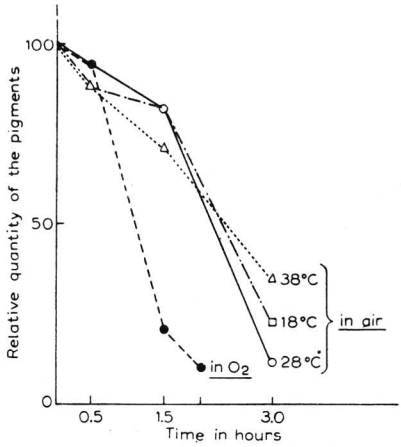

Fig. 3. Action of the temperature on bleaching in air. (See text; initial quantity of the tota pigments = 100; light intensity: about 100,000 lux.)

II. The behaviour of some major pigments during the bleaching

Five pigments have been followed separately during the bleaching: the chlorophylls (a) and (b), the carotenes ($\alpha + \beta$), and carotenols: luteine and epoxides. The data for luteine and epoxides are presented together (carotenols).

a. Chlorophylls (a) and (b)

Before their exposure to light the algae used for the measurements contain approximately 2.5 mg of chlorophyll (a + b) per 100 mg of dry weight; the average ratio (a/b) is 2.48. During the illumination, the decrease in the total chlorophyll content follows the curves of Fig. 1, but the two forms a and b do not disappear at exactly the same rate. This is particularly clear in an atmosphere of oxygen.

The modification of the ratio (a/b) in two different experiments, carried out in pure oxygen is shown in Table II. In the two cases, the ratio falls progressively, especially during the rapid bleaching following the first thirty minutes of illumination. In one case, the ratio goes from 2.82 at the beginning of the illumination to 1.08 three hours later, and in the other case, it falls from 2.39 to 0.94 during the same length of time. Thus, the final ratio is less than a half of the initial one. This means that chlorophyll (a) is much more sensitive to the photoxidative process than chlorophyll (b).

Analogous figures are obtained in air; but the decrease in the ratio appears later and is less marked. In pure nitrogen, however, the ratio does not decrease, as is shown in Fig. 4.

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TABLE II

CHANGES OF THE RATIO $\frac{\text{quantity of chlorophyll a}}{\text{quantity of chlorophyll b}}$ DURING THE BLEACHING IN AN ATMOSPHERE OF O_2

Light intensity: 100,000 lux.

Period of illumination	*Experiment No. 1*	*Experiment No. 2*
0	2.82	2.39
30 min	2.85	2.13
1 h 30 min	1.92	1.16
3 h*	1.08	0.94

* At this time, only traces of chlorophyll are detectable.

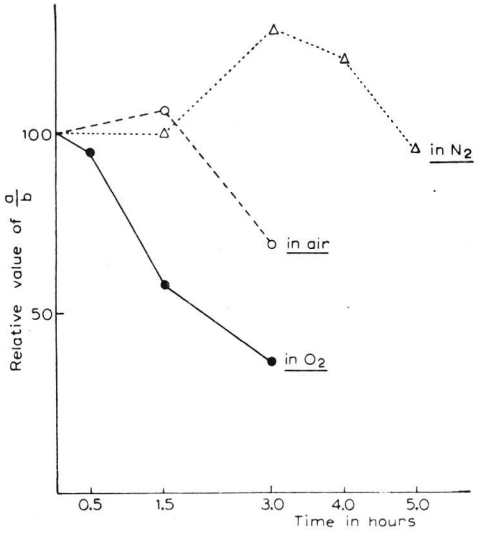

Fig. 4. Changes of the ratio $\frac{\text{Quantity of chlorophyll a}}{\text{Quantity of chlorophyll b}}$ during the bleaching in high intensity light (about 100,000 lux; 100 = ratio a/b at the beginning of the illumination; in N_2, naturally no bleaching).

b. Carotenes and carotenols

The approximate quantity of carotenes is 0,20 mg/mg dry weight, principally of the β form. There is three times as much carotenols (luteine + epoxides) as carotenes. The average ratio $\frac{\text{luteine + epoxides}}{\text{carotenes}}$ is 2.85.

In an atmosphere of oxygen, this ratio does not change greatly at the beginning of the exposure to intense light. It rises about 10 % in the first thirty minutes of the induction phase.

But after that a very drastic change takes place. After half an hour, the ratio approaches infinity. This is due to the extremely rapid disappearance of the carotenes: 1 ½ hours is ample for the complete photoxidation of these pigments, whereas the carotenols are much more stable. The carotenols need 2–3 hours of light, in oxygen, for their total destruction.

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In air there is the same tendency but it is less marked than in oxygen. Here also the carotenes are destroyed more rapidly than the carotenols. However, in pure nitrogen, the picture changes: the high light intensity does not diminish the carotenoid content, and it even favours the non-oxidised carotenes (see Fig. 5).

If we compare the four pigments studied, it appears that their sensitivity to photoxidative processes is as follows: the highest sensitivity is found in the carotenes, followed by chlorophyll a, the chlorophyll b and the carotenols (luteine + epoxides) which have the same sensitivity (Table III).

This means that the oxidised forms are more resistant to light than the reduced ones.

TABLE III

PHOTOSENSITIVITY OF THE DIFFERENT PIGMENTS: % REMAINING AFTER A GIVEN LENGTH OF ILLUMINATION TIME

Light intensity: 100,000 lux.

Nature of the gaseous phase	*Period of illumination*	*Carotenes*	*Carotenols*	*Chlorophyll a*	*Chlorophyll b*
Air	3 h	0	34	18	31
Oxygen	1 h 30 min	0	24	11	25

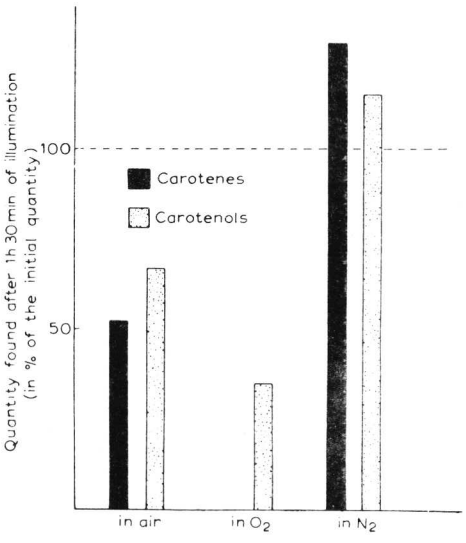

Fig. 5. Behaviour of the carotenes and carotenols in the high intensity light (about 100,000 lux).

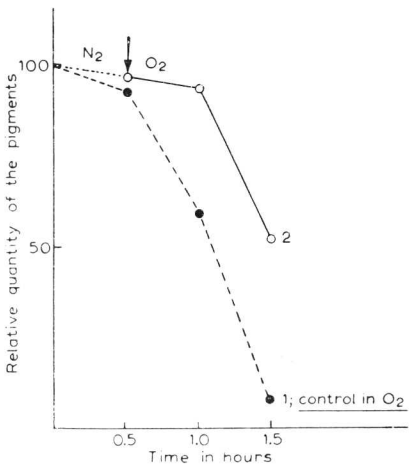

Fig. 6. The lack of induction in an atmosphere of nitrogen. The series (curve 2) received high intensity light (100,000 lux) for 30 minutes in N_2; under these conditions, induction did not take place; it began only with the application of O_2. (Initial quantity of total pigments = 100.)

III. The nature of the induction phase

The occurrence of an induction phase during the first part of the bleaching process is very striking. In what way does the induction prepare the way for the subsequent accelerated bleaching?

We plan to discuss this question more fully in another paper. Here we state

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briefly some facts indicating the photoxidative nature of the induction phase and its relation to unknown modifications of the properties of the living matter.

a. Need for oxygen during the induction phase

If pure oxygen is given in the gaseous phase from the beginning of the illumination in high light intensity, the induction phase is achieved after about 30 minutes (see Fig. 1), provided that a sensitive *Chlorella* strain is used. In air the induction phase is longer.

In the complete absence of oxygen, *e.g.* in a pure nitrogen atmosphere, the induction does not take place. This is illustrated in Fig. 6; nitrogen was given during the first 30 minutes of illumination, and then replaced by pure oxygen. The induction phase begins only with the oxygen treatment, and a new period of 30 minutes is necessary before the rapid bleaching phase occurs.

It is clear, therefore, that the presence of oxygen in the gaseous phase is absolutely necessary for the induction. In all probability, oxygen is essential for some unknown transformations preliminary to the bleaching process itself.

b. Reversibility of the induction by darkness

In order to test whether or not the induction of the bleaching process by intense light in the presence of oxygen is reversible by a dark period, the following experiment was carried out.

After 30 minutes of induction by intense light in pure oxygen *Chlorella* cells (strain P) were placed in the dark. The dark period varied from 1 to 3 hours in the various series (preliminary work had shown that 30 minutes darkness did not reverse the induction); then the high-intensity light was again applied, also with pure oxygen.

In all 3 cases in which a dark period was given, we obtained almost the same result: after the dark period, the high-intensity light does not immediately cause the rapid bleaching that could be expected after the first 30 inductory minutes of light. On the contrary, there has to be a new induction period of about 30 minutes in order to prepare for the final strong photoxidation of the pigments (Fig. 7). For the 3 series the reversibility of the light induction by darkness seems to be very close to completion.

After 1½ hours of continuous light, the control series has lost 80 % of its initial pigment content, while the series where the first and the second induction phases are separated by a dark period, have lost only 20–35 % of their content after the same 1½ hours of exposure (Fig. 7).

The reversibility of the induction by darkness indicates that the induction process probably consists of some change sufficiently weak to permit a recovery when the high-intensity light is removed. Probably a normal low-intensity light would also allow a recovery, but we have not carried out any experiments in this direction.

c. Suppression of the induction phase in killed cells

From the data described above, the induction appears as a photoxidative dark-reversible alteration of the cells. The question that now arises is: do these alterations concern, as is very probable, the living properties of the protoplasm?

A simple method for discovering this consists in studying the kinetics of photoxidation in killed cells (boiled for 10 minutes in a water-bath at 100° C). Fig. 8 shows the result of such an experiment, the initial pigment content being arbitrarily

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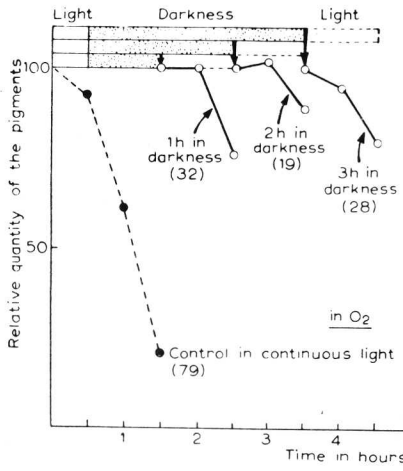

Fig. 7. Reversibility of induction by darkness. The figures in parentheses express the quantity of total pigments destroyed in 1 h 30 min of illumination. For the series in darkness, the total pigment content has been fixed at 100 at the beginning of the second period of illumination. This method permits a better comparison with the control in continuous light.

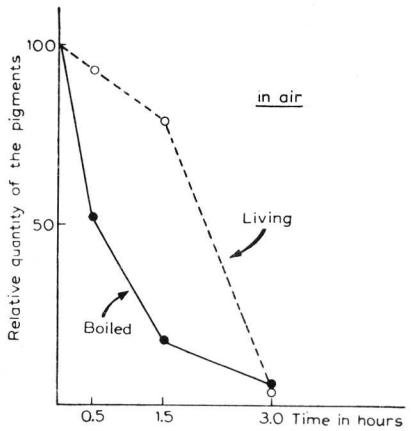

Fig. 8. Absence of induction in the boiled *Chlorella* cells. (Initial quantity of total pigments = 100; light intensity: about 100,000 lux.)

taken as at 100 for the living and the boiled cells. It is easy to see that the main effect of boiling is the total disappearance of the induction phase. In the killed cells the bleaching begins immediately on exposure to intense light and the curve found is an exponential one, which corresponds theoretically to a simple photochemical process.

It is evident that the living matter protects itself to a certain degree against the damaging effects of the intense light and that the induction phase reflects this resistance. During the induction, the properties of the living matter are modified in such a way that rapid bleaching finally becomes possible. We may conclude that the pigment destruction is not in any way a *primary* effect of the oxidizing action of intense light.

The pigment loss is a secondary effect, resulting from other preliminary photoxidizing dark-reversible changes.

DISCUSSION AND CONCLUSIONS

The occurrence of an induction phase for the bleaching process can be explained in 3 main ways:

1. First, we may assume that the oxidation bleaching of the pigments proceeds only after a certain reserve of various oxidisable material in the cell has been used up.

2. It can also be supposed that the bleaching becomes possible only when the original pigment protein complex is broken down.

3. One can assume more generally that some processes of the normal metabolism are necessary in order to protect the pigments from bleaching, and that these protective processes are altered by intense light in such a way that the final destruction becomes possible.

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The first hypothesis is difficult to maintain if we consider that the induction phase is absent in boiled cells, though a sufficient amount of oxidisable material is still present after the boiling.

The alteration of several basic metabolic functions by high-intensity light is a well-known fact. MYERS AND BURR[14] have demonstrated that the photosynthetic apparatus is strongly inhibited by intense light (see also KOK[15], and our own data that will be published in a subsequent paper); at the same time the respiration without substrate is activated (see the subsequent paper). It is very probable that intense light causes many inhibitions and activations of this kind.

The exact relationship between these metabolic alterations and the final bleaching is still difficult to assess. However, one can point for instance to a theory proposed by AACH[7]. This author has insisted on the particularly high sensitivity of the pigments to light in the case of N deficiency. He supposes that the photoxidation occurs more readily when the pigments are not sufficiently stabilised, and that the stabilisation is due to molecules rich in N, as for example in the protein fraction. It is possible that the alteration of the metabolic functions by intense light results in some modification of the properties of the chloroplast proteins necessary for the stability of the pigments.

In this respect our data on the specific behaviour of each particular pigment during bleaching could be explained by differences in the efficiency of their corresponding stabilising systems. However, it might well be that the increase of the ratio a/b during bleaching is due, not so much to a greater stability of b, but rather to its increased production as the result of the oxidation of a. If this is true, chlorophyll b could be derived from chlorophyll a during the bleaching process. It is interesting that HAGER[9] working with 50,000 lux, also found that chlorophyll a disappears before chlorophyll b, and that the same phenomenon was observed by AACH[6] when he illuminated N-deficient algae.

It is remarkable that, in our experiments, not only the oxidised chlorophyll b form is more resistant than the reduced chlorophyll a, to high-intensity light, but also that the oxidised carotenols are more resistant than the reduced carotenes. This is a point in favour of the hypothesis of a photoxidation both of chlorophyll a to b and of carotenes to carotenols. In his experiments with a 50,000 lux source, HAGER[9] has carefully analysed the carotenol content of the illuminated plants. He has found a series of compounds ("Starklicht-Oxyde") that appear to correspond to various degrees of oxidation in intense light. However, especially in the case of the chlorophylls, the above hypothesis needs to be carefully tested by the use of a suitable radioactive tracer technique.

Another fact to be discussed here is the influence of temperature on the photoxidation process. We did not find any obvious differences between the kinetics of bleaching at 18, 28 and 38°. This is in contradiction with much of the literature, which indicates that bleaching is influenced by the temperature. For instance, under solar light, the leaves of *Fragaria vesca* lose their pigments when grown at 20° (SIRONVAL[1]); at higher temperatures (25 to 35°) the pigments are not lost; this effect is due to the coincidence of low temperature and high-intensity light. HAGER[8] finds a similar temperature effect: the day temperature must be high in order to prevent a decrease in the pigment content in light. It is probable that our results can be attributed to the drastically high light intensity used (100,000 lux approximately): we may assume

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that at such an intensity the temperature has only a very slight effect *i.e.*, at least within the temperature range of the experiments.

After NOAK had published his results (1925–1926), it was generally agreed that the effect of CO_2 is a protective one. In our experiments the photoxidation of the pigments was in fact retarded in the presence of CO_2. However, our results indicated only a relatively *weak protection*. This is of course due to the fact that we were working with very high intensities where the action is clearly visible within a few hours, while NOAK was using lower intensities applied for several days. In our case, an obvious protective effect of CO_2 would be rather difficult to interpret since, as has been pointed out, photosynthesis is strongly inhibited some minutes after the beginning of the exposure.

ACKNOWLEDGEMENTS

One of the authors is greatly indebted to the "Institut pour l'encouragement de la Recherche Scientifique appliquée à l'Industrie et à l'Agriculture; IRSIA", Belgium, for financial aid which made it possible for him to participate in this work.

We are also indebted to the "Deutsche Forschungsgemeinschaft" for supplying equipment.

SUMMARY

The authors describe the kinetics of pigment bleaching in *Chlorella* cells exposed to very intense light (100,000 lux). They distinguish two phases: an induction phase preceding bleaching, and a bleaching phase. Both phases require oxygen. During the bleaching phase, carotene disappears, then chlorophyll (a), and finally chlorophyll (b) and the carotenols. The induction of bleaching is dark-reversible and seems to reflect important changes in the cell metabolism. It has been suggested that the induction is concerned with some modifications of the properties of the chloroplast proteins that are essential for the stability of the pigments.

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Received January 14th, 1958