# PHOTOXIDATION PROCESSES IN NORMAL GREEN *CHLORELLA* CELLS

## II. EFFECTS ON METABOLISM

O. KANDLER* AND C. SIRONVAL**
*Botanical Institute of the University, Munich (Germany)*
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SUMMARY

The effect of high light intensity on the metabolism of *Chlorella* was studied. During the induction phase of the bleaching, photosynthesis is strongly inhibited, the endogenous respiration is increased, and the oxidative assimilation decreased. As shown by determining the level of inorganic phosphate within the cell, the phosphorylation rate is decreased very strongly. These effects are similar to those reported for X-ray and u.v. radiations. It is therefore assumed that all types of radiations have the same primary action on metabolism, probably by forming radicals and subsequently peroxides. The bleaching of the green cells is a secondary effect, which takes place only if the stabilising chloroplast structure is partly destroyed as a result of the inhibition of metabolism.

## INTRODUCTION

It has been shown in a previous paper (SIRONVAL AND KANDLER[1]) that the bleaching of *Chlorella* cells in very intense light occurs only after a certain dark-reversible induction phase. The length of the induction phase depends on the oxygen tension, the light intensity and the specific photostability of the strain of *Chlorella* used. The present paper deals with the metabolic changes occurring during the induction.

## MATERIAL AND METHODS

The *Chlorella* strains K and P and the conditions of illumination were the same as those described in the preceding paper[1]. The less photosensitive strain K was used in these studies in order to get a longer induction phase, which is more convenient for metabolic measurements.

Gas exchange during photosynthesis and respiration was measured by the usual manometric methods.

* Present address: Milchbakteriologisches Institut Weihenstephan, Post Freising/Obb., Germany.
** Present address: Botanical Institute, Laboratory of Plant Physiology and "Centre des Hormones Végétales", Liège, Belgium.

*References p. 215.*

Glucose determination was carried out by the method of FOLIN AND WU[2], the phosphate determination according to MARTLAND AND ROBISON[3].

Catalase activity was measured by permanganate titration of hydrogen peroxide (0.05 *N*) incubated with *Chlorella* for 1 h.

To measure the ascorbic acid oxidase activity the difference of oxygen uptake by *Chlorella* cells with and without 1% ascorbic acid was determined. Using the two-vessels method we found that no additional $CO_2$ was produced if ascorbic acid was added. This indicates that ascorbic acid is only oxidised, but not further metabolised in an appreciable amount.

## RESULTS

### *Relations between the inhibition of photosynthesis and pigment bleaching*

The inhibition of photosynthesis by very intense light in *Chlorella* has been carefully studied by MYERS AND BURR[4] and more recently by KOK[5]. According to the data of these authors, the inhibitory effect increases with the light intensity. When extremely high light intensities are applied the inhibition starts almost simultaneously with the illumination. In KOK's experiments, the saturation rate of $O_2$ evolution persists for only a few minutes, and is followed by inhibition.

With our conditions (carbonate–bicarbonate buffer; gaseous phase: air; highest light intensity: about 100,000 lux) the saturation rate is maintained for about 10 min. During the following 20 min a steep decrease leads to values close to zero. However, we do not observe any absorption of oxygen by the illuminated cells. On the contrary, there is still a weak $O_2$ evolution until the chlorophyll is destroyed completely.

When using the more stable strain K, we often observed a certain recovery of photosynthesis after about 90 min, as shown in Fig. 1. While photosynthesis dropped strongly during the first hour and rose again slowly during the following 2 h, the pigment content decreased slowly. Subsequently the pigment bleaching continued at a much higher rate. Only when most of the pigments were destroyed did photosynthesis decrease definitely. On comparing the two curves on Fig. 1 it becomes clear that there is no parallelism between photosynthesis inhibition and pigment bleaching.

The same fact could be shown by measuring the effect of different light intensities on both photosynthesis and pigment destruction. In contrast to the experiments shown in Fig. 1 the more sensitive strain P was used here.

Equal amounts of *Chlorella* suspension (carbonate–bicarbonate buffer) were poured into Warburg vessels. Through screens of different densities fixed on the bottom of the vessels, each sample received a different intensity from the same source of light. The oxygen evolution was measured continuously. After the experiment had been running for 30 min, the oxygen evolution became nearly constant for 30 min. Then it remained constant in the case of very weak light, increased again in the middle range of light intensity, or dropped to zero in the highest light intensity. The values of the second 30 min period were used to measure the inhibition of photosynthesis by increasing light intensity. The pigment destruction was clearly visible only after a period of 3 h. Therefore the pigment content of the cells after 3 h exposure was used in Fig. 2.

Comparing both curves in Fig. 2, there is no doubt that photosynthesis is much more sensitive than the pigments to increasing light intensity. This means that the

*References p. 215.*

inhibition of photosynthesis is not the consequence of the pigment destruction but rather that the bleaching occurs only after a certain degree of metabolic inhibition has been reached.

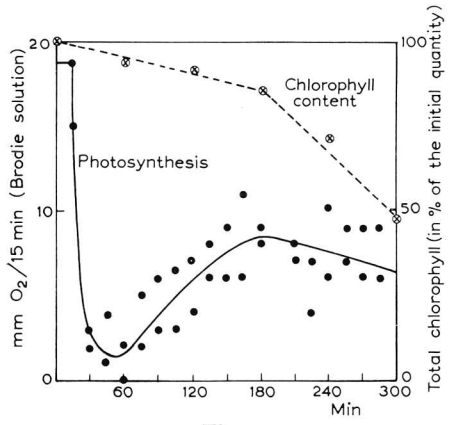


Fig. 1.

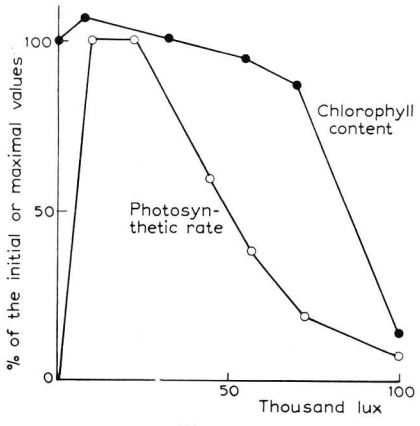


Fig. 2.

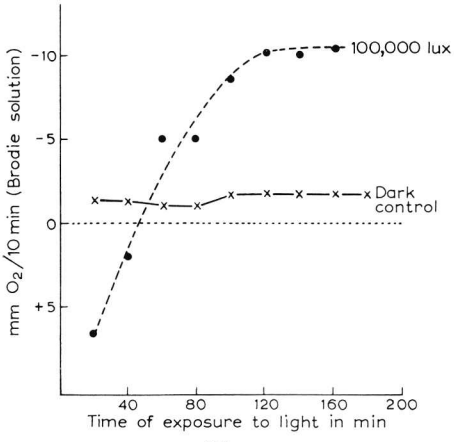


Fig. 3.

Fig. 1. Course of photosynthesis and chlorophyll bleaching in high-intensity light. (100,000 lux; *Chlorella pyrenoidosa*, strain K.)

Fig. 2. Effect of increasing light intensity on chlorophyll bleaching and on photosynthetic rate in *Chlorella vulgaris*. (Strain P; chlorophyll content after 3 h exposure; photosynthesis after 30–60 min of light.)

Fig. 3. The $O_2$ consumption of a very thin *Chlorella* suspension receiving intense light in the absence of $CO_2$. (*Chlorella pyrenoidosa*, strain K; compared with an identical thin suspension in darkness.)

*Respiration during and after intense illumination*

MYERS AND BURR[4] have found oxygen evolution to be replaced by an oxygen consumption at their highest light intensities. In our experiments the light intensity was not high enough to produce this effect in the presence of $CO_2$. But without $CO_2$ we also got an increase in oxygen uptake exceeding the normal endogenous respiration as shown in Fig. 3. There was first a weak oxygen evolution at the beginning of the exposure to intense light, but it was soon replaced by an oxygen consumption exceeding the dark endogenous respiration.

Using the two-vessels method an R.Q. around 1.0 was found. This indicates that the increased oxygen uptake is due to a genuine increase in endogenous respiration and not to a simple photoxidation.

Turning off the light when the oxygen consumption is maximum has a weak effect on oxygen uptake. A typical experiment is shown in Table I. A large amount

*References p. 215.*

of thin *Chlorella* suspension (strain K) was illuminated at 100,000 lux for 2½ h (end of the induction phase). Within this period the chlorophyll content dropped only 10% compared with that of a parallel sample kept in the dark. After 2½ h light the suspension was centrifuged, suspended in a much smaller volume of *M*/30 phosphate buffer and pipetted into Warburg vessels. (It was necessary to concentrate the original suspension in order to get high respiration rates for easier measuring.) To determine the R.Q. the two-vessels method was applied. 30 min after the end of the illumination the first respiration reading in the dark was taken (the first hour in Table I really means the period between 30 and 90 min after the illumination).

TABLE I

EFFECT OF PREILLUMINATION ON THE ENDOGENOUS AND THE GLUCOSE RESPIRATION OF *Chlorella* CELLS IN THE DARK

(*Chlorella pyrenoidosa*, strain K; preillumination: 2½ h, 100,000 lux; $O_2$ and $CO_2$ expressed in $mm^3/h$; glucose concentration: 1%).

| *Time (h)* | *Preilluminated algae* | | | | | | | *Dark control* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *without glucose* | | | *+ glucose* | | | $\frac{+\text{glucose}}{-\text{glucose}}$ $O_2$ | *without glucose* | | | *+ glucose* | | | $\frac{+\text{glucose}}{-\text{glucose}}$ $O_2$ |
| | $O_2$ | $CO_2$ | *R.Q.* | $O_2$ | $CO_2$ | *R.Q.* | | $O_2$ | $CO_2$ | *R.Q.* | $O_2$ | $CO_2$ | *R.Q.* | |
| 1 | 44 | 44 | 1.00 | 54 | 53 | 0.98 | 1.2 | 18 | 16 | 0.89 | 71 | 86 | 1.21 | 4.0 |
| 2 | 38 | 39 | 1.02 | 76 | 78 | 1.02 | 2.0 | 18 | 16 | 0.89 | 113 | 147 | 1.30 | 6.3 |
| 3 | 33 | 32 | 0.97 | 99 | 113 | 1.14 | 3.0 | 17 | 15 | 0.89 | 114 | 153 | 1.34 | 6.7 |
| 4 | 27 | 25 | 0.92 | 95 | 113 | 1.18 | 3.5 | 15 | 15 | 1.00 | 107 | 160 | 1.49 | 7.1 |

The data in Table I show that in a subsequent dark period the endogenous respiration of preilluminated cells is still 2.5 times higher, the R.Q. being 1.0. This stimulation diminishes with time, but very slowly, and is still noticeable even after 4 h.

If glucose is added, the dark sample immediately shows a 4-fold and later a 7-fold increase, while the preilluminated sample shows a slower and weaker effect. This almost equalises the $O_2$ consumption of the two samples when glucose is added. The R.Q. of the dark control increases during glucose respiration to about 1.4, which is caused by the synthesis of lipids and proteins from a part of the assimilated glucose. In the case of preilluminated cells the increase is much smaller, indicating an inhibition of synthetic processes.

We may emphasise that the behaviour of living cells is very different from that of killed cells (boiled for 10 min in a water-bath at 100°). When killed cells are exposed to intense light there is immediately a rapid bleaching of the pigments and, at the same time, a rapid oxygen consumption with an R.Q. close to zero. If one turns off the light, the oxygen uptake decreases immediately to a very small rate. This accounts for only simple photoxidative processes in the case of killed cells.

*Glucose assimilation after intense illumination*

To demonstrate the inhibition of the synthetic capacity of the living *Chlorella* cells during the induction phase of the bleaching, the glucose assimilation was studied in an experiment similar to the one above.

After illuminating a large volume of a very thin *Chlorella* suspension (strain K) for 2½ h (end of the induction phase) the cells were concentrated, suspended in a

*References p. 215.*

glucose solution and placed in Warburg vessels. The oxygen uptake was measured continuously and, after 1 ½ and 3 h respectively, samples were taken out to determine the glucose uptake.

As shown in Table II, the preilluminated cells consumed 7.2 μmoles glucose/ml suspension/1 ½ h, the control cells 11.6 μmoles. The oxygen uptake was about the same in both samples. The ratio oxygen uptake/glucose consumed gives an idea of the efficiency of the oxidative assimilation of glucose. It is evident that in the first 1 ½ h dark period after the induction by intense light the efficiency of the oxidative assimilation of glucose in preilluminated cells is considerably less than in non-preilluminated cells. During the following 1 ½ h period the inhibition is much smaller, corresponding to a recovery effect (see ref. [1]).

TABLE II

EFFECT OF PREILLUMINATION ON GLUCOSE UPTAKE IN THE DARK

(*Chlorella pyrenoidosa*, strain K; preillumination: 2 ½ h, 100,000 lux; initial concentration of glucose: 5 mg/ml).

| *Time (h)* | *Preilluminated algae* | | | *Dark control* | | |
|---|---|---|---|---|---|---|
| | *Glucose consumed (μmoles/ml)* | *$O_2$ consumed (μmoles)* | *$O_2$ used per μmoles glucose consumed* | *Glucose consumed (μmoles/ml)* | *$O_2$ consumed (μmoles)* | *$O_2$ used per μmoles glucose consumed* |
| 1.30 | 7.2 | 16.3 | 2.26 | 11.6 | 16.1 | 1.39 |
| 3.00 | 13.0 | 15.0 | 1.15 | 14.9 | 13.9 | 0.93 |

*Influence of photoxidation on the phosphorylation rate*

The increase of endogenous respiration and the simultaneous decrease of photosynthesis and oxidative assimilation ressemble the effect of 2,4-dinitrophenol (DNP) on the metabolism of *Chlorella* (KANDLER[6]). It is well known, that DNP uncouples the oxidative phosphorylation, which leads to all the other secondary effects. It was tempting, therefore, to study the effect of the photoxidation on the phosphate turnover.

For this, the changes in the level of inorganic phosphate ($P_i$) in the cells were measured. The addition of glucose to a starved *Chlorella* suspension in the dark results in a decrease of $P_i$ in the cells (KANDLER[7]). The decrease persists for about 2 min, after which a steady state level, 30% lower than the original level, is reached. If one adds high concentrations of KCN (0.1–0.01 *M*) the $P_i$ increases quickly above the original level. The increase is linear for about 20 sec. According to LYNEN AND KÖNINGSBERGER[8] the dephosphorylation rate calculated from the linear part of the curve is equal to the phosphorylation rate prior to the addition of KCN. By this method, the P/O ratio in whole cells can be measured (LYNEN AND KÖNINGSBERGER[8]; ZÖLLNER[9]; KANDLER[10–11]).

Fig. 4 shows the effect of preillumination on the $P_i$ changes after glucose and KCN addition.

A large volume of a thin *Chlorella* suspension in phosphate-free water was exposed for 2 ½ h (end of the induction phase) to high light intensities (100,000 lux) and then concentrated by centrifugation. Simultaneously, another part of the same phosphate-free suspension was shaken for 2 ½ h in the dark and also concentrated.

*References p. 215.*

30 min after, 5 ml were withdrawn from both samples and immediately killed in trichloracetic acid (TCA, end concentration: 6%). The $P_i$ content of the TCA extract was measured. Then glucose was immediately added to both dark and preilluminated suspensions (final glucose concentration: 1%), and further samples were taken.

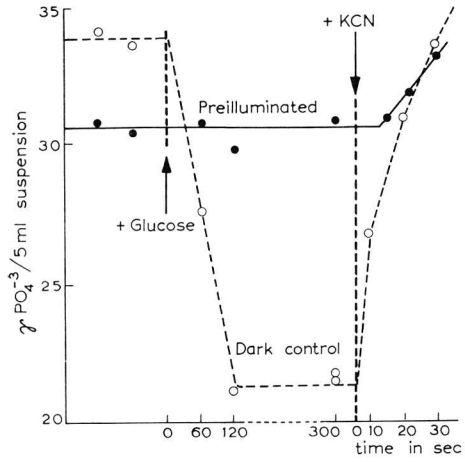


Fig. 4. Effect of illumination by the high intensity light on the phosphorylating apparatus of the cells. (*Chlorella pyrenoidosa*, strain K; exposure to intense light: 2½ h; pigment lost at the end of the exposure: about 10%; for explanations, see text.)

Although in the dark control the $P_i$ decreases very rapidly, the preilluminated cells show no significant response to the glucose feeding. The addition of KCN 5 min after the addition of glucose increases the $P_i$ level many times more in the dark control than in the preilluminated cells. The slopes show a phosphorylation rate of 36 μg $PO_4^{-3}$/min/5 ml suspension for the control and only of 8 μg $PO_4^{-3}$/min/5 ml suspension for the preilluminated cells (Fig. 4).

The very strong decrease of the phosphorylating rate of the preilluminated cells is remarkable. It contrasts with the still high oxygen consumption in these cells in the dark when glucose is available (see Table I). This results in a strong decrease in the P/O ratio, indicating that the oxidative phosphorylation is very likely a main site of action of photoxidation during the induction phase of the bleaching process.

*Catalase and ascorbic acid oxidase activity*

The catalase activity of illuminated and normal *Chlorella* cells was compared. Hydrogen peroxide was added to samples taken at different times during the induction phase. As shown in Table III, there is only a very weak decrease of the activity at the end of the induction phase.

If added to *Chlorella* in the dark, ascorbic acid is oxidised at a certain rate without $CO_2$ release. Therefore one can assume that it is oxidised by its specific oxidase. If the suspension is preilluminated for 30 min or 3 h, the oxidation is much lower (Table III). It is probable that the ascorbic acid oxidase is inactivated during the induction phase.

*Photoxidation of ascorbic acid*

If added to *Chlorella* suspensions in the absence of $CO_2$, in very intense light (100,000 lux), ascorbic acid at the concentration of 1% increases very strongly the

*References p. 215.*

TABLE III

EFFECT OF INTENSE LIGHT (100,000 LUX) ON CATALASE AND ASCORBIC ACID OXIDASE ACTIVITY

(*Chlorella pyrenoidosa*, strain K; the catalase activity is measured during the illumination; initial $H_2O_2$ concentration: 0.05 *N*; the ascorbic acid oxidase activity is measured during the first hour in the dark following the illumination; initial ascorbic acid concentration: 1 %; pH of the medium: 6.0).

| | | *Catalase activity (in % of the dark control)* | *Ascorbic acid oxidase activity (in % of the dark control)* |
|---|---|---|---|
| Dark control | | 100 | 100 |
| Illuminated *Chlorella* | Measurements made after 30 min of illumination | 96 | 44 |
| | Measurements made after 3 h of illumination | 94 | 70 |

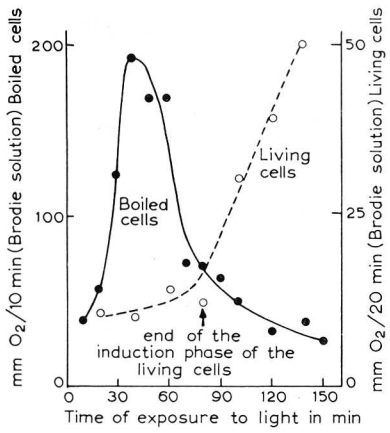


Fig. 5. Course of photoxidation of ascorbic acid in very intense light in the case of boiled and living *Chlorella* cells. (*Chlorella vulgaris*, strain P; light intensity: 100,000 lux; the light and the ascorbic acid are given at 0 time.)

oxygen uptake. The uptake is much higher than one would expect, if one adds up the effect of oxidation of ascorbic acid by its specific oxidase in the dark and the normal increase of the endogenous respiration in intense light. The increased uptake is very clear as soon as the pigments disappear (during the bleaching phase) as shown in Fig. 5, but it still exists during the induction phase.

When boiled *Chlorella* suspensions are illuminated, the photoxidation of ascorbic acid starts immediately just as the photoxidation of the pigments does. However, after 3 h the suspension is completely colourless and the photoxidation ceases. When only the yellowish green supernatant of boiled *Chlorella* suspensions is used, ascorbic acid is also oxidised very rapidly in the light. This indicates that the photoxidation of ascorbic acid is photosensitised by chlorophyll or decomposition products of chlorophyll, as already shown by WESSELS[12].

There are two explanations for the fact that, in contrast to the behaviour of killed cells, in living *Chlorella* the photoxidation of ascorbic acid reaches its full rate only after the induction phase (during the bleaching phase):

1. Normal cells are only slightly permeable for ascorbic acid. Thus photoxidation is limited by the amount of ascorbic acid available in the cell. As a secondary effect of the photoxidative inhibition of metabolism during the induction phase, the osmotic

*References p. 215.*

barrier at the cell surface is increasingly damaged. As a result, the ascorbic acid penetrates much faster and its concentration is no longer limiting.

2. The photoxidation is not sensitised by chlorophyll itself but by its decomposition products, normally present only at a very low concentration. Only when the pigment destruction begins does their concentration rise, leading to a high rate of ascorbic acid oxidation, until further photoxidation destroys this photosensitising intermediant also.

## DISCUSSION

Our experiments show that during the induction phase of the bleaching process the metabolism of *Chlorella* cells is very affected by intense light. The disturbances observed appear before the bleaching process. The most important effect might be the uncoupling of the oxidative phosphorylation, which results in a drastical decrease in the synthetic activities, as shown by measuring the glucose assimilation.

The great sensitivity of the oxidative phosphorylation system to different kinds of radiation is already known (see BACQ AND ALEXANDER[13]). ASHWELL AND HICKMAN[14] as well as MAXWELL AND ASHWELL[15] reported an inhibition of phosphorylation by X-ray radiation in spleen homogenates, although the oxidation of succinate was not inhibited. A similar effect was reported by GIESE AND SWANSON[16] using u.v. light. They observed an increase of endogenous respiration combined with a decrease in assimilation and storage of carbohydrates. This is the same effect we find using visible light.

As pointed out by SCHENK[17], ionising radiation and visible light both lead to the same primary products as excited molecules, radicals and biradicals respectively, which give rise to organic peroxides. It is understandable therefore that *the different types of radiation cause the same physiological effects.*

The fact that the bleaching of the pigments in very intense light begins only after a certain induction phase can therefore be explained as follows:

1. Normally the pigments are protected in the chloroplasts by a structural arrangement which allows a rearrangement of the excited stages without autoxidation.

2. But the peroxides formed by these rearrangements are strong inhibitors of basic metabolic processes, such as photosynthesis and oxidative phosphorylation, and when formed in great quantity in intense light, lead to a decrease of the synthetic capacity of the cells.

3. If we assume a certain turnover of the stabilising protein–pigment complex, the inhibition of the synthetic processes increases the level of "free" or incompletely protected pigments, which accordingly become sensitive to photoxidation. Some of the early decomposition products of chlorophyll may even be more photodynamically active than the original pigments and may accelerate the photodestruction. One passes from the induction to the bleaching phase.

Some of the physiological effects of illumination by intense light as described in the present paper for normal cells were also found in *Chlorella* and *Oenothera* mutants (KANDLER AND SCHÖTZ[18]). The only difference is in the light intensity needed. The higher photosensitivity of the mutants is perhaps caused by a genetic block of the synthesis of the protective structure.

*References p. 215.*

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REFERENCES

1 C. SIRONVAL AND O. KANDLER, *Biochim. Biophys. Acta*, 29 (1958) 359.
2 G. FOLIN AND H. WU, *J. Biol. Chem.*, 82 (1929) 83.
3 M. MARTLAND AND R. ROBISON, *Biochem. J.*, 20 (1926) 848.
4 J. MYERS AND G. BURR, *J. Gen. Physiol.*, 24 (1940) 45.
5 B. KOK, *Biochim. Biophys Acta*, 21 (1956) 244.
6 O. KANDLER, *Physiol. Plantarum*, (1958), in the press.
7 O. KANDLER, *Z. Naturforsch.*, 5b (1950) 423.
8 F. LYNEN AND R. KÖNINGSBERGER, *Ann. Chem.*, 573 (1951) 60.
9 N. ZOELLNER, *Z. physiol. Chem.*, 291 (1952) 157.
10 O. KANDLER, *Naturwissenschaften*, 42 (1955) 390.
11 O. KANDLER, *Z. Naturforsch.*, 12b (1957) 271.
12 J. WESSELS, *Rec. trav. chim.*, 74 (1955) 833.
13 Z. BACQ AND m. ALEXANDER, *Principes de Radiobiologie*, Sciences et Lettres, Liège, 1955.
14 G. ASHWELL AND J. HICKMAN, *Proc. Soc. Exptl. Biol. Med.*, 80 (1952) 407.
15 E. MAXWELL AND G. ASHWELL, *Arch. Biochem. Biophys.*, 43 (1953) 389.
16 A. GIESE AND W. SWANSON, *J. Cellular Com. Physiol.*, 30 (1947) 283.
17 O. SCHENK, *2éme Congr. intern. photobiol., Torino, 1958, Compt. rend.* in the press.
18 O. KANDLER AND F. SCHÖTZ, *Z. Naturforsch.*, 11b (1956) 708.