Photooxydative bleaching of chlorophyll pigments *in vivo* caused by chromatic radiation

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INTRODUCTION

Research on the bleaching of chlorophyll pigments under the influence of light was carried out both in chlorophyll solution (cf. Rabinovitch 1945, Livingston 1960) and chloroplastine preparations Wolken and Mellon 1957) as well as *in vivo* (Sironval and Kandler 1958 and others). Chlorophyll in a natural state in a living cell is much more resistant to the action of light than in the state of solution. The necessity of applying very high intensities of light for the investigation of the bleaching process *in vivo* constitutes a serious technical difficulty in the research. It concerns especially the determining of particular spectrum regions in the bleaching process. The investigations which have been carried out so far on the influence of various spectral ranges were based chiefly on the method of differential filters (Differenzfilter-Methode) (Montfort and Zollner 1942, Ges-sner and Diehl 1951, Munding 1952, Montfort, Felgner and Muller 1952) and they have a number of negative characteristics such as lack of the energy equalization of applied radiation and the possibility of the appearance of cumulative effects (see discussion). Only in very few cases processes of bleaching were obtained which were caused by the action of isolated chromatic ranges of radiation (Kandler and Schötz 1956, Hager 1956).

In the course of research on the contraction of chloroplasts (Zurzycki 1964) very high intensities of chromatic radiation (in the visible range up to 10^7 erg/cm² sec) were obtained which caused complete bleaching of the chloroplasts in a short time. The present paper contains data concerning the course of this process in the leaves of the moss *Mnium undulatum*.

MATERIAL AND METHOD

The investigations were carried out on mature leaves of *Mnium undulatum*. The material was collected from the bottom of a shady damp gorge and kept in the laboratory in a damp atmosphere in light of 150 luxes. Isolated leaves were illumined by an optical system set ac-

Fig. 1a. Bleaching of the fragments of a leaf illuminated by blue light (range III) with intensity $5.10^6 \text{ erg/cm}^2 \text{ sec}$ for 90 minutes. On the left a preparation under a cover glass, on the right leaf in contact with air; Fig. 1b. Scheme of the preparation used for exposure to light;

S — slide; C — damp chamber; L — leaf; P — piece of cover glass glued by silicone paste.

cording to the Köhler principle. The diameter of the light spot on the surface of the preparation was 1.6 mm. Isolation of particular spectral regions was obtained by means of glass and liquid filters (Table 1). For details concerning the sources of radiation and measurements of the intensity of radiation see Zurzycki 1964. As was indicated by preliminary trials the process of bleaching the leaf which is in water in a normal microscopic preparation takes place non-homogeneously within the illuminated spot, the fastest near its edges and much slower in the centre, though the distribution of intensity within the spot is uniform (Fig. 1a). Such a phenomenon may be caused by the lack of sufficient access of oxygen whose diffusion in the microscopic preparation is made difficult. The illumination of a damp leaf which is, however, in contact with the air gives much quicker and more or less uniform bleaching (Fig. 1a). For the investigation on the destruction of chlorophyll the illumination of material prepared according to the scheme presented in Fig. 1b was

used. The leaf, spread on a slide in a thin drop of water was placed by adhesion at top and bottom with a small amount of silicone paste (Rhodorsil S 428) pressed by a piece of cover glass several millimeters long. The preparation was secured against drying up by a small humid chamber set over the slide. The layer of water in which the preparation lay was supplemented in case of loss during periodical spectral measurements.

Table 1
Characteristic of the spectral regions used for irradiations

No.	Range in nm	Glass filters	Liquid filters
I	240–310	UG 5/2 mm	NiSO ₄ ·7H ₂ O 492 g/l 1 cm CS ₂ 7 ml/l C ₂ H ₅ OH 1 cm
II	310–405	UG 2/1 mm	CuSO ₄ ·7H ₂ O 10 g/l 5 cm
III	380–500	BG 1/2 mm GG 13/2 mm	CuSO ₄ ·7H ₂ O 10 g/l 5 cm
IV	475–600	BG 23/2 mm GG 11/2 mm	CuSO ₄ ·7H ₂ O 40 g/l 5 cm
V	600–850	RG 1/2 mm KG 1/5 mm	Water 5 cm
VI	700–1150	BG 1/2 mm RG 8/2 mm	Water 5 cm
VII	1100–3000	BG 23/2 mm UG 8/2 mm	—

Operating with strong intensities of radiation gives the possibility of a considerable increase of temperature in the illuminated object. The measurements of fluctuations of temperature were made using a micro-thermoelement made of copper and constantan wires with a diameter of 0.10 mm whose sensitive point was placed on the illuminated leaf in the centre of the light spot. Exemplary results of measurements of the changes in temperature in time are shown in Fig. 2a. In all cases the initial increase in temperature of Δt was stated (depending on the intensity of radiation and spectral range) and, further, the following stabilization or slight displacements in + or — resulting from very slow changes in temperatures of the whole apparatus. Fig. 2b shows the average values Δt occurring when the preparation is illuminated with various ranges of the spectrum (Table 1) and various intensities. The temperature of the apparatus fluctuated from 22 to 25°C during the measurements.

Changes of chlorophyll pigments were measured in situ without

extraction, using the opal glass method (Shibata 1958, 1959). Before being exposed to light and at various times in the course of it the preparation was covered with opal glass which was placed on pieces of cover glass (Fig. 1b) and semi-integral transmittance was measured (Zurzycki 1961) in the place corresponding to the centre of the exposed surface. The measurement was performed under a dif-

Fig. 2a. Change in temperature in the centre of the illuminated spot; axis X — time, axis Y temperature; arrows denote the moment of switching on and off the light; Fig 2b. Dependence of the increase in temperature of the illuminated object (axis Y) on intensity of radiation (axis X); figures denote spectral range (of Table 1).

ferent microscope from the exposure. The measurement microscope was connected with a photomultiplier RCA 921 whose output was measured by a Zeiss scale galvanometer. The illumination arrangement of the microscope, determined according to the Köhler principle, gave a light spot on the surface of the object with a diameter of 0.48 mm. The measurements were made in red light using the interference filter λ_{max} 679 nm. The leaves chosen for the measurements had shown before being exposed to light semi integral transmittance 70—80%. Complete bleaching of pigments caused a decrease of this value to 12—18%.

RESULTS

The picture of decomposition of chlorophyll presented as semi-integral transmittance changes (Zurzycki 1961) for three ranges of visible light (blue III, green IV and red V — cf. Table 1) is shown on Figs. 3, 4 and 5. Changes occurring in the course of exposure to light lasting up to 6 hours were registered. In order to cause a measurable decomposition of chlorophyll during this time it is necessary to exceed a certain limit intensity $5 \cdot 10^5$ erg/cm² sec for the range III and V or 10^6 for the range IV. In higher intensities of light the decomposition of chlorophyll appears

Fig. 3. Blue light (range III). Change in transmission (axis X) depending on the time of exposure to light; figures by the curve denote intensity of radiation in erg/cm² sec.

Fig. 4. Green light (range IV). Explanations as for Fig. 3.

Fig. 5. Red light (range V). Explanations as for Fig. 3.

faster where the radiation energy is higher. In the course of the curves showing the decomposition of chlorophyll an inductive phase is noticeable, whose duration depends on the light intensities applied, during which the concentration of green pigments does not undergo any essential

changes. There also occurs a bleaching phase in which occurs the decomposition of green pigments which is measurable by the optical method. The rate of decomposition again depends on the radiation intensity. When applying very high light energy 10^7 erg/cm² sec bleaching leads to complete discolouration of the exposed fragment of the leaf already after one hour (in blue light) or after 1.5—2 hours (in red or green light).

A comparison of the course of the bleaching process with cytophysiological processes occurring during exposure to light (Zurzycki 1964) shows that transition from the stationary phase to the bleaching phase is connected with drastic damage to the cell. At the moment when the distinct decomposition of chlorophyll begins, the majority or all the cells show no plasmolysis and their protoplasmic structure is established to such an extent that centrifugation of the chloroplasts is impossible.

In the remainder of the applied spectrum regions decomposition of chlorophyll was not obtained. The results of measurements for the highest intensities applied are shown in fig. 6. Both ranges of infrared radiation.

Fig. 6. Infra-red and ultra-violet light.
Explanations as for Fig. 3.

proved to be completely inactive. Even when an intensity of radiation $50 \cdot 10^6$ erg/cm² sec, causing already very considerable heating of the preparation, was applied the translucence values show slight fluctuations ranging within the error limits, the cells retaining full vitality. A similar result is obtained with illumination by lower intensities. Near UV (range II) applied in an intensity up to $8,8 \cdot 10^5$ erg/cm² sec also caused no measurable decomposition of chlorophyll in the course of 6 hours exposure to light. On the basis of the previously determined (Zurzycki 1964) similar character of the action of this range on blue light but with lower activity, it may be supposed that near ultra-violet is capable of causing bleaching, only the limited efficiency of the applied source of light determining the upper limit of the applied intensities does not allow it to show. This supposition may be supported by the fact that with the highest intensity ($8,8 \cdot 10^5$ erg/cm² sec) after 6 hours exposure to light.

only a part of the cells loses the capacity for plasmolysis. Such a state in the case of blue light is still within the range of the inductive phase. Far UV (range I), however, seems not to cause decomposition of chlorophyll at all. Cytological tests show that the higher intensity applied ($1.15 \cdot 10^5$ erg/cm²sec) causes, after 1.5 to 2 hours, the death of all the cells. Nevertheless in the course of a further four hours' exposure no essential translucance changes were observed.

Table 2

Time of semi-decomposition of chlorophyll (time from the beginning of illumination to the decrease of transmission up to 45%)

Radiation intensity in erg/cm ² sec $\times 10^6$	Spectral region		
	III	IV	V
1	6 h	∞	7 h 30 min
2	1 h 55 min	6 h	3 h 35 min
5	48 min	2 h 35 min	1 h 15 min
10	25 min	55 min	42 min

In summing up it may be stated that all the chromatic ranges of visible light (III—V) and possibly near ultra-violet (range II) have the capacity to cause bleaching of chlorophyll in *Mnium undulatum*, but far ultra-violet (range I) and infra-red light (range VI and VII) do not exert such action. In the visible range blue light has the greatest action and green the smallest. For the sake of comparison of the relative activities of particular ranges of visible light the time necessary for semi-decomposition of chlorophyll (for reaching 45% semi-integral translucance) i.e. the time embracing the inductive phase and half the bleaching phase, is presented in Table 2.

DISCUSSION

Semi integral translucance, accepted in this paper as the criterion of decomposition of chlorophyll, is, contrary to optical methods based on extracts of pigments, only a relative measure of changes of chlorophyll pigments. However, the application of this method permitted the carrying out of numerous measurements of the amount of chlorophyll in the same point of the leaf, which is not possible when using the methods based on extraction, and which reduced considerably individual variability. Besides changes in the concentration of chlorophyll the displacements or changes in the shape of the chloroplasts may influence translucance changes. As, however, in the intensities used in these investigations the movements of chloroplasts are in practice retarded (Zurzycki 1964) and the contraction appearing already in the first period of the inductive phase did not exert any essential influence on the trans-

luctance changes, the determined changes of this value should be attributed only to the decomposition of chlorophyll. The investigated species, though it belongs to the ecological type of shade plants, shows a considerable resistance to the action of light. In Table 3 the intensities of several ranges of chromatic radiation are assembled, represented in natural sunlight. From the comparison of Table 2 and 3 it can be seen that no range of chromatic radiation isolated without an additional concentration from sunlight is able to cause a distinct decomposition of chlorophyll during several hours action.

Table 3

Intensities of spectral regions of sunlight for the angle of incidence 90° and mass of air = 1 (according to Moon 1940)

Spectral region in nm	Intensity in erg/cm ² sec
320–400	37 000
400–500	136 000
500–600	153 000
600–700	139 000
400–700	428 000
700–1100	307 000
1100–1500	93 000
1500–	65 000
	<u>930 000</u>

The decomposition of chlorophyll exposed to a strong illumination is a process of photooxydative character and, as has been determined many times, does not take place in anaerobic conditions (Pringshein 1879, Noack 1926, Kandler and Schö tz 1956). As was observed by Sironval and Kandler (1958) the oxygen produced by green cells during very strong illumination is not sufficient to cause bleaching. The phenomenon is quite understandable when we take into account that in strong light considerably before the beginning of the decomposition of chlorophyll inactivation of photosynthesis takes place (Kok 1956, Zurzycki 1957, Steemann-Nielsen and Jørgensen 1962) which causes a fall to zero of the production of oxygen in the tissue exposed to light. On the basis of this phenomenon it may be explained that the course of bleaching of cells under a cover glass is much slower than that in a leaf exposed to the air (Fig. 1). Non-homogeneous decomposition of pigments (strongest on the edges of the illumined spot) should then be connected with the production of photosynthetic oxygen in the part of the leaves lying beyond the spot of strong light where the diffused light does not act inhibiting on the photosynthesis and at the same time enables it to take place. Diffusion of oxygen causes the most

abundant supplying of it to the parts at the edge of the light spot. This experiment shows that inactivation of photosynthesis may be an essential mechanism protecting the cell from the action of too strong illumination (Stee mann - Nielsen 1962): however the importance of the mechanism seems to be essential in the medium with a slow diffusion of oxygen (water) though considerably less for the leaves of land plants.

In all the series of measurements in which the decomposition of chlorophyll was determined, the kinetics of the process is similar in principle. In accordance with the results of Sironval and Kandler (1958) obtained by exposure to white light of *Chlorella* suspension the destructive action of light takes place in two phases i.e. the induction phase in which changes of metabolism occur, but the concentration of chlorophyll is still constant, and the bleaching phase embracing the proper decomposition of pigments. In induction phase there occurs the loosening of the stable complex chlorophyll-substrate. Thanks to this loosening it is possible for the photooxydative decomposition of pigments to follow (Egle 1960). The similarity of the course of the bleaching process in short- and long-wave light (cf. Figs. 3 and 5) points to the fact that the mechanism of the phenomenon of photooxydation is in the two cases alike.

The role of particular spectral ranges for the phenomenon of photooxydation in vivo has not yet been determined precisely. On the basis of the method of differential filters the early works of Montfort (1941, 1942) attributed the decomposition action only to ultra-violet light, but they did not determine the decomposition of chlorophyll when a region of wave length under 400 nm was excluded from radiation. Munding (1952) determined that also the long wave part of the spectrum (red light) may cause the decomposition of chlorophyll, this being also confirmed by Montfort (1953). While Munding does not attribute an important part to the action of ultra-violet light, Gessner and Diehl (1951) and Montfort (1953) consider that it operates in a most active way in the process of bleaching.

The method of defferential filters compares the effects of exposure by using white light (sunlight or UV lamp) out of which a broader and broader short-wave range is gradually eliminated. The object is thus illumined by radiation with various intensity which does not provides a basis for comparative results. Another more serious objection to this method is the possibility of cumulation of effects. The action of radiation of UV was determined always together with exposure to visible light. Munding (1952) however, stressed that using lamps, rich in ultra-violet light but producing little visible radiation (Vitalux), severe damage of cells with a very slight decomposition of chlorophyll was obtained.

It is only possible to avoid the appearance of cumulation effects when using chromatic or monochromatic radiation. Earlier works carried out on this principle gave contrary results, this probably being due to a lack of measurements and control of the intensity of particular ranges of spectrum. Thus Pringsheim (1879) found that destructive effects on pigments are caused by blue, green, and yellow radiation but the red one is inactive; on the contrary Ursprung (1917) determined the strongest intensity of red light in the range of 656—678 nm. Kandler and Schötz (1956), applying equal energetic intensity of blue and red radiation, determined that both ranges cause the decomposition of chlorophyll though the short-wave range of the spectrum is more active. Photooxydative action of red light on photosynthetic pigments was also found by Hager (1957) who based on it a supposition that it is the amount of absorbed energy which is decisive in this process and not its kind.

The results of the present work are in principle in agreement with the data of Kandler and Schötz (1956) and Hager (1957). The action on bleaching is caused by the whole range of radiation absorbed in photosynthetic pigments and the action of particular spectral ranges is more or less in agreement with their absorption. In near UV which is still strongly absorbed in photosynthetic pigments the decomposition of chlorophyll was not obtained, but this phenomenon should be attributed to the fact that the radiation applied was of too weak energy. As results from the investigations of Metzner (1930) the range 350—400 nm causes an especially strong destructive action to chlorophyll. But far UV (250—300 nm), possessing a very strong biological action, causes earlier death of the cell though the decomposition of chlorophyll does not take place during the irradiation. It was shown by Sironval and Kandler (1958) that in killed cells the photooxidative decomposition of pigments takes place earlier than in living ones with a complete omission of the inductive phase. This may explain the destructive action of far UV by the effect of cumulation of two processes many times determined by means of the differential filter method, i.e. destructive action of UV on the structure of the chloroplast leading to splitting or loosening of the complex chlorophyll-protein which makes possible the shortening of the inductive phase together with the photooxydative action of visible radiation.

SUMMARY

1. The decomposition of chlorophyll in the cells of *Mnium undulatum* caused by strong intensities of chromatic radiation was investigated. Changes in the amount of chlorophyll was measured using the method of Shibata opal glass.

2. Photooxidative bleaching is caused by all ranges of visible radiation, their action being proportional to absorption in photosynthetic pigments (the most active

is the blue range, red is less, the weakest being green). Far UV applied in strong intensity causes quick death of the cell not causing decomposition of pigments in the course of the radiation applied. Infra-red light does not cause any decomposition effect.

3. Photooxydative action is caused only by radiation with intensity exceeding the definite (different for each spectral range) limit intensity. In the course of decomposition an inductive phase and a bleaching phase may be differentiated. In all spectral ranges in which chlorophyll decomposition was obtained the kinetics of decomposition is similar.

4. In the light of the experimental results obtained, the data from the literature concerning the spectral action of radiation on decomposition of photosynthetic pigments based on the method of differential filters were discussed.

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(Entered: 15. IV. 1963)

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Fotooksydatywny rozkład barwników chlorofilowych in vivo wywołany promieniowaniem chromatycznym

Streszczenie

Badano rozkład barwników chlorofilowych wywołany *in vivo* w komórkach liścia *Mnium undulatum* przez naświetlanie promieniowaniem chromatycznym wysokiej intensywności. Stwierdzono, że cały zakres widzialny promieniowania wywołuje fotooksydatywny rozkład barwników, przy czym największą aktywność wykazuje promieniowanie niebieskie, mniejszą czerwone, a zielone działa najslabiej. W przeciwieństwie do zakresu widzialnego daleki nadfiolet nie wywołuje rozkładu chlorofilu, mimo że w krótkim czasie powoduje śmierć komórek. Promieniowanie podczerwone nie wywołuje ani uszkodzenia komórek ani rozkładu chlorofilu.

Destrukcyjne działanie promieniowania na barwniki stwierdzono tylko wówczas, gdy natężenie promieniowania przekracza pewną graniczną wartość (różną dla każdego zakresu spektralnego). W przebiegu rozkładu barwników wyróżnić można fazę wstępną i fazę właściwej fotooksydacji. Kinetyka opisanych procesów jest podobna we wszystkich aktywnych zakresach widma.

Na podstawie uzyskanych wyników przedyskutowano krytycznie rezultaty badań oparte na metodzie filtrów różnicowych.