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Investigation on the contraction of chloroplasts in *Mnium undulatum* (L.) Weis

II. Studies on isolated chloroplasts

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INTRODUCTION

Many research works, carried out in the last years, have shown that the isolated chloroplasts undergo changes in volume and shape under illumination (Packer 1963; Itoh, Izawa and Shibata 1963; Belsky, Siegenthaler and Packer 1965; Lundegardh 1965). The experiments were made with different methods like determination of the average size on the basis of light scattering effect, by determination of the packed volume of chloroplasts, the electric measurements of the volume distribution with the Coulter counter and by studying the size of chloroplasts measured by electron microscopy. The results of investigations showed clearly that the volume of isolated chloroplasts preincubated in darkness decreases under illumination of about 20—50% and simultaneously their area shows slight increase. The phenomenon of shrinkage in volume is accompanied by the effect of flattening and the last one manifests itself in the change of the axial ratio of the cross section of a chloroplast (Itoh, Izawa and Shibata 1963).

The described effect of shrinkage is reversible and seems to be a general phenomenon for lamellar chloroplast systems because it was found not only in higher plants but also in algae (Belsky, Siegenthaler and Packer 1965) and even in purple bacteria (Packer, Marchant and Mikohata 1963). Shrinkage in volume is closely connected with photosynthetic phosphorylation. This was demonstrated by a study of cofactors necessary for the light induced shrinkage (Packer 1963; Belsky, Sieganthaler and Packer 1965) and by poisoning of photophosphorylation by specific inhibitors (Packer 1963; Itoh, Izawa and Shibata 1963). The action spectrum of shrinkage points also to the role of photosynthesis in this process (Izawa, Itoh and Shibata 1963).

Light induced changes in the shape and volume of chloroplasts were also found in intact cells by the method of area measurements (Zurzycki 1964) or by measurements of the linear dimensions studied in living cells (Hilgenheger and Menke 1965) as well as in electron microscope (Kushida, Itoh, Izawa and Shibata 1964).

In the previous study (Zurzycki 1964) it was found that when investigations are made in a wide range of light intensities some differences in response of chloroplasts for the short and long wave range of the spectrum are noticable. In red light only the expansion of area was found, this phenomenon occurs in blue light

exclusively in weak and medium intensities of radiation. In strong blue the area of chloroplasts is largely reduced. The aim of the present work has been to check whether these differences are to be found in isolated chloroplasts and to study in detail the reaction to strong blue light.

MATERIAL AND METHODS

The experiments were carried out on chloroplasts of the moss *Mnium undulatum*. The plants were collected from shady and damp bushes and stored in the laboratory in humid air and weak light conditions.

The reactions of chloroplasts in intact cells were studied according to the previously described methods (Zurzycki 1964). The leaves were pretreated in darkness for 24 hours before experiments started. Only the behaviour of chloroplasts on the cell side turned to the light (proximal) was studied.

The isolation of chloroplasts was performed by grinding the leaves (pretreated 24—36 hours in darkness) in a mortar with a glazed surface with a pestle of perplex provided with many small incessions in a small amount of cold 0.3 M/l sucrose solution in M/15 phosphate buffer pH 7.3. The green suspension was succed with pipete several times in the course of grinding and collected into centrifuge tubes. After first centrifugation 2 min. 1000×g supernatant was discharged and centrifuged for 15 min. 3500×g. The sediment was resuspensed in a small amount of the same medium and centrifuged for 15 min. 3500×g in small tubes. When special inhibitors were used they were added to the medium in which the last centrifugation took place.

The sediment was used for microscopic examination. The cover glass of the preparation was tightened with hot vaseline. The preparation contained besides whole chloroplasts some chloroplast fragments and other contaminations. For observation only whole chloroplasts were chosen according to the criteria of Leech (1963, 1964). The chloroplasts were photographed in weak light under an immersion objective Apo. 90×, NA 1.30. Negatives were magnified to 4000× and the contours of chloroplasts were carefuly traced. The areas of chloroplasts were measured planimetricaly on these drowings. Each measurement was based on 4—6 photographs of the same chloroplast. The change of the area of a chloroplast was calculated in percentage of the initial area i.e. after pretreatment in darkness. Table I gives an example of measurement of one chloroplast.

The values presented in the graphs are the means of measurements of 8—15 individual chloroplasts.

As it was shown in the preliminary experiments, the illumination of chloroplasts during the time of exposure does not induce any significant changes in their area. During 20—40 minutes following preparation the area of some chloroplasts undergoes certain changes (usually decrease an area), but subsequently the area stabilizes. Therefore 1 hour preincubation of microscopic preparations in darkness was applied for all experiments.

The light system of the microscope was based on the Köhler's principle, using an incadescent lamp 750 W, 110 V supplied through a variable auto-transformer. Most experiments were made with interference filters 484 and 668 nm. For the determination of the action spectrum following filters were used (half widths in brackets): 333 (16), 351 (15), 362 (20), 382 (14,2), 400 (11,1), 429 (8,9), 442 (8,4), 454 (6,0), 470 (10,2), 484 (6,8), 506 (6,7), 523 (9,3), 541 (9,5). A 5 cm thick layer of $CuSO_4$ solution or water was used as heat filters.

Measurements of the intensity of radiation were performed by means of a microthermoelement according to the method described in an earlier paper (Zurzycki 1961).

RESULTS

The first experiments concerned the comparison of intact and isolated chloroplasts in the reaction to blue light, in order to find out if the last ones undergo a decrease in area under strong radiation intensity. The time course of changes

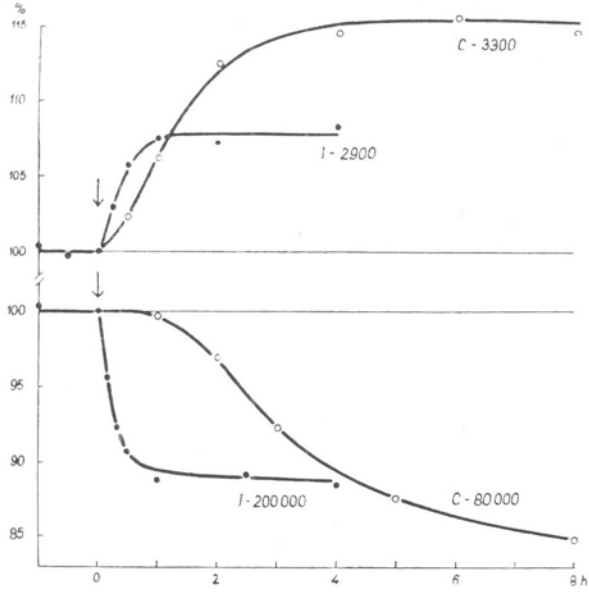

Fig. 1. Changes in area of intact (*C*) and isolated (*I*) chloroplasts under illumination by blue light (484 nm).

X-axis – time in hours, Y-axis – area of chloroplasts in percent of the value for darkness. Figures by the curve show the light intensity in erg/cm² sec.

of isolated and intact chloroplasts area is shown in fig. 1. As it is to be seen the reactions of both kinds of chloroplasts are qualitatively the same. In both cases blue light of low intensity causes the expansion of area and in strong intensity of the same kind of radiation the area of chloroplasts decreases considerably. The differences between intact and isolated chloroplasts concern the speed of changes and their absolute values. The change of area of isolated chloroplasts produced

much quicker and are completed in 1/2 to 1 hour of illumination whether weak or strong light intensity was used. On the other hand changes of chloroplasts in intact cells undergo at a lower rate and become stabilized after 5—8 hours of exposure. The changes in area are slightly bigger in chloroplasts in living cells than in isolated ones. Clear differences are to be stated in the changes of shape. During the increasing or decreasing of area the isolated chloroplasts do not undergo any

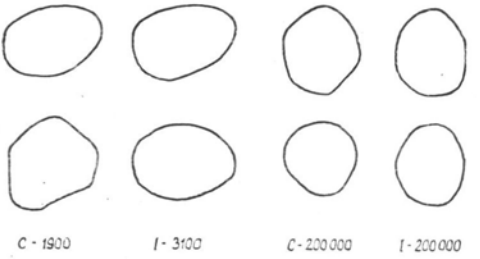

Fig. 2. Typical changes in shape and area of intact (*C*) and isolated (*I*) chloroplasts under illumination by blue light.

Upper row — chloroplasts in darkness, lower row — the same chloroplasts after 1 hour (for *I*) or after 5 hours (for *C*) illumination. Figures give the intensity of radiation in erg/cm² sec.

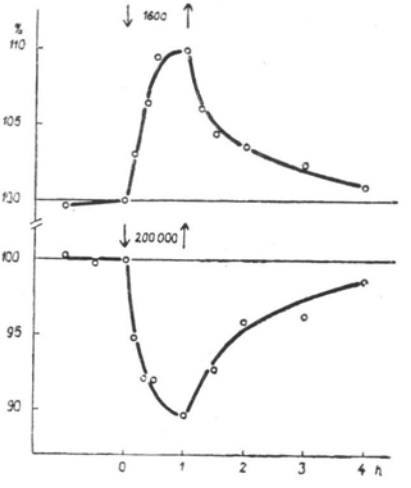

Fig. 3. Change in area of isolated chloroplasts upon illumination during 1 hour with blue light of the intensity 3000 erg/cm² sec (upper curve) and 200 000 erg/cm² sec (lower curve). Chloroplasts are kept in darkness before and after illumination. Time of illumination is marked by arrows.

significant changes in shape, though in intact chloroplasts a decrease in area is accompanied by rounding of contours and with an increase of area the chloroplasts become more irregular or polygonally shaped (Fig. 2).

Both reactions: contraction and expansion of area are reversible in the isolated chloroplasts. In fig. 3. the results of an experiment in which the chloroplasts were illuminated for one hour and kept in darkness before and after illumination is

shown. Independently on the kind of light action (increase or decrease of area) the area of chloroplasts returns in darkness slowly to its former level. It may be concluded that the contraction of area is not connected with irreversible destructions in chloroplast structures in spite of the use of a very high light intensity.

The response of chloroplasts to 1 hour irradiation with different intensities of blue (484 nm) and red light (668 nm) is shown in fig. 4. When after 1 hour dark

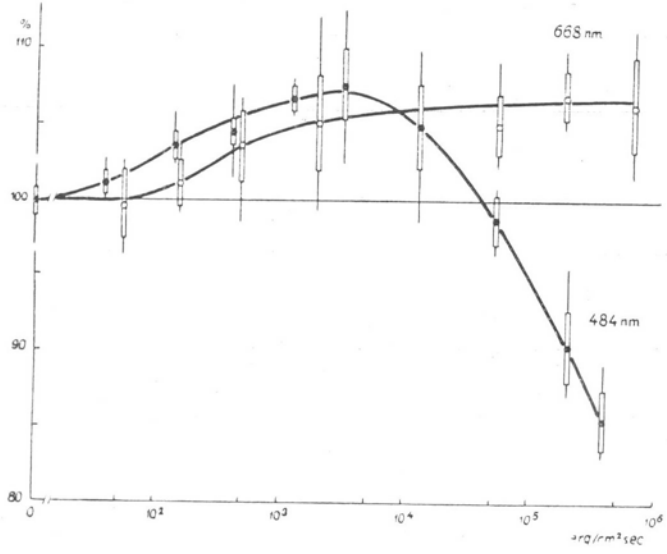

Fig. 4. Dependence of the mean change of area of isolated chloroplasts on the intensity of blue and red radiation as measured after 1 hour exposition. X-axis — light intensity, Y-axis — area of chloroplasts in percent of the value for darkness. Every point represents the mean of 8—15 recordings. Vertical lines mark the extreme dispersion, the rectangles — mean error.

incubation chloroplasts were kept in darkness for one following hour no significant changes in their area occured, though if after dark pretreatment chloroplasts were illuminated with an intensity higher than 50—100 erg/cm² sec. some changes in area can be stated after 1 hour. These changes consist in the enlarging of area to the mean maximum value about 7% (maxima of individual chloroplasts to 13—15%). Red light causes only an increase of area till the highest intensities used in the experiments i.e. about 10^6 erg/cm² sec., in blue light of intensities higher than 5000 erg/cm² sec. however the increase is smaller and in still higher intensities a decrease instead of increase of area as compared to the starting state can be noticed. The higher light intensity the bigger the reduction of area. When highest intensities were used i.e. about $5 \cdot 10^5$ erg/cm² sec. the reduction of area attained about 15%.

The described behavior of isolated chloroplasts may suggest that the area of chloroplast is controlled by two different systems, one causing the enlarging and operating in both short and long wave lenght parts of the spectrum and the othre causing the reduction of area, sensitive only to short wave radiation and operating

in much higher light intensities than the first ones. In strong blue the action of the second system supresses the tendency to flattening of chloroplasts and cause a decrease of their area.

Table 1

Area of a chloroplast pretreated in darkness and after 1 hour of illumination with blue light: 484 nm, 1900 erg/cm² sec.
Figures present the area in planimeter units, 1 unit = 0,435 μ²

No. of picture	1	2	3	4	5	Mean	%
Darkness	58,5	57,5	58,5	58,0	58,5	58,2	100,0
Blue light	63,5	64,5	63,0	64,0	63,5	63,1	108,4

As it was shown in many research works mentioned above the shrinkage in volume is controlled by photosynthetic phosphorylation. In order to check to what an extent the changes in area are connected with this process two inhibitors of photophosphorylation were used: NH_4-ions and o-phenantroline. In fig. 5 the dependence of area on blue light intensity in some concentration of inhibitors is

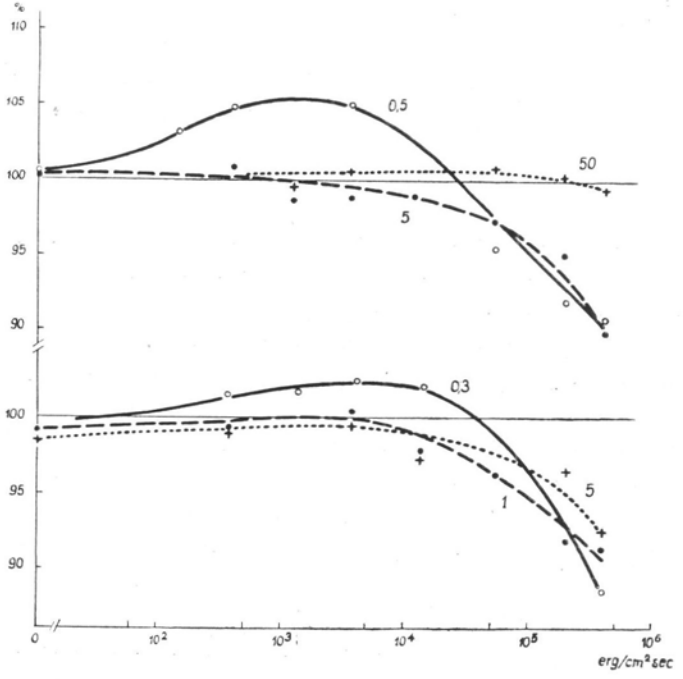

Fig. 5. Influence of NH_4Cl and o-phenantroline on the changes in area of isolated chloroplasts caused by blue light. X-axis — light intensity, Y-axis — area of chloroplasts. The numbers by the curves give the concentration of inhibitors in mM/l.

shown. As it is to be seen both poisons in a proper concentration block completely the enlarging of area, while in the same concentration strong intensities of radiation cause still a markable decrease of area. The conclusion may be drawn that

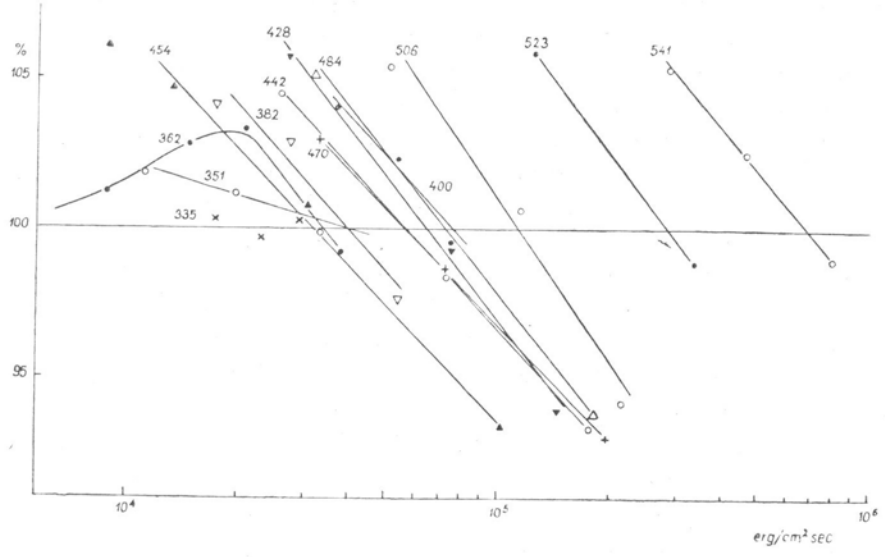

Fig. 6. Contration of chloroplasts area in the radiation of different wave lengh. X-axis — light intensity in erg/cm² sec, Y-axis — chloroplast area. Numbers by the curves show the wave length of radiation.

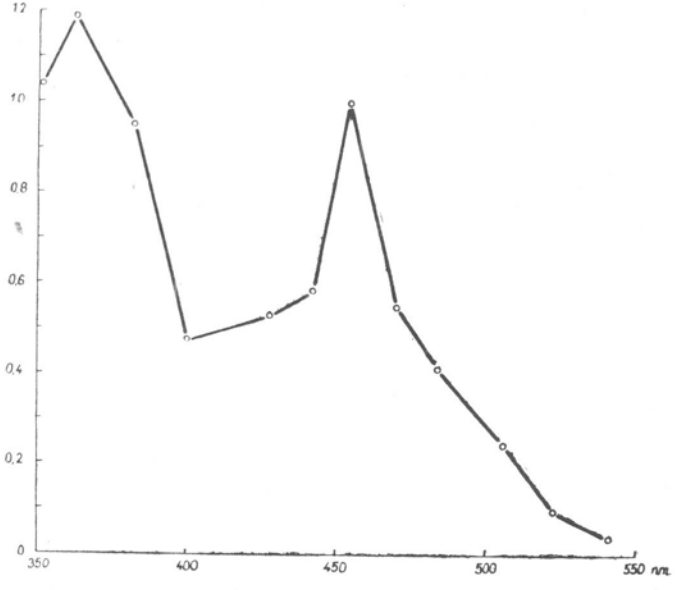

Fig. 7. Action spectrum for the reaction of contraction of chloroplasts area. X-axis — wave length in nm, Y-axis — relative activity of radiation.

photosynthetic phosphorylation is responsible for flattening of chloroplasts and increase of their area but the contraction of area is controlled by another process independent on photophosphorylation.

In order to obtain more data about the strong light reaction the wave length response of this process was studied. The reaction in various wave lengths of light is shown in fig. 6. Those radiation intensities which already do not induce the enlarging and yet do not induce the contraction were chosen as the criterion of activity. These intensities were read from the graph by interpolation at the point of crossing of the curves with the 100% axis. The obtained action spectrum calculated into the quantum number is presented in fig. 7. The spectrum shows two peacks at about 450 nm and between 360 and 380 nm. The activity of radiation is lowered between 400 and 430 nm and drops considerably above 500 nm. Below 350 nm the assumed criterion of activity can not be applied because no enlarging of area in this part of the spectrum can be noticed. The activity obtained in the region 350—380 nm is charged with some error for two reasons. The intensity of radiation obtainable in this region was too low to cause a distinct contraction. In higher intensities the area of chloroplasts is about 100% or a little lower only — and this fact may diminish the accuracy of interpolation. The second reason is connected with low activity of this spectral region in enlarging of the area (compare the curves for 362 nm fig. 6 and for 484 nm fig. 4). In this case the phenomenon of contraction may appear distinctly in lower light intensities because the tendency to flattening to be overcome is not so great as in longer wave lengths of light. The above mentioned reasons may only induce some error in the determination of absolute activity of UV region but would not change the general shape of the action spectrum. The calculated action spectrum is quite similar to the absorption spectrum of riboflavine and may suggest that this pigment is active as light absorbent for the contraction of chloroplasts area.

Table 2

The area of chloroplasts (in percent of the dark stage) in some light intensities of blue light (484 nm)

Light intensity in erg/cm² sec.	3800	56000	360000
Control	104,2 ± 1,8	98,8 ± 3,2	91,6 ± 1,5
KJ 10^{-3} M/l	106,0 ± 2,3	105,7 ± 1,9	101,9 ± 2,1

A succesful use of flavine quencher for inhibition of riboflavine dependent intracellular movements (Mayer 1965) made us investigate the influence of KJ on the contraction phenomena. The changes in area of isolated chloroplasts in addition of 10^{-3} M/l KJ to the medium are shown in table 2. The result show that the expansion of area is not inhibited by KJ but the contraction process in high intensities of light is almost completely blocked.

DISCUSSION

The response of isolated chloroplasts to light may be illustrated by the scheme in fig. 8. The scheme is based on the results of Itoh, Izawa and Shibata (1963) and on the data obtained in the present study. Light controls the structural changes in chloroplasts by two reactions: I and II. The reaction I is depended on the photosynthethic absorpsion system and leads to the increase of area i.e. expension

Fig. 8. Schematic representation of the structural changes of chloroplasts caused by reaction I and II.

of lamellar system and simultaneously to the decrease in volume. The range of light intensities active in this process is comparable in *Mnium* and in Spinach chloroplastes (Itoh, Izawa and Shibata 1963). However the measurements of chloroplasts long axis in the investigation for intact cells (Kushida, Itoh, Izawa and Shibata 1964) did not show any significant increase of this value upon illumination, but for the condition in living cells, where the shape of chloroplasts may vary considerably, the criterion of area seems to be more important than axis measurements. The reaction II leads to the contraction of lamellar system which expresses itself in the decrease in area (and probably further decrease in volume). This reaction is controlled by the short wave part of the spectrum only and riboflavine seems to be active pigment in this case. Characteristics of both reaction is summariesed in table 3.

Table 3

Some features of the systems controlling structural light reactions in chloroplasts

System	Change in area	Change in volume	Active spectral range nm.	Pigment system	Intermediate	Lowest intensities erg/cm² sec	Blocked by
I	Increase	Decrease	350—700	Chlorophylls	ATP	100	o-phenantroline, NH_4
II	Decrease	?	350—540	Riboflavine	?	20000	KJ

It is worth to notice that a similar double system controls the displacements of chloroplasts inside the cell. Absorption by riboflavine seems to control the position of chloroplasts ("phototactic" displacements) and this system is active as well in low as in high light intensities (Zurzycki 1962; Haupt und Schönfeld 1962; Fischer-Arnold 1963; Mayer 1964) though absorption in chlorophyll system is important for the tonal condition in the cells and expresses itself in the speed of displacements (Zurzycki 1962; Fischer-Arnold 1963) by the way of photosynthetic phosphorylation (Zurzycki 1965). Quite simmilar, in the phe-

nomenon of photodinesis, controlled by riboflavine system, red light exerts a specific influence (Seitz 1964).

Studies on the shape of chloroplasts in living cells showed that the shape is highly dependet on the intensity and wave length of radiation (Zurzycki 1964). In the case of isolated chloroplasts changes in shape are neglegible. One may suppose that if the mechanism controlling the area is localized inside the chloroplasts and may be active after their isolation — the changes in shape are the result of interaction between chloroplasts and cytoplasm. Especially obtaining of irregular or polygonal shapes seems to be connected with a kind of anchoring (binding) the chloroplasts by the cytoplasm. The bounds between chloroplasts and cytoplasm are greatest in weak intensities of the short wave part of the spectrum (Zurzycki 1962) and only under this condition and only in living cells the chloroplasts attain irregular and polygonal shapes.

SUMMARY

Light depending structural response of isolated chloroplasts of *Mnium undulatum* was studied, by the method of measurements of chloroplasts area.

The isolated chloroplasts undergo reversible changes in area upon illumination. Red light acts on the increase of area only. Blue light causes the increase of area in low radiation intensities and decrease in high intensities.

It was shown that the increase in area is controlled by the process of photosynthethic phosphorylation and may be inhibited by o-phenantroline or NH_4-ions, and the process of contraction of area is under control of riboflavine system and can be selectively blocked by KJ.

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Zmiany strukturalne izolowanych chloroplastów Mnium undulatum II

Streszczenie

Przedmiotem badań były zmiany strukturalne izolowanych chloroplastów *Mnium undulatum* wywołane działaniem światła. Stwierdzono, że powierzchnia izolowanych chloroplastów wykazuje pod wpływem oświetlenia podobne zmiany jak powierzchnia chloroplastów w żywych komórkach, tj. ulega zwiększeniu lub zmniejszeniu w zależności od intensywności i długości fali działającego promieniowania. Zmiany te są odwracalne. Światło czerwone o natężeniu przekraczającym 100 erg/cm^2 sek. wywołuje zawsze zwiększenie powierzchni. Światło niebieskie działa w podobny sposób, ale tylko w ograniczonym zakresie intensywności. W wysokich intensywnościach tego promieniowania obserwuje się skurcz powierzchni.

Powyższy sposób działania światła można wytłumaczyć zakładając istnienie dwu systemów kontrolujących powierzchnie chloroplastów. System I powoduje ekspansję lamellarnych struktur chloroplastu i jest uzależniony od absorpcji światła w barwnikach fotosyntetycznych. Jego aktywność można zahamować stosując inhibitory fotofosforylacji jak o-fenantrolina lub jony NH_4. System II wywołujący kontrakcję powierzchni regulowany jest przez energię świetlną absorbowaną w riboflawinie i może być zablokowany działaniem KJ.