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Properties and localization of the photoreceptor active in displacements of chloroplasts in *Funaria hygrometrica*

III. Cytochemical studies

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INTRODUCTION

It was concluded from the results of the previous studies (Zurzycki 1967 a and b) that the photoreceptor active in the light induced displacements of chloroplasts in the cells of *Funaria hygrometrica* has the properties of flavin pigment, is localised in the outer parts of the cell and that its oscillator responsible for the absorption in blue region is oriented parallel to the cell walls. The action spectra of the rearrangements of chloroplasts obtained for other object (Haupt 1963, Seitz 1964), as well as the inhibition of the displacements by the quencher of riboflavin — kaliumjodid (Meyer 1966, Seitz 1967) suggest that riboflavin (or riboflavoprotein) plays the main and general role as a photoreceptor in the mentioned phenomena.

In the present study an attempt of the cytochemical detection of the photoreceptor has been made. Taking into consideration the chemical and physical properties of riboflavin its detection may be based on chemical reaction, absorption or fluorescent properties of this compound.

METHODS AND RESULTS

1. Microchemical reaction. The microchemical reaction suitable for the detection of riboflavin was described by Chévremont and Comhaire (1939). It involves the reduction of riboflavin in the fixed tissue to leucoflavin and further reoxidation resulting in the appearance of red granules of rhodoflavin. The procedure used was performed according to Glick (1949). Detached leaves were fixed in formol-basic lead acetate solution (5 days) or in 80% etanol (1 day), then treated for 30 mins. with a reductant solution. After rinsing in water the leaves were treated with 0,5, 3 or 10% hydrogen peroxide and examined under microscope.

Independent on the kind of fixation in no case any granules of rhodoflavin could be detected. From the negative results of the

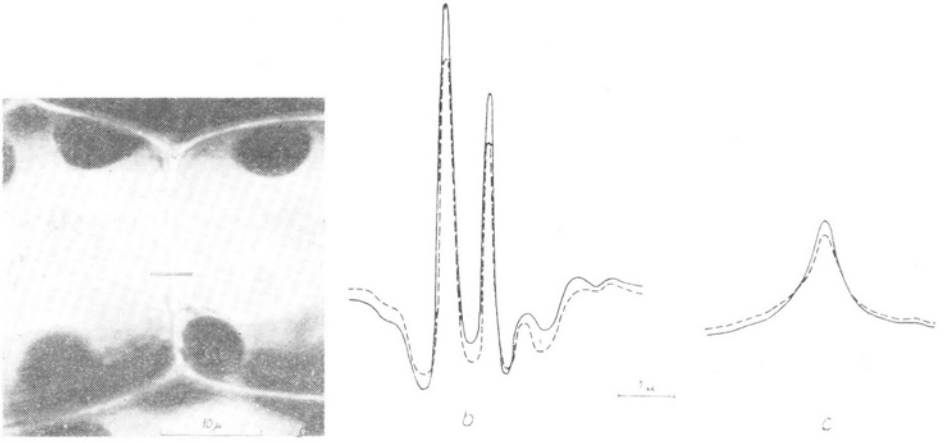


Fig. 1. *a* — Shorter side wall of the cell devoid of chloroplasts and photographed in monochromatic polarized blue light. The square denote the area of densitometric measurements. *b* — densitometric curves of the picture of cell walls as photographed in vivo; ——— with light vibrating parallel to the wall and — — — perpendicular to it. *c* — a similar densitometric curve for the cells fixed in formol basic lead acetate solution and embedded in bromobenzol.

microchemical reaction it may be concluded that if riboflavin is present in the cells its concentration or amount is too low to make the chemical detection possible.

2. Absorption of light. Because of low concentration of the yellow photoreceptor in the cell the direct microspectrophotometrical method would be certainly unsuccessful for its detection. In the present study we tried to make the advantage of the absorption anisotropy which is to be supposed from the reaction of chloroplasts to polarized light.

The leaves were first irradiated with polarized blue light of high intensity. When the vibration plain of the light is perpendicular to the longer axis of the cell, all chloroplasts situate in a profile position on the longer side walls leaving the shorter ones quite free (Zurzycki 1967b). The last were photographed in blue monochromatic polarized light by using interference filter (max 442 nm) and Bernotar polarizer, under water immersion objective (apo 90 ×). The primery magnification on the photographic plate was 285 ×. Each object was photographed twice, with the vibration plane of the light parallel to the cell wall and perpendicular to it. The fine grain negative material (Foton-Microfilm 6 DIN) was developed in a contrast working developer. The obtained density gradation was $\gamma = 2$. The example of the obtained picture is shown in fig 1a. The density of a small fragment of the picture running across the cell wall was measured. For densitometry the pictures were magnified

65×. The measured area (8 × 2 mm) corresponds to the area (0,086 × 0,0215 μ in the microscopic object.

Fig 1b shows the examples of density curves. As it can be seen the two peaks corresponding to the dark margin of the optical cross section of cell wall are somewhat lower when the light vibrates perpendicularly to the cell wall as compared with parallel vibrations. This behaviour was found in most cases but the differences were mostly rather small and in some cases no clear differences could be detected. The interpretation of these results as the sign of absorption anisotropy of the cell wall border layer should be made with great caution. Appearance of dark lines can be induced not (or not only) by absorption properties of the structures in question but by the refraction of light at the surface between two phases of different refractive indexes such as cell wall (and cytoplasm) and cell sap. For elimination of the phenomena connected with light refraction a similar set of experiments was made in which the leaves after the first illumination bringing chloroplasts to the proper position were fixed. Three kinds of solutions were used for fixation: formol-basic lead acetate, osmium tetroxide (2%) in phosphate buffer pH 7,2 and Navashin solution. After washing the leaves were transfered gradually through etanol and benzol to bromobenzol. Bromobenzol was used as an embedding medium because of its high refractive index ($n_D^{20} = 1{,}5598$) which is nearly in the middle of the highest and lowest refractive index of the native cellulose cell walls ($n_\alpha = 1{,}525$, $n_\gamma = 1{,}596$) (Frey Wyssling 1935). The photographs of cells embeded in bromobenzol were made and their densitometry was performed as in the above case described. The preparates fixed in OsO_4, were not used for further investigations because of a heavy darkening of their cell walls and their neigbourhood. Cells fixed in formol-basic and Navashin solutions showed a similar appearance of the cell walls. The example of densitometric curves is shown in fig 1c. The density is much smaller as for the cell walls in vivo and the peak of density is situated in the middle of the cell wall cross section. No distinct dense layer could be observed at the border of the cell walls.

3. Fluorescence. A high pressure mercury lamp (HBO 50) was used as a source of radiation. The primer filter set consists of $CuSO_4$ solution (80 g/l, 2,5 cm) and one or two UG 11/2mm filtres. GG 14/2mm usually with BG 23/2mm served as secondary filters. The BG 23 filter was introduced for elimination of chlorophyll fluorescence. The fluorescence of chloroplasts was observed by use of a RG 1/2mm secondary filter. Transmission curves of the filters as well as the absorption spectrum of riboflavin and the fluorescence spectrum of riboflavin and chlorophyll pigments is presented in fig 2. Microscopic observations were made with 40 × objective. Pictures were taken on a high speed film (ORWO-NP 27, 27DIN) and developed in Kodak D8 developer.

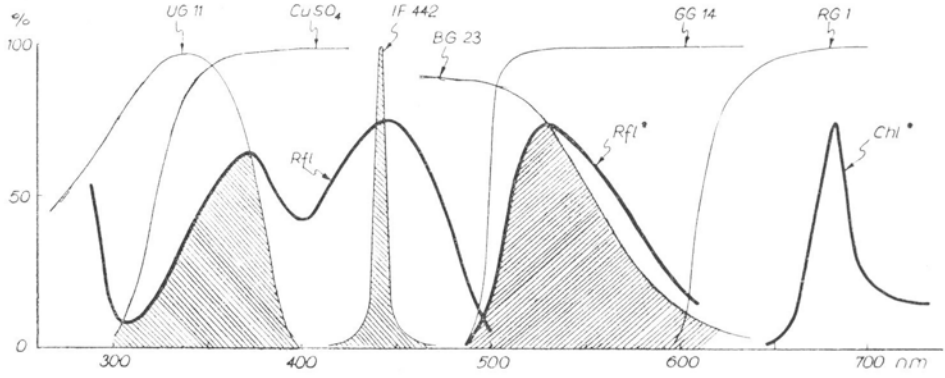


Fig. 2. Transmission curves of the filters used as primary and secondary filters for fluorescence microscopy. $CuSO_4$ — concentration 80 g/l. thickness 2,5 cm., denotation of glass filters according Schott, IF — interference filter 442 nm.

Rfl — absorption spectrum of riboflavin (after Whitby 1953); *Rfl** — fluorescence spectrum of riboflavin (after Yagi at all 1955, from Beinert 1960); *Chl** — fluorescence spectrum of spinach chloroplasts (after Goedheer 1966).

The leaves of *Funaria* irradiated by the spectral range 300—400 nm show a bright two colour fluorescence. By using GG 14 as a secondary filter choroplasts show red colour and the cell walls fluorescence yellow. After introducing of RG 1 filter the fluorescence of cell walls desappeares completely. Using the GG 14 and BG 23 filter set the chloroplasts are dark and yellow fluorescence of the cell walls remains without any apparent decrease in its intersity. (Plate I fig 3 *a*, *b* and *c*). It may be concluded that the spectral range of fluorescence of the cell walls is nearly entirely placed within the limits of GG 14 and BG 23 filter transmission. The question arouses what a more precise localization of the fluorescent substance is (cell wall, its border layer or thin layer of cytoplasm?). The observations made with immersion objective of high resolution show that the fluorescence is equally distributed along the optical cross section of the walls (Plate I fig 4 *a*, *b*). Because of a very small thickness of the protoplasm layer nothing can be concluded about its fluorescence. The last problem was studied in plasmolised cells. After 1 hour plasmolysis in 0,8 KNO_3 it can be found that the fluorescent properties remain in the walls and no traces of yellow fluorescence can be found in the layer of cytoplasm detached from the cell walls (Plate II fig 5 *a*, *b*).

The properties of the yellow fluorescent substance of the cell walls were studied by treating the leaves with different chemicals. Results are summarized in Tab. I. As it can be seen, the substance in question is very resistant against high temperature, acids, alkali and lipid solvents. It should be stressed that KJ in the concentration 10^{-1} M/l causes a very

Plate I

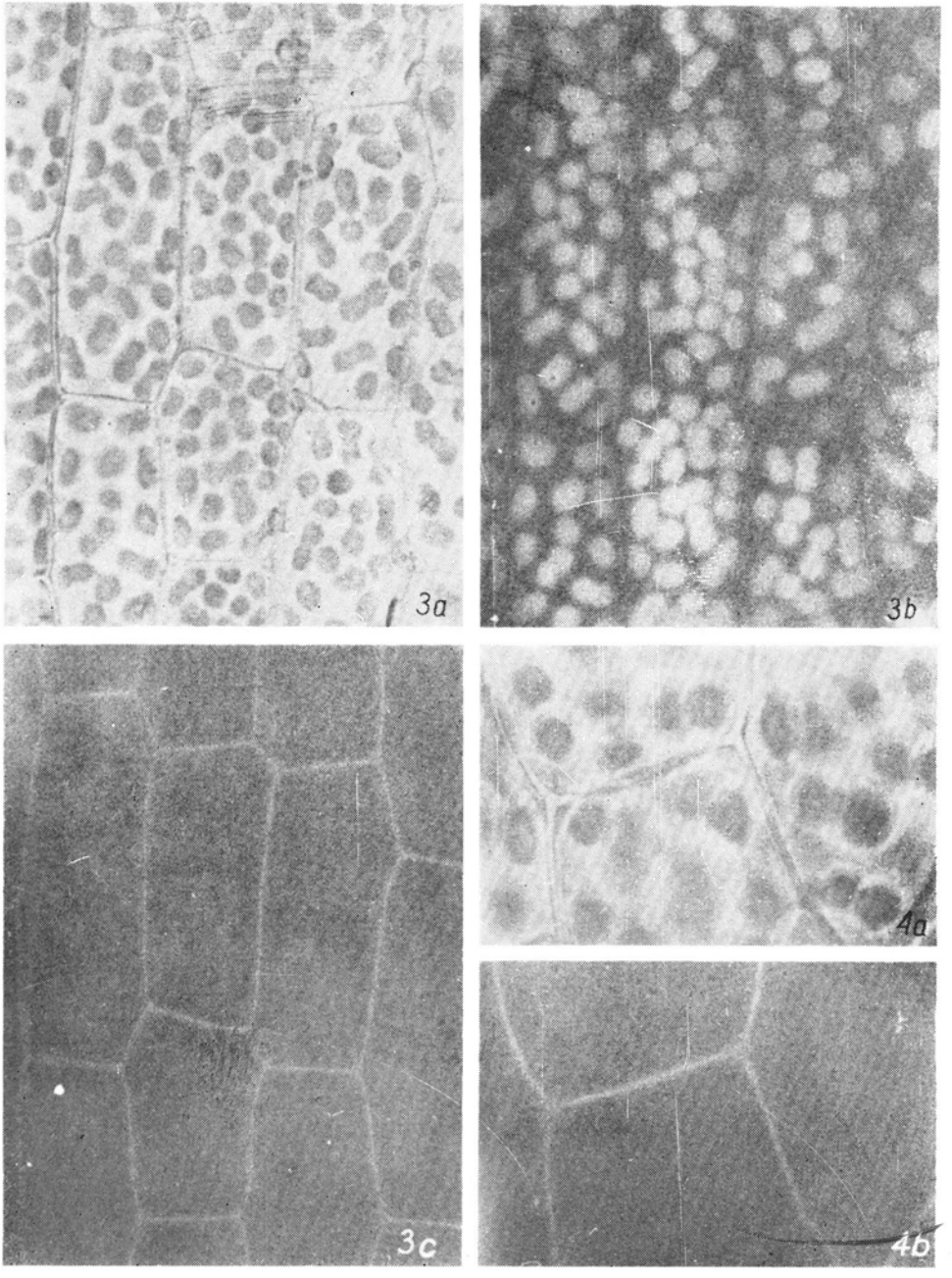
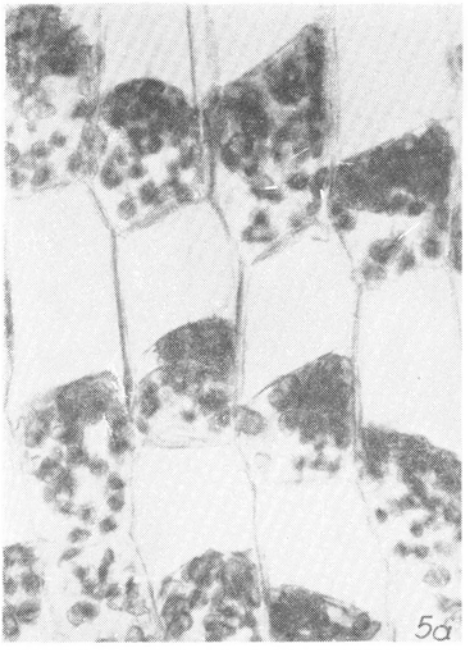
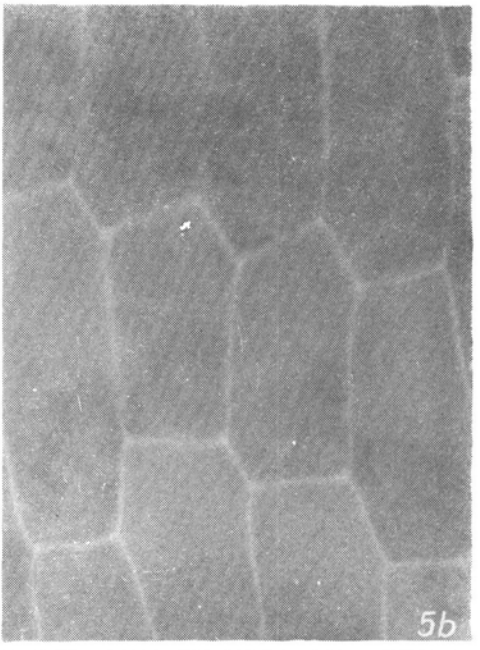
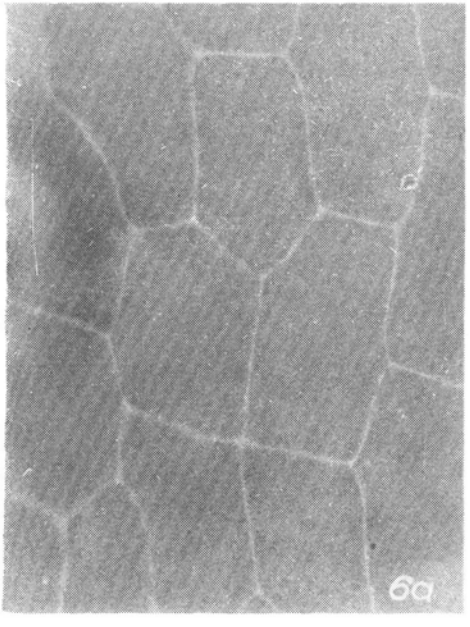


Fig. 3. Cells of the leaf of *Funaria* (*a*) and their fluorescence by using RG 1 (*b*) and GG 14+BG 23 (*c*) secondary filters.

Fig. 4. Fragment of the cell under immersion objective (*a*) and its fluorescence (*b*).







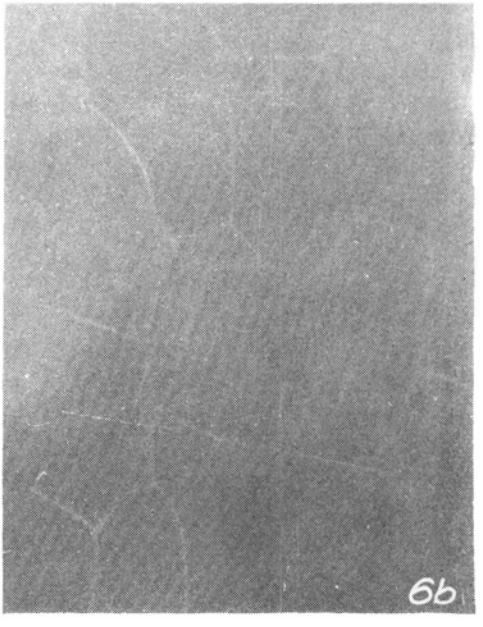


Fig. 5. The cells plasmolised in 0,8 M KNO_3 (*a*) and their fluorescence (*b*).

Fig. 6. The fluorescence of cell walls before (*a*) and after 24 hours treatment with 10^{-1} M/l KJ (*b*).

distinct decrease of the yellow fluorescence as well in vivo (Plate II fig 6 *a, b*) as in killed cells. The most effective quenching effect was obtained by $AgNO_3$ solution. The pH spectrum of fluorescence, made with the solutions according K a v a n a g h and G o o d w i n (1949) shows that in the wide range of pH between 4,5 and 11,3 the intensity of fluorescence is stable and its marked decrease can be found only in very acid and very alkaline solutions.

Table 1

Fluorescence of the cell walls of *Funaria* leaves after 4 hours and 24 hours treatment with different chemicals

Treatment of the leaves		4 hours	24 hours
Water (control)		+++	+++
5 min. boiled		+++	++
Etanol 96%		++++	++++
Ethyl ether		+++	+++
Chloroform		+++	+++
Formal basic lead acetate soln.		+++	+
10^{-1} M/l KJ in vivo		++	+
10^{-1} M/l KJ after 5 min boiling		+	+
10^{-1} M/l $AgNO_3$		o	o
1 n HCl	pH = 0	o	o
0,3 n H_2SO_4	pH = 1,2	+	o — +
Mc Ilwain buffers	pH = 2	++	++ — +
	pH = 4,5	+++	++
	pH = 6	+++	+++
	pH = 7,5	+++	+++
0,2 M Na_2HPO_4 /Na_2CO_3	pH = 9	+++	+++
	pH = 10	+++	+++ — ++
	pH = 11,3	+++	++
0,01 n NaOH	pH = 12,1	++	+
0,1 n NaOH	pH = 13,1	+	+ — o
1 n NaOH	pH = 14	o	o

+++ normal fluorescence, ++ weaker, + very weak, O = no fluorescence.

A specific reaction of chloroplasts displacements to polarized blue light made us to investigate the fluorescence excited by this kind of radiation. An interference filter (λ max 442 mm) and BG 1/2 mm glass filter were used for selection of the range of exciting light. Yellow fluorescence remains under illumination with blue light, although its intensity is weaker. The lowering of intensity of fluorescence should be at least partly explained by much lower energy of exciting radiation (interference filter) as it was in the previous case. When the exciting light was polarized, the intensity of fluorescence shows no visible changes in relation to the direction of polarization.

DISCUSSION

The yellow photoreceptor active in the light induced displacements of chloroplasts is certainly present in the cell in a very small amount. It makes the negative results of its detection by a microchemical reaction for riboflavin as well as by direct observations comprehensible. The detection of the yellow pigment based on the dichroism of light absorption which can be deduced from the study of chloroplasts displacements in polarized light (Zurzycki 1967b) seemed to be more probable. The behaviour of chloroplasts shows that light absorption in the active pigment must be much greater when light vibrates parallel to the cell wall and much smaller when the plain of light vibration is perpendicular to it. The differences — judging from the light intensity curves — should be of the order of ten times. The densitometry of the pictures obtained with monochromatic blue polarized light in vivo showed differences in the density of the border layer of cell wall in dependence on the direction of polarization. The sign of these differences is in accordance with expectations but their extent is neglegible (maximum 0,3—0,8 ×). The appearance of the dark margin by the optical cross section of the wall must be to a great extent (if not quite) connected with differences in the refractive index of the studied structures. After fixation and embedding of the cells in the medium of high refractive index no absorption in the margin layer of the cell walls was detacted. It may speak against the explanation of the results obtained for living cells as differences of absorption in active pigments but on the other hand eluation and destruction of the pigment in the course of fixation and embedding can not be excluded.

The fluorometric method is much more sensitive for the detection of some compounds than the spectrophotometric one (Loofbourow 1943). Riboflavine can be identified and localized in the tissue by its yellow fluorescence (Glick 1946). Although it may be expected that riboflavin as the active photoreceptor is bound with other substances (i.e. protein) even for some flavoproteins the fluorescent properties are retained (Weber 1950). The leaves of *Funaria* show in the fluorescent microscope a distinct yellow fluorescence of the cell walls. The fluorescence of the cell walls is a rather common phenomenon, but usually the colour of fluorescence is whitish blue or violet, seldom bluish green (Klein and Linser 1930). Yellow fluorescence of the cell walls was found only in exceptional cases i.e. for cuticles in *Tilia* (Klein and Linser 1930), *Coelogyne faccida* (Schindler and Thots 1950) *Aneimia phyllitidis* (Lukan 1947), xylem in *Zea* (Klein and Linser 1930), endoderm of some ferns (Lukan 1947). In most cases the chemical nature of the substance responsible for yellow fluorescence is unknown. Among the known biological substances which exhibit

fluorescence properties (reviewed by Goodwin 1953) yellow colour of fluorescence was found in some quinones, isoalloxazines, and glycosides. The reaction of fluorescence of the cell walls of *Funaria* to different chemical factors shows a great coincidence with the behaviour of riboflavin. Not only the spectral range of fluorescence but also the insolublness in lipid solvents, resistance against high temperature and chemical factors (Beinert 1960, Lambooy 1963) are in agreement with the properties of riboflavin. A distinct quenching of fluorescence by KJ as well as strong quenching by Ag-ions are also typical of this substance (Weber 1959, Szent-Györgyi 1957). The influence of pH strongly resembles the pH fluorescence curve given by Kavanagh and Goodwin (1949). The fact that yellow fluorescence can be excited not only by UV but also by blue light is in agreement with the absorption spectrum of riboflavin.

In spite of the fact that the yellow fluorescence of the cell walls may by induced by the existence of riboflavin pigment in them, the detected fluorescent pigment is certainly not the active photoreceptor. Three arguments speak against such a supposition:

1. the fluorescent pigment seems to be localized within the whole thickness of the cell wall whereas the active photoreceptor should be localized in the cytoplasm or at least in the close neighborhood of the cytoplasm,
2. the outher layer of the plasmolised protoplast show no fluorescence but it can be shown that in that place the active and orientated photoreceptor is still localized (Zurzycki 1967c),
3. after excitation with polarized blue light the intensity of fluorescence does not depend on the direction of polarization, which should be expected in the case of an active and oriented photoreceptor.

It must be stated that with the method used in the present study no sure cytochemical detection of the photorecptor was possible.

SUMMARY

An attempt of the cytochemical detection of photoreceptor active in photo--displacements of chloroplasts in *Funaria* was made. The microchemical reaction for riboflavin according Chévremont and Comhaire was negative. The densitometric measurements of the picture of the cell walls made in blue polarized light show only small differences in the density of border layer of the cell in relation to the direction of polarization. The last phenomenon desappeares after fixation and embbeding in the medium of high refractive index. The cell walls show yellow fluorescence. Properties of the substance responsible for this effect are in good agreement with the properties of riboflavin. Localization of this substance among the whole thickness of the wall and only in the cell wall as well as lack of orientation of its molecules speak against the supposition that it may be an active photoreceptor. With the above mentioned method no sure cytochemical detection of the photoreceptor was possible.

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Własności i lokalizacja fotoreceptora czynnego w ruchach chloroplastów u Funaria hygrometrica.

III. Badania cytochemiczne

Streszczenie

Podjęto próby wykrycia fotoreceptora czynnego w przemieszczeniach chloroplastów u *Funaria hygrometrica.*

Reakcja barwna stosowana dla wykrywania riboflawiny dała wynik negatywny.

Pomiary densytometryczne zdjęć komórki wykonanych w niebieskim świetle spolaryzowanym wykazały tylko nieznaczne różnice w gęstości optycznej negatywu przy brzegach ścian komórkowych związane z kierunkiem polaryzacji światła. Różnice te zanikają po utrwaleniu komórek i wysyceniu obiektu ośrodkiem w wysokim współczynniku załamania.

Ściany komórek liścia *Funaria* wykazują żółtą fluorescencję. Występująca w ścianach substancja fluoryzująca posiada własności zbliżone do riboflawiny. Lokalizacja tej substancji ograniczona wyłącznie do ścian komórkowych oraz brak orientacji jej cząstek wskazuje że nie jest to fotoreceptor czynny w przemieszczeniach chloroplastów.

Przy pomocy zastosowanych metod: reakcji mikrochemicznej, pomiarów absorbcji i fluorescencji nie udało się w sposób jednoznaczny stwierdzić rozmieszczenia fotoreceptora w komórce.