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EXPERIMENTAL ARTICLES

Biogenesis and Possible Modification of Carotenoid Composition in the Eyespot of *Chlamydomonas reinhardtii* Mutants

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Abstract—Biogenesis of the eyespot ultrastructure in the chloroplasts of the mutants of unicellular green algae *Chlamydomonas renihardtii* was studied. Development of the eyespot ultrastructure was found to correlate with carotenoid accumulation. Depending on their content, the eyespot formed 1 to 5 layers of lipid–carotenoid globules. Accumulation of carotenes in the eyespot globules was shown. It was found that carotene composition in the eyespot of the mutants could vary depending on their composition in the chloroplast membranes.

Keywords: *Chlamydomonas reinhardtii*, mutants, chloroplast, eyespot, ultrastructure, carotenoids, xanthophylls

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Flagellate unicellular green algae possess phototactic properties and form a specific organelle of the chloroplasts: the eyespot, which is often called a stigma. Structurally, it contains one or several (usually 2 or 3) layers of tightly packed hexagonal carotenoid-rich lipid globules localized inside the chloroplast. In *Chlamydomonas reinhardtii*, it is usually located directly under the chloroplast envelope, which tightly adjoins the cytoplasmic membrane but does not merge with it [1–3]. Under a light microscope, the stigma can be seen as a yellow, orange, or light red dot or spot [1, 4]. Initially, the eyespot was believed to consist of only 2 or 3 layers of lipid–carotenoid globules. However, further studies showed that the lipid globules were surrounded with protein membranes on the outside [3], which were formed during eyespot development inside the chloroplast. As a rule, thylakoids and eyespot are formed almost simultaneously. Each of the 2–3 layers of lipid–carotenoid globules of the eyespot is located on one of the thylakoids [2, 5–7].

The previously obtained pigment mutants of *Ch. reinhardtii* are highly valuable tools both for the identification of photoreceptor pigments and for the analysis of eyespot structure and function [8, 9].

In one of our publications we emphasized the very interesting fact that the area of the maximum spectral sensitivity of phototaxis for the eyespot of the mutant CC1101 *ey, mt*(–) was shifted to the short-wave region compared to the wild type strain, indicating a complex combination of the absorption spectra of eyespot photoreceptors and photosynthetic pigments involved in photo-orientation [10]. These data suggest that the maximum of phototaxis may be shifted due to the changes in carotenoid composition both in the chloroplast membranes and in the eyespot globules. We were interested in whether the carotenoid composition of the eyespot (as well as of the chloroplast membranes) could change in the mutants. In order to answer this question, we have studied the pigment composition of the eyespot in the previously selected [9] series of *Ch. reinhardtii* mutants with different carotenoid composition in the chloroplast membranes.

The following two problems were posed in the present work: (1) to study the biosynthesis of the eyespot, from formation of the first globules to development of the maximally structured organelle; and (2) to determine carotenoid composition in the eyespot globules of the initial wild type strain and, using the mutants, to prove for the first time the possibility of experimental modification of their composition. Both problems could be solved only with the application of pigment mutants from white to dark green in the former case and of the mutants with the modified carotenoid composition in the chloroplast membranes in the latter case. The presence of a wide range of both types of mutants [9] made it possible to solve both problems. All stages of eyespot development were determined and the possibility of experimental modification of carotenoid composition in the eyespot globules of *Ch. reinhardtii* mutants was demonstrated.

MATERIALS AND METHODS

The research subjects were the unicellular green alga *Chlamydomonas reinhardtii* Dang. of the wild type K(+) strain and the mutants: white B-1 accumulating

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no pigments; yellow Zh-4 containing carotenoids only; light green C-41 with the high content of zeta-carotene; green non-photosynthesizing A-90 with the high content of alpha-carotene; and dark green T-8 containing 2 to 3 times more pigments than the wild type cells [9].

For the experiments, all strains were grown for 4–5 days on solid acetate medium [8] at 23–25°C under 24-h illumination with LB-40 luminescent lamps (Russia) at 1000–3000 lx.

The eyespot globules and chloroplast membrane fragments were obtained as follows: the cells were suspended in the medium containing 0.3 M sucrose, 0.05 M Tris-HCl, 0.01 M $MgCl_2$, pH 7.5, and disintegrated with ultrasound in UZDN-1 (Russia) at 4°C by two 20-s pulses at 15 kHz and the current of 0.2 A. Undestroyed cells were separated by centrifugation at 1500 *g* (3000 rpm) for 5 min in a TsUM-1 centrifuge (Russia). The chloroplast fragments from the supernatant were washed with 0.05 M Tris-HCl and 0.001 M EDTA, pH 8, and precipitated by centrifugation at 14000 *g* for 20 min. Then repeated centrifugation at 14000 *g* for 20 min in the sucrose density gradient of 0.5 : 1.0 : 1.5 : 2.0 M was performed to separate the lipid–carotenoid globules of the eyespot from the chloroplast membrane fragments. The fraction of eyespot globules (fraction 1) was observed as a yellow or orange band at the interface between the zones of 0.5 and 1.0 M sucrose solutions. In both cases, we accurately collected the yellow band with a pipette into a separate test tube and then used it for biochemical, spectral, and electron microscopic studies.

The content of chlorophylls and carotenoids was determined spectrophotometrically using the absorption spectra of 100% acetone extracts [11].

The qualitative composition of carotenoids was analyzed by paper and thin-layer chromatography [12, 13]. The bands of individual carotenes and xanthophylls were extracted with chloroform and 96% ethanol, respectively. Carotenoid extracts were analyzed by spectrophotometry using the absorption spectra and their second and forth derivatives obtained on Shimadzu-UV-160 or Hitachi-557 spectrophotometers (Japan).

Electron microscopy of the eyespot of the intact cells, and of the isolated lipid–carotenoid globules, was performed after fixation with 2.5% glutaraldehyde, either followed by fixation with 1% OsO_4 solution or without it. The samples were dehydrated in a series of 20–100% ethanol solutions and 100% acetone and embedded in Epon-812. After polymerization of the samples, the sections were made in an LKB microtome (Sweden) and examined under a JEM-7A microscope (Japan) [2].

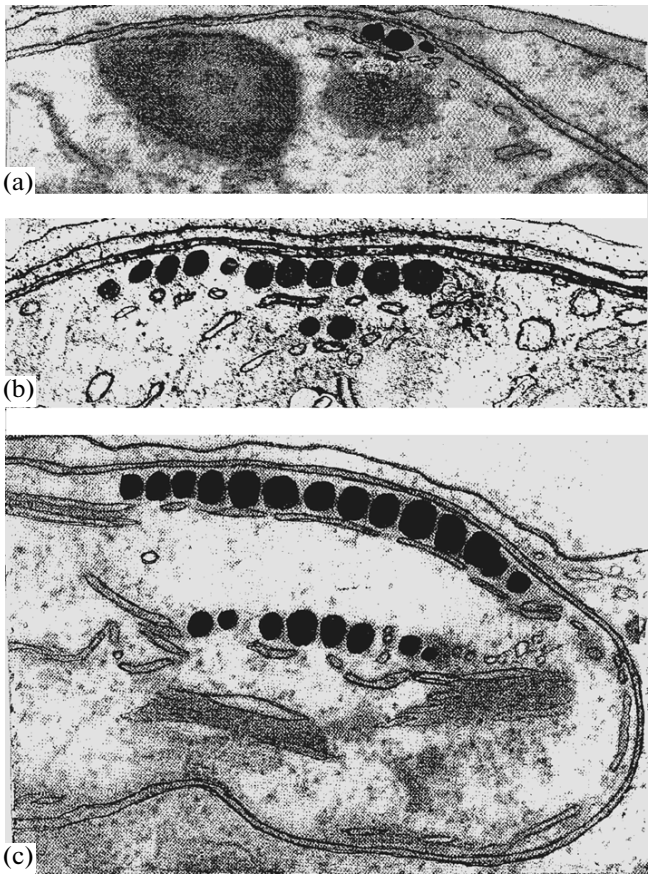

Fig. 1. Ultrastructural organization of the eyespot on cross sections of the chloroplasts of the mutants: white B-1 (a), white light-sensitive B-3 in the dark (b), and yellow Zh-4 in the light (c). Magnification: ×80000.

RESULTS

Biogenesis of the eyespot ultrastructure. Due to ability of the of wild type *Ch. reinhardtii* cells to synthesize chlorophyll and carotenoids in the dark, its chloroplasts always exhibit normal development of the membrane system and the eyespot both in the light and in the dark. Therefore, it was necessary to artificially block the early stages of pigment biosynthesis in order to study different stages of formation of the eyespot and the chloroplast membranes. To this effect, the white, yellow, light green, and dark green mutants were obtained by the method of mutagenesis [8, 9].

The earliest stages of eyespot formation were found using the white mutant B-1 containing neither chlorophyll nor carotenoids. The eyespot of the mutant B-1 consisted of only two or three lipid–carotenoid globules (Fig. 1a), while the light-sensitive mutant B-3 was shown to have almost the entire first layer of globules (Fig. 1b).

We obtained three types of yellow mutants. The mutant Zh-1 is yellow in the dark and turns green in the light, similar to higher plants. The mutant Zh-3 is green in the dark and phenotypically yellow in the

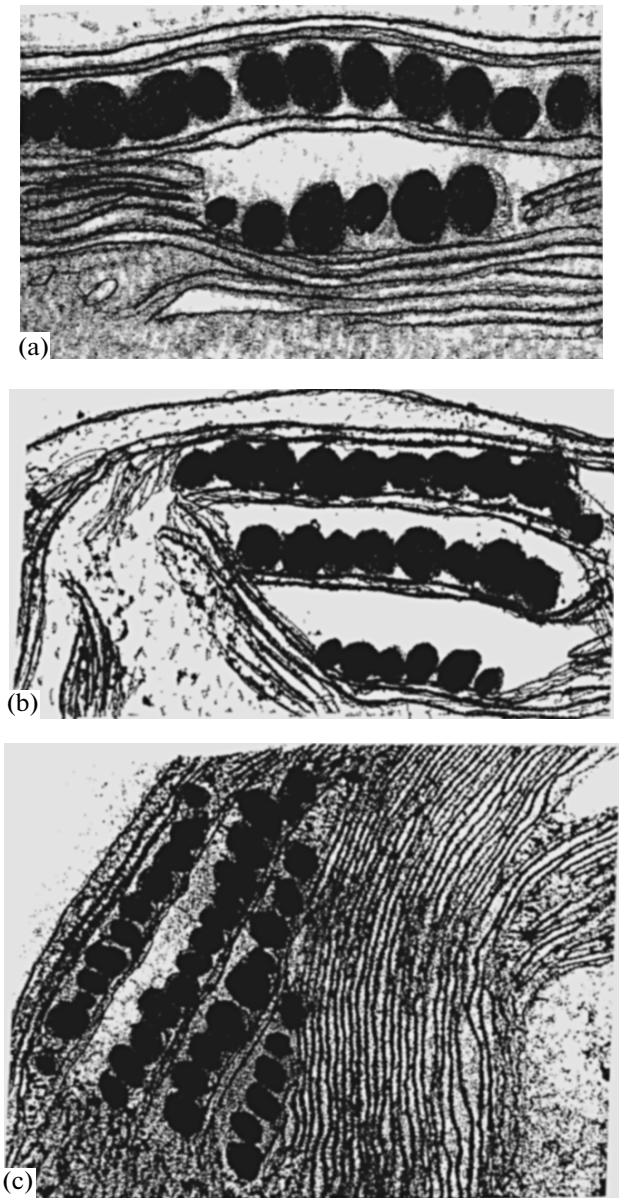

Fig. 2. Ultrastructural organization of the eyespot on cross sections of the chloroplasts of the light green mutant C-48 (a), wild type K(+) cells (b), and mutant T-8 (c) after glutaraldehyde–osmium fixation. Magnification: ×80000.

light. This mutant lost chlorophyll in the light only in the presence of O_2. When stab-inoculated into the agar column, this mutant remained green in the light too, i.e., photodestruction and discoloration occurred only in the presence of O_2 in the light. The mutant Zh-4 was permanently yellow, both in the light and in the dark [2, 9]. Investigation of yellow mutants revealed that the degree of eyespot development distinctly correlated with the content of carotenoids. The presence or absence of chlorophyll and the respective degree of development of the membrane system had a lesser effect on eyespot formation. We observed a well-developed eyespot, usually consisting of two layers of globules, in the chloroplasts of yellow mutants of nearly all variants (Fig. 1c). Only the initial stages of thylakoid formation were observed in these mutants.

A similar level of eyespot development, although one with well-developed thylakoids, was observed in the group of light green mutants with different contents and ratios of chlorophylls *a* and *b*, which accumulated nearly the same amount of carotenoids as the yellow mutants (Fig. 2a).

The eyespot consisting of two or three layers of lipid–carotenoid globules, depending on accumulation of the pigments, was formed in the wild type cells K(+) (Fig. 2b).

In the dark green mutant T-8 accumulating 2- or 3-fold more carotenoids and chlorophylls than the control, the eyespot could form up to four layers of globules (Fig. 2b). This phenomenon has not been reported previously.

Thus, we succeeded in defining the early stages of eyespot biogenesis using the series of pigment mutants. Moreover, we have shown for the first time that the degree of development of eyespot globules correlates well with the accumulation of carotenoids and, to a lesser extent, depends on the content of chlorophylls. At the same time, the mutants made it possible to study the biogenesis of the eyespot from 2–3 globules to the formation of 4 layers of lipid–carotenoid globules.

After fixation with glutaraldehyde, it was discovered that each lipid–carotenoid globule was surrounded with a protein membrane [3]. The membranes around eyespot globules can be detected by different methods: either by (1) fixation of the proteinaceous membrane components with glutaraldehyde, followed by extraction of lipids and pigments with ethanol and acetone during dehydration of samples for electron microscopy, in which case we observed the remaining rounded protein components surrounding the eyespot globules (Figs. 3a, 3b), or (2) by fixing very old cells (grown in the light for more than 30 days), where partial degradation of the lipid–carotenoid components of the chloroplast, including those of the eyespot, occurred. As a result, we observed the destruction of the internal structure of eyespot globules and preservation of the surrounding protein membranes. The shapes of surrounding membranes completely maintained the contours of all layers of eyespot globules [3]. It should be particularly noted that the shapes of eyespot globules on cross sections were rounded or oval (Figs. 3a, 3b).

At the same time, longitudinal sections of the eyespot (Figs. 3c, 3d), especially after extraction of the lipid–carotenoid components, clearly show that the membranes within each layer of globules have a distinct hexagonal structure (Fig. 3d). The structure of the longitudinal section of the eyespot (plan view) resembles the comb structure (Fig. 3d). Such eyespot

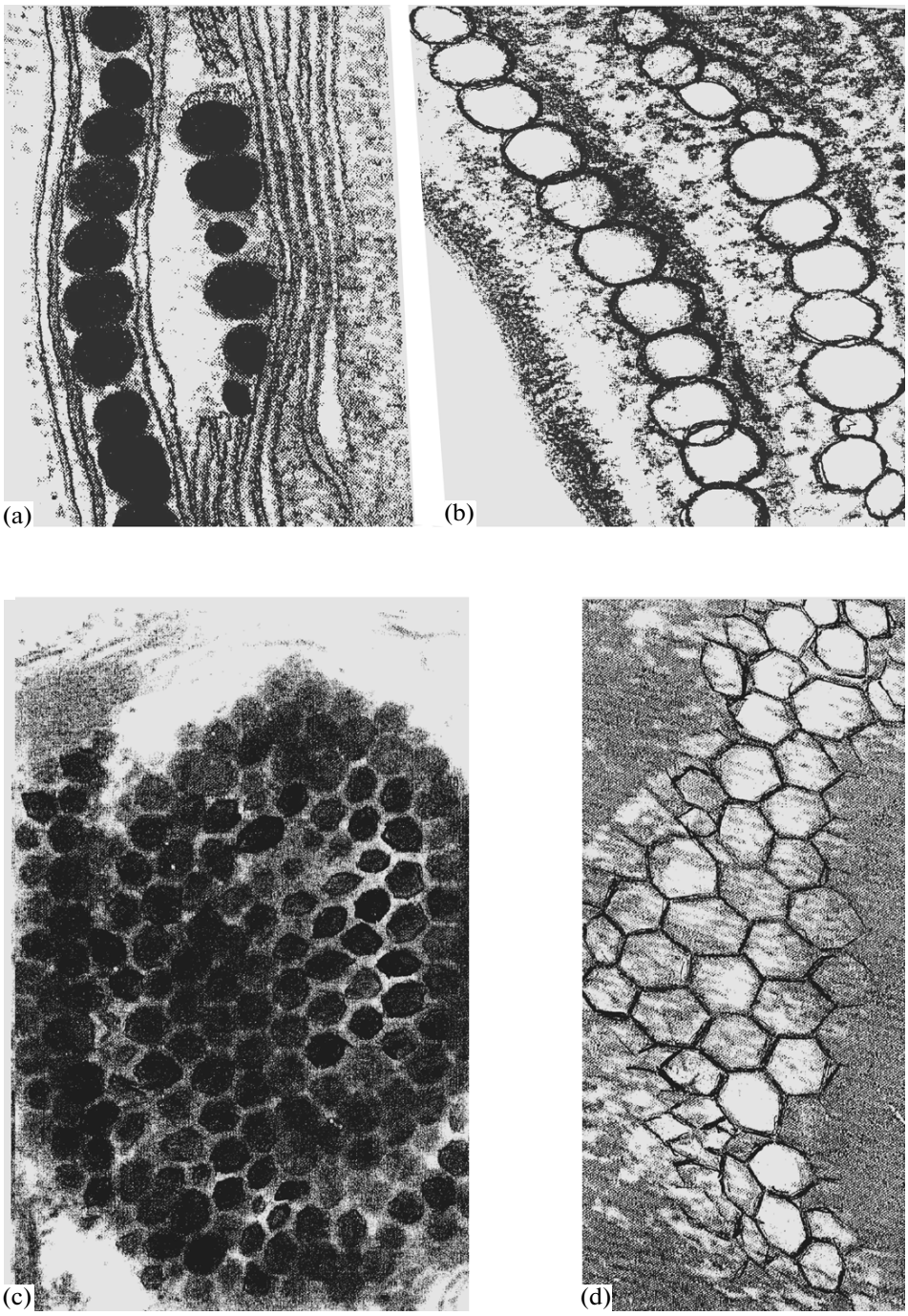

Fig. 3. Ultrastructural organization of the eyespot on cross sections after glutaraldehyde–osmium (a) and glutaraldehyde fixation (b), as well as on longitudinal sections after glutaraldehyde–osmium (c) and glutaraldehyde fixation (d) of wild type K(+) cells in the light. Magnification: ×100000.

structures have also been found in the green alga *Spermatozopsis similis* [14].

Composition of carotenoids and possibility of their modification in the eyespot globules. Two issues were to be elucidated in the present work: (1) to identify the major carotenoids comprising eyespot globules and (2) to find out whether their composition could change in case of substantial variation of carotenoid composition in the chloroplast membranes of *Ch. reinhardtii* mutants. For this purpose, we isolated the fraction of lipid–carotenoid eyespot globules from destroyed chloroplasts by differential centrifugation in sucrose density gradient (Fig. 4a). The yellow or orange band no. 1 of eyespot globules was collected into a separate test tube. Then the fraction of eyespot

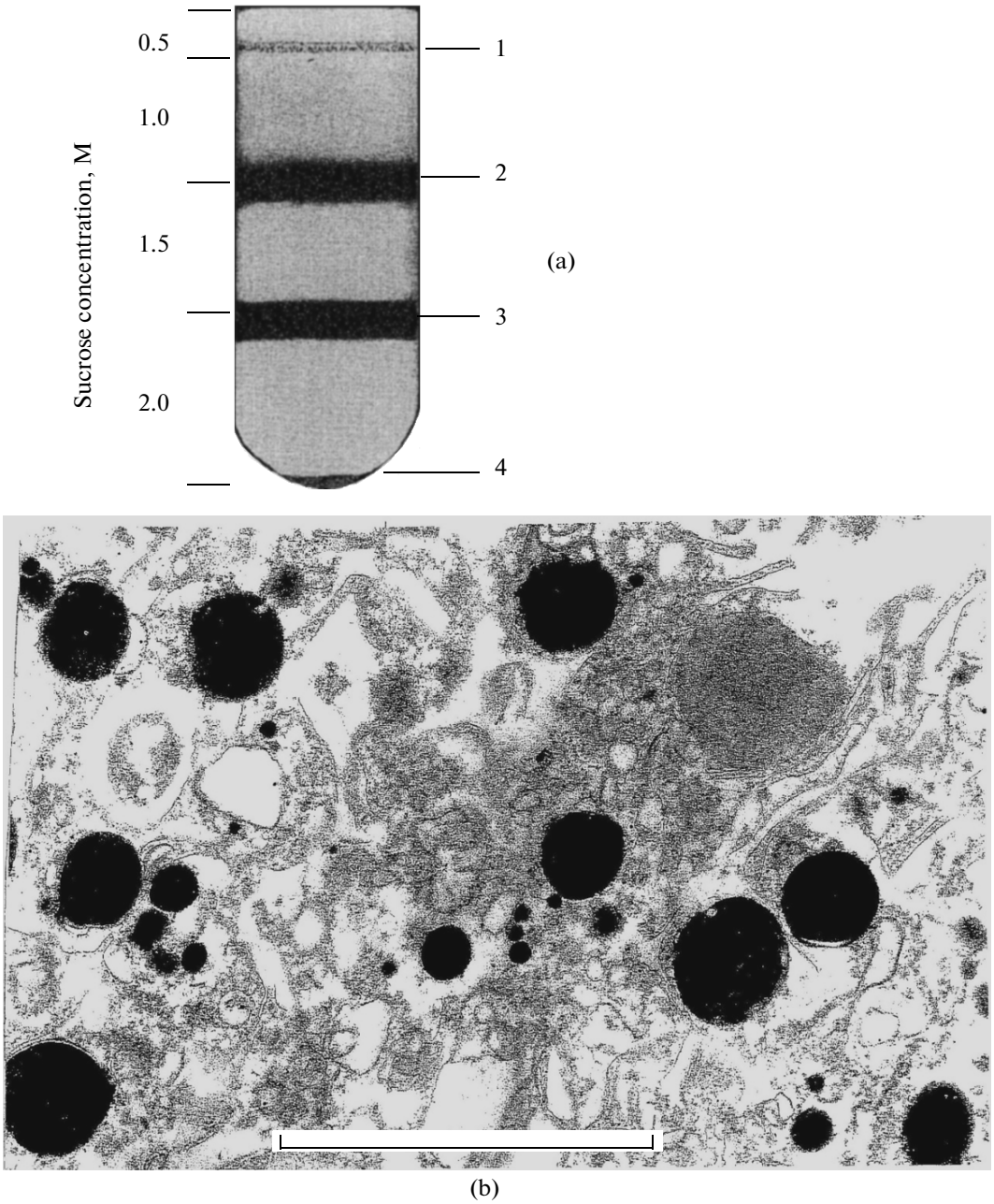

Fig. 4. Isolation of eyespot globules by centrifugation in sucrose density gradient (a, fraction 1) and the ultrastructure of isolated lipid–carotenoid eyespot globules after glutaraldehyde–osmium fixation (b, black globules). Magnification: ×300000 (b).

globules was used for the biochemical, electron microscopic, and spectrophotometric analysis. Each eyespot globule is a membrane bubble filled with lipids and carotenoids.

Electron microscopy showed that fraction no. 1 isolated by centrifugation actually indeed contained the lipid–carotenoid globules of the eyespot (Fig. 4b). Under osmium fixation, they appeared as black rounded globules often linked into aggregates of different sizes. Similar structures were described previously for the eyespots of *Spermatozopsis similis* [14]. They are formed at different extents of ultrasound treatment of the eyespot apparatus.

Our preliminary studies showed that eyespot globules comprised mostly carotenes. Therefore, in the present work we paid special attention to this group of carotenoids. The biochemical and spectrophotometric studies on eyespot globules revealed that β-carotene with the absorption maximum at 464 nm was the major carotenoid in wild type K(+) cells (Fig. 5a). Since the ratio of α- and β-carotenes in the chloroplast membrane of wild type K(+) cells is 5 : 95, we

may conclude that the major pigment of eyespot globules of the K(+) strain is β-carotene (Tables 1 and 2). It is difficult to say whether α-carotene is a component of wild type cells because of its very low content in the K(+) strain. We have not found substantial amounts of xanthophylls in the eyespot globules. Our results suggest that carotenes are the components of eyespot globules and β-carotene is the major component of the wild type K(+) cells.

We examined the A-90 mutant accumulating up to 60% of α-carotene in chloroplast membranes, with the content of β-carotene decreased to 40% (Table 1), in order to find out whether α-carotene could be a component of eyespot globules. The pigment analysis of eyespot globules of the mutant A-90 showed that α-carotene with the absorption maximum at 457 nm was the major pigment (Figs. 5, 6). Consequently, it may be concluded that the carotene composition of eyespot globules may vary simultaneously with their changing composition in the chloroplast membranes. Obviously, the eyespot of the mutant A-90 contains approximately the same percentage of α-carotene (up to 60%) as the chloroplast membranes of this mutant. These results give grounds for asserting that the major pigments of the eyespot globules are carotenes and that the composition of α- and β-carotenes changes simultaneously with the variation of their composition in the chloroplast membranes of each strain, as may be seen from the absorption maximums (Table 2).

Another study with the mutant C-41 accumulating up to 38% of ζ-carotene, 19% of β-zeacarotene, and 43% of β-carotene in the chloroplast membranes was performed for final verification of this hypothesis (Table 1). High content of ζ-carotene in the chloroplast membranes of the mutant C-41 made it possible to study the possibility of its integration into the eyespot globules. The results clearly demonstrated that the eyespot globules of the mutant C-41 contained ζ-carotene, and its content seemed to be as high (Fig. 5c) as in chloroplast membranes. One can see that ζ-carotene with the absorption maximums at 409 and 433 nm (Fig. 6) and β-carotene with the maximum at 464 nm make a substantial contribution to the absorption spectrum of the eyespot of the mutant C-41.

We isolated chromatographically pure β-carotene and ζ-carotene from the mutant C-41 cells to verify the correctness of our reasoning concerning the affiliation of specific absorption bands with specific carotenes (Fig. 6b). The results show good coincidence of absorption maximums of carotenoids from the eyespot globules (Fig. 5) and pure individual carotenes from the chloroplast membranes of intact cells (Fig. 6).

Thus, based on the results for wild type K(+) cells, mutant A-90 cells with the high content of α-carotene, and mutant C-41 cells with the high content of ζ-carotene, it may be concluded that carotenes are the major pigments of the eyespot. It was established for the first time that the composition and ratio of carotene isomers in the eyespot globules of the mutants may vary depending on the changes in their composition and ratio in the chloroplast membranes of *Ch. reinhardtii*.

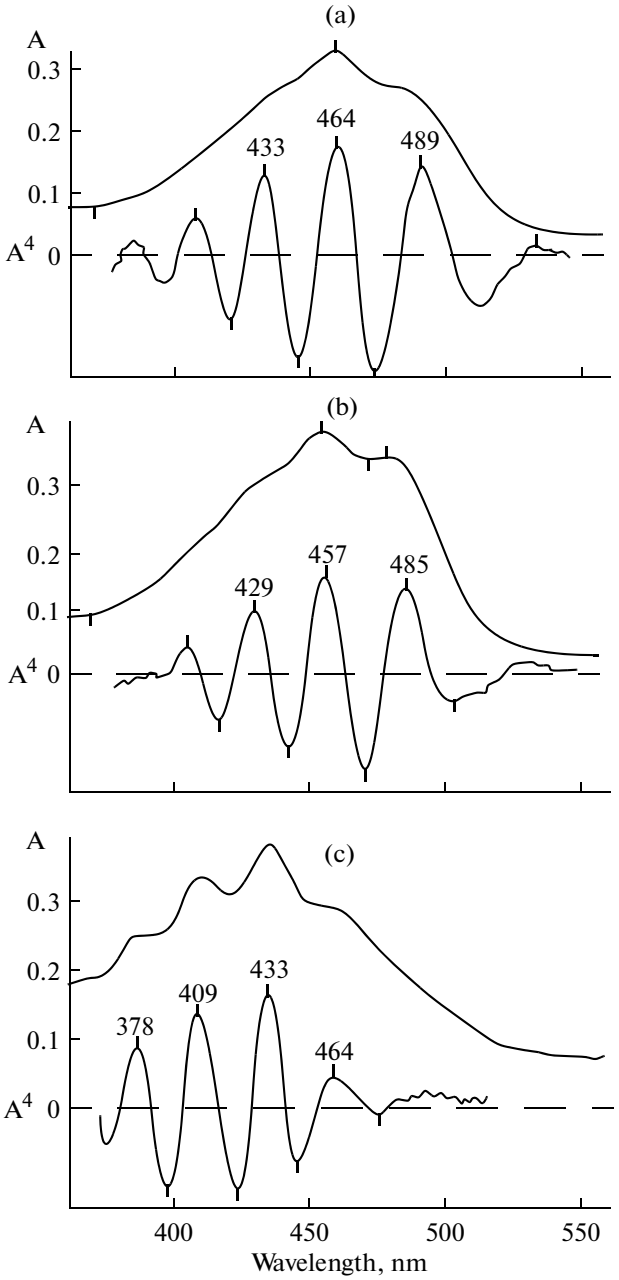

Fig. 5. Absorption spectra of carotenes in chloroform from eyespot globules (A) and their quaternary derivatives (A^4) of the wild type K(+) cells (a), the mutant A-90 with the high content of α-carotene (b), and the mutant C-41 with the high content of ζ-carotene (c).

DISCUSSION

It was shown that the eyespot of *Ch. reinhardtii* consisting of 1–4 layers of globules is located in the

Table 1. The percentage of carotenes in the chloroplast membranes of the wild type K(+) cells and the mutants A-90 and C-41 of *Chlamydomonas reinhardtii*

Strain	Content of carotenes, %			
	α-carotene	β-carotene	β-zeacarotene	ζ-carotene
Wild type K(+)	4.9 ± 1.7	95.1 ± 3.2	0	0
Mutant A-90	57.4 ± 3.9	42.6 ± 2.8	0	0
Mutant C-41	0	46.4 ± 2.6	17.1 ± 2.7	36.5 ± 2.7

upper part of the chloroplast. The eyespot globules of each layer are located on one of the thylakoids directly under the chloroplast envelope tightly adjoining the cytoplasmic membrane. They were believed to consist of adjacent lipid–carotenoid globules not separated from each other. It was subsequently shown that they are surrounded by membranes.

Association of different forms of retinal and rhodopsin with the surrounding membranes of the eyespot compartments is assumed [15–17]. The internal structure of each compartment is filled with lipid–carotenoid globules. Although the composition of the pigments included in these globules is of considerable interest, it has not yet been finally determined.

The observations of phototaxis in the mutants with impaired biosynthesis of carotenoids upon exogenous addition of retinal or some retinal analogues (11-*cis*-retinal [18, 19] or *all-trans*-retinal [15, 20]) revealed the receptor pigments required for phototaxis in *Ch. reinhardtii*. In these studies, the results were obtained using high performance liquid chromatography (HPLC) of retinoids extracted from whole cells or from the membrane fractions of *Ch. reinhardtii* wild type strains and mutants. It has been established that retinal may exist as *all-trans* [16, 17] or *all-trans* and 13-*cis* [14] isomers from whole cells of *Ch.reinhardtii*, as well as from isolated intact eyespots of *Spermatozopsis similis all-trans*- and 11-*cis*-retinal [21]. These photoreceptor pigments are probably localized in the membranes surrounding the eyespot globules [5, 6].

Some authors believe that the eyespot is not a photoreceptor for green alga phototaxis but only plays an important secondary role in the signal generation. They suppose that rotation of the cells during their motion is associated with the modulation of the signal of the photoreceptor locus by means of its periodic illumination and shading [5, 22, 23]. Some authors consider that phototactic orientation of the cells of *Ch. reinhardtii* mutant *ey 627, mt*(–) (in which the eyespot is lacking or strongly reduced) is realized due to the shading properties of the chloroplast and is required for signal generation [4]. At the same time, they do not deny that an intact eyespot is necessary for more accurate phototactic orientation [21, 24]. The extension of the phototactic signal and range of sensitivity at low smooth rates of cell movement is probably an additional function of the eyespot [4].

Table 2. Absorption maximums of carotenes from the wild type K(+) cells and the mutants A-90 and C-41 of *Chlamydomonas reinhardtii*

Carotenes	Absorption maximums in chloroform, nm		
α-Carotene	432	457	485
β-Carotene	433	464	493
β-Zeacarotene	410	433	460
ζ-Carotene	378	409	434

In all studies of the eyespot of flagellate green algae, the phototactic activity in the light was observed when incident light was perpendicular to the eyespot [5, 25, 26]. It is presently considered that accurate phototactic orientation in green algae depends on both reflecting and absorbing properties of the eyespot [4, 26, 27]. The chemical changes in the eyespot that affect its functional properties have not yet been identified.

Disturbance of the eyespot structure in the *Ch. reinhardtii* mutant *ey 627, mt*(–) under conditions of rapid cell division is an ideal model for studying these effects. Analysis of the reflecting properties of the eyespot of this mutant in synchronous cultures by confocal laser scanning microscopy has shown [4] that they are similar to those of the wild type strain and determine the orientation of the cell population and the photoelectric response. Such studies were performed also on other *Ch. reinhardtii* mutants, including eyespot-lacking mutants [4, 28, 29], where light flashes at the wavelengths of 500 and 440 nm did not result in phototactic orientation of the cells if the eyespot was absent. The functional state of the photoreceptor pigments also had little effect on the behavior of the mutant cells. At the same time, incubation of the mutant cells with a high concentration of *all-trans*-retinal (10 mM), independent of carotenoid biosynthesis, resulted in enhancement of the eyespot reflectivity [4]. These data demonstrate the significance of the eyespot both for absorption of the phototactically active light and for the distinct orientation of the cells toward the light in the course of phototaxis [4].

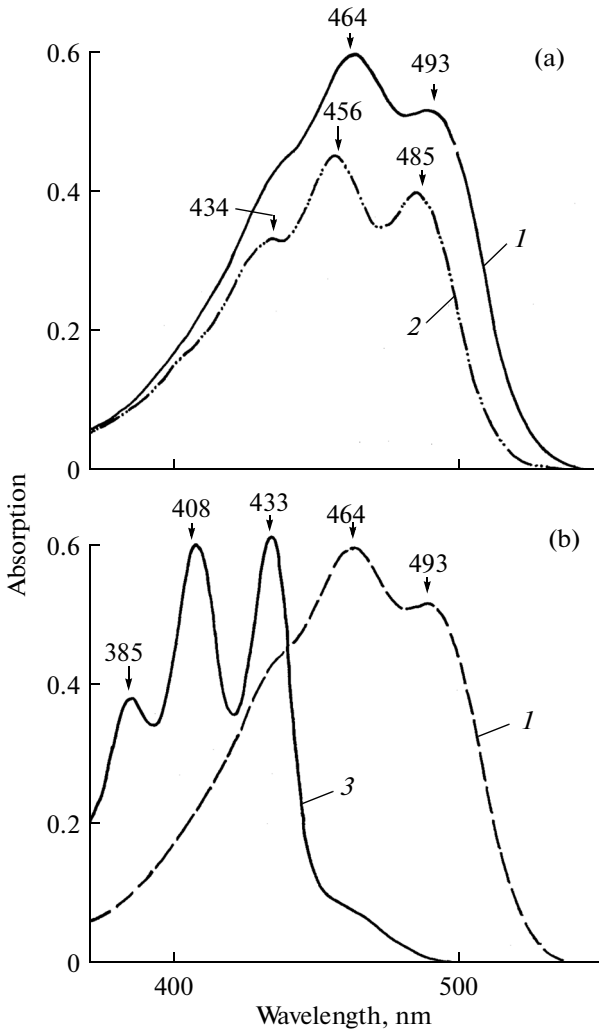

Fig. 6. Absorption spectra in chloroform of chromatographically isolated β-carotene (*1*) and α-carotene (*2*) from the mutant A-90 (a), as well as β-carotene (*1*) and ζ-carotene (*3*) from the mutant C-41 (b).

The biochemical composition of the proteins of the eyespot membranes has been actively investigated in the recent decade [30]. Particular attention was paid to the study of rhodopsin and retinal isomers [14, 29]. The carotene and eyespot reduction in a white light-sensitive mutant, with blocking the synthesis of phytoene synthase, was described recently [1]. These results confirm our conclusion that carotenoids are required for eyespot development. Depending on the content of carotenoids in *Ch. reinhardtii* chloroplasts, the eyespot may contain 1 to 4 layers of lipid–carotenoid globules. Each layer of lipid–carotenoid globules is surrounded by membranes with the longitudinal section showing a hexagonal structure similar to the honeycomb. The cross section of each globule of the eyespot shows its oval or rounded shape. The eyespot of the green alga *Spermatozopsis similis* has the same structure, and its globules demonstrate predominant accumulation of carotenes [14].

Our studies lead to a conclusion that the lipid–carotenoid globules of the eyespot of the unicellular green microalga *Chlamydomonas reinhardtii* contain carotenes. It was shown for the first time that the composition of carotenes in the mutants may vary depending on the carotene composition in the chloroplast membranes.

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