Large Scale Isolation and Purification of Eyespot Granules from *Euglena gracilis* var. *bacillaris*

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**ABSTRACT**

Large volumes of eyespot granules were isolated from homogenates of *Euglena gracilis* Klebs var. *bacillaris* Pringsheim by flotation centrifugation in a Beckman Ti-15 zonal rotor, and were further purified by centrifugation in a swinging bucket rotor. Examination with the electron microscope showed the eyespot granules to be free from other cellular material. Freezing had no apparent effect on the structure or on the absorption properties of the eyespots. Absorption spectra of pure fractions of eyespot granules free of chloroplast contamination showed the previously reported curves in the range of 360 to 520 nanometers, as well as a peak at 660 to 675 nanometers. The procedure for the large scale isolation of eyespot granules from *Euglena gracilis* is compared with other methods which have employed conventional centrifugation, and the significance of the use of zonal rotors for isolating large quantities of pure eyespot granules is discussed.

The development of zonal centrifuge rotors (1) has made possible the isolation of considerably greater quantities of subcellular components than can be obtained by more conventional methods (2, 3, 12). Since the orange-red eyespots in euglenoid flagellates comprise only a very small fraction of the total volume of the cells, the desirability of using mass cultures and large scale isolation techniques to obtain large quantities of granules for subsequent biochemical analysis is readily apparent.

Although the isolation of eyespot granules has been reported previously (5), conventional centrifugation techniques were employed and smaller quantities of granules were obtained. We report here the development of a method for isolating, with high resolution, large quantities of eyespot granules in pure fractions for subsequent biochemical analyses, which should provide new information on the function, origin, development, and possible evolution of eyespots in algal flagellates.

**MATERIALS AND METHODS**

**CULTURE METHODS AND FRACTIONATION PROCEDURES**

**Step 1.** Axenic cultures of *Euglena gracilis* Klebs var. *bacillaris* Pringsheim were grown in 200-liter quantities and harvested as described previously (4). Cells were suspended in cold 0.25 M buffered sucrose solution and were broken in a Gaulin press at 4000 p.s.i. The homogenate was filtered twice through a G-10 Sephadex column to remove aggregates of cellular material. Breakage, filtration, and subsequent centrifugation procedures for separation of chloroplasts from the homogenate were carried out in the cold, as described by Brown (6, 7). After chloroplasts were isolated, 2.5 liters of effluent were obtained. The effluent was free of chloroplasts but not smaller particles such as chloroplast fragments, mitochondria, peroxisomes, and eyespots.

**CENTRIFUGATION AND PURIFICATION PROCEDURES**

**Step 2.** Isolation of the eyespot fraction from the effluent was effected by flotation centrifugation in a Beckman Model L ultracentrifuge, with a Beckman Ti-15 zonal rotor, for 1 hr at 35,000 rpm (65,000g; 4,960 × 10^5 αt). The gradient consisted of 100 ml of buffer overlay, 1060 ml of sample homogenate in 8.5% sucrose, edgeloaded at 1500 rpm, followed by a step gradient of 150 ml of 17% sucrose and 350 ml of 30% sucrose (w/w). The rotor effluent was monitored at 640 nm through a continuous flow cell with a Beckman DU spectrophotometer. Fractions of 60 ml were collected. The percentage of sucrose for each fraction was determined with a Bausch and Lomb refractometer. Samples of selected fractions were examined with a phase contrast microscope. These fractions were correlated with their absorbance peaks and positions in the sucrose gradient prior to their additional processing for further purification or preparation for electron microscopy.

**Step 3.** The eyespot fractions collected from the zonal rotor were purified further by centrifugation for 20 min at 16,000 rpm (31,000g) in a Sorvall RC-2B centrifuge (rotor radius = 4.25 inches). The eyespot fraction (fraction A) consisted of a floating orange layer which was removed with a Pasteur pipette.

**Step 4.** A 3.5-ml sample of fraction A (in 8.5% sucrose) was layered on 15 ml of 30% sucrose and overlaid with 7 ml of phosphate buffer; the buffer solution consisted of 50 mM PO₄ (pH adjusted to 7.0 with NaOH), 2 mM cysteine, and 1 mM EDTA. This preparation was then centrifuged at 22,000 rpm (50,000g) for 2 hr in a Spinco SW-25 rotor, after which the eyespot granules banded (fraction B) at the interface of sample and buffer. Aliquots of fraction B were either fixed immediately for electron microscopy or frozen for subsequent use in biochemical analysis.

**SPECTROPHOTOMETRY**

Eyespot granules were extracted with either 100% acetone or a mixture of petroleum ether (boiling point 30–75 C) and...
made on a Sorvall MT-2 ultramicrotome, stained with uranyl acetate followed by lead citrate, and examined and photographed with a JEM 6C or a Siemens Elmiskop I electron microscope.

**ALTERNATE METHOD: SMALL-SCALE ISOLATION PROCEDURE**

**Culture Methods and Fractionation.** Axenic cultures of *E. gracilis* var. *bacillaris* were grown in 2-liter, cotton-stoppered flasks, containing 1300 ml of Difco *Euglena* Broth, for 5 to 6 days under constant illumination of 100 to 125 ft-c on a New Brunswick gyrotory shaker. Cells were harvested by centrifugation for 15 min at 3000 rpm (1085g) in a Sorvall RC-2B centrifuge (Type GSA rotor, radius = 5.75 inches). The cells were suspended in iced distilled water, recentrifuged, and then suspended in a 0.25 m buffered sucrose solution. Procedures for cell breakage were identical to those outlined previously. The homogenate was filtered through glass wool to remove cellular aggregates and then was centrifuged in a Sorvall RC-2B centrifuge at 16,000 rpm (30,900g) for 20 min. The eyespot fraction formed a floating orange layer and was removed with a Pasteur pipette.

**Purification.** Procedures for purification were identical to those described in the preceding sections.

**RESULTS**

We have obtained fractions of eyespots of *Euglena gracilis* in larger quantities and with greater purity than heretofore reported, by the methods described above. The first 60 ml collected at the core of the zonal rotor (fraction 1; Fig. 1, region 1) was bright orange and constituted a nearly pure fraction of eyespot granules. The position of the eyespot-containing fraction in the sucrose gradient in relation to the absorbance at 460 nm is shown in Figure 1. Examination of this fraction (Fig. 1, region 1) with phase-contrast and electron microscopy (Fig. 4) confirms the presence of eyespot granules only slightly contaminated with small cellular fragments. Fractions 2 and 3 (Fig. 1, regions 2 and 3) contained some eyespot granules, but these fractions were more highly contaminated with fragments of chloroplasts and other cellular components. Later fractions, in 17 to 20% sucrose, also contained some eyespot granules but contained larger amounts of cellular fragments and debris (Fig. 1, region 4; Fig. 5).

The granules obtained from the final step (No. 4) of the purification procedure (fraction B, Fig. 2) appeared, upon examination by electron microscopy, to be a pure fraction of eyespots, free of any contaminating particles (Fig. 6). Absorption

**ELECTRON MICROSCOPY**

Samples of fractions obtained after centrifugation in the zonal rotor (Fig. 1, region 1) and after subsequent centrifugations (fractions A and B above) were fixed for 1 to 48 hr at 6 C in 3% glutaraldehyde, buffered to pH 7.5 with sodium cacodylate. They were subsequently washed in distilled water, centrifuged, overlaid with 1% agar, and recentrifuged at 15,000 rpm (27,000g) for 20 min. After solidification the agar was removed from the test tubes, and the pellets containing eyespot granules were excised, diced into 1-mm³ blocks, and placed in vials containing buffered glutaraldehyde for 30 min at room temperature. Samples were rinsed in several changes of buffer over a 1-hr period and postfixed in 2% OsO₄ in the same buffer. Dehydration in a graded series of ethanol and acetone was followed by embedding in a mixture of Epon and Araldite, according to the procedures of Mollenhauer (9). Sections were

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**Fig. 1.** Separation of eyespot granules from other subcellular components in a homogenate of *Euglena* after removal of the chloroplast fraction by prior centrifugation and after centrifugation of the homogenate effluent for 1 hr at 35,000 rpm in a Beckman Ti-15 zonal rotor (step 2). The gradient consisted of 400 ml of 30% sucrose as a cushion with 200 ml of 17% sucrose added. Sample homogenate, 1060 ml, in 8.5% sucrose was placed above the gradient. The density curve was determined from refractive index measurements of samples of the fractions. Numbers 1 through 4 represent the positions of samples examined by electron microscopy (see Figs. 5 and 6).

**Fig. 2.** Final purification (step 4). Samples (0.35 ml) of fraction 1 from step 2 were layered over 3 ml of 25% sucrose. Distilled water or phosphate buffer (1.0 ml) was used as an overlay. The tubes were centrifuged at 28,000 rpm for 1 hr.
FIG. 3. Absorption spectrum of extracts of purified isolated eyespot granules (fraction B). Identical absorption spectra were obtained from preparations of granules from fraction 1 immediately after zonal centrifugation and from fractions 1 and B after freezing. All preparations were extracted in a 4:1 (v/v) mixture of petroleum ether and ethanol.

FIG. 6. Isolated eyespot granules after purification by two successive centrifugations through a sucrose density gradient. × 49,500. spectra of extracts of isolated eyespot granules were obtained after initial isolation in the zonal rotor, after purification, and after freezing. Neither freezing nor the addition of cysteine to the fraction had any significant effect on the absorption properties of eyespots (Fig. 3). Additional biochemical determinations of their purity are in progress. The granules from the purified fraction (fraction B) were collected and frozen for use in later experiments. Freezing had no deleterious effect either on eyespot structure (Fig. 7) or on their absorption properties. For purposes of comparison, the structure and density of intact eyespot granules are shown in Figures 8 and 9.

DISCUSSION

The isolation of eyespots from algal flagellates is of interest particularly with regard to their postulated roles in phototaxis and photoreception. Although, they appear to function in concert with the paraflagellar body, flagellum and microtubules
concerning their photoreceptor primary and phototaxis. Tollin (5) isolated small quantities of eyespot granules and reported a close correlation between the action spectrum of phototaxis and the absorption spectrum of the isolated granules. They demonstrated the presence of lutein, β-carotene, and cryptoxanthin. Since that report Tollin and Robinson (10) and

In order to resolve this controversy and other questions concerning their origin and development, eyespot granules must be isolated in pure fractions and characterized with regard to their biochemical composition. Using conventional sucrose density gradient centrifugation techniques, Batra and Tollin (5) isolated small quantities of eyespot granules and reported a close correlation between the action spectrum of phototaxis and the absorption spectrum of the isolated granules. They demonstrated the presence of lutein, β-carotene, and cryptoxanthin. Since that report Tollin and Robinson (10) and

Diehn (8) have suggested a presumptive role for a flavin or flavoprotein in photoreceptor, and Wolken (14) has shown that the age of the culture, pH, and light-dark adaptation of the organism are important considerations in the ultimate identification of the photoreceptor molecule. In addition to the peaks in the 360 to 520 nm range reported by others (5), our data show the presence of a peak at 660 to 675 nm. Batra and Tollin (5) reported the presence of a similar peak but attributed it to contamination with chloroplast fragments. Although that is a reasonable assumption, the fact that our purest fraction contained no chloroplast fragments (by the criterion of electron-microscopic examination) leads us to believe that the peak is not due to direct contamination with chloroplasts but possibly to a pigment system actually located in the eyespot granules. Further characterization of eyespot pigments is in progress.

The procedures described here make possible the isolation, with reasonable purity, of large quantities of eyespots of Euglenoid flagellates. With this capability, these organelles can now be characterized biochemically in various phases of growth, in senescence or after various kinds of experimental treatment, which should yield information relating to their structure and function.

Acknowledgments—We thank Drs. A. H. Haber, W. E. Barnett, O. L. Miller, Jr., and N. G. Anderson, and Ms. Mary Long and Mr. E. F. Phares for the use of their laboratory facilities for portions of this study. We are grateful to Dr. J. P. Breilat and to Mr. J. N. Brantley, Mr. L. L. Triplett, and Mr. L. M. Craig for technical assistance.

This work was supported in part by National Science Foundation Grant GB 12423 and by Biomedical Sciences Support Grants 1181R-01 and 1111R-01 from the National Institutes of Health to Dr. Walne; by Grant HD0208 from the National Institutes of Health, National Institute of Child Health and Development to Dr. Schwarz; by the Molecular Anatomy Program, which is supported by the National Cancer Institute, the National Institute of General Medical Sciences, the National Institute of Allergy and Infectious Diseases, and the United States Atomic Energy Commission to Dr. Brown; by the United States Atomic Energy Commission under contract with the Union Carbide Corporation; Contribution No. 376 from the Department of Botany, University of Tennessee, Knoxville, Tennessee.

LITERATURE CITED
