To the Graduate Council:

I am submitting herewith a thesis written by Lula Lail Hilenski entitled "Characterization of Lipids from Isolated Eyespot Granules of Euglena gracilis var. bacillaris." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Arts in College Teaching, with a major in Biology.

Patricia L. Walne, Major Professor

Otto J. Schwarz, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

CHARACTERIZATION OF LIPIDS FROM ISOLATED EYESPOT GRANULES OF *EUGLENA GRACILIS* VAR. *BACILLARIS*

A Thesis

Presented for the

Master of Arts in College Teaching

Degree

The University of Tennessee

Lula Lail Hilenski

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ABSTRACT

Isolated eyespot granules of Euglena gracilis var. bacillaris were analyzed for lipids. The quantity of total lipids extracted from one milliliter of purified eyespot sample was 0.062 µg of lipid/µg of protein. Total lipids were separated into lipid classes by thin-layer chromatography in an 80:20:1 (hexane:diethyl ether:acetic acid) solvent system. The main fractions detected were: (1) waxes, (2) triacylglycerols, (3) free fatty acids and (4) polar lipids which remained at the origin. Phospholipids were not detected by thin-layer chromatography in either acid or basic solvent systems used to resolve phospholipids.

Each lipid fraction was isolated by preparative thin-layer chromatography and then methylated with methanol containing 2% sulfuric acid. The fatty alcohols derived from the wax fraction were acetylated with acetic anhydride in pyridine (4:1 v/v).

Methyl esters of the fatty acids and acetates of the fatty alcohols were characterized by gas-liquid chromatography. Identification of peaks was based on comparison with retention times of known standards. Additional information about the composition of the methyl esters of the fatty acids was obtained by fractionation of

saturated and monounsaturated fractions on silver nitrate-impregnated silica gel chromatoplates. The following methyl esters of fatty acids were identified: the saturate series consisted of 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, and 18:0. The monounsaturates consisted of 16:1, 17:1, and 18:1. The higher unsaturates included 18:2, 18:3, 20:2, 20:3, 20:4, either 22:2 or 20:5, 24:1, either 24:2 or 22:5, and 22:6. The acetates of the fatty alcohols were identified as 11:0, 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, and 18:0.

No differences in major lipid components of crude and purified eyespot preparations were revealed by thin-layer chromatography. Thawing and refreezing of the eyespot sample before lipid extraction did not alter the major lipid components.

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LIST OF ABBREVIATIONS*

C	centigrade
cm	centimeter
ft	foot
ft-c	foot candle
g	gram
g	gravity
hr	hour
in	inch
M	molar (moles per liter)
mg	milligram
min	minute
ml	milliliter
μ	micron
μg	microgram
N	normal
nm	nanometer
OD	optical density
psi	pounds per square inch
r	radius
R_f	$\frac{\text{distance moved by sample}}{\text{distance moved by solvent front}}$

*These abbreviations are recommended by the Committee on Form and Style of the Conference of Biological Editors and are listed in Style Manual for Biological Journals.

rpm revolutions per minute

v volume

Chemicals

$AgNO_3$	silver nitrate
$CuSO_4 \cdot 5H_2O$	copper sulfate
EDTA	ethylenediaminetetraacetic acid
Folin-Ciocalteau reagent 2N	contains lithium sulfate, sodium tungstate, sodium molybdate, phosphoric acid, hydrochloric acid, bromine
K_2PO_4	potassium phosphate
KOH	potassium hydroxide
Na_2CO_3	sodium carbonate
Na K tartrate	sodium potassium tartrate
NaOH	sodium hydroxide
Vitride	$NaAlH_2$ $(OCH_2CH_2$ $OCH_3)_2$

CHAPTER I

INTRODUCTION

Light has been shown to have diverse effects on living organisms; responses range from phototropism and phototaxis to vision and hormonal stimulation. Early investigators observed phototaxis in unicellular microorganisms and speculated how the response to light is elicited (Table I). Some thought that the bright red spot found in motile cells of many algae was responsible for light perception; thus, the name eyespot was given to this structure, suggesting an analogy to the vertebrate eye.

Whether the eyespot is the structural equivalent to the photoreceptor is unknown at present, but the eyespot continues to be the subject of many investigations as scientists seek to unravel the questions concerning light perception in "primitive" forms of life. Dodge (1969) stated that all eyespots have two things in common: (1) a normally definite position in the cell, and (2) a red or orange carotenoid pigment located in lipid (osmiophilic) granules. He classified eyespots into five categories (Table II). The fine structure and some physiological characteristics of eyespots have been

TABLE I

A PARTIAL SUMMARY OF PERTINENT EARLY LITERATURE ON PHOTOTAXIS

Date	Author	Contribution
1817	Treviranus	Observed that zoospores of Draparnaldia and Ulothrix avoided high light intensity.
1878	Strasburger	Demonstrated that colorless forms of algae showed phototaxis; thus, sensitivity to light was a property of entire protoplasm.
1882	Englemann	Observed that shadow passed over Euglena had no effect until eyespot was covered.
1893	Francé	Concluded that eyespot was light-receiving organelle.
1900	Wager	Observed enlargement at base of flagellum next to eyespot; the latter cast shadow on enlargement.
1903	Pfeffer	Showed that zoospores without eyespots were phototactic.
1927	Mast	Suggested that eyespot was photoreceptor and directed flagellar movement.
1936	Tchakhotine	Found that micro-beam irradiation elicited phototaxis only when directed on eyespot.
1937	Pringsheim	Concluded that stigma was light-perceiving organ because action spectra agreed with absorption spectra of eyespot pigments.
1948	Pohl	Demonstrated a suppressive effect of light on phototaxis.
1953	Hartshorne	Showed that lack of eyespot was not associated with complete inability to respond to light.

TABLE I (continued)

Date	Author	Contribution
1957	Gössel	Concluded that eyespot was not involved in negative phototaxis and positive phototaxis was due to periodic darkening of paraflagellar swelling by eyespot.
1958	Wolken and Shin	Concluded that more than one light-absorbing pigment was present in eyespot or that other pigments in cell were involved in light absorption.

TABLE II

STRUCTURAL CATEGORIES OF ALGAL EYESPOTS (DODGE, 1969)

Type	Class	Characteristics
A	Chlorophyceae Prasinophyceae Cryptophyceae	Eyespot is part of chloroplast but has no apparent association with flagella.
B	Chrysophyceae Xanthophyceae Phaeophyceae	Eyespot is part of chloroplast but is associated with swollen portion on one flagellum.
C	Euglenophyceae	Eyespot is extrachloroplastidic--an independent cluster of osmiophilic granules at anterior end of cell and positionally related to swelling on emergent flagellum in reservoir.
D	Dinophyceae	Eyespot consists of osmiophilic granules situated near flagellar bases, and has membranous lamellar structure nearby.
E	Dinophyceae (only in family Warnowiaceae)	Eyespot is a large complex consisting of a lens, retinoid and pigment cup.

investigated in the hope of correlating structure with function. A partial review of recent literature on eyespot ultrastructure is presented in Table III.

Identification of the photoreceptor molecules or pigments is another area of research that has received widespread attention. Although there have been studies on total pigments of Euglena (Table IV), only one study has dealt solely with characterization of three pigments--lutein, cryptoxanthin, and β-carotene--from isolated eyespots (Batra and Tollin, 1964). Bound and Tollin (1967), using polarized light, showed that the carotenoid molecules in the eyespot of Euglena are aligned with the long axes parallel to the long axis of the organism. The pigments in the photoreceptor, however, may or may not be the same as those in the eyespot, depending upon whether the eyespot is determined to be a primary or secondary photoreceptor. Tollin and Robinson (1969) demonstrated a photosuppression of phototactic activity in Euglena and suggested the photoreceptor molecule to be a protein-bound flavin in the photosensitive portion of the flagellum. Diehn (1969c) suggested that in Euglena, both a carotenoid and a flavoprotein cooperated in the shading of a photoreceptor. More recently, he postulated that phototaxis (Table V) is mediated by inverse photophobic reactions which occur when the shadow of the eyespot periodically

TABLE III

A PARTIAL SUMMARY OF RECENT PERTINENT LITERATURE ON EYESPOT ULTRASTRUCTURE

Date	Author	Organism	Type*	Contribution
1963	Lang	Volvocacean species	A	Observed multistigmatic cells; suggested hyaline area between eyespot and chloroplast as possible "refractive lens" seen by early workers.
1965	Lembi and Lang	Carteria	A	Reported 2 (or 3) rows of granules in eyespot.
1965	Lembi and Lang	Chlamydomonas	A	Reported one layer of granules; suggested that eyespot granules are chemically modified osmiophilic granules from chloroplasts.
1967	Arnott and Brown	Tetracystis excentrica	A	Reported a thickened zone of the plasmalemma outside eyespot which could contain photoreceptor pigments.
1967	Walne and Arnott	Euglena granulata	C	Observed microtubules in close association with eyespot granules; one or several granules surrounded by membranes.
1967	Walne and Arnott	Chlamydomonas eugametos	A	Observed thickened plasmalemma as in Tetracystis.
1967	Mornin and Francis	Nematodinium armatum	E	Observed that eyespot consists of lens, pigment cup and retinoid.

TABLE III (continued)

Date	Author	Organism	Type*	Contribution
1967	Belcher and Swale	Chromulina placentula	B	Observed that eyespot is in a short process of the chloroplast and is adjacent to a short flagellum.
1969	Dodge and Crawford	Glenodinium foliaceum	D	Identified a lamellar body similar in structure to outer segments of retina.
1971	Walne	Euglenoid flagellates (4 species)	C	Examined 4 euglenoid flagellates with EM and light microscopy and found that eyespot intermittently shades parabasal body.
1971	Hibberd	Chrysamoeba radians	-	Observed no eyespot but reported characteristic structural relation between vestigial flagellum and chloroplast.
1971	Belcher and Swale	Phaeaster pascheri	A (?)	Reported that short flagellum is modified as photoreceptor.
1972	Belcher and Swale	Chrysococcus cordiformis	-	Observed no association between second flagellum and chloroplast and no pigment globules in eyespot.
1972	Kivic and Vesk	Euglenoid flagellates	-	Found eyespot ultrastructure altered by cycloheximide; red-orange pigment sensitive to chloramphenicol; all or most stigma globules contained within a common envelope.
1972	Hibberd and Leedale	Eustigmatophycean species	C	Described new algal class, Eustigmatophyceae, which contains motile cells with large eyespot of Type C.

*According to Dodge, (1969).

TABLE IV

A PARTIAL SUMMARY OF PERTINENT LITERATURE ON EUGLENOID PIGMENTS

Date	Author	Organism	Pigment Identified	Contribution
1960	Krinsky and Goldsmith	Euglena gracilis	Major: β-carotene antheraxanthin neoxanthin Minor: γ-carotene cryptoxanthin echinenone euglenonone hydroxyechinenone xanthophyll mon-epoxide	Found no astaxanthin, astacene, or vitamin A.
1963	Green	Trachelomonas volvocina	β-carotene astaxanthin ketocarotenoids lutein violaxanthin neoxanthin	Found astaxanthin in form of an ester and suggested that astaxanthin production similar to that of metazoans.
1964	Batra and Tollin	Isolated eyespots from Euglena	lutein cryptoxanthin β-carotene unknown	Isolated eyespot granules by centrifugation.
1967	Wolken	Euglena species	-	Observed pigmented structures called "hematochrome flecks" in old Euglena cultures.

TABLE V

PROPOSED TERMINOLOGY FOR ORGANISMAL RESPONSES TO STIMULATION (DIEHN, 1973)

Date	Author	Type of Response	Stimulus Intensity	Change in Degree or Direction of Response	Examples*
		Kinesis: Change in Linear Velocity			
1969a	Diehn	Inverse	Decrease (Increase)	Increase (Decrease)	Photokinesis: Euglena
1972	Berg and Brown	Direct	Increase (Decrease)	Increase (Decrease)	Chemokinesis: Escherichia coli
		Phobic Response: Nondirectional Shock Reaction			
1883	Englemann	Inverse	Decrease	Stop/(backup)/ spin	Photophobic: Bacterium photometricum
1969	Naitoh and Eckert	Direct	Increase	Stop/(backup)/ spin	Thigmophobic: Paramecium
		Taxis: Orientation and Directed Motion			
1969b	Diehn	Negative	High	Away from source	Phototaxis: Euglena
1972	Berg and Brown	Positive	Low	Toward source	Phototaxis: Chlamydomonas

*The prefixes photo-, chemo-, and thigmo- refer to stimulation by light, chemical, and mechanical means, respectively.

falls upon the photoreceptor proper (Diehn, 1973). Hand, Forward, and Davenport (1967) cited evidence for a single pigment system, with one pigment shifting from a blue- to a red-absorbing state. The red system may act as a "cocking" device for phototaxis, while the response itself is "triggered" by blue light.

The location of the photoreceptor molecule(s) and the functions of the eyespot are both controversial in the following ways:

(1) Is the eyespot the primary photoreceptor? This theory is based on the fact that the action spectrum for phototaxis is similar to the absorption spectra for eyespot carotenoids (Wolken, 1971). Although no direct connection between the eyespot and flagellum has been observed, Walne and Arnott (1967) and Walne (1971) observed in different species of Euglena that microtubules are associated with the base of the reservoir, where the flagella are inserted and the eyespot is located. If the eyespot is the primary photoreceptor, then microtubules could provide a link between the eyespot and flagellum. Wolken (1971) calculated that the energy absorbed by carotenoids in the eyespot of Euglena is sufficient to cause forward movement by the flagellum.

(2) Is the eyespot a shading device for the paraflagellar swelling at the base of the emergent flagellum

in Euglena? Leedale (1967) reviewed the evidence for this theory, but no one has identified a pigment in the swelling. This theory would only hold true for organisms having both a close association of the eyespot-flagellum and a paraflagellar swelling.

(3) Is the photoreceptor located in a membrane--the plasmalemma or the flagellar membrane? Arnott and Brown (1967) observed that in Tetracystis, the eyespot was situated to shade a thickened zone of the plasmalemma. Walne and Arnott (1967) observed a similar thickened zone in the plasmalemma in Chlamydomonas. Bouck (1970) suggested that in Fucus sperm, the paraflagellar swelling and the eyespot are secondary devices which focus light and shade a plasma membrane-bound photoreceptor.

Although there is information on eyespot ultrastructure, their chemical components are not well characterized. With the development of a technique for large-scale isolation of eyespot granules from Euglena (Bartlett, Walne, Schwarz and Brown, 1972), the biochemistry of the eyespot can now be investigated more readily. The research presented in this thesis concerns the characterization of lipids from isolated eyespots of Euglena gracilis var. bacillaris. Although there have been studies on euglenoid lipids (Table VI), none has dealt exclusively with those of the eyespot. Information gained from this

TABLE VI

A PARTIAL SUMMARY OF PERTINENT LITERATURE ON EUGLENOID LIPIDS

Date	Author	Contribution
1962	Erwin and Bloch	Identified a large amount of ∝-linolenate in light-grown Euglena but little, if any, in dark-grown cells.
1963	Rosenberg	Observed that fatty acids of green Euglena were mainly unsaturated, whereas in etiolated cells, they were mostly saturated fatty acids of fewer than 17 carbons.
1964	Korn	Identified 51 fatty acids; concluded that Euglena gracilis synthesizes ∝-linoleic and ∝-linolenic acid and thus has a fatty acid biosynthesis of both plants and animals.
1964	Hulanicka et al.	Observed accumulation of C_{20}, C_{22}, and C_{24} polyunsaturated fatty acids in heterotrophic cells; also observed that fatty acid composition of Astasia longa was similar to that of etiolated Euglena.
1964	Guehler et al.	Found that Euglena gracilis var. bacillaris, when grown on a medium containing ethanol, produced a wax composed of esters of long, straight-chain acids and alcohols, with a high percentage of odd-numbered acids (15.4%) and odd-numbered alcohols (20.1%).
1965	Nagai and Bloch	Concluded that photoauxotrophic Euglena synthesizes monounsaturated fatty acids by modifications of two mechanisms that occur in nonphotosynthetic organisms.
1968	Erwin	Found that the major saturated fatty acids in all strains of Euglena were myristic and palmitic; the major monounsaturated fatty acids were oleic and hexadecenoic.

study, together with other biochemical, physiological and ultrastructural studies on eyespots, hopefully will lead to a better understanding of the intriguing phenomenon of light perception in these unicellular organisms.

CHAPTER II

MATERIALS AND METHODS

I. CULTURE METHODS

Cultures of Euglena gracilis var. bacillaris were grown in 5-liter toxin bottles, each containing 7 g Difco Euglena broth (Appendix A-1) dissolved in 800 ml distilled water. After the medium was sterilized, approximately 100 ml inoculum of cells were added to each of ten bottles that were then turned on sides and placed on an Eberbach reciprocating shaker. Cells were kept in suspension by gentle agitation (70 complete strokes/min). Cultures were grown at 20-22° C for 5-7 days under constant illumination with General Electric warm-white fluorescent bulbs (800-1000 ft-c).

Cultures were checked for bacterial contamination by inoculation of aliquots into proteose peptone and thioglycollate media two days prior to harvesting. Only axenic cultures were used in subsequent procedures.

II. ISOLATION AND PURIFICATION OF EYESPOT GRANULES

Cells were harvested at 5° C in a Sharples Superspeed centrifuge, Type T-1, at 35,000 rpm. The sedimented material was suspended immediately in 0.25 M sucrose

buffered with pH 7.0 0.05 M potassium phosphate, containing EDTA and cysteine (5 ml/g wet weight of cells). The composition of the phosphate buffer can be found in Appendix A-2.

Cells suspended in the buffered sucrose solution were ruptured in the cold (5° C) with a Gaulin press at 4,000 psi. The eyespot fraction was separated from the homogenate by sucrose density gradient centrifugation in a Beckman Model L3-50 ultracentrifuge equipped with a Ti-15 zonal rotor (Bartlett, et al. 1972). The following gradient was loaded from the edge of the rotor at 2,000 rpm: (1) an overlay of 300 ml 0.05 M phosphate buffer (Appendix A-2), (2) 1,000 ml of sample in 8.5% (0.25 M) phosphate-buffered sucrose, (3) a cushion of 365 ml of 30% unbuffered sucrose. After spinning for 2 hr at 20,000 rpm (39,827 x g) the rotor was slowed to 2,000 rpm for unloading. Fifteen milliliter fractions were collected from the center of the rotor in test tubes that were covered immediately with aluminum foil to prevent photooxidation and were stored at 0° C.

Fractions from the zonal rotor that contained large concentrations of eyespots were further purified (described below) either by a single centrifugation (Centrifugation Step 1) or by two sequential centrifugations (Step 1 followed by Step 2) in a Beckman Model L3-50

ultracentrifuge equipped with an SW 27.1 (16.6 r in cm) swinging bucket rotor.

Centrifugation Step 1

The following gradient was added to each of three centrifuge tubes (35-ml capacity): (1) a cushion of 13 ml of 30% unbuffered sucrose, (2) 9 ml of eyespot fraction with the sucrose concentration increased to 10%, and (3) 7 ml of 0.05 M phosphate buffer overlay (Appendix A-2). The prepared tubes were centrifuged for 2 hr at 22,000 rpm (63,423 x g). The eyespot fraction, which banded at the interface of the sucrose and buffer overlay, was collected with a Pasteur pipette and dispensed into small screw-cap glass vials. The vials were wrapped in aluminum foil and stored at 0° C.

Centrifugation Step 2

The sucrose concentration of the eyespot fraction collected in Centrifugation Step 1 was increased to 15%, and the fractions were re-centrifuged in the following gradient: (1) a cushion of 19 ml of 30% unbuffered sucrose, (2) 4 ml of the eyespot fraction, and (3) 4 ml of 0.05 M phosphate buffer overlay (Appendix A-2). Centrifugation, collection, and storage were the same as for Centrifugation Step 1.

III. LIPID ANALYSES

All procedures and assays were carried out at least twice. Similar results were obtained each time.

Sources of Materials and Reagents

Cholesterol ester, fatty acid, fatty alcohol, triacylglycerol, cholesterol and phospholipid standards used in thin-layer chromatography were purchased from Supelco, Inc. Methyl esters for gas-liquid chromatography were obtained from Applied Science Laboratories. Alcohol acetates for gas-liquid chromatography were prepared by acetylation of fatty alcohols from Supelco, Inc.

Silica Gel G and Silica Gel HR used in the preparation of thin-layer chromatoplates were purchased from Brinkman Instruments, Inc.

Eastman Organic Chemicals supplied Vitride [$NaAlH_2(OCH_2CH_2OCH_3)2$] and Sigma Chemical Co. supplied Folin-Ciocalteau reagent. Diethyl ether (anhydrous grade) from Fischer Scientific Co. and ether (anesthesia grade) from Mallinckrodt Chemical Works were found to contain impurities (Figure 1). Diethyl ether (glass-distilled) from Burdick and Jackson Laboratories was free from impurities and was thus used in lipid analyses.

Extraction of Lipids

Lipids from crude and purified eyespot samples were

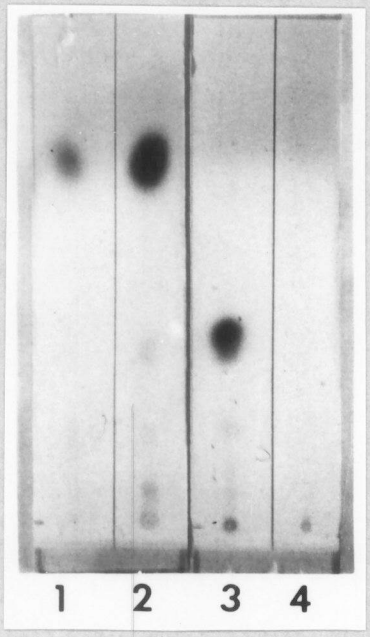


Figure 1. Thin-layer chromatogram of impurities found in solvents. Lanes 1 and 2 show impurities in ether anhydrous grade (Fischer Scientific Co.) and ether anesthesia grade (Mallinckrodt Chemical Works), respectively. Lane 3 reveals impurity found in chloroform (Burdick and Jackson Laboratories, Inc.) from bottle without teflon-lined cap. Lane 4 shows no impurity (i.e., no spot) in glass-distilled ether (Burdick and Jackson Laboratories, Inc.).

Adsorbent was Silica Gel G with development in solvent system I.

extracted by either of two methods: (1) a modified procedure of Bligh and Dyer (1959) (Appendix B-1) or (2) a modified procedure of Folch, Lees, and Stanley (1957) (Appendix B-2). The weight of the extract was determined on a Cahn G2 electrobalance.

Separation of Lipids by Thin-Layer Chromatography

Analysis of nonpolar lipids. Total lipid extracts were separated into major classes of lipids according to the nature of their functional groups (e.g., esters, acids, alcohols) on layers of Silica Gel G (see Appendix C-1 for information on preparation of chromatoplates). Nonpolar lipid standards were chromatographed with the eyespot lipids under identical conditions. The plates were developed in several different solvent systems: (1) system I (80:20:1-hexane:diethyl ether:acetic acid), (2) system II (70:30:5:1-hexane:diethyl ether:methanol: acetic acid), and (3) system III (98:2:1-chloroform: methanol:acetic acid) (see Appendix C-2 for information on solvent systems). After development, components resolved on the chromatogram were visualized by first spraying the plate with concentrated sulfuric acid, then by charring for 20 min in an oven at 180° C.

Analysis of phospholipids. Total eyespot lipid

extracts were applied to two thin-layer chromatoplates layered with Silica Gel HR(B) (see Appendix C-1 for information on preparation of plates). Commercially available phospholipids were used as standards. One plate was developed in solvent system IV (50:25:8:2-chloroform:methanol:acetic acid:water) and the other in solvent system V (60:35:5-chloroform:methanol: ammonium hydroxide) (Appendix C-2). Visualization was the same as that described for nonpolar lipids.

Isolation of lipid components by preparative thin-layer chromatography. When total eyespot lipid extracts were applied to Silica Gel G layers and developed in solvent system I (Appendix C-2), four main components were detected. These components, designated Fractions I-IV, were isolated by preparative thin-layer chromatography (see Appendix C-3 for general procedure).

Chemical Fractionation of Lipids and Analysis of Products by Thin-Layer Chromatography

Saponification of ester-linked lipids produces fatty acids and free alcohols. Total lipid extracts and lipid fractions thereof (designated Fractions I-IV), separated by thin-layer chromatography, were saponified according to the procedure outlined in Appendix D-1. The products were analyzed by thin-layer chromatography on Silica Gel G in solvent system I (Appendix C-2).

Vitride [$NaAlH_2$ $(OCH_2CH_2OCH_3)_2$] reduces fatty acid esters to the corresponding fatty alcohols. Total lipid extracts and lipid fractions thereof (I-IV) were reacted with Vitride and were analyzed according to the method of Snyder, Blank, and Wykle (1971) (Appendix D-2).

Lipid fractions I-IV were each methylated by the procedure outlined in Appendix D-3. The methyl esters of the fatty acids from each fraction were purified by preparative thin-layer chromatography (Appendix C-3).

Methylation of Fraction 1 produced three products: methyl esters, fatty alcohols, and an unknown component. The fatty alcohols were acetylated by the procedure outlined in Appendix D-4. The unknown component, thought to be a pigment, was chromatographed along with β-carotene on a Silica Gel G layer in solvent system VI (95:5-hexane:diethyl ether).

Gas-Liquid Chromatography of Methyl Esters of Fatty Acids and Acetates of Fatty Alcohols

The methyl esters of fatty acids obtained from Fractions I-IV were dissolved in carbon disulfide. Aliquots from each sample were injected into a Model 4000 Victoreen Gas Chromatograph equipped with a dual flame ionization detector. The methyl esters were separated on columns (6 ft x 1/8 in) of 10% EGSS-X (organosilicone

polymer of ethylene succinate and methyl silicones) on Gas-Chrom P, 100/120 mesh, with a helium flow of 20-30 ml/min. Chromatography was carried out at 155° C or 185° C or by using temperature programming from 140-200° C (2°/min). Methyl ester standards of 14:0, 16:0, 18:0, 18:1, 18:2, 20:0, 22:0, 20:4, 24:0, 24:1, and 22:6 were used to plot retention time graphs. In the designation of fatty acids and their methyl esters or fatty alcohols and their acetates, the first number refers to the number of carbon atoms in the molecule and the second number to the number of double bonds.

Alcohol acetates obtained from the acetylation of the fatty alcohols of Fraction 1 were chromatographed at 175° C. Retention time graphs were based on data obtained with 14:0, 16:0, and 18:0 fatty alcohol standards.

The area of each of the peaks in the chromatograms of the total methyl esters and acetates was calculated by 1/2 x base x height in order to determine relative percentages of each component.

Separation of Saturated and Unsaturated Methyl Esters of Fatty Acids by Chromatography and Subsequent Gas-Liquid Chromatography

To obtain more information about the fatty acid composition, the methyl esters were separated according to degree of unsaturation on Silica Gel G layers impregnated

with 12.5% $AgNO_3$. Methyl esters obtained from Fraction III were applied in lane 1 and a mixture of the following methyl ester standards was chromatographed in lane 2: 14:0, 16:0, 18:0, 18:1, 18:2, 20:0, 20:4, 22:0, 24:1 and 22:6. The plate was developed in solvent system VII (90:10-hexane:diethyl ether) (Appendix C-2). After development, lane 1 was covered with a clean glass plate, and components in lane 2 were sprayed with 2'4'dichlorofluorescein and visualized under ultraviolet light.

The components in lane 1 representing the saturates, the monoenes, and the dienes were each scraped into fritted funnels, then eluted with 20% methanol in ether. The filtrate was added to hexane and washed with water. The hexane-ether was evaporated, and the methyl esters were dissolved in 50 µl carbon disulfide.

Saturated and monounsaturated methyl esters from Fraction III were analyzed by gas-liquid chromatography at 150° C.

IV. PROTEIN DETERMINATION

The amount of protein in 1 ml of purified eyespot sample was measured by a modified method of Lowry, Rosebrough, Farr and Randall (1951) (Appendix D-5). Bovine albumin was used as a standard in the following amounts: 100 µg, 50 µg, and 25 µg.

V. QUANTITATIVE DETERMINATION OF PHOSPHORUS

Although as little as 1 μg of phospholipid in a sample can be detected by thin-layer chromatography, a more precise test for phospholipids is the method of Rouser, Siakotos, and Fleischer (1966) (Appendix D-6) which measures the amount of inorganic phosphate in a lipid sample. Total eyespot lipids, along with known amounts of K_2PO_4 and distilled water blanks, were examined for phosphorus content.

CHAPTER III

RESULTS

The results indicate that lipids of eyespot granules of Euglena gracilis var. bacillaris consist mainly of non-polar lipids that contain a wide variety of fatty acids ranging from 12:0 to 22:6. Although the presence of polar lipid constituents is suggested, phospholipids were not detected by the experimental procedures used in this study.

Characterization of Constituents from Total Lipid Extracts

Presence of pigments in eyespot lipid extracts. Extracts of total lipids of eyespots were dark orange, suggesting the presence of pigments. When large amounts of lipid extracts (up to 2 mg) were analyzed by thin-layer chromatography in a nonpolar solvent system, pigments were immediately detectable as a series of colored bands from the origin to the solvent front. The pigments varied in polarity and it was not possible to separate them completely from the other eyespot lipids. The characterization of eyespot lipid pigments constitutes another research problem, so no attempts were made to study them in the present thesis.

Presence of nonpolar lipids in eyespots. Four lipid

components were detected as faint spots by chromatography in nonpolar solvent system I (Figure 2, lanes 1-5). The only components that could be tentatively identified were free fatty acids. Amounts of total lipids in lanes 1-5 varied, so quantitative comparisons could not be made. No qualitative differences in lipid class composition were apparent in lipids extracted by the methods of Bligh and Dyer (1959) or of Folch, et al. (1957) (Figure 2, lanes 2 and 5). Thawing and refreezing of eyespot samples before lipid extraction had no effect on major components of eyespot lipids (Figure 2, lanes 1 and 3).

To resolve the more polar lipids that remained near the origin (Figure 2), eyespot lipids were chromatographed in solvent systems II (Figure 3) and III (Figure 4). In both systems, polar constituents remained at the origin; fatty acids were also present. Remaining components could not be identified because of the small quantity of lipid that was chromatographed. When the amount of lipid applied to silica gel layers was increased, the results indicated that cholesterol esters or waxes, triacylglycerols, free fatty acids and more polar lipids were present in eyespot lipids (Figure 5).

Analysis of lipid derivatives revealed the presence of nonpolar lipids in eyespots. Vitride reduction produced fatty alcohols (Figure 6, lane 4) and saponification

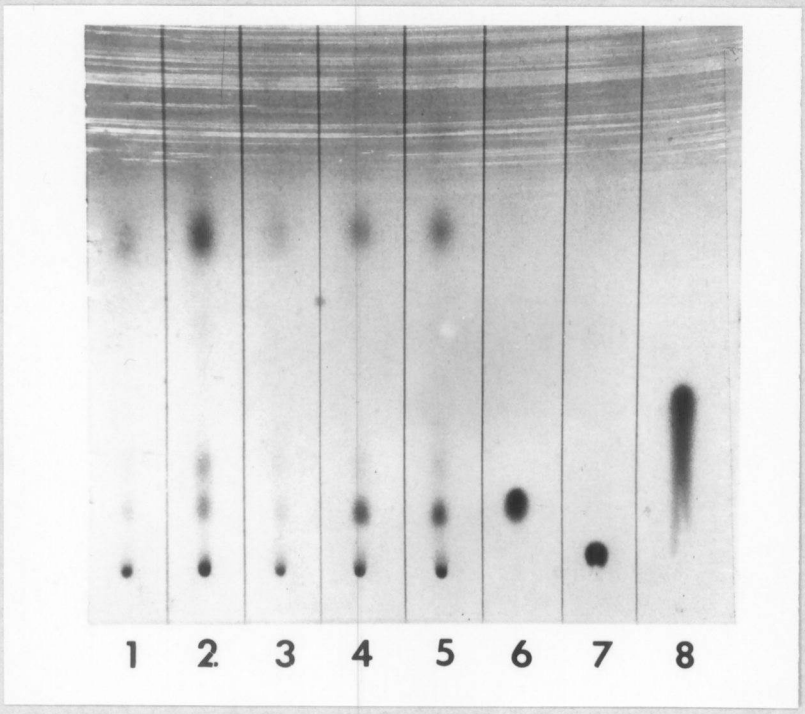


Figure 2. Thin-layer chromatogram of total eyespot lipid extracts (lanes 1-5). Lipids extracted by the Bligh and Dyer method (lanes 1, 2, and 4) showed the same four major components as those extracted by the Folch method (lanes 3 and 5). No differences were evident in lipids extracted from refrozen sample (lane 1). Standards were oleic acid (lane 6), cholesterol (lane 7) and triolein (lane 8). Adsorbent was Silica Gel G with development in solvent system I.

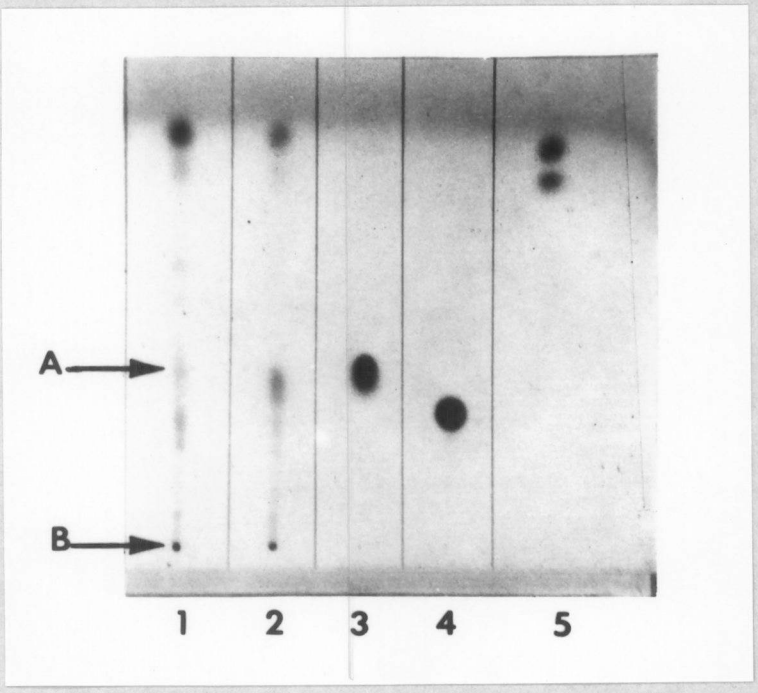


Figure 3. Thin-layer chromatogram of total eyespot lipid extracts (lanes 1 and 2) indicating fatty acids (A) and polar lipids (B). Standards were oleic acid (lane 3), cholesterol (lane 4) and triolein (lane 5). Adsorbent was Silica Gel G with development in solvent system II.

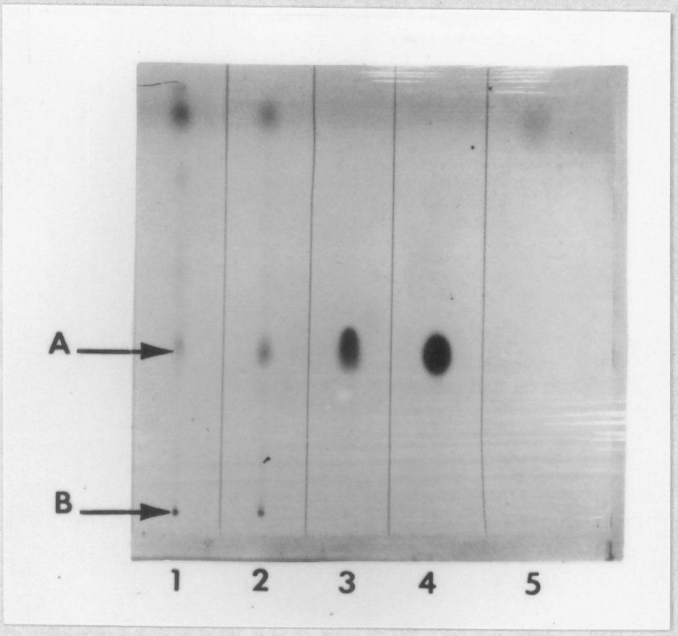


Figure 4. Separation of total eyespot lipid extracts on Silica Gel G in solvent system III. Fatty acids (A) and polar lipids (B) were evident in eyespot lipids (lanes 1 and 2). Standards were oleic acid (lane 3), cholesterol (lane 4) and triolein (lane 5).

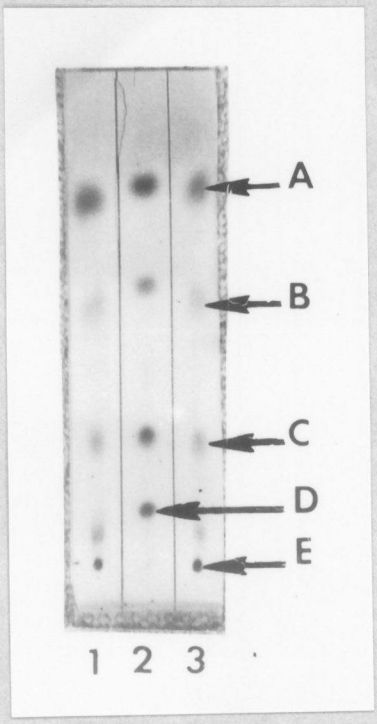


Figure 5. Thin-layer chromatogram of lipids from purified eyespot sample (lanes 1 and 3). Sample, when compared with standards in lane 2, appeared to contain cholesterol ester or wax (A), triacylglycerol (B), free fatty acids (C), and polar lipids (E). Standards in lane 2 also contained cholesterol (D), which appeared to be absent from sample. Adsorbent was Silica Gel G with development in solvent system I.

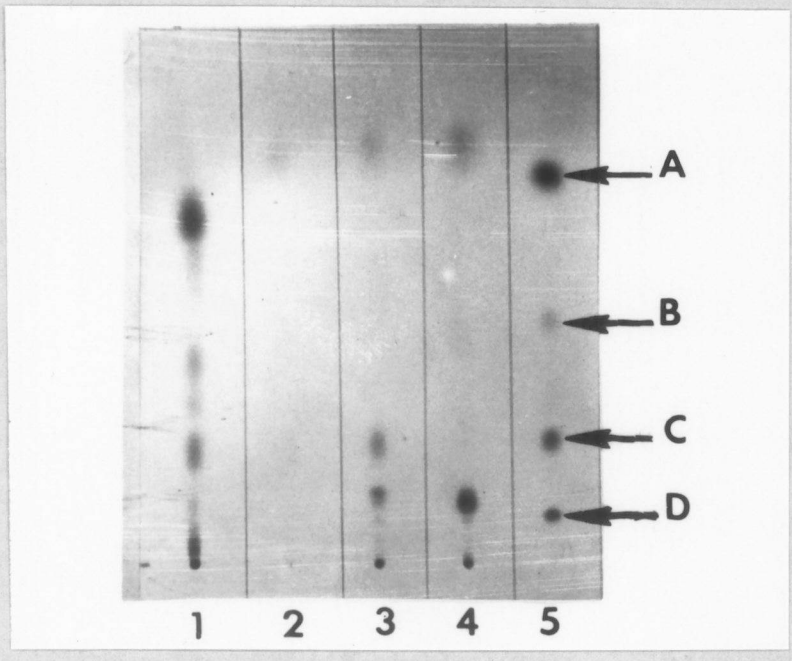


Figure 6. Thin-layer chromatogram of products formed after saponification and Vitride reduction of total eyespot lipid extracts. Fatty alcohols were products after Vitride reduction (lane 4) and fatty acids were products after saponification (lane 2), indicating that esters were present in eyespot lipids.

Also shown are total eyespot lipids (lane 1) and standards (lane 5), cholesterol oleate (A), triolein (B), oleic acid (C), and cholesterol (D). The presence of fatty acids in the nonsaponifiable products (lane 3) was due to incomplete extraction.

The saponifiable products in lane 2 were too dilute; thus, they were concentrated and re-chromatographed in Figure 7.

Adsorbent was Silica Gel G with development in solvent system I.

producted fatty acids (Figure 6, lane 2 and Figure 7, lane 1), demonstrating that fatty acid esters were present in eyespot lipids.

Absence of phospholipids in eyespots. Phospholipids in total eyespot lipids were not detected by thin-layer chromatography in either an acid (Figure 8) or a basic (Figure 9) phospholipid system. The colorimetric phosphorus assay (Appendix D-6) indicated the presence of phosphorous, but the data are considered to be inconclusive and untenable because an insufficient amount of eyespot lipid was available.

Effect of further purification on eyespot lipids. When lipid extracts of crude and purified eyespot samples were analyzed by thin-layer chromatography, no major qualitative differences were found (Figure 10). Because varying amounts of eyespot lipids were applied to the plates, no quantitative differences could be determined.

Determination of the amount of lipids in eyespots. The quantity of lipids extracted from one milliliter of purified eyespot sample was based on the protein content. The lipid weight was 0.062 μg of lipid/μg protein.

Characterization of Constituents from Lipid Classes

Because eyespot lipids were composed of several

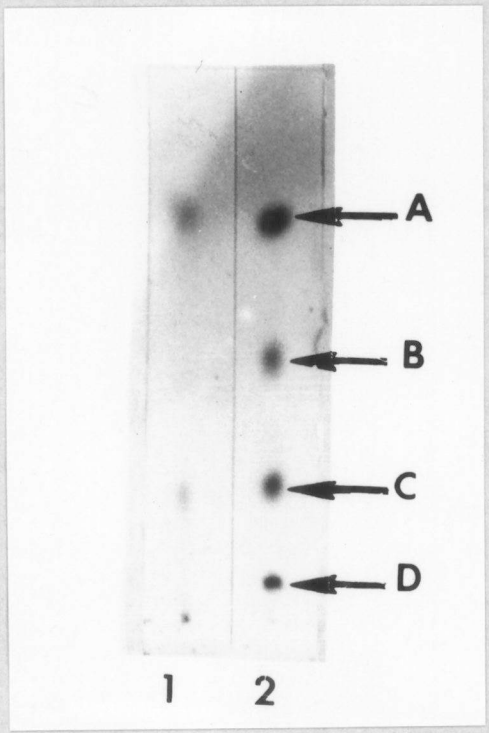


Figure 7. Thin-layer chromatogram of products after saponification of total eyespot lipid extracts showing fatty acids (lane 1) (C). Conditions and standards were the same as for Figure 6, page 31.

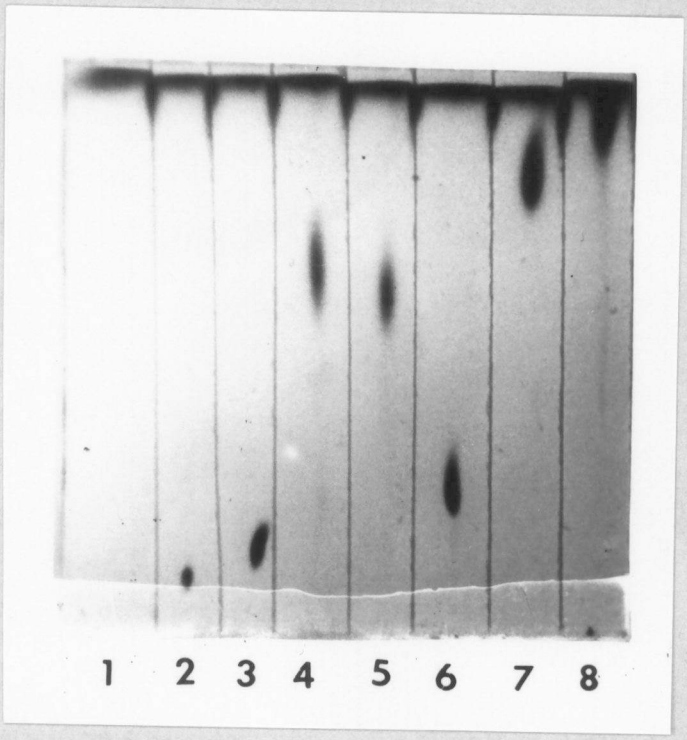


Figure 8. Thin-layer chromatogram of eyespot lipid extracts (lane 1) showing absence of phospholipids. Standards were lysophosphatidylcholine (lane 2), sphingomyelin (lane 3), phosphatidylserine (lane 4), phosphatidylinositol (lane 5), phosphatidylcholine (lane 6), phosphatidylethanolamine (lane 7), phosphatidic acid (lane 8). Adsorbent was Silica Gel HR(B) with development in solvent system IV.

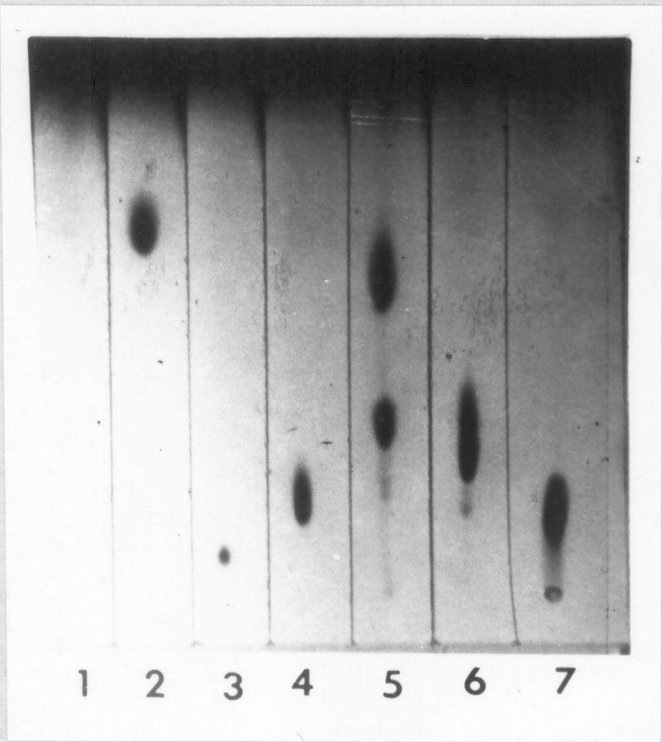


Figure 9. Separation of eyespot lipids on Silica Gel HR(B) in solvent system V. No phospholipids were evident in sample (lane 1). Standards were fatty acid (lane 2), lysophosphatidylcholine (lane 3), sphingomyelin (lane 4), phosphatidylserine (lane 5, lower spot), phosphatidylethanolamine (lane 5, upper spot), phosphatidylinositol (lane 6) and phosphatidic acid (lane 7).

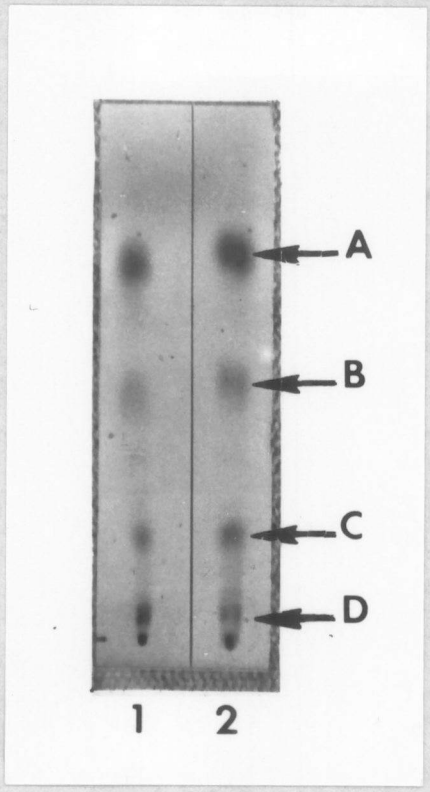


Figure 10. Thin-layer chromatogram of total lipids from crude (lane 1) and purified eyespot preparations (lane 2) showing no major differences in four components: cholesterol ester or wax (A), triacylglycerol (B), free fatty acids (C) and polar lipids (D). Adsorbent was Silica Gel G with development in solvent system I.

different classes, it was necessary to isolate and analyze each class separately. Lipids were separated into four fractions in solvent system I (Figure 11, lane 2). After isolation by preparative thin-layer chromatography, components in each fraction were saponified and reduced with Vitride.

Fraction I Constituents

The saponifiable products revealed a spot at the fatty acid R_f (Figure 12, lane 3); the nonsaponifiable products contained a distinct spot between the cholesterol and fatty acid R_f's, suggesting the presence of fatty alcohols (Snyder, personal communication). The appearance of fatty alcohols in the saponifiable products (Figure 12, lane 3) was due to incomplete extraction of nonsaponifiable components. Because fatty acids and fatty alcohols were components of Fraction I after saponification, Fraction I represented wax esters.

Fraction II-IV Constituents

The saponifiable products of Fractions II-IV (Figure 12, lanes 5, 7, and 9, respectively) also consisted of fatty acids, suggesting that each of the fractions contained esters. Further evidence that esters were components in each fraction was the presence of fatty alcohols after Vitride reduction (Figure 13, lanes 2-5).

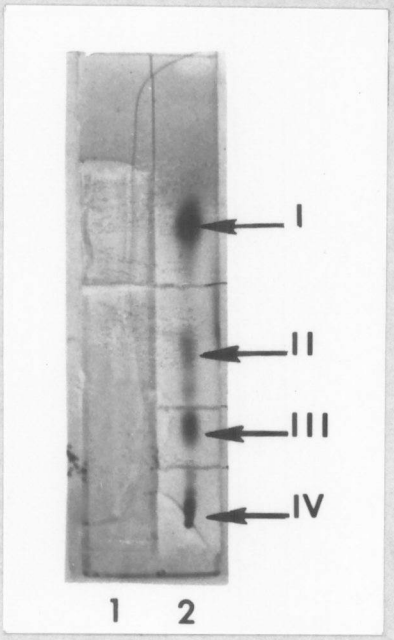


Figure 11. Thin-layer chromatogram of total eyespot lipid extracts (lane 2) showing Fractions I-IV which were isolated by preparative thin-layer chromatography.

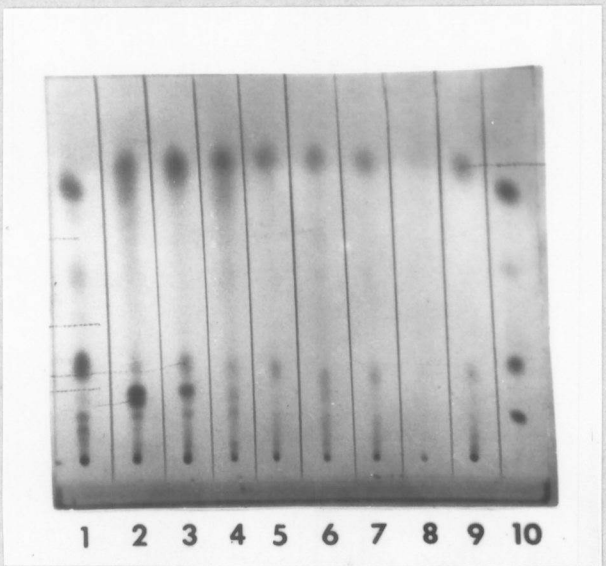


Figure 12. Thin-layer chromatogram of products formed after saponification of Fractions I-IV. Fatty acids were saponifiable products of Fractions I-IV (lanes 3, 5, 7, and 9, respectively). Fatty alcohols appeared to be nonsaponifiable products of Fraction I (lane 2). Lanes 4, 6, and 8 contained nonsaponifiable products of Fractions II, III, and IV.

Also shown are total eyespot lipid extracts (lane 1) and standards as in Figure 6, page 31. Upper spot in lanes 2-9 was solvent impurity (see Figure 1, page 18).

Adsorbent was Silica Gel G with development in solvent system I.

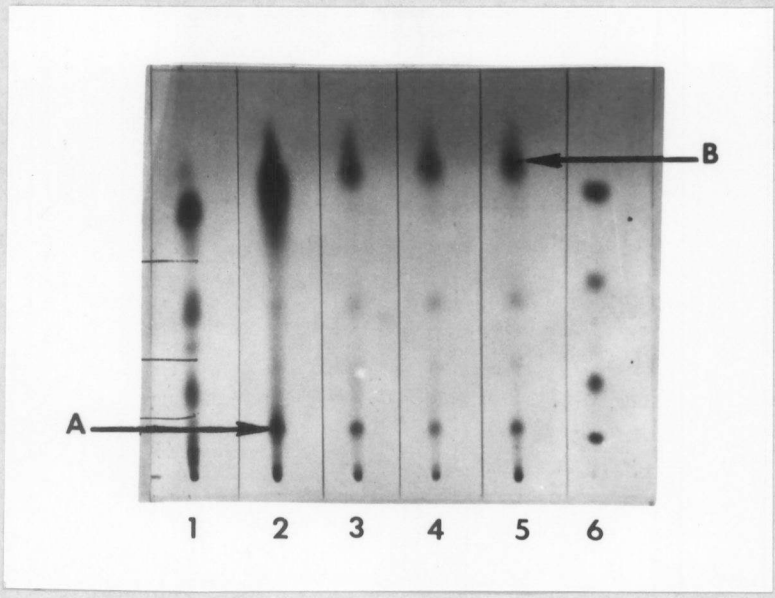


Figure 13. Thin-layer chromatogram of products formed after Vitride reduction of Fractions I-IV (lanes 2-5, respectively). All four fractions contained fatty alcohols (see arrow A), indicating that esters were present in eyespot lipids. Lane 1 contained total eyespot lipids and lane 6 contained standards as in Figure 6, page 31. Upper spot in lanes 2-5 (arrow B) was solvent impurity (see Figure 1, page 18).

Adsorbent was Silica Gel G with development in solvent system I.

Characterization of Methyl Esters and Alcohol Acetates by Gas-Liquid Chromatography

Components in each fraction were methylated to obtain methyl esters of fatty acids for gas-liquid chromatography (Figure 14, lanes 1-4). Components derived after methylation of Fraction I were methyl esters, fatty alcohols, and a component of unknown composition near the solvent front (Figure 14, lane 1). Acetylation of the fatty alcohols of Fraction I produced alcohol acetates (Figure 15, lane 1) for characterization by gas-liquid chromatography. In solvent system I, the unknown component from Fraction I (Figure 14, lane 1) had an R_f similar to a hydrocarbon. Since β-carotene is a hydrocarbon found in eyespots (Batra and Tollin, 1964), the R_f of the unknown component was compared to the R_f of β-carotene by thin-layer chromatography. The results indicated that the unknown component was not β-carotene (Figure 16).

Fraction I Constituents

Gas-liquid chromatograms of methyl esters of fatty acids and alcohol acetates of fatty alcohols derived from Fraction I (wax fraction) are shown in Figures 17 and 18. Constituents of these fractions are summarized in Tables 7 and 8. Although 27 peaks are discernible in Figure 17,

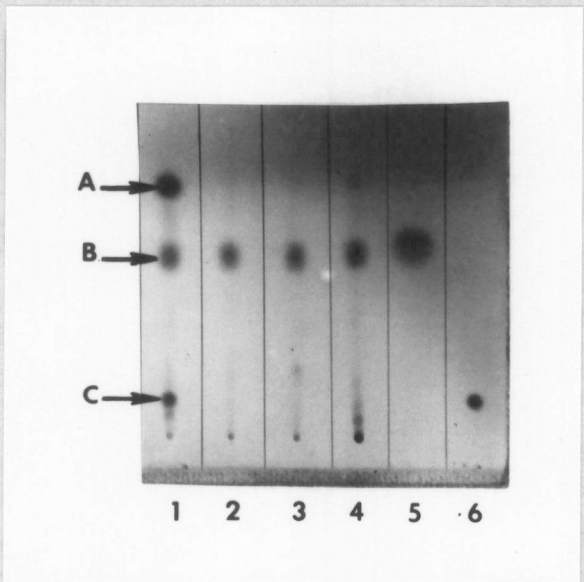


Figure 14. Thin-layer chromatogram of products formed after methylation of Fractions I-IV (lanes 1-4). Fraction I (lane 1), in addition to methyl esters of fatty acids (B), contained an unknown component (A) and fatty alcohols (C). Standards in lanes 5 and 6 were methyl oleate and hexadecanol. Adsorbent was Silica Gel G with development in solvent system I.

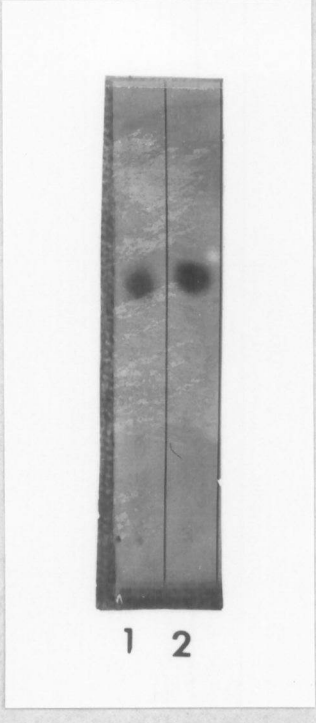


Figure 15. Thin-layer chromatogram of alcohol acetates prepared from the fatty alcohols isolated from Fraction I (lane 1) with standard shown in lane 2. Adsorbent was Silica Gel G with development in solvent system I.

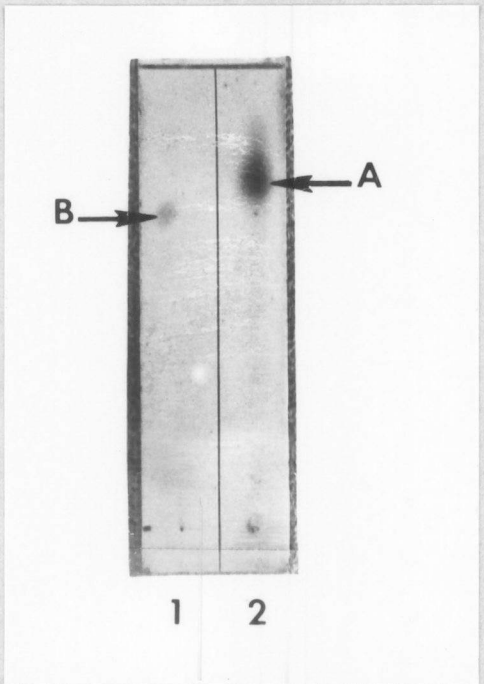
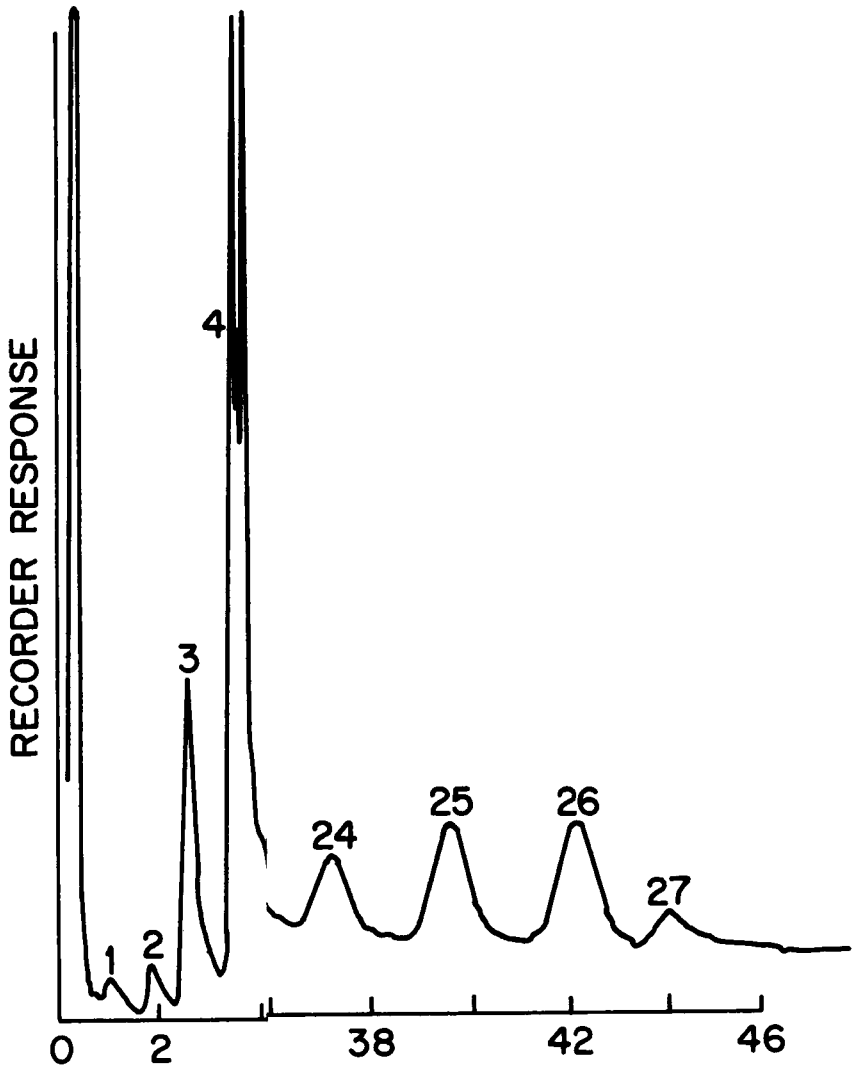


Figure 16. Thin-layer chromatogram showing that unknown component of Fraction I (A) did not have same R_f as β-carotene (B), an eyespot pigment. Adsorbent was Silica Gel G with development in solvent system VI.



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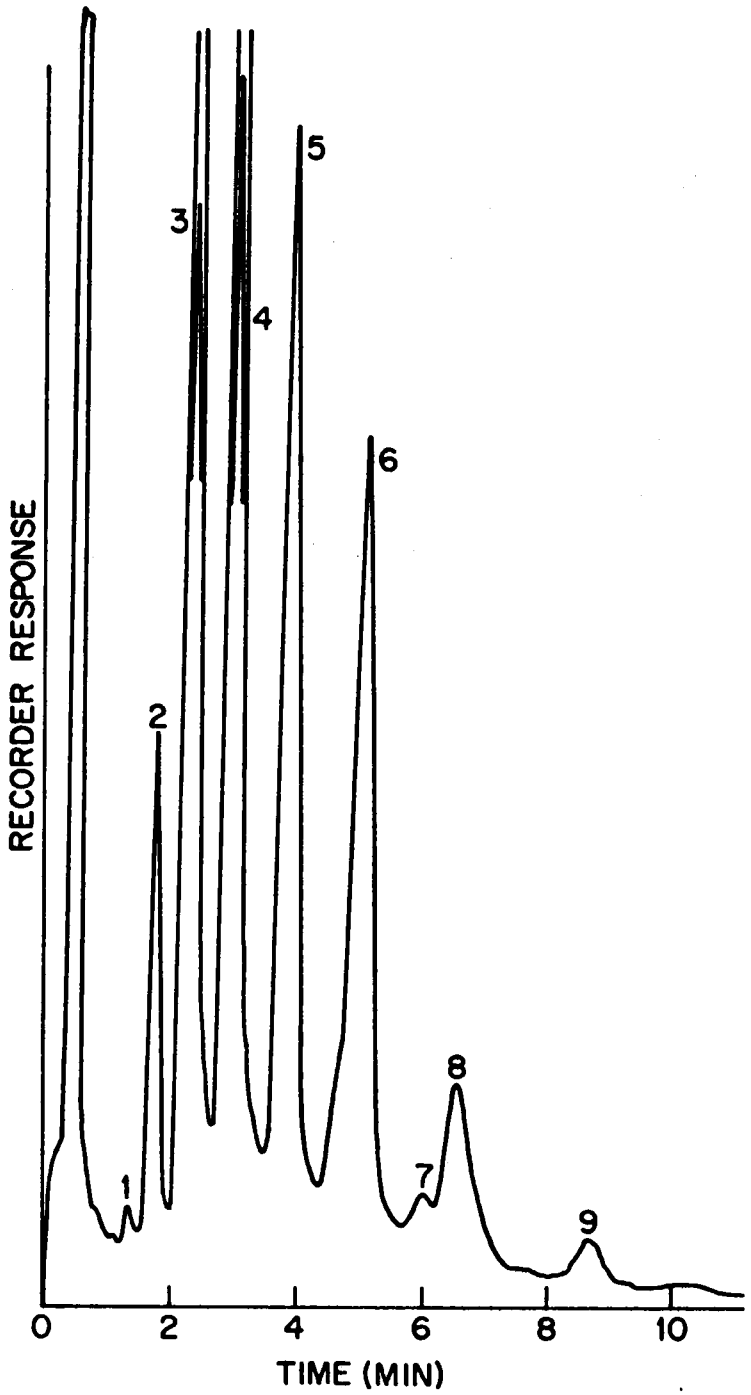


Figure 18. Gas-liquid chromatogram of the alcohol acetates of fatty alcohols derived from Fraction I (waxes). Peak components are identified in Table VIII, page 48. Chromatography was carried out on columns, 6 ft x 1/8 in, packed with 10% EGSS-X coated on Gas-Chrom P at a temperature of 175° C.

TABLE VII

PERCENTAGES OF FATTY ACIDS, DETERMINED AS METHYL ESTERS, IN THE FRACTION I (WAXES) OF EYESPOT LIPIDS

Peak	Fatty Acid	Percent of Total
1	?	0.12
2	?	0.33
3	12:0	2.43
4	13:0	10.33
5	14:0	17.27
6	15:0	2.43
7	16:0	9.12
8	16:1	2.34
9	17:0	0.70
10	16:2	0.63
11	17:1	0.24
12	18:0	6.88
13	18:1	4.05
14	?	1.45
15	18:2	1.91
16	?	1.45
17	18:3	3.16
18	20:2	2.48
19	20:3	0.91
20	20:4	8.86
21	22:2 or 20:5	1.27
22	22:2 or 20:5	7.66
23	24:1	3.92
24	24:2 or 22:5	1.92
25	24:2 or 22:5	3.04
26	22:6	3.89
27	?	1.00

TABLE VIII

PERCENTAGES OF ALCOHOLS, DETERMINED AS ACETATES, IN FRACTION I (WAXES) OF EYESPOT LIPIDS

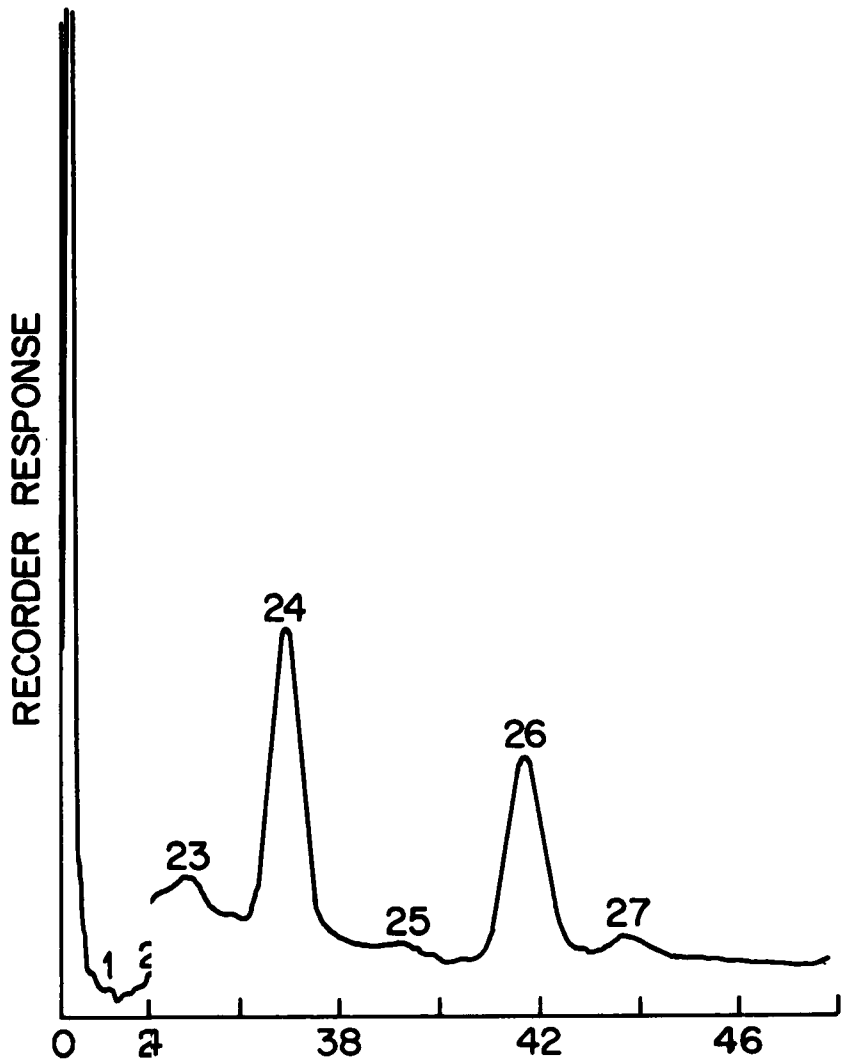
Peak	Fatty Alcohol	Percent of Total
1	11:0	0.35
2	12:0	4.95
3	13:0	20.90
4	14:0	34.65
5	15:0	15.82
6	16:0	15.50
7	?	1.12
8	17:0	5.10
9	18:0	1.57

chromatograms such as these are difficult to evaluate since one peak could possibly contain more than one component. The identification of the peaks is tentative and based solely on comparison with retention times of known standards, which were chromatographed under identical conditions.

The mixture of fatty alcohols in Fraction I (waxes), as shown in Figure 18, page 46, is not as complex as the methyl esters from the same fraction. Only the saturated fatty alcohols from 11:0 to 18:0 appear to be present, with 14:0 being the major component.

Fraction II-IV Constituents

Chromatograms of methyl esters of fatty acids derived from Fraction II (triacylglycerols), Fraction III (free fatty acids) and Fraction IV (polar lipids) are shown in Figures 19, 20, and 21. Constituents of each fraction are identified in Tables IX, X, and XI. The fatty acid pattern from the three fractions (Fractions II-IV) is similar to that found for the fatty acids of the wax fraction (Fraction I), with 27-29 peaks being evident in each. Although the similarity suggests that the fatty acid components from each fraction are identical, it is not known whether the peaks in the four chromatograms represent the same components. A detailed study of each fraction was not made, and the complexity of the fractions



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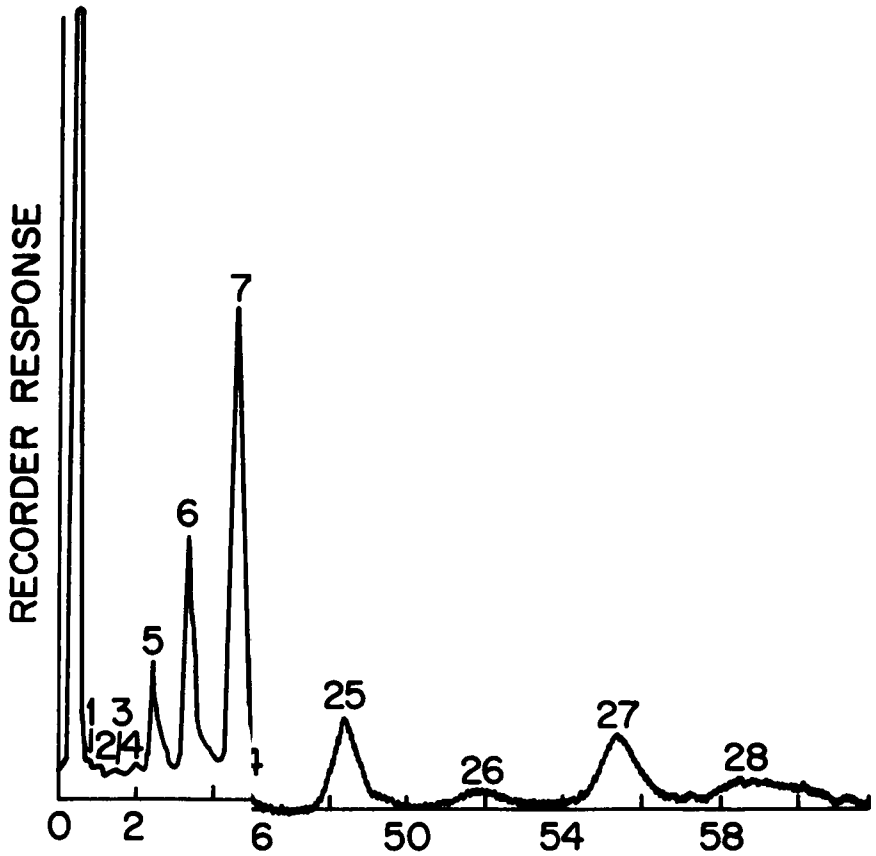
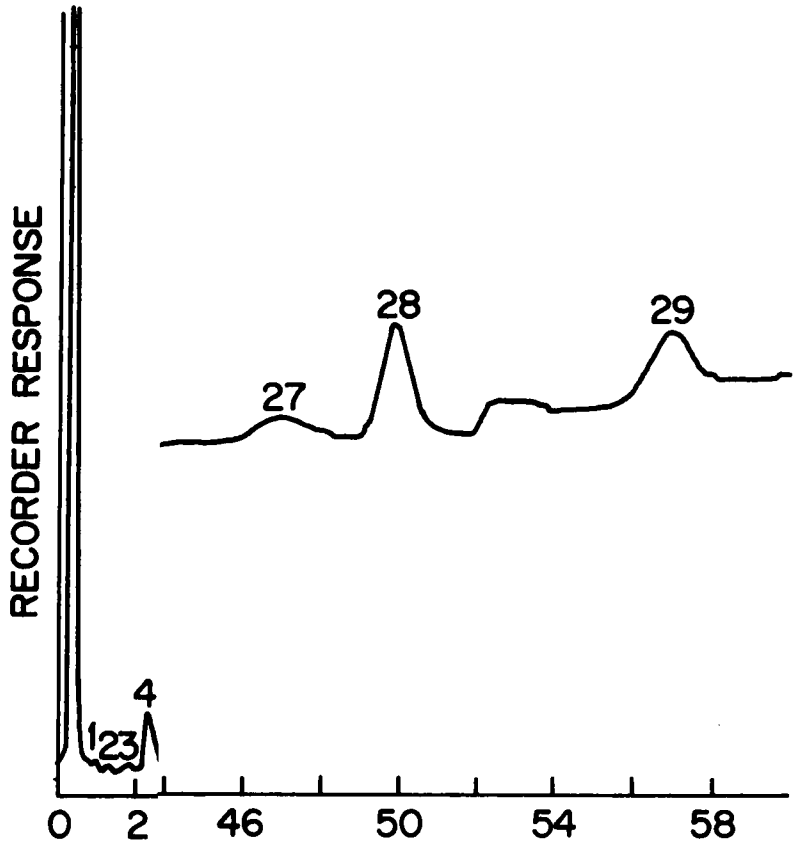


Figure III
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out on colif 155°
and 200° C.



raction IV
(polar same as
Figure

TABLE IX

PERCENTAGES OF FATTY ACIDS, DETERMINED AS METHYL ESTERS, IN FRACTION II (TRIACYLGLYCEROLS) OF EYESPOT LIPIDS

Peak	Fatty Acid	Percent of Total
1	?	0.04
2	?	0.18
3	12:0	1.74
4	13:0	4.82
5	14:0	13.66
6	15:0	2.52
7	16:0	9.67
8	16:1	4.84
9	17:0	0.34
10	16:2	0.23
11	17:1	0.45
12	18:0	3.10
13	18:1	8.92
14	18:2	2.39
15	?	1.08
16	18:3	1.70
17	20:2	6.21
18	20:3 (?)	0.71
19	20:3 (?)	0.71
20	20:4	10.51
21	22:2 or 20:5	1.02
22	22:2 or 20:5	8.42
23	24:1	2.39
24	24:2 or 22:5	7.68
25	24:2 or 22:5	0.34
26	22:6	6.38
27	?	0.95

TABLE X

PERCENTAGES OF FATTY ACIDS, DETERMINED AS METHYL ESTERS, IN FRACTION III (FREE FATTY ACIDS) OF EYESPOT LIPIDS

Peak	Fatty Acid	Percent of Total
1	?	0.01
2	?	0.34
3	?	0.34
4	?	0.13
5	12:0	1.31
6	13:0	3.89
7	14:0	10.39
8	15:0	2.51
9	16:0	12.88
10	16:1	3.18
11	17:0	0.86
12	16:2	0.10
13	17:1	0.86
14	18:0	5.20
15	18:1	10.91
16	?	2.07
17	18:2	2.85
18	18:3	5.10
19	20:2	4.93
20	20:3	1.21
21	20:4	8.41
22	22:2 or 20:5	1.20
23	22:2 or 20:5	6.00
24	22:4	2.00
25	24:2 or 22:5	6.20
26	24:2 or 22:5	0.97
27	22:6	6.20
28	?	4.71

TABLE XI

PERCENTAGES OF FATTY ACIDS, DETERMINED AS METHYL ESTERS, IN FRACTION IV (POLAR LIPIDS) OF EYESPOT LIPIDS

Peak	Fatty Acid	Percent of Total
1	?	0.03
2	?	0.03
3	?	0.09
4	12:0	1.24
5	13:0	1.49
6	14:0	7.47
7	?	0.18
8	15:0	3.19
9	16:0	16.45
10	16:1	4.36
11	17:0	1.49
12	16:2	0.77
13	17:1	1.55
14	18:0	3.11
15	18:1	9.81
16	18:2	3.27
17	?	1.40
18	18:3	3.81
19	20:2	4.20
20	?	0.66
21	20:3	3.36
22	20:4	8.83
23	?	0.43
24	22:2 or 20:5	1.40
25	?	0.49
26	22:2 or 20:5	4.05
27	22:4	3.50
28	24:2 or 22:5	6.68
29	22:6	6.51

made absolute identifications on the basis of retention times untenable.

To facilitate the identification of the components in Fraction III (free fatty acids) (Figure 20, page 51), the methyl esters of the fatty acids were first separated according to degree of unsaturation on $AgNO_3$-impregnated Silica Gel G chromatoplates and then each subfraction was characterized by gas-liquid chromatography. The results are shown in Figures 22 and 23. Constituents are identified in Tables XII and XIII. Logarithms of retention times of saturated and monounsaturated fatty acids fell on a straight line when plotted against the number of carbon atoms in the molecule (Figure 24) with the exception of components of peak 1 (Figure 23) which was unidentified.

In summary eyespot lipids contain a complex mixture of saturated and mono-, di-, tri-, tetra-, penta-, and hexa-unsaturated fatty acids ranging from 12:0 to 22:6 and saturated fatty alcohols ranging from 11:0 to 18:0.

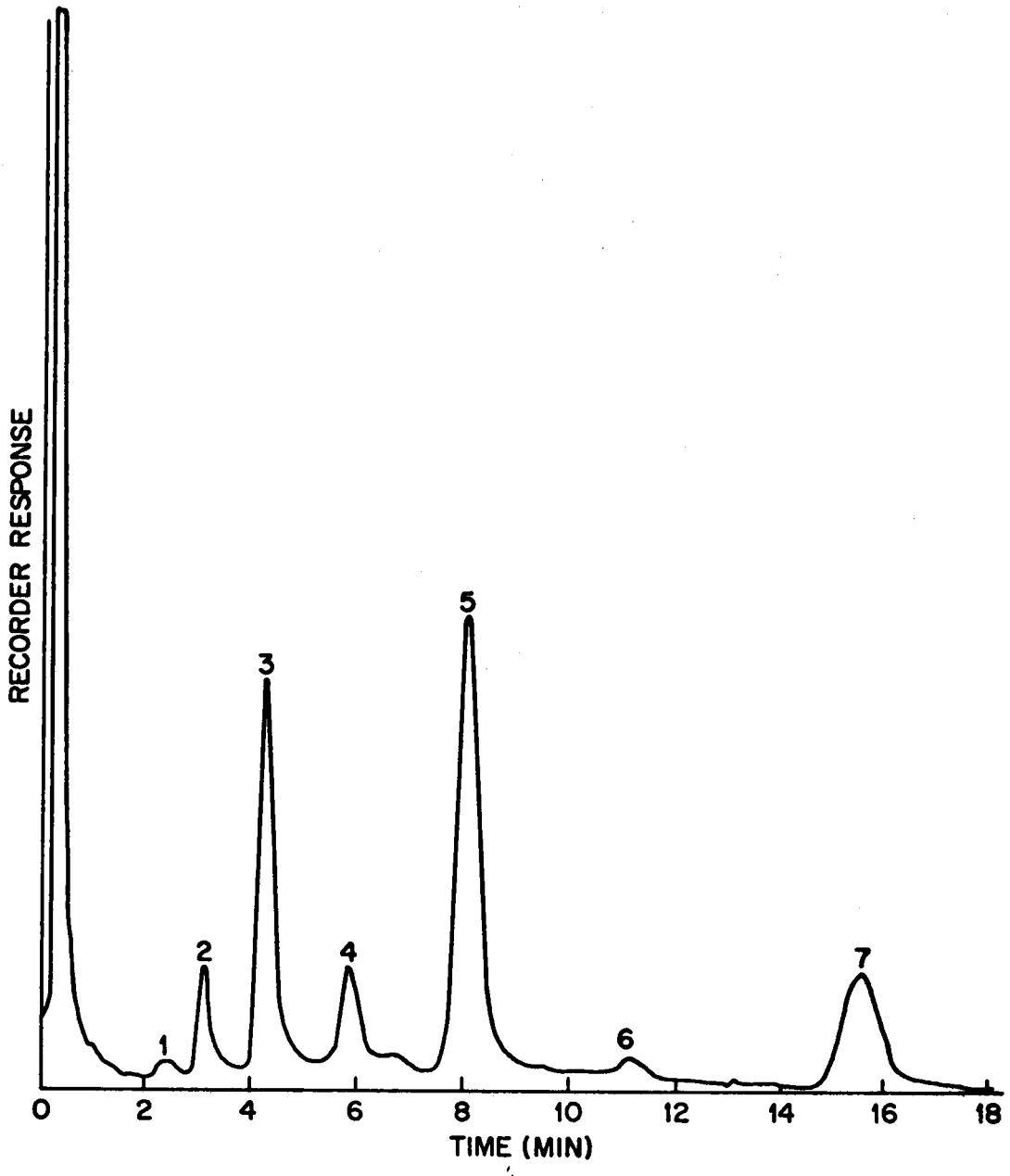


Figure 22. Gas-liquid chromatogram of the methyl esters of saturated fatty acids derived from Fraction III (free fatty acids). Peak components are identified in Table XII, page 59. Conditions were the same as Figure 20, page 51.

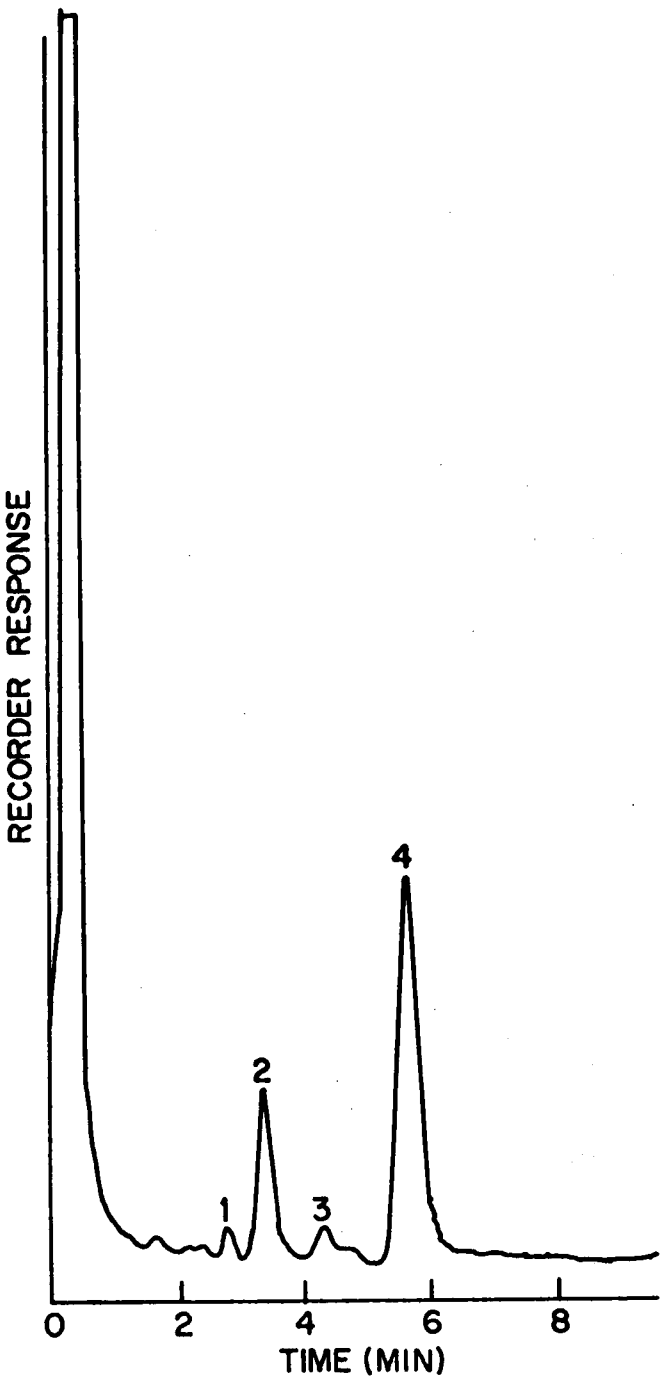


Figure 23. Gas-liquid chromatogram of methyl esters of monounsaturated fatty acids derived from Fraction III (free fatty acids). Peak components are identified in Table XIII, page 60. Conditions were the same as Figure 20, page 51, except the temperature was 185° C.

TABLE XII

PERCENTAGES OF SATURATED FATTY ACIDS, DETERMINED AS METHYL ESTERS, IN FRACTION III (FREE FATTY ACIDS) OF EYESPOT LIPIDS*

Peak	Fatty Acid	Percent of Total
1 (5)	12:0	1.53
2 (6)	13:0	4.99
3 (7)	14:0	23.80
4 (8)	15:0	7.67
5 (9)	16:0	41.84
6 (11)	17:0	2.23
7 (14)	18:0	17.91

*Peak numbers indicated parenthetically refer to the peaks in Figure 20, page 51.

TABLE XIII

PERCENTAGES OF MONOUNSATURATED FATTY ACIDS, DETERMINED AS METHYL ESTERS, IN FRACTION III (FREE FATTY ACIDS) OF EYESPOT LIPIDS*

Peak	Fatty Acid	Percent of Total
1	?	2.21
2 (10)	16:1	19.92
3 (13)	17:1	5.90
4 (15)	18:1	71.95

*Peak numbers indicated parenthetically refer to the peaks in Figure 20, page 51.

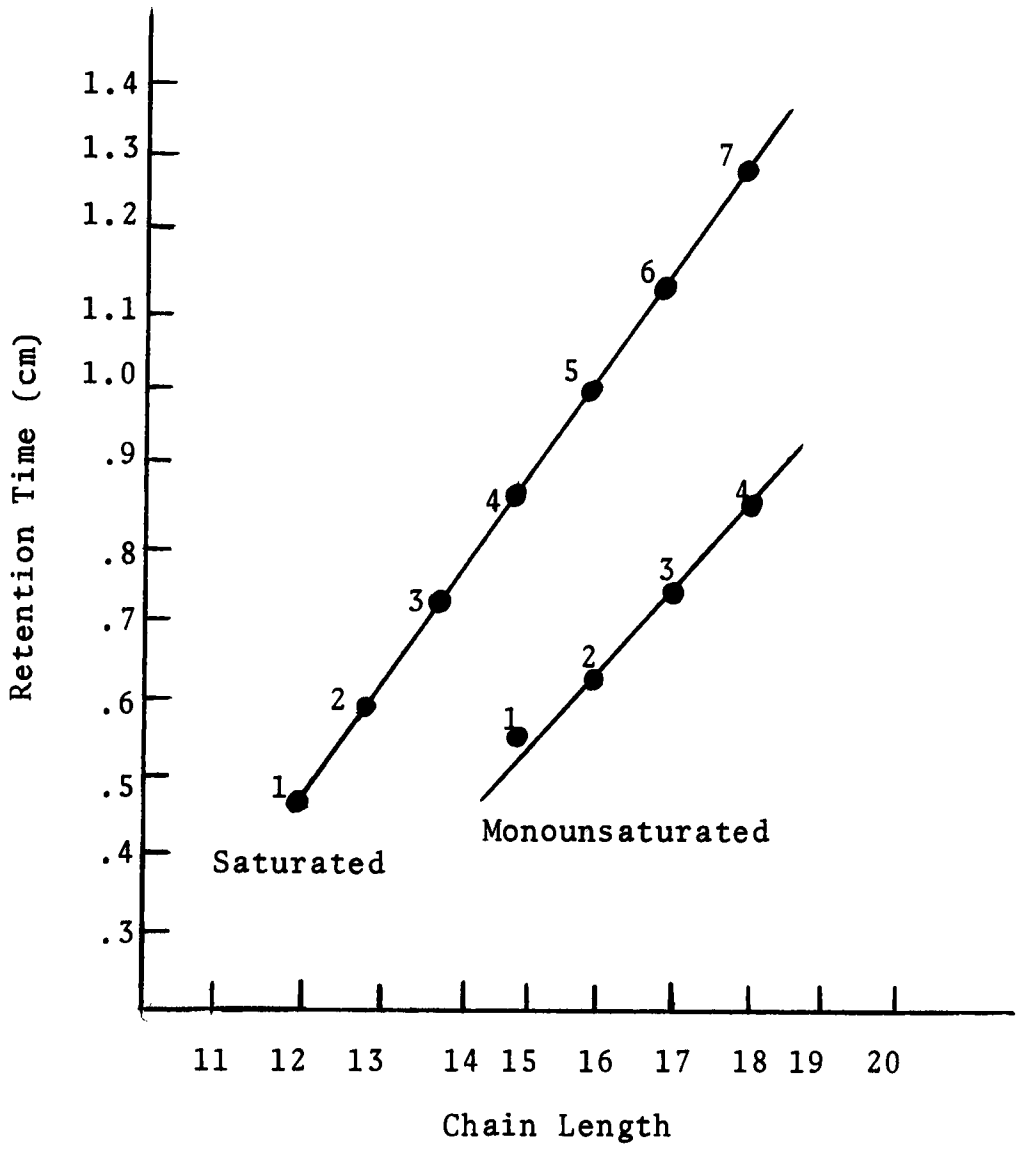


Figure 24. Semilogarithmic plot of the retention times of the methyl esters of the saturated and monounsaturated fatty acids derived from Fraction III (free fatty acids). Number 1 in the monounsaturated series does not fall on the straight line and could not be identified.

CHAPTER IV

DISCUSSION

Although the difference between plants and animals is sometimes difficult to define, especially among unicellular organisms, the two groups have been delineated on the basis of their different fatty acid compositions. Higher plants characteristically synthesize ∝-linolenic acid (18:3), whereas animals synthesize polyunsaturated fatty acids of 20 or more carbons, such as arachidonic acid (20:4). Euglena has the ability to grow photosynthetically in the light or heterotrophically on synthetic media in the dark. It combines both the plant-like ability to synthesize 18:3 fatty acid and the animal-like ability to form 20:4 and longer-chain, more highly unsaturated fatty acids (Korn, 1964).

Moreover, Euglena also synthesizes the widest variety of fatty acids yet reported in either plants or animals (Korn, 1964). Over 50 different fatty acids from light-grown cells of Euglena gracilis 'Z' were identified by Korn (1964). These 50 fatty acids were isolated from an initial group of 29 constituents that were detected by gas-liquid chromatography. The 29 constituents reported by Korn coincide, at least upon superficial examination, with the 27-29 fatty acids that

have been detected in Euglena eyespot lipids. Additional analysis of the 27-29 peaks may reveal that eyespots also are composed of more complex mixtures of fatty acids.

Studies of the wax fractions of Euglena indicate that waxes are formed under a variety of conditions. Guehler (1964) reported that cultures of Euglena gracilis var. bacillaris grown in a medium containing ethanol produce a wax of saturated fatty acids and fatty alcohols from 12:0 to 18:0, with 14:0 the major component in both. Rosenberg (1967) showed that waxes extracted from Euglena gracilis grown in the dark consisted of fatty acids and fatty alcohols of mainly 12:0 and 13:0. Khan and Kolattukudy (1973) reported that in cell-free preparations of etiolated Euglena gracilis, the major fatty alcohols in the wax esters produced by microsomes and mitochondria were 16:0 and 14:0, respectively. The present data indicate that 14:0 is the major fatty alcohol in the wax fraction of eyespots. The fatty acid composition of the wax component from eyespots is, however, more complex than that reported previously for the wax obtained from extracts of whole cells of Euglena. In the latter, only fatty acids ranging from 12:0 to 18:0 were found, whereas in eyespots, fatty acids ranging from 12:0 to 22:6 have been detected.

In attempting to define the role of waxes in

Euglena, Rosenberg (1967) also reported that wax reserves were rapidly depleted when well-nourished etiolated organisms were transferred to a strictly mineral medium in the dark. Addition of glucose to the medium slowed the disappearance of waxes in starving organisms. Thus, he concluded that waxes served as storage products for metabolic energy. Inferences may be drawn from this observation to the effect that waxes, along with triaclyglycerols in the eyespot, could perhaps function in a similar capacity.

Eyespot granules of Euglena, as seen by electron microscopy, are surrounded by membranes (Walne and Arnott, 1967); however, phospholipids, which are components of membranes, were not detected in this analysis of eyespot lipids. If phospholipids are present, then they are in amounts too minute to be detected by the thin-layer chromatographic and colorimetric phosphorus methods used in this study.

Pigments from vertebrate retinal cells also contain little or no phospholipids (Young, 1970). Although the analogy between the eyespot of Euglena and the vertebrate eye may seem remote, there are two striking similarities: (1) both contain carotenoid pigments [lutein, cryptoxanthin and β-carotene in the eyespot (Batra and Tollin, 1964) and vitamin A aldehyde in the retina] and (2) both are

associated with flagellar-like structures [the eyespot with the flagella (Walne and Arnott, 1967) and rods and cones of the eye with flagellar-like fibrils which connect the inner segment of the retina with outer segments containing photoreceptor pigments (Young, 1970)]. Although these parallels in composition and structure have been demonstrated, parallels in function between the eyespot and the vertebrate eye have yet to be documented.

The function of polyunsaturated fatty acids in Euglena was examined by Rosenberg (1967). He found that in starving, dark-grown cells, arachidonic and other polyunsaturated fatty acids concentrate in the pellicle, thus providing an expanded membrane system receptive to the influx of hydrated metabolites from the surrounding environment. It is possible that the polyunsaturated fatty acids in eyespots are components of membranes surrounding eyespot granules. Rosenberg also reported that in photosynthesizing organisms, arachidonic acid was found only in chloroplasts. The fact that arachidonic acid is found in eyespots could support the theory that eyespots are evolutionary remnants of chloroplasts and/or the theory that eyespot granules originate in the chloroplast (Lembi and Lang, 1965; Walne and Arnott, 1967). Other support for these theories may come from further

analysis of the polar lipid constituents in the eyespot. Although the polar lipids were not identified in this study, it is possible that they are glycolipids or sulfolipids, which are found predominantly in chloroplasts (Salisbury and Ross, 1969).

Examination of fractions of purified eyespot granules by electron microscopy showed no contamination by other organelles (Bartlett, et al. 1972). It is possible, however, that some lipids from other parts of the cells, released during fractionation or subsequent procedures, were collected with the eyespot fraction and analyzed along with eyespot components. Because pigments are extracted with other lipids, it is also possible that some of the fatty acids identified in this study are components of lipid pigments. This possibility can be neither accepted or negated until the chemical composition of the pigments is known.

Because the isolation and purification of eyespot granules are time-consuming procedures, it is usually more convenient to store the isolated eyespots at 0° C until further analysis; thus, it is necessary to determine if eyespot composition is altered by storage conditions. Bartlett, et al. (1972) found that freezing had no effect on eyespot ultrastructure or on absorption properties. The results in this study indicate that

thawing and refreezing do not seem to affect the major lipid components as revealed by thin-layer chromatography. This procedure, however, separates only major lipid classes and does not yield information on specific molecular species within each class. Additionally, even though comparative ultrastructure of purified and crude eyespot preparations reveal the latter to contain fragments of membranes (Bartlett, et al. 1972), no differences in major lipid components of the two preparations were revealed by thin-layer chromatography. More detailed analyses of fatty acid structure and composition by gas-liquid chromatography and other analytical techniques (e.g., infra-red or mass spectroscopy) may reveal more components in crude eyespot fractions than in purified preparations.

In summary, this investigation revealed that eyespot granules contain waxes, triacylglycerols, free fatty acids and polar lipids (excluding phospholipids) with at least 27-29 different fatty acids ranging from 12:0 to 22:6. Saturated fatty alcohols from 11:0 to 18:0 are present in the wax fraction. Future studies on the function of these lipids, together with information on eyespot structure, on organelle interrelationships and on other chemical constituents present in the eyespot, may reveal the role of the eyespot in photoreception.

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APPENDICES

APPENDIX A

MEDIUM AND BUFFER COMPOSITIONS

APPENDIX A-1

Composition of Difco Euglena broth medium (Greenblatt and Schiff, 1959)*

COMPONENT	AMOUNT
KH_2PO_4	0.04 %
$CaCO_3$	0.02 % (as Ca)
$(NH_4)_2HPO_4$	0.02 %
L-Glutamic acid	0.5 %
dl-Malic acid	0.2 %
$MgSO_4 \cdot 7H_2O$	0.05 %
$FeCl_3 \cdot 6H_2O$	0.5 mg % (as Fe)
Thiamine·HCl	0.1 mg %
Vitamin B_{12}	0.02 µg %
Trace metals solution containing the following per liter	2 ml per 100 ml
$ZnSO_4 \cdot 7H_2O$	4.4 g
$MnSO_4 \cdot 4H_2O$	4.08 g
$Na_2MoO_4 \cdot 2H_2O$	1.008 g
$CoCl_2 \cdot 6H_2O$	0.0807 g

*The components constitute the powdered medium prepared by Difco. In the routine preparation of the medium in the laboratory, the directions on the bottle were followed.

$CuSO_4 \cdot 5H_2O$	0.039 g
H_3BO_3	0.0283 g
NaI	0.00118 g

pH of the medium is 3.3 at 25° C.

APPENDIX A-2

Composition of phosphate buffer (Bartlett, et al. 1972)

Potassium phosphate	0.05 M
Cysteine	0.002 M
EDTA	0.001 M

pH was adjusted to 7.0 with 4 N NaOH.

APPENDIX B

LIPID EXTRACTION PROCEDURES

APPENDIX B-1

Lipid extraction (Bligh and Dyer, 1959) (modification according to F. Snyder, personal communication)

1. Add 3 ml of the eyespot fraction to a vial (25-ml capacity) equipped with teflon-lined screw cap.*
2. Add 7.35 ml of methanol containing 2% acetic acid. Then add 3.67 ml of chloroform and shake vigorously. Allow to stand for 30-60 min.
3. Add 3.72 ml of chloroform, shake, and add 3.56 ml of distilled water.
4. Centrifuge at 1500 rpm for 3 min in an International Refrigerated Centrifuge. Three phases should develop: (1) a clear chloroform phase at the bottom; (2) a clear methanol-water phase at the top; and (3) a thin white salt-protein interphase.
5. Collect the chloroform phase with a syringe (10-ml capacity), leaving some chloroform behind. Extract again with 3 ml chloroform and centrifuge as in #4.
6. Repeat #5 and collect chloroform layer.
7. Pool the extracts into a pear-shaped flask. Remove most of the solvent in a rotary evaporator at room

*Only glass and teflon should be allowed to come in contact with the solvents and lipids.

temperature; residual amounts can be evaporated with nitrogen gas.

8. Dissolve the lipids in chloroform and transfer to a small glass vial. Seal the vial with teflon-lined screw cap and store at 0° C.

APPENDIX B-2

Lipid extraction (Folch, et al. 1957) (modification according to F. Snyder, personal communication)

1. Add the eyespot sample (5-10 ml) to a freeze-dry flask.
2. Freeze the sample in liquid nitrogen, making sure that a thin layer of material is distributed evenly on the inner surface of the flask.
3. Attach the flask to a freeze-dry unit, and allow it to remain until sample is dry.
4. Remove the flask and add 5 ml of methanol. Shake by hand for a few seconds.
5. Add 10 ml chloroform and shake sample at room temperature for 30 min on a mechanical shaker.
6. Filter the extract through Whatman No. 1 filter paper on a Buchner funnel. The filter paper should be wet with chloroform before filtering the lipids.
7. Repeat #4, 5, and 6 two more times.
8. Transfer the extract to a small beaker and add distilled water until beaker is nearly filled. Place the small beaker carefully into a 1-liter beaker of distilled water and allow it to stand overnight.
9. Remove the small beaker, carefully decanting part of

the contained water layer. The remaining water in the small beaker should be removed with a syringe.

10. Transfer the chloroform layer to a pear-shaped flask. Remove most of the solvent in a rotary evaporator; residual amounts can be evaporated with nitrogen gas.
11. Dissolve the lipids in chloroform and transfer to a small glass vial. Seal the vial with teflon-lined screw cap and store at 0° C.

APPENDIX C

THIN-LAYER CHROMATOGRAPHY PROCEDURES

APPENDIX C-1

Preparation of thin-layer chromatography plates (F. Snyder, personal communication)

I. Silica Gel G plates

1. Prepare a slurry using 30 g of Silica Gel G in 60 ml water. Apply a uniform layer of 250μ on glass plates (20 x 20 cm) using a modified Colab applicator (No. 2810).
2. Air dry chromatoplates for 30 min.
3. Next dry the chromatoplates for 15 or 30 min in an oven at 110° C.
4. Cool plates and store over a desiccant until ready for use.

II. Silica Gel HR(B) plates

1. Prepare a slurry of 40 g Silica Gel 60 HR in 80 ml of 0.001 M Na_2CO_3. The "B" in HR(B) refers to the base, Na_2CO_3, which is added to eliminate tailing of phospholipids during chromatography.
2. Follow the procedure as for Silica Gel G plates, except dry the HR(B) plates for 1 hr in an oven at 110° C.

III. Silver nitrate plates

1. Prepare a slurry of 30 g Silica Gel G in 60 ml of a solution of 12.5% silver nitrate ($AgNO_3$) in

28-30% ammonium hydroxide.

2. Follow the procedure as for Silica Gel G plates, except dry the $AgNO_3$ plates for 1 hr in an oven at 110° C.
3. Store the plates over a desiccant in the dark. Use within 3-4 days.

APPENDIX C-2

TABLE XIV

SOLVENT SYSTEMS USED IN THIN-LAYER CHROMATOGRAPHY (F. SNYDER, PERSONAL COMMUNICATION)

Solvent System	Components (v/v/v)	Thin-layer Plates	Major Use
I	Hexane:diethyl ether:acetic acid 80 : 20 : 1	Silica Gel G	Separation of cholesterol, triacylglycerols, fatty acids and other nonpolar lipids
II	Hexane:diethyl ether:methanol:acetic acid 70 : 30 : 5 : 1	Silica Gel G	Separation of lipids having an intermediate polarity
III	Chloroform:methanol:acetic acid 98 : 2 : 1	Silica Gel G	Separation of fatty acids and fatty alcohols
IV	Chloroform:methanol:acetic acid:water 50 : 25 : 8 : 2	Silica Gel HR(B)	Separation of phospholipids
V	Chloroform:methanol:ammonium hydroxide 60 : 35 : 5	Silica Gel HR(B)	Separation of phospholipids
VI	Hexane:diethyl ether 95 : 5	Silica Gel G	Separation of very nonpolar lipids
VII	Hexane:diethyl ether 90 : 10	Silica Gel G impregnated with $AgNO_3$	Separation of methyl esters of fatty acids according to degree of unsaturation

APPENDIX C-3

Preparative thin-layer chromatography (Silica Gel G)

(F. Snyder, personal communication)

1. With small amounts of sample, use 2 x 20 cm plates and apply sample in one lane of the plate and the standards in the other. With large amounts of sample, use 20 x 20 cm plates and apply standards in one lane and the sample as a series of spots along the origin on the remaining part of the plate.
2. Develop the plate in solvent system I (Appendix C-2, page 87).
3. After development, place a clean glass plate over the portion of the plate containing the sample. Then visualize the standards by one of the following methods:
   (a) Drop iodine crystals into a heated beaker on a hot plate in a fume hood. Hold the portion of the plate containing the standards over the iodine vapors and mark the brown spots that appear.
   (b) Spray the standards with 2'4'dichlorofluorescein and mark spots that can be visualized under ultraviolet light.

4. Using the standards as references, scrape each corresponding sample component into separate beakers (50-ml capacity) and add chloroform. Stir each with a clean glass rod to break up silica gel particles.
5. Pour the chloroform containing the silica gel into fritted funnels attached to a vacuum.
6. Elute with chloroform and then with 2:1 chloroform: methanol.
7. Transfer the eluate into a pear-shaped flask. Remove most of the solvent in a rotary evaporator; residual amounts can be evaporated with nitrogen gas.
8. Dissolve the sample in chloroform and transfer to a small glass vial. Seal the vial with teflon-lined screw cap and store at 0° C.

APPENDIX D

CHEMICAL PROCEDURES

APPENDIX D-1

Saponification procedure (F. Snyder, personal communication)

1. Prepare saponification reagent by dissolving 1 g of potassium hydroxide in 10 ml of 75% ethanol.
2. Add 2 ml of the reagent to the dried lipid extract in a test tube and seal tightly with a teflon-lined screw cap.
3. Heat the tube at 100° C for 1 hr in a boiling water bath with occasional shaking.
4. Cool the tube and add exactly 2 ml of distilled water. Extract the nonsaponifiable fraction three times using about 4 ml of 1:1 hexane:diethyl ether each time.
5. Acidify the bottom aqueous layer with 2 ml of 6 N hydrochloric acid. Extract the fatty acids by the same procedure as that described for the non-saponifiable fraction (#3 above).
6. Pool the extracts from #4 into a pear-shaped flask. Remove most of the solvent in a rotary evaporator; residual amounts can be evaporated with nitrogen gas.
7. Dissolve the nonsaponifiable lipids in chloroform and transfer to a small glass vial. Seal the vial

with teflon-lined screw cap and store at 0° C.

8. Repeat #6-7 for the extracts obtained from procedure carried out in #5 above.

APPENDIX D-2

Vitride reduction procedure (F. Snyder, et al. 1971)

1. Add lipid sample to a small glass vial equipped with a teflon-lined screw cap. Evaporate solvent.
2. Add 2 ml of diethyl ether and 0.5 ml of benzene to the vial.
3. Add 0.5 ml of Vitride to vial and shake before putting cap on to release hydrogen gas. Heat at 37° C with shaking for 30 min.
4. Cool vial. Carefully add 3-4 drops of water, one drop at a time.
5. Add 10 ml of 30% ethyl alcohol (aqueous) and extract three times with 6-8 ml of ethyl ether. Centrifuge the vial at 1000 rpm for 3 min.
6. Pool the ether extracts into a pear-shaped flask. Remove most of the solvent in a rotary evaporator; residual amounts can be evaporated with nitrogen gas.
7. Dissolve the sample in chloroform and transfer to a small glass vial. Seal the vial with teflon-lined screw cap and store at 0° C.

APPENDIX D-3

Methylation of fatty acids (F. Snyder, personal communication

1. Scrape silica gel fraction directly into test tubes (8-ml capacity) equipped with teflon-lined screw caps.
2. Add 2 ml of methanol containing 2% (v/v) sulfuric acid.
3. Flush with nitrogen gas for a few seconds and immediately cap the tubes tightly. Place tubes in boiling water bath for 1 hr.
4. Cool tubes. Add 3 ml of distilled water and extract the methyl esters of fatty acids three times with 1:1 hexane:diethyl ether.
5. Wash the combined extracts twice with distilled water. Discard the water and then add a small amount of Na_2SO_4 to the organic solvent extract.
6. Transfer the extract to a pear-shaped flask. Remove most of the solvent in a rotary evaporator; residual amounts can be evaporated with nitrogen gas.
7. Dissolve the sample in chloroform and transfer to a small glass vial. Seal the vial with teflon-lined screw cap and store at 0° C.

APPENDIX D-4

Acetylation of fatty alcohols (F. Snyder, personal communication)

1. Prepare reagent by adding 4 ml of acetic anhydride to 1 ml of pyridine.
2. Scrape silica gel fraction directly into test tubes (8-ml capacity) equipped with teflon-lined screw caps.
3. Add 0.5 ml of reagent (#1 above).
4. Place tubes in boiling water bath for 1 hr.
5. Cool tubes. Add 1.5 ml distilled water and shake.
6. Extract three times with 5 ml of 2:1 hexane: diethyl ether.
7. Transfer the extracts to a pear-shaped flask. Remove most of the solvent in a rotary evaporator; residual amounts can be evaporated with nitrogen gas.
8. Dissolve the sample in chloroform and transfer to a small glass vial. Seal the vial with teflon-lined screw cap and store at 0° C.

APPENDIX D-5

Protein determination (Lowry, et al. 1951) (modification according to F. Snyder, personal communication)

1. Prepare Reagent A by mixing the following just before use:
   (a) 100 ml of 2% Na_2CO_3 in 0.1 N NaOH
   (b) 1 ml of 1% $CuSO_4 \cdot 5H_2O$
   (c) 1 ml of 2% Na K tartrate
2. Prepare Reagent B by mixing the following just before use:
   (a) 1 part 2 N Folin-Ciocalteau reagent
   (b) 1 part 1 N NaOH
3. Add 1 ml of sample and standards to separate test tubes (15-ml capacity).
4. Add 5 ml of Reagent A (#1 above) to each tube and mix immediately.
5. After 5-10 min, add 0.5 ml of Reagent B (#2 above) to each tube and mix immediately.
6. Develop color for 30 min and read OD at 730 nm on a Beckman Spectrophotometer (Model 2400).
7. Prepare a standard curve with bovine albumin and determine the amount of protein in the sample from the curve.

APPENDIX D-6

Quantitative determination of phosphorus (Rouser, et al. 1966) (modification according to F. Snyder, personal communication)

1. Use only acid-washed test tubes (15-ml capacity).
2. Transfer the total lipid sample to test tubes and evaporate solvent. Add 0.9 ml of 70% (or 1 ml of 60%) perchloric acid. Work in designated perchloric acid hood.
3. Insert tubes into aluminum block which has been preheated to 160-180° C. Place a marble on each tube and then reflux for 20 min.
4. Cool tubes. Add 3 ml distilled water to the sample.
5. Prepare standards of K_2PO_4 in duplicate series in the following concentrations: 0.25 µg, 0.5 µg, 0.75 µg, 1.0 µg, 1.5 µg and 2.0 µg. The total volume in the standards should be brought to 3 ml with distilled water. Prepare blank tubes with 3 ml distilled water.
6. Add perchloric acid to the standards (as in #2 above).
7. Add 1 ml of 2.5% ammonium molybdate to the sample and standards. After addition, mix immediately with Vortex mixer.
8. After 5 min, add 1 ml 10% ascorbic acid. Mix with Vortex mixer as in #7 above.

9. Heat for 5 min in hot water bath (80-100° C).
10. Wipe tops of tubes with cotton swabs to remove any blue residue that may have formed.
11. With Pasteur pipette, transfer sample and standards to cuvettes.
12. Read OD at 820 nm on Beckman Spectrophotometer (Model 2400), using the water blank as a reference cell.

VITA

Lula Lail Hilenski, daughter of Julius L. and Catherine H. Lail, was born in Gastonia, North Carolina, on December 5, 1947. She attended elementary school in that city and was graduated from Hunter Huss High School in June, 1966. The following August, she entered Pfeiffer College, Misenheimer, North Carolina, and in May, 1970, graduated cum laude with a Bachelor of Arts degree in Biology.

In September, 1970, she entered Graduate School at The University of Tennessee, Knoxville, and began study toward a Master of Arts in College Teaching degree with a major in Biology. She held a fellowship through this program administered under the Education Professions Development Act (1970-72) and a Graduate Teaching Assistantship from the Department of Botany (1972-74). She is a member of Phi Kappa Phi National Honorary Society, the Phycological Society of America and the Botanical Society of America.

She is married to Ferdinand Alexi Hilenski II of Knoxville, Tennessee.