Fatty Acids in the Pellicles and Plastids of Light-grown and Dark-grown Cells of *Euglena gracilis**

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ABSTRACT: Green and etiolated cells of *Euglena gracilis* were collected late in the stationary phase of growth, disrupted, and separated into three fractions: pellicles, plastids, and the remainder of the cell. A study was made of the fatty acids in each fraction. Arachidonic acid and its homologs were the major fatty acid components of the etiolated cell. A higher percentage of long-chain polyenoic acids was found in the pellicle and in the plastid than elsewhere in the etiolated cell. In the green cell, trienoic fatty acids comprised the major fatty acid fractions. There was a roughly equal concentration of trienoic fatty acids throughout the green cell, with twice as much 16:3 as 18:3, except in the chloroplast where there was an equal concentration of each. Arachidonic acid and its homologs were found in small quantity and only in the pellicle and in the plastid.

Alterations in fatty acid composition mark the light-induced transition of *Euglena gracilis* from a heterotrophic to an autotrophic existence (Rosenberg and Pecker, 1964). The well-nourished dark-grown heterotrophic cell stores a wax composed mainly of tetradecanyl tetradecanoate and tridecanyl tridecanoate (Rosenberg, 1963). Illumination of the dark-grown cell induces the formation of functional chloroplasts (Wolken, 1961). The appearance of the photosynthetic mechanism is associated with a rapid removal of the wax and therefore of its constituent fatty acids from the cell (Rosenberg and Pecker, 1964). The photoautotrophic cell accumulates doubly and triply unsaturated fatty acids of the 16- and 18-carbon series (Erwin and Bloch, 1962; Rosenberg, 1963). When the etiolated cell is deprived of exogenous metabolites, the wax fraction is rapidly depleted, and arachidonic acid emerges into quantitative predominance (Rosenberg and Pecker, 1964). This report compares the species of fatty acids localized in the pellicles and in the plastids isolated from starved heterotrophic and photoautotrophic *Euglena* cells.

Experimental Methods

E. gracilis, strain Z, was grown on a fully defined medium and was harvested late in the stationary phase of growth before loss of viability was detectable (Rosenberg, 1963; Rosenberg and Pecker, 1964). All manipulations were carried out at a temperature as near to 4° as possible. Harvested cells, washed three times in distilled water, were suspended in an equal volume of a salt solution which contained 5.2 g KCl, 1.75 g NaCl, and 2.5 g Na citrate in 1 liter of 0.01 M potassium phosphate buffer, *p*H 7.3. The suspension was ground manually for 3 minutes in an ice-cooled glass mortar with a glass pestle, with approximately 10 g of washed and ignited sea sand (Fisher, S-25) per ml of suspension. A slow addition of 20 ml of salt solution was made, with stirring, and the slurry was centrifuged in a Lourdes Model A centrifuge. Fractions were collected as follows: (1) 200 × *g* for 10 minutes: whole cells, nuclei, sand, and debris; (2) 1000 × *g* for 7 minutes: chloroplasts or leucoplasts; (3) 12,000 × *g* for 10 minutes: pellicles; (4) 25,000 × *g* for 30 minutes: fragmented plastids; (5) 40,000 × *g* for 20 minutes: the remainder of the cell.

The course of cell disruption and cell fractionation was followed in a Zeiss GFL microscope fitted with a phase-contrast arrangement. Step 1 was repeated until the supernatant fluid was free from whole cells. The pellicle preparation was washed thoroughly in 0.067 M phosphate buffer, *p*H 7.0, to remove adhering cytoplasmic material.

The fatty acids were completely released from the whole cells and from the isolated fractions by heating at 55° for 5 hours under nitrogen in 10% (w/v) KOH in methanol. Nonsaponifiable material was removed by extraction five times with a 2-fold volume of hexane and diethyl ether (3:1, v/v). The samples, cooled in ice and diluted with an equal volume of concd HCl, were immediately extracted three times with an equal volume of hexane. The hexane was evaporated with nitrogen. The fatty acids were converted to methyl esters by heating for 3 minutes in a boiling bath with boron trifluoride in methanol (Metcalfe and Schmitz, 1961). An equal volume of water was added to the cooled sample, and the methyl esters

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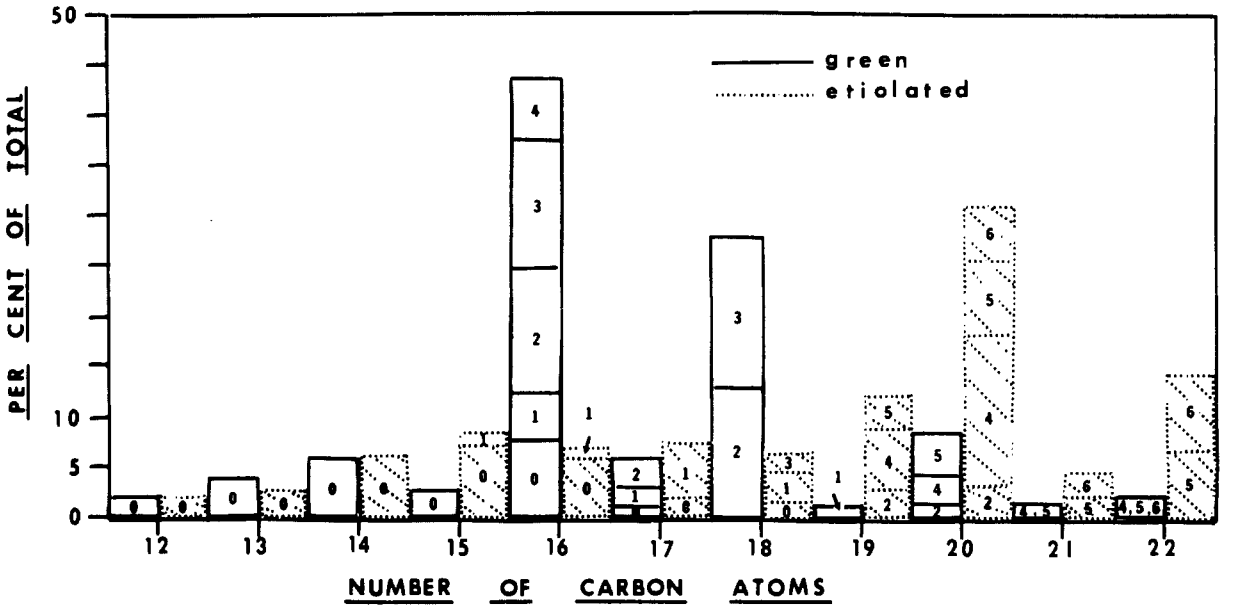

FIGURE 1: A comparison of the fatty acid distributions in green and in etiolated cells of *E. gracilis* in the late stationary phase of growth. The subdivisions of each bar show the relative quantities of each species according to degree of unsaturation. The numbers within the bars indicate the number of double bonds in the molecule.

were extracted three times with an equal volume of hexane.

A Barber-Coleman Model 10 instrument was used for gas-liquid chromatographic identification of the methyl esters. The column was maintained at 173° with argon as the mobile phase and Chromosorb W coated with 17% ethylene glycol succinate as the stationary phase. Fully saturated esters were readily identified by comparison with known standards. Unsaturated esters were present in great variety. They produced many composite elution bands. As an initial guide to identification, all of the methyl ester samples were reduced to the completely saturated analogs by treatment with 3 atm of hydrogen over Pt in hexane at room temperature for 1 hour. A comparison of the relative areas of the gas-liquid chromatographic elution peaks of reduced and of nonreduced samples indicated the nature and quantities of the species that were to be detected. For identification, methyl esters were separated according to their degree of unsaturation by the use of two thin-layer chromatographic arrangements. The acetoxymercurimethoxy adducts were made (Jantzen and Andreas, 1959). These were chromatographed in two dimensions on thin-layer plates of Merck Silica Gel G. In the first dimension, irrigation with hexane and diethyl ether (4:1, v/v) removed the saturated esters. Irrigation in the second dimension with 1-propanol and acetic acid (100:1, v/v) resolved the adducts into separated fractions which originally had one, two, three, and four and more double bonds. The solvents were evaporated from the plate under nitrogen. The spots were made visible by spraying with fluorescein (Malins and Mangold, 1960). The spots were scraped from the plate, and the samples were treated with 1 ml of 2% (v/v) concd HCl in methanol to break the adducts. After the addition of 1 ml of water, the liberated esters were extracted with hexane. In the second method, the esters were separated on plates of Silica Gel G impregnated with silver nitrate (Barrett and Padley, 1962). The isolated fractions were divided. One half of each was analyzed by gas-liquid chromatography directly. As a control, the other half was analyzed by gas-liquid chromatography after reduction to the readily identifiable saturated analogs.

To quantify their components, composite elution bands were collected individually from the gas-liquid chromatograph. The collected material was resaponified to remove contaminants arising from the column. The fatty acids were isolated, and esters were formed again. One portion of the latter was subjected to the protocol of thin-layer fractionation to verify identification. Another portion was reduced, the reduced peaks were identified, and the relative peak areas were calculated.

The position of the first double bond with respect to the carboxyl group was established for most of the isolated unsaturated ester fractions by oxidation to the dicarboxylic acid monomethyl esters (James and Webb, 1957), followed by gas-liquid chromatographic identification of the dimethyl esters formed by heating in methanol and boron trifluoride as described. Fractions separated on thin-layer plates could be oxidized without loss in the presence of the silica gel scraped from the plate. To obtain more efficient recovery of the resultant dicarboxylic acids it was necessary to methylate in sealed tubes and to run the gas-liquid chromato-

TABLE I: Types and Relative Quantities of Fatty Acids in Etiolated Cells of *Euglena gracilis*.[a]

Fraction	Elution Time (min)	Chain Lengths in Reduced Fraction	Mass % of[b] Each Chain in Fraction	Identification	Mass % of Total
1	1.4	[c]		12:0	0.2
2	1.8	[c]		13:0	0.9
3	2.4			14:0	5.6
4	3.4			15:0	8.0
5	4.0			15:1	0.4
6	4.8			16:0	4.8
7	5.6	16	100	16:1	0.4
8	6.5		100	17:0	0.9
9	7.5	17	100	17:1	4.7
10	8.4	18	100	18:0	0.5
11	10.2	18	100	18:1	3.2
12	12.8	17	50	17:3	0.4
		18	50	18:2	0.4
13	17.3	18	37	18:3	0.6
		19	63	19:2	1.1
14	22.6	19	32	19:4	2.2
15	23.4	20	68	20:2	4.7
16	30.0	19	2	19:5	0.4
		20	98	20:4[d]	19.6
17	34.8	19	11		0.2
		20	89	20:4	1.2
18	40.0	20	100	20:5[d]	11.2
19[e]	49.5	20	72	20:5	2.2
		21	28	21:5	1.0
20[e]	61.5	20	31	20:6	2.9
		21	12	21:5	1.1
		22	57	22:5	5.5
21[e]	81.6	20	34	20:6	4.1
		21	8	21:6	1.0
		22	57	22:6	7.0

[a] Calculated from relative areas of gas-liquid chromatographic peaks; values less than 0.2% are not shown. [b] Calculated from relative areas of peaks in reduced isolated fraction. [c] Not isolated as a separate fraction. [d] Arachidonate series; first double bond at carbon 5. [e] Proposed identifications based only on reduced products and elution time; no oxidation products isolated.

graphic column at a high flow rate to avoid ester interchange between sample and column. Degraded ethylene glycol succinate reacts with dimethyl succinate and can cause serious losses. A column must be checked for complete recovery each working day.

While this manuscript was in preparation, a report appeared on the identification of the fatty acids of green *E. gracilis* cells (Korn, 1964). Since our results with such cells are generally in close agreement with those published therein, this report will contain only the identifications of the etiolated cell fatty acids, and details which augment those already published on the green cell fatty acids. Unlike that of the green cell, the total lipid of the etiolated cell was not readily extracted with chloroform-methanol (2:1, v/v) at a low temperature (Korn, 1964; Entenman, 1961). The etiolated cell pellicle lipid was especially resistant. All of the fatty acids were liberated by prolonged direct saponification of the cellular material in methanolic KOH under nitrogen.

The relative distributions of fatty acids by chain length in the green and in the etiolated cell forms is indicated by the heights of the bars in Figure 1. The values were obtained from total cellular fatty acid samples hydrogenated as described. In the green cell, 16- and 18-carbon fatty acids predominated with emphasis on the former. In contrast, the etiolated cell had mainly 19-, 20-, and 22-carbon fatty acids. The subdivisions of the bars in Figure 1 present the distributions of fractions within each chain-length species according to degree of unsaturation. Fatty acids with two and three double bonds predominate in the green cell. Fatty acids with four, five, and six double bonds predominate in the etiolated cell. There was no evidence

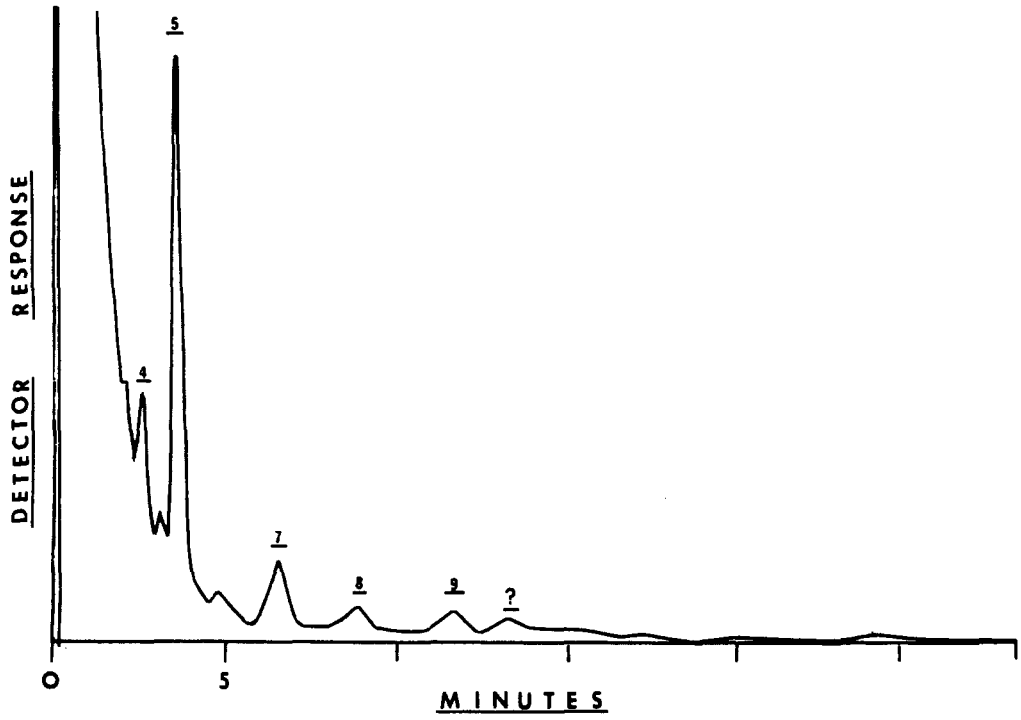

FIGURE 2: Gas-liquid chromatogram of the dimethyl esters of the dicarboxylic acids produced on permanganate oxidation of the etiolated *Euglena* cell fatty acid fraction containing four and more double bonds. The numbers over the peaks indicate the number of carbon atoms in the fractions. Some minor peaks are unidentified.

of 24-carbon or longer-chain fatty acids in either cell type.

Table I lists in detail the typical distribution of fatty acids of the starved etiolated *E. gracilis* cell grown and harvested as described. Fatty acids containing four and more double bonds comprise the major fraction. This fraction, on oxidation, gave rise to the relative quantities of dicarboxylic acids shown in Figure 2. Glutaric acid was the main product of oxidation of the total fraction, with lesser amounts of the 4-, 7-, 8-, and 9-carbon analogs. The single most abundant fatty acid in the starved etiolated cell was a 20-carbon fatty acid with four double bonds. It comprised about 20% of the total fatty acid content of the cell. It migrated as arachidonic acid. When collected from the gas-liquid chromatograph and oxidized separately, it gave rise almost entirely to glutaric acid, the oxidation product of arachidonic acid, 20:4 (Δ5, 8, 11, 14). The bulk of the polyunsaturated fatty acids of the etiolated cell was made up of arachidonic acid and its homologs. In contrast, the green cell shows (Korn, 1964) much less 20:4, with a roughly equal division between the Δ5, 8, 11, 14 and the Δ8, 11, 14, 17 isomers.

There is very little trienoic fatty acid in the starved etiolated cell. Oxidation of 20:2, the major diene, gave sufficient 11-carbon dicarboxylic acid to account for all of the 20:2 fraction as the Δ11, 14 isomer. Much 11- and little 10-carbon dicarboxylic acid was given by the 19:2, the second most abundant diene. Thus the cell could not have produced this odd-chain acid mainly through α-oxidation of the Δ11, 14, C_{20} acid.

Upon oxidation, 17:1, the major monoenoic fraction gave the 9-carbon dicarboxylic acid exclusively. The monoenoic and dienoic fatty acids together constituted a small fraction. Within this fraction, the bulk of the dienoic fatty acids had the first double bond at the eleventh carbon atom from the carboxyl end of the molecule, and the bulk of the monenoic fatty acids had the first double bond at the ninth, regardless of an odd or an even numbering of carbon atoms in the chain.

Green cells in the stationary phase of growth accumulate an unidentified fatty acid. This fatty acid is not indicated in Figure 1. The rate of elution of its methyl ester was slightly lower than that of 16:4 (Δ7, 10, 13, 16). The fraction originally was considered to be an isomer of the latter (Klenk and Knipprath, 1959). Reduction of the total fatty acids of the green cell was accompanied by a disappearance of the unidentified peak. There was a concomitant appearance of a new peak in equal quantity. The rate of elution of the new peak was slightly but measurably lower than that of palmitic acid. Attempts at isolation of the unreduced compound failed since it was almost completely destroyed during collection. In thin-layer chromatographic separations, it accompanied the fraction having four or more double bonds. Further attempts at identification have not yet met with success.

Excessive destruction of subcellular particles re-

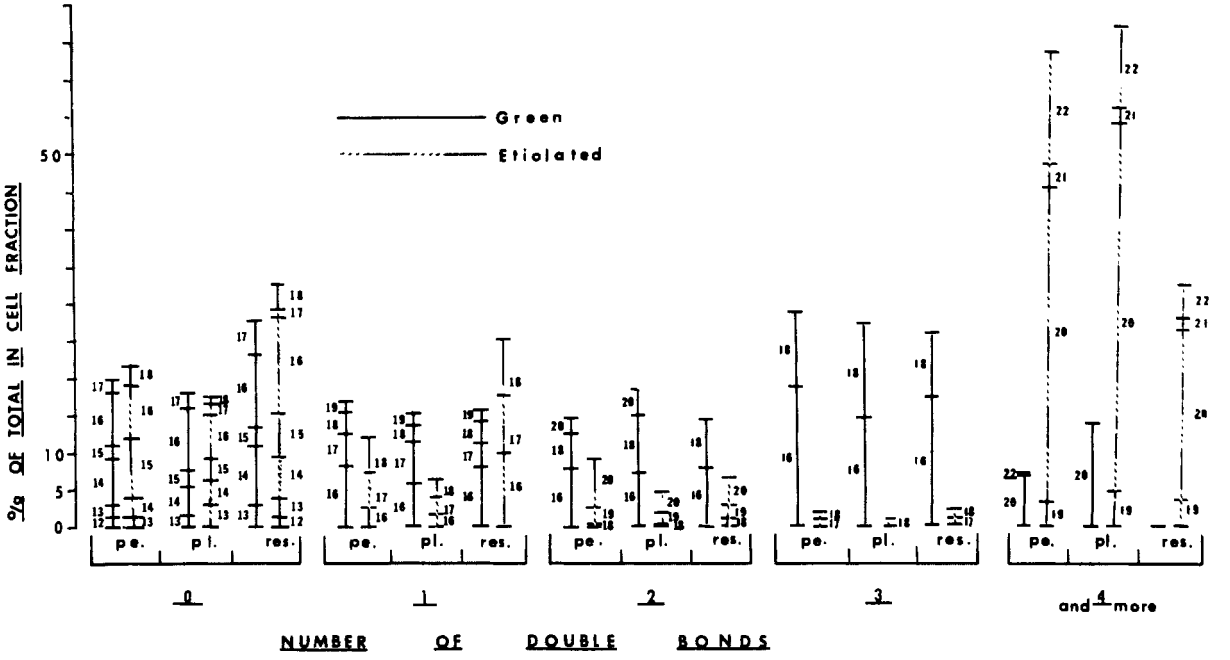

FIGURE 3: Distribution of fatty acids in the subcellular fractions of green and of etiolated *Euglena* cells. The fatty acids are grouped according to degree of unsaturation. The divisions of the lines show the relative quantities of each chain length indicated by the adjacent numbers. Values less than 0.5% are not shown. Abbreviations: Pe., pellicle; pl., plastid; res., residue of the cell.

sulted when cells were disrupted by shearing forces in a Waring Blendor or by vibration in a sonic oscillator. Extrusion of the cells through a Hughes press (Hughes, 1951) was a sufficiently gentle procedure, but yields of broken cells were too low. Manual grinding with fine sand at low temperature ruptured most of the cells and permitted the recovery of an estimated 60% of the pellicles and an estimated 40% of the plastids in apparently intact form. A buffered salt solution (Claude, 1946) containing citrate was useful in the centrifugal fractionation of the ruptured cells. Control of the *p*H was mandatory. Larger subcellular particles clumped irreversibly when a *p*H lower than 7 was allowed to persist, and the isolation of clean fractions became difficult. It should be emphasized that the plastids of the etiolated cell are defined operationally. It is not known whether these bodies become chloroplasts upon illumination of the cell.

The isolated pellicles, plastids, and the remainders of green and of etiolated cells were examined for their fatty acid compositions. The results are summarized in Figure 3. It may be seen that fully saturated fatty acids comprise a roughly equal portion of the total fatty acids of both cell types. The highest concentration of saturated acids was in the lipid found outside of the pellicle and the plastid. Measurable quantities of stearic acid were found in the etiolated cell, but this fatty acid is present only in trace amounts in the green cell in the late stationary phase of growth. Monoenes were found mostly outside the pellicle and the plastid, the latter having the smaller quantity. No significant quantity of monoenes with more than 18 carbon atoms was found in the starved etiolated cell. In the green cell, up to 19-carbon fatty acids were found in the monoenoic fraction, and there was roughly equal distribution of monoenes in all of the fractions studied. There was less dienoic fatty acid in the etiolated than in the green cell. In the latter, 16, 18, and 20 carbon atoms predominated in this fraction. In the etiolated cell, little 18:2, but significant amounts of 19:2 were found. While the highest concentration of dienes was found in the chloroplast of the green cell, the smallest concentration was found in the leucoplast of the etiolated cell. Very little trienoic fatty acid was found in the etiolated cell fractions. Almost half was a 17-carbon species essentially absent from the green cell. In the latter, trienoic fatty acids constituted the major fraction. As a group, trienes formed a roughly equal percentage of the fatty acids of all of the green cell fractions studied. However, in the chloroplast there were roughly equal quantities of 16:3 and 18:3, while there was twice as much 16:3 as 18:3 in the other cell fractions. Fatty acids with four and more double bonds were found only in the chloroplast and, to a smaller extent, in the pellicle of the green cell. In contrast, fatty acids with four and more double bonds constituted major fractions throughout the etiolated cell where polyunsaturated fatty acids with 19, 20, 21, and 22 carbon atoms were found. The most abundant species was that containing 20 carbon atoms. The lipids of the pellicle

and the plastid had a concentration of polyunsaturated fatty acids which was twice that found elsewhere in the etiolated cell.

Discussion

Euglena cells produce fatty acids with a rich and varied distribution of molecular species. The relative quantity of each species present in the cell is influenced by many factors. Some of these are stages of growth, level of illumination, and the nature of the medium. A classification of microorganisms according to the fatty acids they produce in major amount is a useful taxonomic device (Korn, 1964). Photoautotrophic *Euglena* produces far more 16:3 and 18:3 than heterotrophic *Euglena*. Conversely, the latter produces arachidonic acid and its homologs in much greater quantity than does the autotrophic form. The preponderance of arachidonic acid in heterotrophic *Euglena* has prompted the assumption that the fatty acid metabolism of the heterotrophic *Euglena* cell is like that of an animal cell. This may be misleading since animal cells cannot synthesize linoleic acid. The *Euglena* cell readily synthesizes linoleic acid, which it then uses either to make preferentially linolenic acid in the photoautotrophic cell, or preferentially arachidonic acid in the heterotrophic cell (Hulanika, *et al.*, 1964).

Polyunsaturated fatty acids of the arachidonate family were found to be present in highest concentration in the pellicle and in the plastid of the heterotrophic cell. The observed differences in the fatty acid components of the subcellular fractions of *Euglena* may be associated with changes in membrane structure (Van Deenen *et al.*, 1962; MacFarlane, 1964) which can influence the degree and direction of the cellular transfer of chemical potential. The large number of *cis* double bonds in arachidonic acid tends to fix the molecule in a curled shape and leave a series of only three connected methylene groups at both of its ends, a number insufficient to permit formation of a strong bond with other hydrocarbon chains through the interaction of London–Van der Waals dispersion-attraction forces. The membrane systems of the arachidonic acid–rich pellicle of etiolated *Euglena* may therefore have relatively expanded and hydrated lipid layers that allow passage to exogenous metabolites required by the dark-grown cells.

The *Euglena* cell synthesizes linoleic acid (Rosenberg and Pecker, 1964) which may then serve as a precursor (Hulanika *et al.*, 1964) either for linolenic or for arachidonic acids. Yet the source of the mono- and dienoic fatty acids in *Euglena* is not clear. This study indicates that most of the monoenoic fatty acids in *Euglena* have the double bond at position 9 from the carboxyl group. These double bonds apparently do not arise directly from the action of an oxygen-dependent desaturating system on a preformed fully saturated chain (Bloch, 1963). While monoenes and dienes of the oleic and linoleic acid type are apparently favored substrates for the synthesis of arachidonic acid (Sen and Schlenk, 1963), and are so utilized in the heterotrophic cell, introduction of the third double bond toward the methyl end of a Δ-9 monoene renders it unsuitable as a substrate for the synthesis of arachidonic acid. Experimental evidence should be sought to determine whether the abundance of molecular oxygen produced intracellularly by photosynthesis (Arnon, 1961) induces further desaturation of monoenes and dienes to produce the high level of trienes of the α-linolenate type observed in green cells.

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