

# Monogalactosyl and digalactosyl diglycerides from heterotrophic, hetero-autotrophic, and photobiotic *Euglena gracilis*

ABRAHAM ROSENBERG,\* JUNE GOUAUX, and PETER MILCH

Departments of Biochemistry and Medicine, Columbia University, and the Columbia University Research Service, Goldwater Memorial Hospital, New York, N. Y.

**ABSTRACT** The lipid of *Euglena gracilis*, dark-grown in a complete medium, contained 2% galactose. The lipid of *Euglena gracilis*, light-grown in either a complete or an inorganic medium, contained 13–14% galactose. Pure monogalactosyl and digalactosyl diglyceride fractions, isolated by column plus thin-layer chromatography, contained 50% of the lipid-bound galactose of dark-grown cells, and 80% of that of light-grown cells. Molar ratios of monogalactosyl to digalactosyl compounds ranged from 2 to 3. The results show that galactosyl diglycerides, stored in large amount in light-grown cells, persist in small amount in the dark-grown cells.

Fatty acids in both the monogalactosyl and the digalactosyl diglycerides were mainly of the 16- and 18-carbon varieties, with high proportions of trienes. The monogalactosyl diglycerides were rich in hexadecatetraenoic acid. Strictly photobiotic cells had twice as much hexadecadienoic and hexadecatetraenoic acids in their monogalactosyl diglycerides, and three times as much hexadecadienoic and octadecadienoic acids in their digalactosyl diglycerides as did illuminated cells grown in a complete medium. Dark-grown (obligate) heterotrophs contained galactosyl diglycerides with high percentages of monoenes. Great compositional variations in the galactosyl diglycerides are thus induced by light and also by nonlipid exogenous metabolites.

**KEY WORDS** monogalactosyl · digalactosyl · diglycerides · *Euglena gracilis* · isolation · light-grown · dark-grown · organic · inorganic · media · heterotrophic · hetero-autotrophic · photobiotic · fatty acid composition

Abbreviations: GLC, gas-liquid chromatography; TLC, thin-layer chromatography.

\* Recipient of an Investigatorship of the Health Research Council of the City of New York, under Contract I-164.

**G**ALACTOSYL DIGLYCERIDES have been found to comprise a major fraction of the lipids in photosynthesizing plants and green microorganisms (1–5). Their concentration is very high in the chloroplasts of light-grown cells (3). Linolenic acid is often the quantitatively predominant fatty acid in the galactosyl diglycerides of the leaves of higher plants (2) but not, apparently, in photosynthesizing algae (6, 7).

The protist *Euglena gracilis* has proven to be a useful model organism in the study of photosynthetic processes (8). Galactose has been found (1) in abundance (9) in the total lipid extracted from green cells of *Euglena gracilis*, and a galactose-rich “pigment” fraction (10) from *Euglena* has been described. No study has been made of pure mono- and digalactosyl diglycerides isolated from these cells. The present report describes a method of isolation and purification, and compares the fatty acid compositions, of mono- and digalactosyl diglycerides from photosynthesizing and nonphotosynthesizing cells of *Euglena gracilis*. A description is given of the appreciable differences in fatty acid composition of galactosyl diglycerides isolated from cells maintained in the dark, in the light under conditions which permit simultaneous heterotrophy and autotrophy, and in the light under conditions of strict photobiosis.

## MATERIALS AND METHODS

### *Growth of Cells*

*Euglena gracilis*, strain Z, was a gift of Dr. Seymour Hutner, Haskins Laboratories, New York, N. Y. Photosynthesizing cultures were maintained at a light intensity of approximately 30 foot-candles with 15-watt “Cool-

White" fluorescent tubes at a constant temperature of 25°C. Fully green hetero-autotrophic cells were grown in a defined complete medium (8) with successive weekly transfers of inocula over a period of months. Cultures were tested periodically for viability and inspected for the presence of mutant forms. Cultures that reached the stationary phase of growth 10 days after inoculation of the medium were harvested.

Etiolated cells were grown in the same medium but in total darkness. Upon completion of the logarithmic stage of growth, they were collected and washed. One half was used for the study of their lipids. The other half was suspended in a mineral medium (8) and subjected to constant illumination at the level indicated above. These photobiotic cells, which become completely dependent on light as sole source of energy, formed their full complement of functional chloroplasts before 6 days, as measured by chlorophyll content (optical density at 668  $m\mu$ ). They were collected at the end of 7 days of illumination.

#### Extraction of Lipids

Washed and packed cells were agitated in a Waring Blendor for 2 min with 20 volumes of chloroform-methanol 2:1, at approximately 10°C. The resultant slurry was filtered with the aid of suction, and the clear filtrate was dialyzed for 24 hr against running tap water at 4°C in the dark. The lower phase of the dialysate (chloroform) was collected and treated for several hours with anhydrous sodium sulfate. The water-free solution was concentrated under vacuum, transferred to a volumetric flask, and diluted to a known volume with chloroform. The lipids from roughly 1 g of packed cells were contained in 10 ml of chloroform. When not in use, the solutions were stored under nitrogen at -20°C in the dark.

#### Column Separations

Fractions containing the monogalactosyl diglycerides and the digalactosyl diglycerides were separated from most of the other *Euglena* lipids by column chromatography, with the aid of the discontinuous solvent gradient system devised by Vorbeck and Marinetti (11) for separation of the glycosyl diglycerides of *S. faecalis* and *L. plantarum*. A mixture of 15 g of silicic acid ("100 mesh," Mallinckrodt, Analytical Reagent) and 110 ml of *n*-heptane was poured into a glass column to a height of approximately 40 cm. The heptane was drained, and the column was washed with 90 ml of chloroform. An aliquot of the chloroform solution of *Euglena* lipids (usually 5 ml, containing about 40 mg) was applied to the column which was then washed with 90 ml of chloroform, collected as an undivided fraction (fraction A). The column was eluted with 80 ml of chloroform-

acetone 1:1, the eluate being collected in 10-ml portions (fraction B); with 80 ml of acetone collected in 10-ml portions (fraction C); and with 80 ml each of chloroform-methanol 9:1 (fraction D), chloroform-methanol 1:1 (fraction E), and methanol (fraction F), the last three collected as undivided fractions.

#### Galactose, Phosphorus, and Fatty Acid Analyses

Aliquots (0.5 ml) of the total lipid solution and of each column fraction were dried under a stream of nitrogen. The dry residues were analyzed for phosphorus, after wet ashing (12), and for galactose, after hydrolytic separation of the sugar from the lipid (13) *under nitrogen*. Fatty acids were obtained as the methyl esters by methanolysis with boron trifluoride-methanol (14). The esters were analyzed by GLC in a Barber-Colman model 10 instrument (ethylene glycol succinate polyester, argon, 173°C). For quantitative estimation of total fatty acid content of the samples, the esters were reduced (hexane solution, Pt catalyst, 3 atm H<sub>2</sub>, 3 hr, room temperature), then combined with a known quantity of pure methyl behenate as an internal standard. Aliquots of this mixture were analyzed by GLC. Peak areas were estimated by triangulation. The area of each peak was compared with that given by the internal standard. Quantities were calculated by direct proportion. Values obtained with known mixtures of pure compounds (C<sub>14</sub>, C<sub>16</sub>, C<sub>18</sub>, C<sub>20</sub>, and C<sub>22</sub>) were within 3% of theoretical.

#### TLC of Galactosyl Diglycerides

The column fractions containing the monogalactosyl diglycerides (chloroform-acetone eluate, fraction B) and the digalactosyl diglycerides (acetone eluate, fraction C) were freed from pigment and lipid contaminants by two-dimensional TLC on 8 × 8 cm glass plates coated with Silica Gel G (Warner-Chilcott Laboratories, Richmond, Calif.). Plates were developed in the first dimension with chloroform-methanol-acetic acid 100:25:8, and in the second dimension with diisobutyl ketone-acetic acid-water 120:75:11. Removal of the second solvent mixture from the plate was essential for proper visualization of the spots. Accordingly, prior to staining, developed plates were dried overnight at room temperature in a vacuum oven fed continuously with a trickle of nitrogen. Identification of sugar-containing spots was initially made by spraying with 0.2% (w/v) recrystallized anthrone in concentrated sulfuric acid, followed by heating at 70°C for 20 min. For nondestructive location of spots, plates were sprayed with a solution of Rhodamine G prepared on the day of use by mixing 1 ml of a stock solution of 0.1% (w/v) Rhodamine G in methanol (which could be kept indefinitely in the refrigerator) with 25 ml of 2 N ammonium hydroxide (stable at room temperature). The sprayed plates were

viewed while still damp under long-wave UV light (Blak-Ray model XX15, Ultra-Violet Products, Inc., San Gabriel, Calif.). The galactosyl diglycerides are readily distinguishable by their brilliant pink fluorescence.

#### Preparation of Pure Fractions for Infrared Spectrophotometry

Although Rhodamine G did not interfere with any of the foregoing analyses, its presence was undesirable in samples prepared for infrared analysis. Fractions for this purpose were prepared by TLC as described, but the spots were located by very brief exposure to iodine vapor. The galactolipid spots, recognized by their  $R_f$  values, were scraped from the plate. Most of the iodine was removed in a drying pistol (oil pump,  $0.3\mu$ ) overnight over phosphorus pentoxide at room temperature. The scrapings were then transferred to a graduated, glass-stoppered, 15 ml conical test tube. A 5-fold volume of hexane was added. The slurry formed by vigorous mixing was transferred to a small chromatography column made of glass tubing (approximately 1 cm i.d.) that had been tapered and plugged at one end with glass wool overlaid with about 1 cm of clean Silica Gel G. When all the gel had settled, the supernatant hexane was permitted to drain into a graduated test tube, and its volume was measured. The difference between the volume of hexane used to form the slurry and that collected from the column gave the approximate column volume. The column was washed with two column volumes of chloroform, which effectively removed the last traces of iodine but none of the galactosyl diglyceride. Monogalactosyl diglycerides were then eluted with five column volumes of acetone; digalactosyl diglycerides were eluted with five column volumes of methanol-acetone 5:1. Infrared spectra were obtained with a Perkin-Elmer Corp. (Norwalk, Conn.) instrument, 221 PGI, model No. 12000. Lipid samples were either pressed in potassium bromide, or layered on a KRS-5 thallium bromide plate for total attenuated reflectance measurements.

## RESULTS

Although most of the pigment in the lipid extract of the cells was removed from the column in fraction A (chloroform), a small amount remained to be eluted in fraction B (chloroform-acetone) which contained the monogalactosyl diglycerides, and in fraction F (methanol) which contained most of the phospholipid. A typical column elution pattern is given in Fig. 1. Phosphorus analyses indicated that fraction C, which contained the digalactosyl diglycerides, was free from phosphorus, but that fraction B contained some phosphorus. Calculated as phosphatide, this amount corresponded

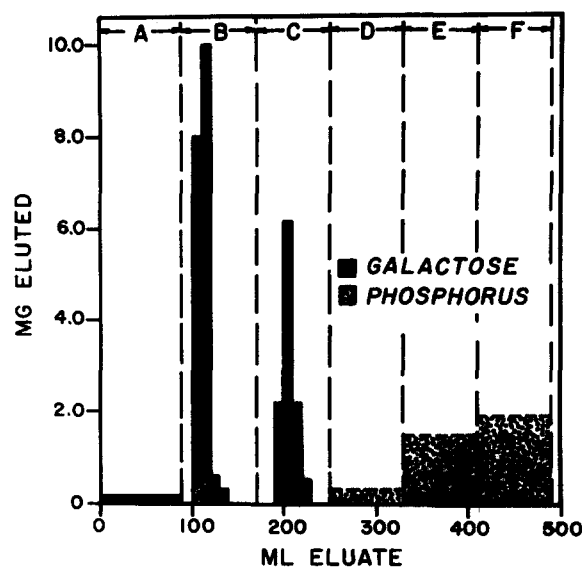


FIG. 1. Separation of the monogalactosyl and digalactosyl diglycerides from the remainder of the lipids of *Euglena gracilis* on a silicic acid column eluted with a discontinuous solvent gradient system (11). The solvent fractions are A, eluted with chloroform; B, with chloroform-acetone 1:1; C, acetone; D, chloroform-methanol 78:2; E, chloroform-methanol 1:1; and F, methanol. The eluate with each solvent mixture was collected as a single fraction except for B and C, containing the monogalactosyl and digalactosyl diglycerides, respectively, which were collected in 10-ml portions.

roughly to a 30% contamination by phosphatide in the monogalactosyl diglycerides from green cells, and roughly a 300% contamination in the smaller amount of monogalactosyl diglycerides from etiolated cells.

Recovery of lipid-bound galactose from the column was 88-90% of that in the total lipid extract. As shown in Fig. 1, a small amount of galactose was also found in fractions A and F. Only the mono- and digalactosyl diglyceride fractions (fractions B and C) were studied further.

TLC permitted a clean separation of the desired lipids from contaminants to be made. The monogalactosyl diglyceride fraction from the column was resolved in the first dimension into a pigment spot running with the solvent front, a monogalactosyl diglyceride spot running close behind, and a phosphorus-containing spot at the origin. In the second dimension, the pigment was resolved into an intense spot and several faint spots running near the solvent front. The monogalactosyl diglyceride ran with an  $R_f$  of approximately 0.7. Digalactosyl diglycerides ran with an  $R_f$  of 0.5 in the first solvent mixture and 0.3 in the second. In the latter system, two minor lipid spots migrating just behind the digalactosyl diglycerides were discernible. The faster-moving of these, which was not studied further, contained galactose. The galactosyl diglycerides isolated from the silicic acid column in fractions B and C gave,

on analysis, a molar ratio of fatty acid to galactose of 2.09 for the former and 1.02 for the latter, which confirmed that these fractions contained, respectively, the mono- and digalacto compounds (11).

In green cells maintained in the light in the complete medium, galactose comprised 13% (w/w) of the total lipid. Approximately 49% of the galactose was found in the pure monogalactosyl fraction and 31% in the digalactosyl fraction. Since the former compounds contain one galactose residue per molecule and the latter, two, the ratio of the quantities of mono- and digalactosyl diglycerides on a molar basis was roughly 3:1. In cells maintained in the light in the mineral medium, there was 14% galactose in the total cellular lipid, with 43% of it in the monogalactosyl fraction and 41% in the digalactosyl fraction, giving a molar ratio of roughly 2:1.

Nonphotosynthesizing cells grown in the dark in the complete medium had only 2.2% galactose in their total cellular lipid, 28% of it in the monogalactosyl fraction and 21% in the digalactosyl fraction. Thus, no more than half of the total lipid galactose was found in the mono- and digalactosyl diglycerides, with a molar ratio of the former to the latter of roughly 3:1.

The fatty acid compositions of monogalactosyl and digalactosyl diglycerides isolated by column chromatography and purified by TLC are shown in Table 1. All of the galactosyl diglyceride fractions had similar, although not identical, distributions of fatty acids with respect to chain length. The percentage of unsaturated fatty acids was high: 87–98% of the total in the green cells, somewhat lower in the etiolated cells. C<sub>16</sub> and C<sub>18</sub> fatty acids predominated; the former were in greater abundance. Appreciable differences were observed in the distribution of unsaturated fatty acids in the mono- and digalactosyl compounds. Monogalactosyl diglycerides tended to have a higher percentage of 18:3 than 16:3 fatty acid, while the reverse was true of the digalactosyl diglycerides. Less palmitic acid was found in the mono- than in the digalactosyl diglycerides, and a quite high percentage of 16:4. The latter was almost absent from the digalactosyl diglycerides. Measurable quantities of C<sub>20</sub> (saturated and polyenoic) acids generally were found only in the monogalactosyl diglycerides.

Strictly photobiotic cells maintained in the light in a mineral medium had a markedly increased percentage of di- and tetraenoic acids in the fractions studied. Thus, monogalactosyl diglycerides from cells in the mineral medium under constant illumination had percentages of 16:2 and 16:4 which were almost twice those of green cells in the complete medium. In the digalactosyl diglycerides from photobiotic cells in the mineral medium, the percentage of 16:2 and 18:2 was twice that of digalactosyl diglycerides from cells in the complete me-

TABLE 1 FATTY ACID COMPOSITION OF MONOGALACTOSYL AND DIGALACTOSYL DIGLYCERIDES FROM CELLS OF *Euglena gracilis* UNDER SEVERAL ENVIRONMENTAL ARRANGEMENTS

Fatty Acid*	Hetero-Autotrophic		Heterotrophic		Photobiotic	
	Mono†	Di†	Mono	Di	Mono	Di
	% total based on peak area					
12:1	1.9	0.8	—	—	—	—
14:0	1.6	0.9	2.3	2.4	0.3	0.6
15:0	1.0	0.4	0.8	1.8	—	—
15:1	0.5	0.5	1.3	—	0.1	0.5
16:0	5.3	11.5	6.5	11.0	1.2	5.6
16:1	8.4	7.9	14.3	13.7	2.7	7.1
16:2	7.3	9.5	7.6	7.3	16.5	25.4
16:3	19.5	26.8	10.3	12.0	15.9	20.4
16:4	10.8	1.5	5.7	—	20.0	—
17:1	0.6	2.5	—	—	4.2	—
18:0	1.4	1.0	2.5	3.5	0.3	3.8
18:1	3.0	9.0	13.0	28.0	1.7	2.0
18:2	7.5	5.9	4.0	4.7	9.3	16.3
18:3	23.8	19.0	17.5	11.8	19.0	17.3
18:4	1.6	—	—	—	—	—
19:0	—	1.3	3.0	3.8	—	1.0
20:0	2.0	1.5	—	—	2.5	—
20:4	2.7	—	—	—	2.5	—
20:5	0.9	—	—	—	3.8	—
22:0	—	—	5.3	—	—	—
22:2	—	—	6.3	—	—	—

Hetero-autotrophic (green) cells were grown in a complete, defined medium (8) under constant illumination. Heterotrophic (etiolated) cells were grown in the same medium in total darkness. Photobiotic (green) cells were developed in an inorganic medium (8) under constant illumination.

\* Chain length and number of double bonds, the positions of which are described elsewhere (15, 16).

† Mono, monogalactosyl diglyceride; Di, digalactosyl diglyceride.

dium. Unlike cells grown in the light, cells grown in the dark produced galactosyl diglycerides rich in 16:1 and 18:1.

Infrared spectra of the *Euglena* monogalactosyl and digalactosyl diglycerides in the solid state are shown in Fig. 2. The spectrum of monogalactosyl diglyceride from bovine brain (Applied Science Laboratories Inc., State College, Pa.) is included for comparison. The spectra of the three compounds are similar. Three overlapping bands attributed to the C–O stretching modes of the ester functions are seen in the 1300–1000 cm<sup>-1</sup> region (17). The characteristic maximum given by diglyceride molecules is seen near 1040 cm<sup>-1</sup> (18). A strong band, attributed to the bending vibration of hydrogen atoms in the *cis* double bond groupings (17), is seen at 1650 cm<sup>-1</sup> in the digalactosyl diglycerides of *Euglena* (curve B') and in the monogalactosyl diglycerides of bovine brain (curve A') pressed in potassium bromide. In the attenuated total reflectance spectrum of the *Euglena* monogalactosyl diglycerides (curve C) the band occurs at 1610 cm<sup>-1</sup>. At lower concentrations of lipid (curves A and B), the band diminishes, and a new band appears

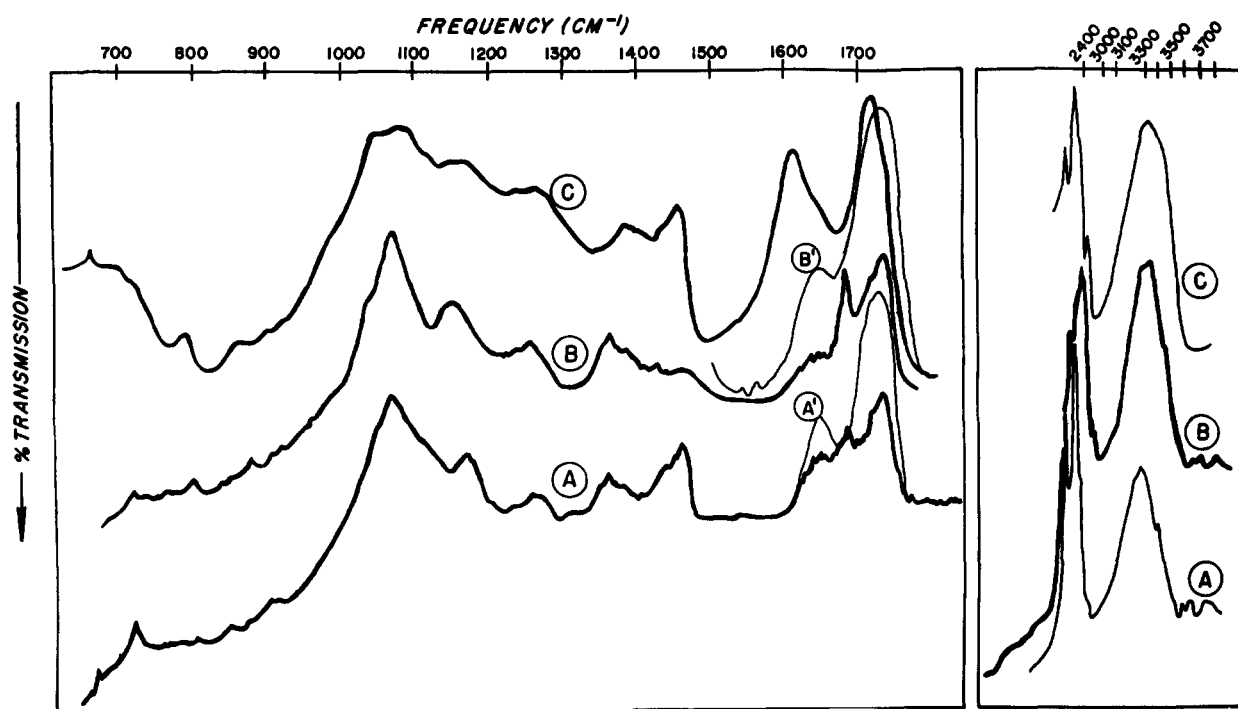


FIG. 2. Infrared spectra of galactosyl diglycerides in the solid state. *A*, monogalactosyl diglyceride (bovine brain) pressed in KBr. *A'*, the same, at higher concentration. *B*, digalactosyl diglyceride (*Euglena gracilis*) pressed in KBr. *B'*, the same, at higher concentration. *C*, monogalactosyl diglyceride (*Euglena gracilis*), total attenuated reflectance measurement of lipid film on thallium bromide plate.

at a somewhat higher frequency,  $1685\text{ cm}^{-1}$ , a possible indication that molecular interactions at high concentrations inhibit the bending mode of hydrogen in the *cis* double bond groupings of the fatty acid residues. The position ( $3400\text{ cm}^{-1}$ ) and breadth of the O-H stretching band indicates, as expected for such molecules, a considerable degree of bonding of O-H hydrogen.

### DISCUSSION

A high percentage of the galactose in the total lipid extract of *Euglena gracilis* can be accounted for as monogalactosyl and digalactosyl diglycerides. The technique of Vorbeck and Marinetti (11) provides a convenient method for the preliminary separation of these compounds from the rest of the cellular lipid. Contaminants which accompany the column fractions can readily be removed by TLC.

There are compositional similarities between the monogalactosyl and digalactosyl diglycerides of green *Euglena* cells. Fatty acids with 16 and 18 carbon atoms predominate and are found in roughly the same proportions in all fractions. The very high percentage of unsaturated fatty acids found in these galactolipids indicates that no more than one quarter of the total can have the structure typical of the phosphatides, namely, with one saturated and one unsaturated fatty acid residue. The galactosyl

diglycerides are thus among the most highly unsaturated lipids known. A salient difference between the mono- and digalacto compounds lies in the high percentage of hexadecatetraenoic acid in the former and the very low percentage in the latter. Another difference lies in the presence of eicosapolyenoic acids in the mono- but not the digalacto compounds. Compositional similarities of the two fractions may support an assumption that monogalactosyl and digalactosyl diglycerides share a common synthetic pathway. However, one must note the substantial quantity of fatty acids that are not common to both fractions. In this, there is an indication that at least a portion of the mono- and digalacto compounds may have independent metabolic fates.

No explanation is readily apparent for the great accumulation of hexadecadienoic and hexadecatetraenoic fatty acids in the monogalactosyl diglycerides, and hexa- and octadecadienoic acids in the digalactosyl diglycerides, of cells that are compelled to rely only on light as an exogenous source of metabolic energy. It is clear from our findings that environmental conditions other than temperature and intensity of illumination may influence the synthesis of these important chloroplast lipids.

GLC analyses of fatty acids before and after hydrogenation permit an assessment of the relative quantities of the paired fatty acid components  $C_{16:3}$ - $C_{18:1}$  and  $C_{16:4}$ - $C_{19:0}$  which tend, respectively, to give single GLC peaks

because of the similar retention times of the members of each pair. Since such hydrogenations are not always done, comparison at the moment with the findings of others is difficult, but it appears that fatty acid distributions in the monogalactosyl and digalactosyl diglycerides of *Euglena gracilis* are very similar to those of *Chlorella* (6), and they are very different from those of green leaves (6, 19). The interesting approach of Korn (15) in comparing fatty acid compositions of organisms as a taxonomic device apparently may be extended with success to the fatty acid compositions of lipid subclasses such as the monogalactosyl and digalactosyl diglycerides of photosynthesizing cells.

This work was supported by PHS Grants GM 09041-05 and HE 00052-19 from the National Institutes of Health, U. S. Public Health Service, and by the Health Research Council of the City of New York under Contract U-1562.

Manuscript received 3 June 1966; accepted 25 July 1966.

#### REFERENCES

1. Carter, H. E., K. Ohno, S. Nojima, C. L. Tipton, and N. Z. Stanacev. *J. Lipid Res.* **2**: 215, 1961
2. Allen, C. F., and P. Good. *J. Am. Oil Chemists' Soc.* **42**: 610, 1965.
3. Benson, A. A. *Ann. Rev. Plant Physiol.* **15**: 1, 1964.
4. Carter, H. E., R. H. McCluer, and E. D. Slifer. *J. Am. Chem. Soc.* **78**: 3735, 1956.
5. McArthur, J. A., T. V. Marsho, and D. W. Newman. *Plant Physiol.* **39**: 551, 1964.
6. O'Brien, J. S., and A. A. Benson. *J. Lipid Res.* **5**: 432, 1964.
7. Holton, R. W., H. H. Blecker, and M. Onore. *Phytochemistry* **3**: 595, 1964.
8. Rosenberg, A., and M. Pecker. *Biochemistry* **3**: 254, 1964.
9. Rosenberg, A. *Biochemistry* **2**: 1148, 1963.
10. Hulanicka, D., J. Erwin, and K. Bloch. *J. Biol. Chem.* **239**: 2778, 1964.
11. Vorbeck, M. L., and G. V. Marinetti. *J. Lipid Res.* **6**: 3, 1965.
12. King, E. J. *Biochem. J.* **26**: 292, 1932.
13. Rosenberg, A., and E. Chargaff. *J. Biol. Chem.* **233**: 1323, 1958.
14. Morrison, W. R., and L. M. Smith. *J. Lipid Res.* **5**: 600, 1964.
15. Korn, E. D. *J. Lipid Res.* **5**: 352, 1964.
16. Rosenberg, A., M. Pecker, and E. Moschides. *Biochemistry* **4**: 680, 1965.
17. Chapman, D. *J. Am. Oil Chemists' Soc.* **42**: 353, 1965.
18. O'Connor, R. T. *J. Am. Oil Chemists' Soc.* **33**: 1, 1956.
19. Sastry, P. S., and M. Kates. *Biochemistry* **3**: 1271, 1964.