References and Notes

1. S. Bergström, *Recent Progr. Hormone Res.* **22**, 153 (1966).
2. P. W. Ramwell and J. E. Shaw, *Amer. J. Physiol.* **211**, 125 (1966).
3. P. W. Ramwell, J. E. Shaw, R. Jessup, *ibid.*, p. 998.
4. F. Coceani and L. S. Wolfe, *Can. J. Physiol. Pharmacol.* **43**, 445 (1965); W. Feldberg and R. D. Myers, *J. Physiol.* **184**, 837 (1966); B. Samuelsson, *Biochim. Biophys. Acta* **84**, 218 (1964); J. E. Shaw, thesis, Birmingham University, Birmingham, England (1964); E. W. Horton and I. H. M. Main, *J. Physiol.* **185**, 36P (1966).
5. J. F. Mitchell, *J. Physiol.* **165**, 98 (1963).
6. P. W. Ramwell and J. E. Shaw, in *Proceedings of the Second Nobel Symposium* (Almqvist and Wicksell, Stockholm, 1967).
7. G. L. Avanzino, P. B. Bradley, J. H. Wolstencroft, *Brit. J. Pharmacol.* **27**, 157 (1966).
8. E. W. Horton and I. M. Main, *J. Physiol.* **179**, 18P (1965).
9. E. G. Gray and V. P. Whittaker, *ibid.* **153**, 2 (1960).
10. E. De Robertis, A. Pellegrino de Iraldi, G. Rodriguez, C. J. Gomez, *J. Biophys. Biochem. Cytol.* **9**, 229 (1961).
11. K. Kataoka and E. De Robertis, *J. Pharmacol. Exp. Therap.* **156**, 114 (1967).
12. K. Gréen and B. Samuelsson, *J. Lipid Res.* **5**, 117 (1964).
13. E. G. Daniels, *et al.*, *Biochem. Biophys. Res. Comm.* **21**, 413 (1965); D. Schneider, J. E. Pike, F. P. Kupiecki, *Biochem. Biophys. Acta* **125**, 611 (1966).
14. Conditions for biosynthesis of prostaglandins by brain microsomes have been discussed by L. S. Wolfe, F. Coceani, C. Pace-Asciak, in *Proceedings of the Second Nobel Symposium* (Almqvist and Wicksell, Stockholm, 1967).

15. Supported by PHS grant FR 05528 and by NIMH TOI MH 10625. Prostaglandin standards, PGE_1-1-C^{14}, and bis-homo-γ-linolenic acids-1-C^{14} were obtained through the kindness of Dr. John Pike, Upjohn Company.

25 May 1967

Euglena gracilis: A Novel Lipid Energy Reserve and Arachidonic Acid Enrichment during Fasting

Abstract. *In* Euglena gracilis *grown in the dark, wax esters, consisting of a combination of medium-chain fatty acids and alcohols that contain both odd and even numbers of carbon atoms, appear to be a reservoir for metabolic energy. When the organisms are fasted, their pellicular membrane systems become quite rich in long-chain polyenoic acids, mostly of the arachidonic acid family.*

The unicellular (or acellular) flagellate, *Euglena gracilis* (*1*), is a useful model for the study of organismic adaptive processes of the kind that can be induced by shifting environmental conditions. One may derive novel information of general interest about such processes from an inspection of the lipids of euglenas in which adaptive changes are taking place. When grown under continuous illumination, euglenas are chloroplast-bearing and behave as photosynthesizing organisms. As such, they are readily capable of an autotrophic way of life. When supplied with exogenous metabolites (*2*), they thrive in complete darkness as obligate heterophytes. Adaptive changes may be seen in the lipid components of euglenas that are starved, whether through deprivation of exogenous metabolites, in the case of dark-grown organisms, or of light, in the case of light-grown organisms kept in a barren medium. Some of the changes that have been observed in the lipids of starved *Euglena gracilis*, grown in the dark, are given in this report. The composition of the fatty acids of mature euglenas grown in both the presence and absence of light was examined (*3*). The organisms were grown without aeration in a synthetic medium (*2*) at 25°C. The light-grown euglenas were illuminated continuously with fluorescent light, generally at 990 lu/m^2; dark-grown euglenas were shielded from light in a light-proof box. Cultures were harvested as soon as their phase of growth at a logarithmic rate had terminated.

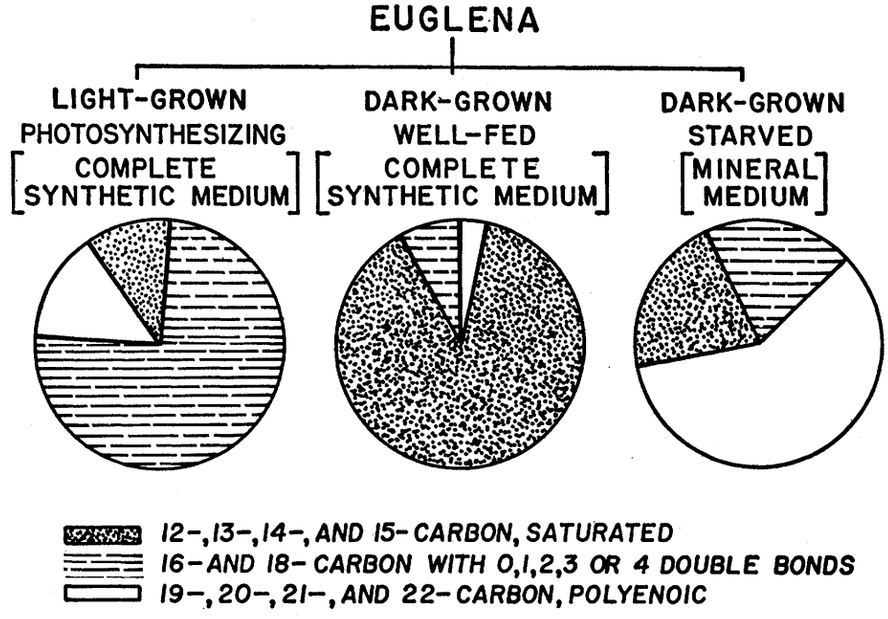

Fig. 1. Distribution of fatty acids by classes in green and etiolated *Euglena gracilis*.

Fatty acids were completely released from lipids of the harvested organisms by lengthy saponification of the whole organisms in methanolic alkali, under nitrogen (*4*) in the dark. The saponified mixture was then rendered strongly acidic with cold HCl, fatty acids were extracted, and methyl esters were made (*5*). The esters were analyzed by a combination of thin-layer and gas-liquid chromatography (*6*).

The fatty acid compositions of light-grown and dark-grown euglenas were quite different quantitatively (Fig. 1). Fatty acids of the light-grown organisms (Fig. 1, left-hand circle) were mainly 16 and 18 carbon atoms in length. The 16-carbon acids had either no double bonds or from one to four double bonds; for the most part, the 18-carbon acids had two or three double bonds. In addition, there were smaller amounts of 20-carbon acids with four or five double bonds, and of acids with shorter chains—12-, 13-, 14-, and 15-carbon acids—with no double bonds.

In contrast, the dark-grown organisms (Fig. 1, center circle) had a preponderance of saturated 12-, 13-, 14-, and 15-carbon acids and a quantity of 20-, 21-, and 22-carbon acids with either four, five, or six double bonds. Acids with 16 or 18 carbon atoms, the major fractions in light-grown organisms, were present, but in much smaller amount.

Attention was drawn to the large amount of 12- to 15-carbon saturated fatty acids in the dark-grown organisms. The occurrence of as large a fraction of shorter-chain fatty acids, especially with odd numbers of carbon atoms, is rare among Protista and higher forms of life. The lipid fraction that contained these acids was analyzed. It migrated on thin-layer plates of silica gel and on columns of silicic acid as a fraction of relatively low polarity (*3*). Consequently, it was readily separable from the other, more po-

Fig. 2. Crystals of the wax ester fraction of *Euglena gracilis*, in acetone. Photographs of crystals were taken in their own refracted light on polaroid color film in a Zeiss microscope fitted with polarizing optics set at full extinction. Optical magnification at the film was 190 diameters.

lar, complex lipids of the organism. Eventually the pure fraction in crystalline form (Fig. 2) was obtained by allowing it to emerge slowly from solution in hot methanol containing a few percent of hexane, or from acetone. The infrared spectrum of the crystalline lipid (*3*) indicated that it was a simple aliphatic ester. Saponification in methanolic alkali split the ester bond and produced equivalent moieties that consisted of (i) a series of aliphatic acids, mainly of 12 and 13 carbon atoms plus smaller amounts of acids of lesser and greater chain length, and (ii) a series of primary aliphatic alcohols with a distribution of chain lengths almost identical with that in the fatty acid moiety (*3*).

Thus the lipid fraction was identified as a family of wax esters composed of fatty acids and alcohols with odd and even numbers of carbon atoms. Such a fraction had not been observed previously in any protist. This wax ester fraction sometimes constituted more than one-half the total lipids in well-nourished dark-grown euglenas, and a smaller fraction of the lipids of well-nourished euglenas grown in the light.

In their molecular packing properties, wax esters should closely resemble triglycerides (*7*). It is not likely that these esters can act as functional components of membrane systems since esters are essentially lacking in polar or ionic terminal groups. Wax esters can, like triglycerides, serve with great practicality as a form of storage for metabolic energy.

It seemed possible that the wax esters of *Euglena* might arise from fatty acids by α-oxidation, an exergonic process known to occur in higher plants (*8*). In this process, fatty acids are oxidized at the α-carbon atom (next to the carboxyl group) by peroxide, the reaction being mediated by peroxidase. Alpha oxidation produces α-keto acids which, upon decarboxylation, give rise to fatty aldehydes with one carbon atom less than the original acids. The aldehydes are readily further oxidized by aldehyde dehydrogenase and pyridine nucleotide to fatty acids and these, once again, are susceptible to the process of α-oxidation. Through α-oxidation, fatty acids are degraded one carbon atom at a time to produce energy, and, in the process, fatty acids and aldehydes, alternately of odd and even numbers of carbon atoms, are the intermediates.

No specific attempt has yet been made to demonstrate this, but it is probable that the process of α-oxidation occurs in *Euglena* since, metabolically, *Euglena* is a plant-like organism. Further, it is conceivable that when the energy requirements of the organism dictate it, the net α-oxidation of fatty acids may be halted by the occurrence of a continuous dismutation of the aldehydic intermediates. Such a dismutation would involve reduction to alcohols, presumably by reduced pyridine nucleotide and alcohol dehydrogenase, of as many molecules of fatty aldehyde as are oxidized to fatty acids by pyridine nucleotide and aldehyde dehydrogenase. The equivalent quantities of fatty acids and fatty alcohols may then be combined chemically to form wax esters for purposes of storage as a metabolic energy reserve that may be hydrolyzed and further oxidized in time of need.

Two experiments were designed to attempt to define the role of wax esters in *Euglena*. Well-nourished etiolated organisms were washed free of medium and transferred to a strictly mineral medium in the dark. The organisms had no exogenous source of energy. Under these conditions, the wax esters were found to disappear rapidly (*4*), and, consequently, the lipid content of the euglenas dropped to roughly one-half. During this process, the organisms displayed loss of neither motility nor viability. Addition of glucose to the medium markedly slowed the disappearance of wax esters in the starving organisms (*4*). Thus, indications are that, in *Euglena*, these esters serve as a form of storage for meta-

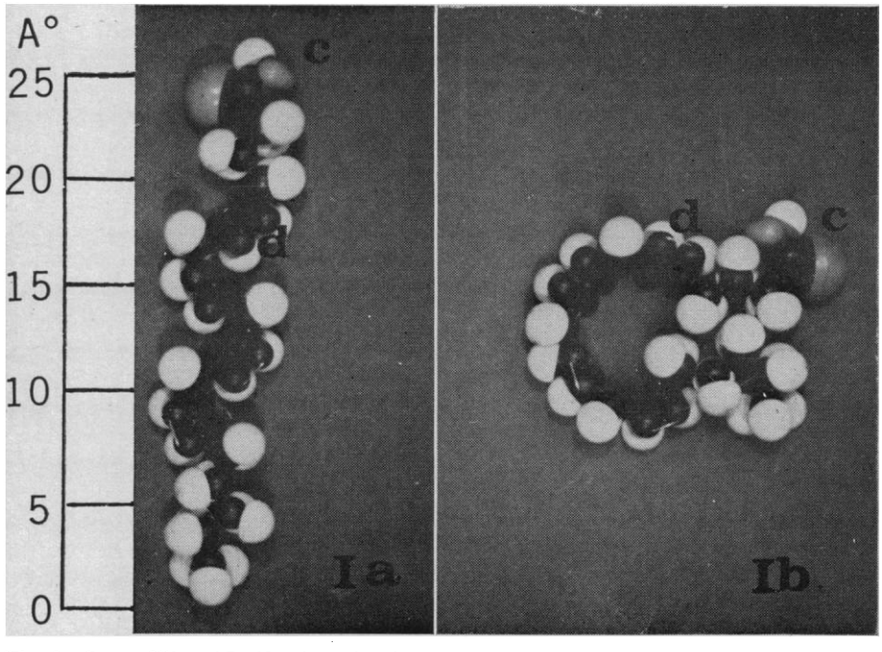

Fig. 3. Space-filling (Godfrey) molecular models. (Ia) Arachidonic acid in unstrained, extended, helical configuration; *c* indicates the carboxyl group and *d*, the first of the four double bonds after the carboxyl group. (Ib) Arachidonic acid in unstrained, quasi-planar, curled configuration; *c* and *d*, the same as for Ia. Ia and Ib represent two extremes of the configurations that can be assumed by the molecule.

bolic energy. This appears to be the first indication that, in some organisms, neutral fatty esters other than triglycerides may serve as the major form of storage of such reserves.

To test whether the mechanism of α-oxidation is involved in the metabolism of wax esters, imidazole, a potent inhibitor of fatty acyl peroxidase (*8*), was added to the culture medium (*4*). Entry of imidazole into the organism should block the α-oxidation of fatty acids, since it is a peroxidase-dependent process. In a culture of well-nourished euglenas growing in the dark in a complete medium, imidazole caused a lowering of the content of medium-chain fatty acids typically found in the wax esters, with a simultaneous accumulation of saturated 16-carbon (palmitic) acid and 18-carbon (stearic) acid, the chief saturated acids to be synthesized *de novo* (*9*) in *Euglena*. In starving dark-grown euglenas, imidazole clearly slowed the removal of their store of preformed wax esters (*4*). These observations are consistent with the interpretation that the wax esters of *Euglena* are produced and metabolized by a peroxidase-dependent process of fatty acid oxidation that can be blocked by exogenous imidazole.

The changing composition of lipids of starving dark-grown organisms provided a clue to an adaptive mechanism of general interest. As the euglenas starved and as their wax esters declined, arachidonic acid (*10*) and its homologs became the quantitatively predominant fatty acids (*4, 6*) in these organisms. The theoretical role of arachidonic acid as a basic component in expanded and readily hydrated membrane systems has recently been the subject of plausible speculation (*6, 11, 12*).

The four, all *cis*, double bonds in the hydrocarbon chain of arachidonic acid oblige the chain to assume a curled configuration when it is packed in a quasi-planar arrangement, or a helical configuration when packed in a linear arrangement (Fig. 3, Ia and Ib). With either configuration, a close packing with other, fully saturated, hydrocarbon chains is not possible, nor can there be strong London–Van der Waals bonding with other, fully saturated chains, since bonding necessitates the close approach of large enough numbers of methylene groups in adjacent chains that can, with such a close approach, form tight "hydrophobic" aggregates, as in myelin (*11, 12*). Rather than the formation of tightly aggregated, hydrophobic, limiting membrane systems, the formation of electron-rich, expanded, limiting membrane systems that are potentially receptive to the influx of metabolites from the surrounding aqueous medium would appear to be a necessity for the starving dark-grown euglenas. The introduction of a high percentage of arachidonic acid or similar long-chain polyunsaturated acids into the membranes of the starving organisms should help to build the needed systems. To examine this possibility, euglenas that had been maintained for a week in a medium that contained only potassium phosphate and magnesium chloride were fractioned and their pellicles isolated. Fully two-thirds of the fatty-acid components of the pellicles of starved etiolated euglenas turned out to be arachidonic acid and its homologs (*6*). Much of this was incorporated into complex lipids that were so firmly bound into the pellicle's structure that it was not easily extracted with a mixture of chloroform and methanol, the usual lipid extractant. Only after destruction of the pellicular architecture by prolonged heating in methanolic alkali, in the dark under nitrogen, was all of the arachidonic acid fraction released. In the pellicles of green photosynthesizing euglenas, there was only half the quantity of arachidonic acid and its homologs that was found in the pellicles of the starving dark-grown organisms. In the photosynthesizing organisms, arachidonic acid was also found in the chloroplasts, but nowhere else in the organisms. In the starving dark-grown euglenas, arachidonic acid and its homologs pervaded the organisms, with a great concentration in the pellicles (*6*). Thus, the long-chain polyenoic acids of *Euglena* were components of lipids that appeared to be located mostly in membrane systems close to the aqueous environment from which presumably hydrated metabolites had to be derived.

ABRAHAM ROSENBERG
Columbia University Research Service, Goldwater Memorial Hospital, New York 10017

References and Notes

1. J. J. Wolken, *Euglena* (Rutgers Univ. Press, New Brunswick, N.J., 1961).
2. S. Hutner, *Anal. Chem.* **30**, 849 (1958).
3. A. Rosenberg, *Biochemistry* **2**, 1148 (1963).
4. ——— and M. Pecker, *ibid.* **3**, 254 (1964).
5. L. D. Metcalfe and A. A. Schmitz, *Anal. Chem.* **33**, 363 (1961).
6. A. Rosenberg, M. Pecker, E. Moschides, *Biochemistry* **4**, 680 (1965).
7. L. Chapman, *J. Amer. Oil Chem. Soc.* **37**, 73 (1960).
8. F. O. Martin and P. K. Stumpf, *J. Biol. Chem.* **234**, 2548 (1959).
9. G. M. Cheniae, *Biochim. Biophys. Acta* **70**, 504 (1963).
10. J. Erwin and K. Bloch, *Biochem. Z.* **338**, 496 (1963).
11. J. S. O'Brien, *Science* **147**, 1099 (1965).
12. F. A. Vandenheuvel, *J. Amer. Oil Chem. Soc.* **40**, 455 (1963).
13. Supported by PHS grants GM 09041-06 and HE 00052-19 from NIH, and by the Health Research Council of the City of New York under contracts U-1562 and I-164.

10 July 1967

Galactosyl Diglycerides: Their Possible Function in Euglena Chloroplasts

Abstract. *Illumination of euglenas grown in the dark induces the formation of chloroplasts characterized by the simultaneous appearance of chlorophyll and galactosyl diglycerides in a relatively fixed ratio. The fatty acyl chains of the galactosyl diglycerides are constructed so that they can provide a stable lock-and-key fit with the phytol chains of chlorophyll in such a way as to localize the porphyrin structures of chlorophyll and space them for efficient photoreception. Light-starved photobiotic euglenas show chloroplast shrinkage with a concurrent partial loss of galactosyl diglycerides.*

The chloroplasts of green euglenas do not replicate in the dark. Once deprived of light, the progeny of these organisms will contain successively smaller stores of chlorophyll and, in the end, an inoculum of green euglenas grown in complete darkness will produce a dark-grown (etiolated) culture that is essentially achlorophyllous. Subsequent subcultures that can be produced repeatedly in the absence of light have the same characteristic. Exposure of etiolated euglenas to light induces them to redevelop chloroplasts. After this, the organisms are capable of an autotrophic existence once again.

Illuminated green euglenas live, but they cannot grow and divide, in a medium that contains no nitrogen, and,