at the concentration employed, it is reasonable to assume that the *N*-acetylneuraminic acid linkage in monosialoganglioside is different from that in neuraminlactose. Several alternative possibilities may be visualized; viz., (1) the *N*-acetylneuraminic acid might be linked to *N*-acetylgalactosamine; (2) the *N*-acetylneuraminic acid may be linked to a different carbon of galactose; or (3), as suggested by studies of the reduction of intact gangliosides with borohydride (Kanfer and Brady, 1963), it might not be ketosidically linked. So far we have not been able to obtain a sufficient quantity of *N*-acetylneuraminyl-*N*-acetylgalactosamine or the various other possible isomers of neuraminlactose or neuramingalactose to test as inhibitors.

Finally, hapten-inhibition assays were carried out with whole antiserum and different inhibitors used in the present study may be bound to different extents to serum albumin. However, the high concentrations of hapten required for inhibition make it seem unlikely that major difficulties due to binding with albumin would be encountered in these experiments.

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REFERENCES

Bogoch, S. (1961), *Nature 190*, 153.
Boyd, W. C. (1956), Fundamentals of Immunology, 3rd ed., New York, Interscience.
Egge, H. (1960), *Bull. Soc. Chem. Biol. 42*, 1429.
Kabat, E. A. (1957), *J. Cell. Comp. Physiol. 50*, supp. 1, 79.
Kabat, E. A. (1958), *in* Chemistry and Biology of Mucopolysaccharides, Wolstenholme, G. E. W., and O'Connor, M., eds., Boston, Little, Brown, p. 42.
Kabat, E. A., and Mayer, M. M. (1961), Experimental Immunochemistry, Springfield, Ill., Charles C Thomas, p. 241.
Kanfer, J. N., and Brady, R. O. (1963), *Biochem. Biophys. Res. Commun. 11*, 267.
Karkas, J. D., and Chargaff, E. (1960), *Biochim. Biophys. Acta 42*, 359.
Klenk, E., and Gielen, W. (1960), *Z. Physiol. Chem. 319*, 283.
Klenk, E., and Gielen, W. (1961), *Z. Physiol. Chem. 326*, 144.
Klenk, E., Gielen, W., and Padberg, G. (1962), *in* Cerebral Sphingolipidoses, Aronson, S. M., and Volk, B. W., eds., New York, Academic, p. 301.
Kuhn, R., and Wiegandt, H. (1963), *Chem. Ber. 96*, 866.
Landsteiner, K. (1945), The Specificity of Serological Reactions, revised ed., Cambridge, Mass., Harvard University Press.
Makita, A., and Yamakawa, T. (1962), *J. Biochem. (Japan) 51*, 124.
Schiffman, G., Kabat, E. A., and Leskowitz, S. (1962), *J. Am. Chem. Soc. 84*, 73.
Svennerholm, L. (1962), *Biochem. Biophys. Res. Commun. 9*, 436.
Trams, E. G., and Lauter, C. J. (1962), *Biochim. Biophys. Acta 60*, 350.
Wagner, A., Dain, J. A., and Schmidt, G. (1963), *Fed. Proc. 22*, 334.
Yamakawa, T., Yokoyama, S., and Kiso, N. (1962), *J. Biochem. (Japan) 52*, 228.
Yokoyama, M., Trams, E. G., and Brady, R. O. (1963), *J. Immunol. 90*, 372.

Lipid Alterations in *Euglena gracilis* Cells During Light-induced Greening*

ABRAHAM ROSENBERG† AND MARC PECKER

From the Departments of Biochemistry and Medicine, College of Physicians and Surgeons, Columbia University, and The Columbia University Research Service, Goldwater Memorial Hospital, New York City

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Etiolated cells of *Euglena gracilis* were subjected to constant illumination in a mineral medium. After a short lag period the cells produced chlorophyll in three successive stages. Sulfolipids accumulated before measurable amounts of chlorophyll. Sulfolipid synthesis occurred in stages corresponding to those of chlorophyll synthesis. In contrast, the cellular galactolipid level increased at a linear rate with the onset of illumination, independently of chlorophyll level. Upon illumination of the cells, the major fatty acids of the well-nourished etiolated cell, C_{13} and C_{14}, rapidly disappeared. The green cell replenished its fatty acid complement by the synthesis of unsaturated fatty acids mainly of the 16- and 18-carbon series.

A study of the lipids of photosynthesizing organisms may result in new information about photosynthetic processes. We have previously reported an accumulation of unsaturated fatty acids, galactolipids, and sulfolipids in photosynthesizing *Euglena gracilis* cells (Rosenberg, 1963). The present experiments were designed to trace these three lipid components during stages of the greening of etiolated cells. Greening was induced by constant illumination of the cells. Chlorophyll content was taken as an index of photosynthetic activity. The cells were examined for a relationship between their chlorophyll content and the levels of newly synthesized lipids. Conditions were arranged to limit the extraneous factors imposed by growth and division of the cells.

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MATERIALS AND METHODS

Cells of *Euglena gracilis* were grown in the dark on a synthetic medium (Wolken, 1961) and harvested. The harvested cells were washed in distilled water. Approximately 4-g batches were suspended in 100 ml

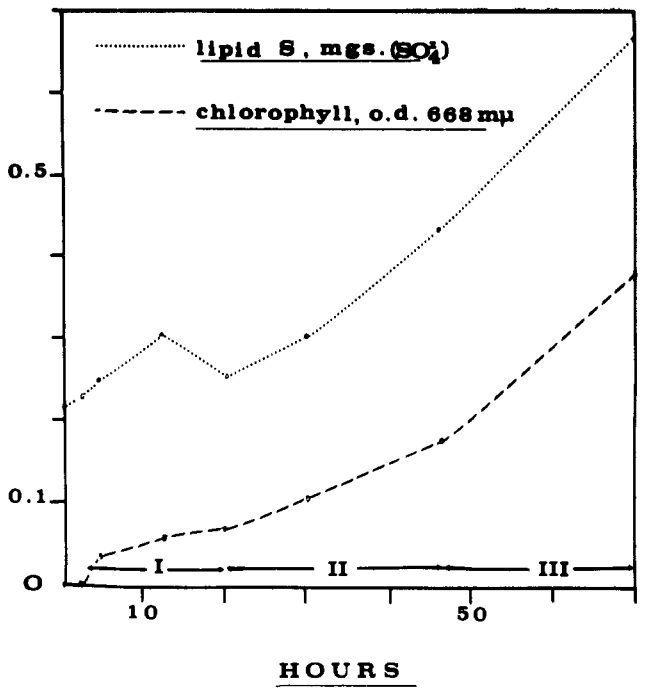

FIG. 1.—The relative quantities of chlorophyll and sulfolipid in greening cells of *Euglena gracilis*. Sulfolipid content is expressed in terms of milligrams of sulfate obtained on ashing the total lipid. Chlorophyll content is expressed in terms of optical density measurements at 668 mμ. The abscissa shows the number of hours of constant illumination of the cells. The roman numerals indicate the stages of greening.

of a mineral medium (0.01 M KH_2PO_4 and 0.01 M $MgCl_2$) supplemented in most experiments with 1% (w/v) glucose. The cultures were agitated under constant fluorescent illumination with 15-w "Cool White" tubes at 90 ft-c at 26°. At intervals, uniform samples of the cultures were removed under sterile conditions. The cells were collected, and their total lipids were extracted as described (Rosenberg, 1963). The extracts were evaporated under nitrogen. Total lipid weights were obtained after drying the lipid residues in a vacuum overnight over $CaCl_2$, $CaSO_4$, and paraffin. Absorption spectra of the total lipid in hexane were traced in a Bausch and Lomb Spectronic 505. Optical densities at 668 mμ were used as a measure of chlorophyll content. The total lipid was saponified under nitrogen at 60° for 5 hours in 5% (w/v) KOH in methanol, yielding the fatty acids. These were titrated (Dole, 1956) or methylated and quantitatively estimated by the hydroxamic acid reaction (Rosenberg and Chargaff, 1958). Identification of the methyl esters of the fatty acids was made by gas-liquid chromatography in a Barber-Coleman Model 10 instrument. An ethylene glycol succinate column at 173° was used with argon serving as the mobile phase. The unsaturated methyl ester fraction was isolated as the methoxy mercuri-acetate adduct (Jantzen and Andreas, 1959) and chromatographed separately. As a further aid to identification, the unsaturated fraction of methyl esters of the fatty acids was reduced and rechromatographed. The reduction was accomplished in 1 hour in hexane solution over platinum oxide with hydrogen gas at room temperature and 2 atm. Lipid hexose analyses and ashing prior to total sulfate analyses were done as before (Rosenberg, 1963).

RESULTS

Mature, well-nourished, dark-grown cells of *Euglena gracilis* in a mineral medium supplemented with glucose manufactured chlorophyll in three successive stages on exposure to light. The course of chlorophyll synthesis during greening is shown in Figure 1. Absorption spectra of the total cellular lipids prior to greening and at each stage of greening are shown in Figure 2. On exposure of the cells to light, greening was preceded by a short lag period. During this period no chlorophyll could be measured in the total lipid extracts. The successive stages of greening are designated I, II, and III. During stage I chlorophyll was produced at a slow rate with an eventual leveling of production. Stage I lasted approximately 15 hours. During stage

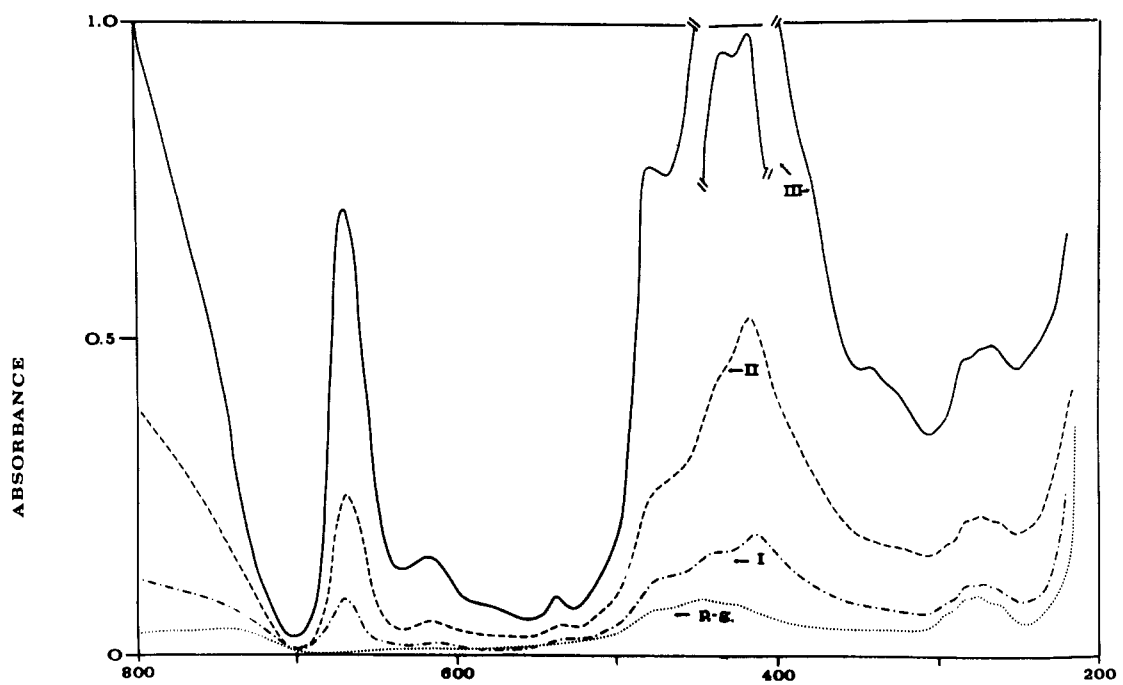

FIG. 2.—Absorption spectra of the total lipid of greening *Euglenia gracilis* cells in hexane. The period before greening is indicated by p.-g. The roman numerals indicate the stages of greening.

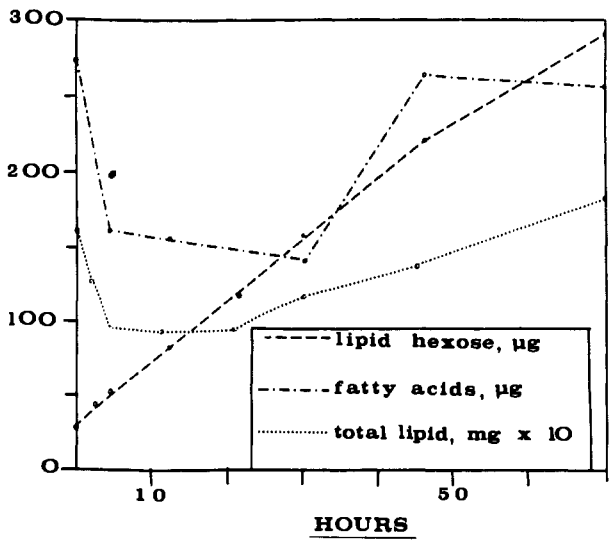

FIG. 3.—The relative quantities of lipid hexose, fatty acids, and total lipid in greening cells of *Euglena gracilis*. The abscissa indicates the number of hours of constant illumination.

II a renewed synthesis of chlorophyll at a linear rate was observed, lasting some 25 hours. Stage III followed with a further increase in chlorophyll synthesis until maximum greening occurred.

The lag period preceding greening was marked by a sharp decrease in the total lipid content of the cell to roughly half the original. The total lipid of the cell remained at the minimal level throughout stage I of greening. Stages II and III showed an increase in total cellular lipid until the original level was regained. These changes are shown graphically in Figure 3.

Fluctuations in the quantity of fatty acids in the cells generally paralleled those of the total lipid. During the pregreening period the total fatty acid content of the cells dropped rapidly. During stage I of greening the loss all but halted. In stage II, the cellular fatty acid content quickly returned to the initial level and remained there throughout stage III. The changes in fatty acid content of the greening cells are shown in Figure 3. Changes in fatty acid content of the cells during the several stages of greening are a reflection of changes in the levels of different individual fatty acids. Changes in cellular content have been calculated for the major fatty acids. These are shown graphically in Figure 4. With the onset of greening, the fatty acids of *Euglena gracilis* changed quantitatively from the predominantly saturated to the predominantly unsaturated variety. The ratios of the weights of the unsaturated to the saturated fatty acid fractions were 0.49 for the pregreening stage and 1.8, 1.3, and 1.7 for stages I, II, and III, respectively, of greening.

The sharp loss of fatty acids during the pregreening period is due to the rapid disappearance of C_{18}, C_{14}, and to some extent C_{16} and C_{18} compounds. C_{18} and C_{14} compounds are quantitatively the major fatty acids in the well-nourished etiolated cell. In stage I of greening a loss of $C_{18:2}$ was offset by a gain in $C_{18:1}$ and $C_{18:3}$, and the total fatty acid content of the cell dropped very little. In stage II of greening, C_{16}, $C_{16:2}$, C_{18}, $C_{18:2}$, and C_{20} fatty acids increased so greatly that the cellular fatty acid level returned to its original point. During stage III, C_{16} and C_{18} fatty acids quickly declined while $C_{16:2}$, $C_{18:2}$, and C_{20} fatty acids continued to rise. An increase in $C_{18:3}$ followed. In cultures maintained under the specified conditions, $C_{16:2}$ became the major fatty acid in the cell.

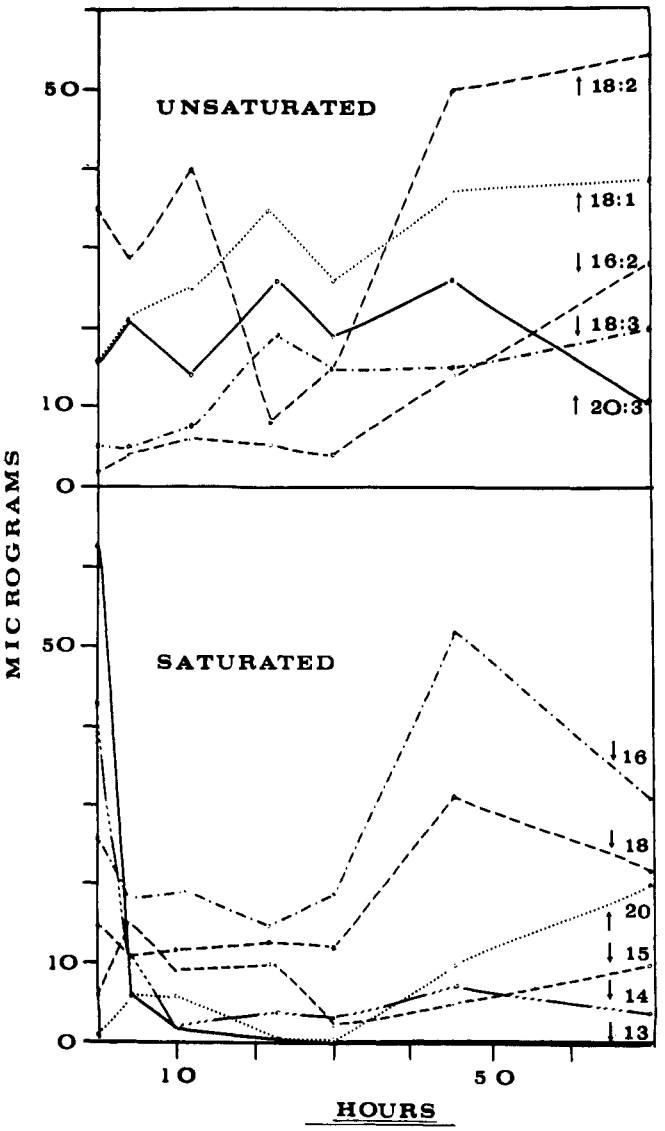

FIG. 4.—The changes in the cellular content of the major fatty acid species in greening cells of *Euglena gracilis*. "20:3" is not fully identified; arachidonate has the same retention time. The abscissa indicates the number of hours of constant illumination.

When etiolated cells were exposed to light, their sulfolipids (Benson, 1961) increased before measurable quantities of chlorophyll could be observed. The quantity of sulfolipids (Abraham and Bachhawat, 1963) trebled during the total greening process. Their production occurred in stages which corresponded to those of chlorophyll synthesis in the illuminated cell. The change in sulfolipid levels with time is shown in Figure 1.

Unlike the sulfolipids, the galactolipids (Carter *et al.*, 1961) showed a linear time rate of increase with the beginning of illumination. During stages I and II the percentage of galactolipid in the total lipid increased very rapidly. It became static during stage III. These results are traced in Figure 3.

Lipid phosphorus declined with the onset of greening. It dropped from 1% (w/w) before greening to 0.7% during stage I. In stages II and III the original level was gradually recovered.

The lipid components measured in the illuminated cell were not influenced to a measurable extent by glucose in the medium at the level specified. In etiolated cultures maintained as controls in the dark, glucose in the medium exhibited a 2-fold influence.

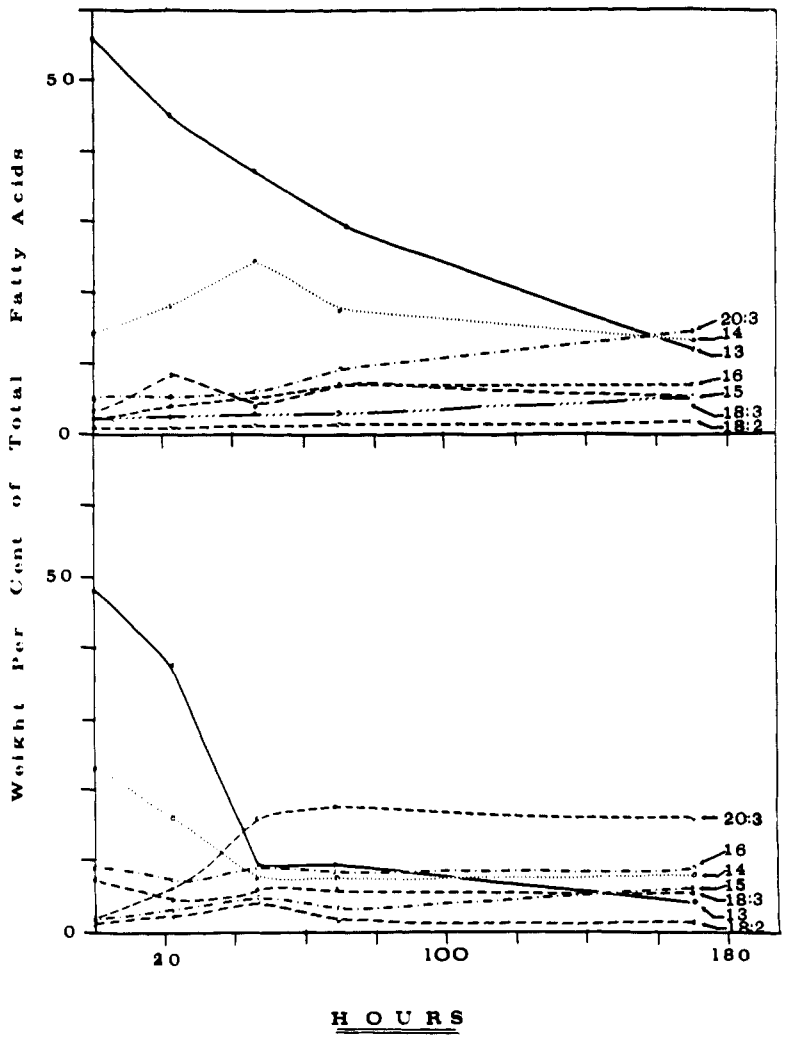

FIG. 5.—The changes in the relative weight percentages of the major fatty acids of *Euglena gracilis* cells kept in the dark in a mineral medium. Upper curves: medium supplemented with 1% (w/v) glucose. Lower curves: medium without energy source. "20:3" is not fully identified; arachidonate has the same retention time. This fraction may be either 20:3, arachidonate, or a mixture of both.

A small increase in lipid hexose occurred, reaching a plateau a few hours after exposure of the cells to the medium. The initial lipid hexose level shown in Figure 3 is the result of an overnight accommodation of the cells to the medium before illumination. A more far-reaching effect of glucose in the medium was noted in the marked sparing effect on utilization of C_{13} and C_{14} fatty acids by cells maintained in the dark. (See Figure 5.) In starved etiolated cells in a glucose-free mineral medium in the dark, $C_{20:3}$ soon became the major fatty acid. It emerged into relative predominance essentially because of the disappearance of C_{14} and C_{13} compounds which originally constituted the greatest fatty acid fraction. However a net increase in $C_{20:3}$ could also be measured.

When returned to the dark, greening cells maintained a stable chlorophyll level for a period of some 70 hours. The chlorophyll level then diminished slowly over a period of about a week. The rate of chlorophyll disappearance in the dark was relatively constant in cells at all stages of greening in spite of their differing chlorophyll content. Green cells kept in the dark beyond 200 hours lost viability and integrity rather than all their chlorophyll. The mineral medium does not support cell division. Reversion to an etiolated culture with total loss of chlorophyll was not possible by this means. During the period of stable chlorophyll level in the dark, the fatty acid content of the cells doubled. The lipid hexose dropped some 30%. The increase in fatty acid content resulted from a 7- to 8-fold accumulation of C_{12}, C_{13}, and C_{14} compounds. This accumulation continued during the ensuing slow process of chlorophyll disintegration.

DISCUSSION

Exposure of etiolated cells of *Euglena gracilis* to light occasions a rapid disappearance of their saturated fatty acids of chain length lower than 16 carbon atoms. These fatty acids predominate in the well-nourished etiolated cell. They occur mainly in a waxy ester fraction composed of equal moieties of fatty acid and fatty alcohol (Rosenberg, 1963). They presumably arise through α-oxidation (Martin and Stumpf, 1959) of fatty acids to aldehydes. It is postulated that the aldehydes undergo rapid dismutation to acids and alcohols which are stored as waxy esters. Etiolated cells transferred in the dark to a medium devoid of energy source slowly lose the fatty acids found in their waxy-ester fraction. The addition to the medium of glucose as a foodstuff markedly slows the disappearance of lower-chain saturated fatty acids

in the dark. The waxy-ester fraction appears to serve the function of a storage of chemical potential in the etiolated cell. When etiolated cells are illuminated, their lower-chain saturated fatty acids almost completely disappear within a few hours. The rate of disappearance in the illuminated cell is not influenced by glucose in the medium. This contrasts markedly with the behavior of cells kept in the dark. The illuminated cell may fail to utilize glucose in the medium and as a consequence may lean more heavily on the waxy-ester fraction for the energy required for a transition from the etiolated to the photosynthesizing state. It appears more likely, however, that induction of the photosynthetic mechanism by light is accompanied by a repression of metabolic pathways responsible for waxy-ester production. Rapid loss of waxy-ester would follow utilization without replenishment. Greening occurs when the lower-chain saturated fatty acid fraction has dropped to a very low static level.

Illumination of the cell marked the onset of synthesis of galactolipid at a linear rate. The rate of galactolipid production does not depend on the level of chlorophyll. The system producing the galactolipids apparently relies on the products of photosynthesis but, nevertheless, it seems to be fully satisfied even at low levels of photosynthetic activity in the cell. Galactolipids increase 10-fold during greening. At maximal greening with constant illumination their level remains static. They are most probably an energy storage form in the green cell with a function analogous to that of the waxy esters in the dark-grown cell. The inverse relationship between accumulation of these two lipid fractions may be observed when green cells are deprived of light. Lipid hexose drops, and the fatty acids typical of the waxy-ester fraction quickly rise. Other fatty acid species in the cell remain relatively undisturbed. The etiolated cell undergoes a substantial loss of fatty acids with the disappearance of the lower-chain saturated fraction upon illumination. It is clear that the green cell remedies this loss by the abundant production of unsaturated fatty acids. These are mainly of the 16- and 18-carbon series. Identification has not yet been attempted of the molecular compounds which contain the unsaturated fatty acids accumulating in the photosynthesizing cell.

Chlorophyll in the granum is considered to be distributed on a layer of lipid (Goedheer, 1955). It may be reasonable to hypothesize that some lipids are among the initial reactants with the photosynthetically supplied reduced pyridine nucleotide, oxygen, and ATP. The widespread fatty acid desaturating system (Bloomfield and Bloch, 1960; Bernhard *et al.*, 1959; Talalay, 1957) which requires these factors (Bloomfield and Bloch, 1960) may also be present in chloroplasts (Stumpf *et al.*, 1963). It is tempting to speculate on the presence in the lipid layers of the grana of a system which utilizes the photosynthetically produced high-energy compounds to build carbohydrate molecules. These may then be bound into lipids (Neufeld, 1963) within the chloroplast for retention and utilization during periods of darkness.

Sulfolipid synthesis appears to bear a temporal relationship to chlorophyll synthesis. Sulfolipid molecules may be necessary for the orientation and possibly the functions of chlorophyll. Their ability to readily undergo oxidation and photoreduction (Ibanez-Martini and Lindstrom, 1959) should not be overlooked as possible clues to the mechanisms involved in the photoinduction of chlorophyll synthesis in etiolated cells.

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References

Abraham, A., and Bachhawat, B. K. (1963), *Biochim. Biophys. Acta 70*, 104.

Benson, A. A. (1961), *in* Light and Life, McElroy, W. D., and Glass, B., eds., Baltimore, Johns Hopkins Press, p. 392.

Bernhard, K., von Bulow-Koster, J., and Wagner, H. (1959), *Helv. Chim. Acta 42*, 152.

Bloomfield, D. K., and Bloch, K. (1960), *J. Biol. Chem. 235*, 337.

Carter, H. E., Ohno, K., Nojima, S., Tipton, C. L., and Stanacev, N. Z. (1961), *J. Lipid Res. 2*, 215.

Dole, V. P. (1956), *J. Clin. Invest. 35*, 150.

Goedheer, J. C. (1955), *Biochim. Biophys. Acta 16*, 471.

Ibanez-Martini, M. L., and Lindstrom, G. S. (1959), *Biochem. Biophys. Res. Commun. 1*, 224.

Jantzen, E., and Andreas, H. (1959), *Chem. Ber. 92*, 1427.

Martin, F. O., and Stumpf, P. K. (1959), *J. Biol. Chem. 234*, 2548.

Neufeld, E. F. (1963), *Fed. Proc. 22*, 464.

Rosenberg, A. (1963), *Biochemistry 2*, 1148.

Rosenberg, A., and Chargaff, E. (1958), *J. Biol. Chem. 232*, 1031.

Stumpf, P. F., Bové, J. M., and Goffeau, A. (1963), *Biochim. Biophys. Acta 70*, 260.

Talalay, P. (1957), *Physiol. Rev. 37*, 362.

Wolken, J. J. (1961), *Euglena*, New Brunswick, N. J., Rutgers University Press, p. 13.