Correlation of α-Linolenate to Photosynthetic O_2 Production in Chlorella[1]

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Summary. Photosynthetic oxygen evolution per milligram of chlorophyll in *Chlorella vulgaris* varies with the age of the culture. The rate of oxygen evolution is low in the starting cells, it rises to a maximum after 24 hours of growth and then declines to the initial low value after 72 to 90 hours. These changes in photosynthetic competence of chlorophyll in *Chlorella* are paralleled by changes in α-linolenate per milligram of chlorophyll. In general the magnitude of the photosynthetic competence of chlorophyll is directly proportional to the magnitude of the ratio of α-linolenate to chlorophyll, regardless of whether high ratios are due to high α-linolenates or low chlorophyll values. This relationship holds when the cultures are grown either under continuous or intermittent illumination.

That chloroplasts of photosynthetic organisms which produce O_2 have a high lipid content is now well established. Sisakyan and Smirnov (14) found that higher fatty acids compose 50 to 60% of the absolute dry weight of the lipid fraction of chloroplasts from higher plants. They also report that isolated chloroplasts are able to synthesize and oxidize fatty acids of the C_{16} and C_{18} types. Crombie (2), working with greening, etiolated leaves as well as with white and green tissue of variegated plants, concluded that the fat formed at the same time as chlorophyll is highly unsaturated and is characterized by a high proportion of linolenic acid. In a recent review Benson (1) summarized critically much of the work on chloroplast lipids. The evidence he presented clearly indicates that the predominant fatty acid of chloroplasts is linolenic and that it occurs primarily as the galactolipids, monogalactosyl dilinolenin and digalactosyl dilinolenin. There is thus general agreement that α-linolenate-containing galactolipids are quantitatively the most important lipids of chloroplast; as to the function of these lipids there is no accepted view.

Crombie (2) and Wolf et al. (15) speculate that linolenic acid may have a specific function in photosynthesis. Erwin and Bloch (4) cite some interesting experimental findings pointing to a direct relationship between the ability to liberate O_2 and α-linolenate (*cis*-9,12,15-octadecatrienoic acid) content of photosynthetic cells or tissues. They postulate that α-linolenate (and perhaps galactolipid) is a necessary lipid component not for photosynthesis per se but for one or more of the steps that lead to O_2 evolution during photosynthesis. Benson (1), on the other hand, speculates that linolenic acid synthesis and accumulation in chloroplasts are consequences of photosynthetic activity. He postulates that linolenic acid is probably derived from the more saturated acids, such as oleic, and that the desaturation process has some O_2-requiring steps.

In a recent paper from this laboratory (13) it was shown that *Chlorella* cells in a nonsynchronous culture in liquid medium go through a characteristic series of physiological changes, depending on the age of the culture used as a starter and the environmental conditions. For the purpose of this paper we need mention only the change in photosynthetic O_2 evolution. The rate of O_2 evolution per unit of total chlorophyll is very low in the old starting cells. It increases and reaches a maximum, roughly 3 times as high as the initial rate, in approximately 24 hours, and then declines to the initial low value at about 72 to 96 hours, depending on the age of the starting cells.

This paper reports on experiments in which the changes in the lipid composition that parallel those in photosynthetic O_2 evolution in growing *Chlorella* cultures were studied.

Materials and Methods

Chlorella vulgaris, originally obtained from Dr. S. Granick of the Rockefeller Institute, was used in all experiments. The method of growing the algae and all the methods for the various determinations not specifically mentioned here are described in a previous publication (13).

All cultures were started so that the initial concentration was 0.5 ml of packed cells (PC) per liter of suspension. At predetermined periods samples of

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suspensions were harvested, and PC concentrations determined. Then the cells were centrifuged and washed once with a fresh culture solution. The washed cells were made up to the desired concentration and aliquots were taken for the determinations of photosynthesis, respiration, total chlorophyll, nitrogen and cell counts. An aliquot equivalent to 200 mm^3 of PC was taken for lipid determination. This aliquot was centrifuged and resuspended in 6 ml of cold distilled water, and delivered quantitatively into a precooled X-press (3). The frozen plug of broken cells was delivered directly from the X-press into 60 ml of cold extraction solvent consisting of chloroform: methanol 2:1. The extraction was hastened by mixing the solvent and broken cells for about 15 minutes with a magnetic stirrer in an atmosphere of N_2. The extract was filtered on a sintered glass filter and the residue was washed on the filter with 5 ml solvent and finally with 10 ml of methanol. All above operations were carried out in a cold room (0–5°). The extract was reduced to a small volume in vacuo on a rotary evaporator (at 0°) and transferred to a small pyrex tube having a teflon-lined screw cap. The extract was reduced to dryness in the tube by blowing dry N_2 over it.

Preparation of Methyl Esters. The lipid residue in the tube was dissolved in 4 ml of 4 % anhydrous HCl in methanol and heated for 6 hours at 80 to 90° in the closed tube. The reaction mixture was then reduced to a small volume on a water bath under a gentle stream of nitrogen and the methyl esters were taken up in 10 ml of *n*-pentane. The pentane solution of the methyl esters was washed several times with 5 ml portions of water, dried over anhydrous $MgSO_4$ and filtered. The crude methyl esters thus obtained were then chromatographed on 1 g of silicic acid (Unisil, 100–200 mesh obtained from the Clarkson Chemical Company, Inc.). Hydrocarbons were eluted with 15 ml of *n*-pentane. The methyl esters were then eluted with 10 ml of 4 % ethyl ether in *n*-pentane. More polar materials such as hydroxy esters, alcohols, and chlorophyll are left on the column in this procedure (6). The methyl esters, thus purified, were weighed and then dissolved in benzene (2 % solution) for gas chromatographic analysis.

Quantitative Analyses of Methyl Esters. Gas chromatographic analyses of the methyl esters were performed with a Barber-Colman Model 10 instrument equipped with a 40 inch ethylene glycol succinate polyester column as described by Rodegker and Nevenzel (11). The percentage composition was determined from the areas under each peak (11). Minor components (less than 0.5 % by weight) were ignored in order to simplify the calculations.

Determination of the Structure of Unsaturated Fatty Acids. Methyl esters obtained by pooling the samples obtained from one experiment were separated into 5 fractions (saturated, mono-, di-, tri- and tetraenoic esters) by chromatography of the mercuric acetate adducts on silicic acid as described by Erwin and Bloch (5). The individual fractions were then analyzed by gas chromatography. In this manner both the chain length and number of double bonds could be determined for each component. The positions of the double bonds were determined by gas chromatographic isolation of individual peaks (9) followed by permanganate-periodate cleavage of the double bonds (12) and analysis of the mono- and dicarboxylic acids thus obtained by gas chromatography of the methyl esters (7,8).

Results and Discussion

Table I gives the results of an experiment which was started from a 3-day-old culture which had a cell concentration of 9.2 ml per liter. The illumination was continuous at 750 ft-c from incandescent bulbs. The culture temperature was maintained at 27 ± 1°.

It is evident that growth starts off at a rapid rate. The increase in the first 12 hours is 235 %. In the same period chlorophyll increases only 45 % and total lipids 180 %. α-Linolenate, however, increases more rapidly than cell volume; its increase is 310 %. If we look at the composition of the cells, i.e. constituents per unit cell volume, we see that due to the initial rapid rate of growth and the slower rates of chlorophyll and total lipid synthesis, the amounts of both chlorophyll and total lipids per unit of cell volume suffer a marked decrease during the first 24 hours of growth. After that, the growth rate decreases while the rates of chlorophyll and total lipid synthesis increase. As a result, the concentrations of

Table I. *Growth, Chlorophyll Content, Total Lipids, α-Linolenate and Photosynthesis as a Function of Time (Continuous Illumination)*

Time hrs	P.C.* ml/liter of suspension	Chlorophyll mg per ml P.C.	Chlorophyll mg per Liter of susp.	α-Linolenate mg per ml P.C	α-Linolenate mg per Liter of susp.	α-Linolenate mg per mg of chlor.	μl of O_2 per mg chlor per min
0	0.5	18.65	9.33	4.96	2.48	0.266	43.7
12	1.68	8.10	13.60	6.08	10.20	0.751	71.3
24	3.40	8.55	29.10	7.20	24.50	0.842	81.6
48	6.80	17.00	115.50	6.72	45.70	0.395	45.5
72	9.67	19.05	118.50	4.48	43.40	0.235	37.8

* P.C. = packed cells.

both chlorophyll and total lipids in the cells increase until at about 72 hours when both of these substances have again reached concentrations approximating those of the starting cells.

The situation with respect to α-linolenate is just the opposite to that of chlorophyll. Its initial concentration in the starting cells is low but its initial rate of increase is even more rapid than the initial growth rate, and it reaches its maximum concentration in the cells at approximately the same time that the chlorophyll concentration is at its minimum. This rapid increase in linolenic acid is generally accompanied by a percentage decrease in the more saturated C_{18} fatty acids. This is evident from the percent composition of the lipids (tables II, IV, VI, VIII). It is tempting to speculate that the linolenic acid is produced by desaturation of the more saturated acids. However, the suggestion of Crombie (2) that the fatty acids synthesized along with the chlorophyll are largely linolenic is equally tenable. The increase in total lipids per liter of suspension (tables I, III, V, VII) is more than sufficient to accommodate the possibility that all the linolenic acid is newly synthesized. The data available at present are not sufficient to indicate the synthetic pathway of linolenate. This phase of the problem may be profitably studied with the use of isotopic tracers. This may also give some clue as to what happens to linolenic acid in very old cells which show an absolute loss of linolenate per cell volume as well as per liter of suspension.

Turning our attention to the photosynthetic activity of the cells we see (table I) that photosynthetic O_2 evolution computed per unit of chlorophyll, as has been observed previously (13), is low in the starting cells and rises to a maximum at about 24 hours when the chlorophyll per unit cell volume is at a minimum and the α-linolenate is at its highest concentration per unit PC as well as per unit of chlorophyll. Figure 1A shows graphically the parallelism of photosynthetic efficiency of chlorophyll and α-linolenate per unit of chlorophyll.

Since in this experiment the cells were grown under continuous illumination, it was of interest to see the relationships of the various components of the photosynthetic system under intermittent illumination. Table III presents the results of an experiment in which all conditions were the same as in the previous experiment with the exception that the cultures were alternately illuminated for 12 hours and kept in darkness for 12 hours. The starting cells were from a 96-hour-old culture which had a cell concentration of 11.3 ml/liter and which was grown under continuous illumination. The samples were taken at the end of

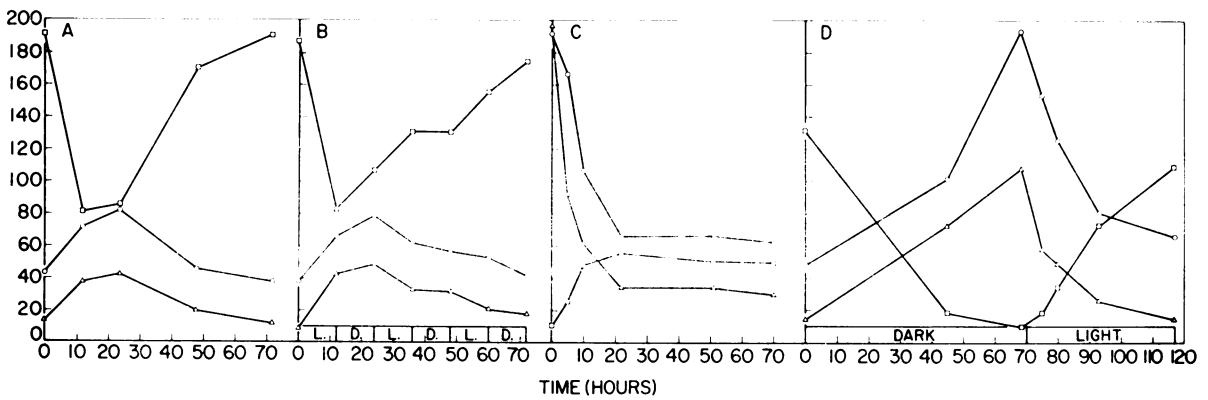

FIG. 1. Changes in chlorophyll-□-mg/ml P.C. × 10, α-linolenate-△-mg/mg chl × 50 and O_2 evolution-○-μl/mg chl/minute during the growth of *Chlorella vulgaris*. A, continuous illumination. B, intermittent illumination. C, Y_1 mutant continuous illumination after 70 hours of dark growth. D, 70 hours dark then continuous illumination.

Table II. *Percent Composition of Fatty Acids (as Methyl Esters) in* Chlorella *as a Function of Time (Data from Experiment in Table I)*

Time hrs	Total methyl esters mg/ml P.C.	Fatty acids (carbon atoms: double bonds) 16:0	16:1	16:2	18:1	18:2	18:3
0	31.20	17.05	1.07	16.97	4.31	44.70	15.90
12	26.05	22.46	1.28	8.73	16.32	27.82	23.35
24	18.82	22.75	1.60	5.30	10.91	21.24	38.20
48	28.65	19.34	1.35	14.15	6.62	35.12	23.42
72*	28.16	16.12	1.00	12.85	5.41	48.62	16.00

* May have been a slight loss of methyl esters from this sample due to prolonged solvent removal under N_2. This would tend to lower the C_{16} esters relative to the less volatile C_{18} esters

each period so that they would reflect the effects of light or darkness as the case may be.

The overall relationships, as may be seen from table III and figure 1B, are similar to those of the experiment under continuous illumination. The effects of the dark periods are of interest to examine. During the first dark period chlorophyll per liter of suspension increases by 30%. There is no increase in total lipids but α-linolenate increases by 50 %. In the subsequent dark periods neither chlorophyll nor lipids are synthesized. While chlorophyll remains unchanged, total lipids as well as linolenic acid show a slight loss. It appears that some factor or condition is produced during the first light period which permits the synthesis of α-linolenate in the subsequent dark period. However, during the second dark period there is no further synthesis of either total lipids or α-linolenate. Actually there is a slight decrease in total lipids per liter of suspension as well as per unit of packed cells. It seems that once a certain density of cells is attained in the culture there is actually a loss of α-linolenate even though lipid synthesis still

Table III. *Growth, Chlorophyll Content, Total Lipids, α-Linolenate and Photosynthesis as a Function of Time (Intermittent Illumination)*

Time hrs	P.C.* ml/liter of susp.	Chlorophyll mg per ml P.C.	Chlorophyll mg per Liter of susp.	α-Linolenate mg per ml P.C.	α-Linolenate mg per Liter of susp.	α-Linolenate mg per mg of chlor.	μl of O_2 per mg chlor per min
0 Light	0.5	18.68	9.34	3.35	1.68	0.179	38.2
12 Dark	1.26	8.24	10.40	6.90	8.70	0.848	66.0
24 Light	1.26	10.68	13.50	10.35	12.93	0.969	78.8
36 Dark	3.05	13.08	40.00	8.65	26.36	0.661	61.8
48 Light	3.05	13.02	39.75	8.30	25.30	0.637	56.7
60 Dark	5.25	15.54	81.50	6.05	31.80	0.415	53.0
72	5.25	17.44	91.60	6.20	32.50	0.355	41.9

* P.C. = packed cells.

Table IV. *Percent Composition of Fatty Acids (as Methyl Esters) in* Chlorella *as a Function of Time (Data from Experiment in Table III)*

Time hrs	Total methyl esters mg/ml P.C.	Fatty acids (carbon atoms: double bonds) 16:0	16:1	16:2	18:1	18:2	18:3
0	32.0	16.93	0.97	20.10	2.89	48.80	10.30
12	31.5	23.25	1.57	10.54	15.90	26.78	21.95
24	32.5	21.98	1.42	8.50	12.32	23.92	31.85
36	26.0	22.05	1.55	10.22	8.40	24.50	33.30
48	25.0	20.84	1.28	12.65	7.77	24.30	33.15
60	27.0	16.95	1.36	22.83	5.42	31.05	22.40
72	25.0	18.72	1.18	15.50	5.24	34.50	24.86

Table V. *Growth, Chlorophyll Content, Total Lipids, α-Linolenate and Photosynthesis as a Function of Time in* Chlorella *(Y_1 Mutant)*

Time after illum. hrs	P.C. ml/liter of susp.	Chlorophyll mg per ml P.C.	Chlorophyll mg per Liter of susp.	α-Linolenate mg per ml P.C.	α-Linolenate mg per Liter of susp.	α-Linolenate mg per mg chlor.	μl per O_2 mg chlor per min
0	6.5	0.97	6.30	3.8	24.7	3.93	192
5	7.0	2.56	17.92	4.7	32.9	1.84	166
10	8.33	4.80	40.0	5.9	49.1	1.23	107
22	12.0	5.54	66.5	3.75	45.0	0.68	66
51	16.5	5.06	83.5	3.45	56.9	0.68	66
70	18.75	4.95	92.8	2.9	54.4	0.59	62

continues at a reasonable rate. This takes place regardless of whether the cells are illuminated or kept in darkness.

The results so far indicate that the highest photosynthetic competence is attained when chlorophyll content per unit of cell volume is at or near the minimum. This coincides with the time when the ratio of linolenate to chlorophyll is at its maximum. In view of these observations it was decided to obtain cells with as low a chlorophyll content as possible for further experiments. Fortunately we have in our algae collection a *Chlorella* mutant, which we originally obtained from Dr. S. Granick, along with the wild type which we used in the above experiments. This mutant, referred to as Y_1 by Granick, is indistinguishable from the wild type when grown on inorganic medium in the light. But when grown in darkness on inorganic medium plus an available carbon source, it grows well, but unlike the wild type it does not synthesize chlorophyll. However, like etiolated higher plants it synthesizes some xanthophyll and thus appears yellow.

The experimental procedure followed was to grow a culture in complete darkness on inorganic medium plus 0.5 % glucose. After approximately 70 hours the cells grew to a concentration of 5 to 7 ml PC per liter. The cells were centrifuged, washed with sterile culture solution, and resuspended in the original volume of fresh culture solution but without glucose and exposed to continuous illumination. Aliquots of cells were taken at stated intervals and growth, photosynthetic O_2 evolution, chlorophyll and lipid content were determined.

Table VI. *Percent Composition of Fatty Acids (as Methyl Esters) in* Chlorella *(Y_1 Mutant) as a Function of Time (Data from Experiment in Table V)*

Time hrs	Total methyl esters mg/ml P.C.	Fatty acids, carbon atoms: double bonds							
		14(?)	16:0	16:1 + ?	16:2	18:0	18:1	18:2	18:3
0	23.0	0.84	24.7	1.35	4.02	3.45	22.15	27.52	16.50
5	21.0	0.80	23.46	2.18	3.34	3.42	16.80	27.08	22.92
10	18.5	0.76	19.95	2.78	3.30	1.46	11.52	28.41	31.80
22	13.5	1.31	24.91	3.10	4.16	1.04	7.68	30.00	27.85
51	12.0	1.31	24.92	2.93	5.58	0.40	4.79	31.50	28.57
70	12.0	1.16	22.95	3.63	7.11	0.31	3.89	36.60	24.32

Table VII. *Growth, Chlorophyll Content, Total Lipids, α-Linolenate and Photosynthesis as a Function of Time in* Chlorella *(Y_1 Mutant)*

Time, hrs	P.C. ml/liter of suspension	Chlorophyll mg per		α-Linolenate mg per			μl per O_2 mg chlor per min
		ml P.C.	Liter of susp.	ml P.C.	Liter of susp.	mg chlor.	
0*	0.5	13.14	6.57	3.90	1.95	0.291	49.0
45	3.3	1.90	6.27	2.75	9.08	1.45	101.8
70	4.8	0.995	4.77	2.15	10.32	2.16	194.0
75	5.3	1.85	10.73	2.15	11.40	1.16	153.5
80	6.0	3.45	20.70	3.40	20.40	0.985	125.3
93	8.8	7.24	63.70	3.75	33.00	0.518	80.2
117	14.8	10.90	161.50	3.90	1.95	0.291	49.0

* From 0 to 70 hours in the dark; after that in continuous illumination.

Table VIII. *Percent Composition of Fatty Acids (as Methyl Esters in* Chlorella *(Y_1 Mutant) as a Function of Time (Data from Experiment in Table VII)*

Time hrs	Total methyl esters mg/ml P.C.	Fatty acids, carbon atoms: double bonds							
		14:1?	16:0	16:1 + ?	16:2	18:0	18:1	18:2	18:3
0*	23.5	1.48	27.76	2.00	10.89	trace	5.88	35.40	16.62
45	24.5	0.67	26.65	2.03	4.63	2.91	26.80	25.20	11.12
70	29.5	0.84	28.18	2.93	3.95	2.80	28.40	25.65	7.23
75	21.0	1.04	34.25	2.59	3.14	3.14	21.81	23.80	10.22
80	19.5	0.92	30.88	2.61	3.06	2.58	15.35	27.00	17.55
93	18.5	1.00	33.20	2.74	5.10	1.11	11.22	25.30	20.30
117	19.0	1.03	27.21	1.81	10.16	0.33	4.93	38.10	16.41

* From 0 to 70 hours in the dark; after that in continuous illumination.

The results of this experiment are given in tables V and VI. The sample taken just before the dark grown cells were illuminated had the lowest chlorophyll content and the highest α-linolenate per mg of chlorophyll. As expected, it had also the highest photosynthetic competence. As the illumination continues, chlorophyll is synthesized at a more rapid rate than linolenate, the ratio of linolenate to chlorophyll decreases and so does the photosynthetic competence.

In order to see what happens during the dark period an experiment was carried out similar in all respects to the previous one except that one sample for analysis was taken during the dark period. The results are given in tables VII and VIII.

During 45 hours of dark growth the cell volume increased almost 7-fold, no new chlorophyll was synthesized but linolenate increased almost 6-fold, thus linolenate per mg of chlorophyll increased 5-fold. Here again the photosynthetic O_2 evolution per mg of chlorophyll doubled. In another 25 hours of dark growth chlorophyll was further reduced, the ratio of α-linolenate to chlorophyll increased and photosynthetic efficiency per unit of chlorophyll doubled again.

Once the dark grown cultures were exposed to light chlorophyll was synthesized at a more rapid rate than α-linolenate, resulting in a lower α-linolenate/chlorophyll ratio and a much slower rate of O_2 evolution per unit of chlorophyll. Figure 1C and 1D show that, as in the previous experiments, the changes in O_2 evolution per mg of chlorophyll parallel those for α-linolenate per mg of chlorophyll. This correlation holds for all the experiments which we performed on *Chlorella*. These results lend support to the views of Erwin and Bloch that α-linolenate is in some way related to the photosynthetic O_2 evolving system of green cells.

It is clear from the data that in all cases the highest photosynthetic competence of chlorophyll occurs when the α-linolenate per unit chlorophyll is highest. This is so regardless of whether the high ratio is due to a low chlorophyll or a high α-linolenate content. It is also apparent (tables III, VI) that light is not required for the synthesis of α-linolenic acid. It is of course possible that there is a light requirement for synthesis of the galactolinolenates, which are the complex glycolipids in which the α-linolenic acid occurs in the chloroplasts.

It is evident from tables II, IV, VI and VIII that lipid components other than α-linolenic acid experience large quantitative changes during the growth of a culture. We have been unable, so far, to correlate these changes with any physiological function. There is undoubtedly a relationship between different lipid fractions. But it will require more sophisticated experiments to study these complex relationships.

We are examining other plants and other systems for the relationship between α-linolenate and photosynthetic efficiency. So far we have found one photoautotrophic organism, *Anacystis nidulans*, that has no linolenic acid and no polyunsaturated lipids (18). However, another blue-green alga, *Anabaena variabilis*, is similar to *Chlorella* in its α-linolenate-photosynthesis relationship. *Anacystis* differs in many respects from other blue-green algae. It is therefore not surprising that it differs also in respect to lipids. It does nevertheless raise the question of what substance takes the place of α-linolenic acid in its supposed role in O_2 evolution.

We are very much interested now in investigating the factors that control the synthesis of α-linolenic acid. What is it that triggers its rapid synthesis as we start a culture and what stops its synthesis when the culture is 2 to 3 days old? We have eliminated the possibility of nutrient deficiency or toxic secretions by aseptically centrifuging an old culture (PC-12 ml/liter) and reinoculating the clear supernatant with the old cells, but to a dilution of 0.5 ml. The cells behave exactly as they do in a fresh solution. Light does not seem to be a direct factor since linolenate is synthesized in the dark. Population density is a possible factor. How it would act in preventing synthesis of α-linolenate is not known.

In summary, we think that our results, and the reports from Bloch's laboratory (4), constitute good presumptive evidence for the concept that α-linolenate participates in photosynthesis. To establish the validity of this concept will require much more work. We feel that the consistency of the correlation we found strongly suggests that further work on these lipids would be fruitful in enhancing our knowledge of the photosynthetic process.

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