Fat Synthesis in Unicellular Algae

Part IV. Nocturnal Effect on Fat Accumulation in *Chlorella*

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Photosynthetic and respiratory quotients of *Chlorella* in nitrogen-deficient growth was investigated. Both quotients were found to be higher than unity, which indicates that the reducing power used in fat synthesis is supplied by both the light energy absorbed and the energy derived by respiration. It being clarified that respiration takes part in fat synthesis to a certain extent, the effect of respiration in dark periods on fat accumulation was investigated in connection with the practical nocturnal effect.

Alternation of light and dark periods once a day gave a favourable effect on fat accumulation, and a distinct difference was observed in fat content of the final crops between the continuous illumination culture and the intermittent illumination culture. And it became clear that the aerated dark periods are effective on fat accumulation especially in the early phase of nitrogen-deficiency.

It was already reported¹⁾ that *Chlorella* cells continue to fix carbon dioxide actively, when transferred into the nitrogen-free medium, at least in the early phase of nitrogen-deficiency of the cells. In the course of growth in that condition, the cells synthesize and accumulate a larger amount of fat than carbohydrate²⁾. It is not clear, however, by what mechanism fat is synthesized in *Chlorella* and whether fat is a primary product of photosynthesis or a secondary one synthesized from carbohydrate in the process of respiration. In other words, it is uncertain if fat is synthesized by the reducing power directly originated in the light energy or by the reducing power released from carbohydrates by respiration. The same question was presented by Barker³⁾ in diatoms which were thought to store oils rather than carbohydrate as reserve materials. Whether diatom oils arise as primary products

of photosynthesis as suggested by Beijerinck⁴⁾ and by Bond⁵⁾ or they are secondary products of metabolism, as is true in many other organisms, was studied by Barker with the medium of photosynthetic and respiratory quotients. And he found the photosynthetic quotient ($\Delta O_2 / -\Delta CO_2$) to be not larger than 1.05, and concluded that fat production on diatoms is a secondary reaction, not directly associated with photosynthesis. It does not always appear to be so, however, since it is possible that the diatom cells were not in the state where active fat synthesis was taking place, because it is likely that the cells transferred from a growing culture into a nitrogen-deficient medium are not in a condition for the immediate rapid synthesis of lipid.

Several workers examined photosynthetic quotients in a wide range of plants and found them to be very close to unity. These determinations have generally been made with

1) H. Iwamoto, G. Yonekawa and T. Asai, This Bulletin, **19**, 240 (1955).

2) H. Iwamoto and N. Nagahashi, *ibid.*, **19**, 247 (1955).

3) H. A. Barker, *Arch. f. Microbiol.*, **6**, 141 (1935).

4) M. W. Beijerinck, *Rec. trav. bot. neel.*, **1**, 28 (1904).

5) R. M. Bond, *Bull. Bingham Oceanographic Collection*, **4**, Art. 4, 1 (1932).

materials taken from growing cultures and it is also uncertain whether they were actually in a fat synthesizing state. Among these workers, however, Kok⁶⁾ and Myers⁷⁾ reported 1.09 for P.Q. of *Chlorella*, Cramer et al⁸⁾ 1.085 for that of *Euglena*, and Frenkel et al⁹⁾ 1.08 for that of *Synechococcus*. These values appear to be significantly higher than unity, indicating that a small proportion of fat is produced.

But it must be noted that most of these values involve the influence of respiration, and if the respiratory quotients were very high, the true photosynthetic quotients are supposed to have been lower than these values.

The authors determined the photosynthetic and respiratory quotients of *Chlorella* cells taken from the culture growing in the state of nitrogen deficiency in which active fat synthesis was undoubtedly taking place, and reached to the conclusion that both photosynthesis and respiration are responsible for fat formation.

It is convincing, therefore, that in the practical outdoor culture of nitrogen-deficient cells, the reducing power for fat synthesis is supplied by both processes in the daytime, and at night fat formation does take place through the process of respiration. In the latter case, the principle of fat formation is considered to be the same as that in heterotrophic microorganisms such as fat yeast. Fat accumulation in those microorganisms requires an ample oxygen supply, and therefore, it is conceivable that aeration during the night might have some good effect on fat accumulation in nitrogen-deficient *Chlorella* cells.

This so-to-speak nocturnal effect was investigated in this study by alternation light and dark periods once a day in the whole course of cultivation.

EXPERIMENTAL METHODS

The organism, the culture apparatus and the medium were the same as described in the previous paper⁽¹⁾. Determinations of nitrogen content, lipid content and dry weight of cells were carried out in the same ways as described in that paper. Warburg's indirect method and direct method were applied to the determination of photosynthetic quotient and respiratory quotient respiratory

RESULTS AND DISCUSSION

The respiratory and photosynthetic quotients in the course of nitrogen-deficient culture are shown in Table I. The quotients were determined with algal suspensions taken from a culture growing in the nitrogen-free medium once a day. Photosynthetic quotients were calculated by correcting the net gas exchange in the light for the gas exchange of respiration determined in parallel dark experiments on the assurance that the rate of respiration and the respiratory quotient are not affected by photosynthesis¹⁰⁾

The initial algal density at the start of cultivation was 1.37 g/l and the incident light intensity was 5,000 lux throughout the cultivation. The culture was diluted twice to half of the algal concentration in the course of cultivation in order to make the culture progress further in nitrogen-deficiency.

The data shows that both quotients are strikingly higher than unity. The rate of respiration per unit weight of cells and that per unit weight of cell nitrogen are shown in the same table. It is interesting that the former (Q_{O_2}) does not show a marked decreasing trend in spite of the extreme decrease in the nitrogen content of the cells, and therefore, the latter ($Q_{O_2(N)}$) shows a remarkable increase along with the progress of nitrogen deficiency of the cells. Table I shows the increase is especially conspicuous at the later phase of nitrogen deficiency. $Q_{O_2(N)}$ at the later phase is three to four times larger than

6) B. Kok, *Enzymologia*, **13**, 1 (1938).

7) T. Myers, in "Photosynthesis in Plants", p. 349, Iowa State College Press (1949).

8) M. Cramer and T. Myers, *Amer. J. Bot.*, **37**, 677 (1950).

9) A. Frenkel, H. Gaffron and E. H. Battley, *Biol. Bull.*, **99**, 157 (1950).

10) A. H. Brown, *Amer. J. Bot.*, **40**, 719 (1953).

TABLE I. THE PHOTOSYNTHETIC AND RESPIRATORY QUOTIENTS AND THE RATE OF RESPIRATION IN NITROGEN-DEFICIENT CULTURE

Culture time (hours)	0	24	48	72	168	240	336
Algal density (g/l)	1.37	2.30	2.60	2.88	4.32*	6.32*	7.28*
Nitrogen content(%)	8.50	5.02	4.45	3.85	2.65	1.81	1.54
P.Q. ($\Delta O_2 / -\Delta CO_2$)	1.18	1.39	1.19	1.28	—	—	—
R.Q. ($\Delta CO_2 / -\Delta O_2$)	1.16	1.20	1.13	1.31	1.27	—	1.12
QO_2 ($\mu l/mg$, hr)	3.2	3.7	2.3	2.0	3.8	2.2	1.9
$QO_2(N)$ ($\mu l/mg(N)$, hr)	37.7	75.0	52.2	52.0	143.5	121.5	120.3

* Corrected for the dilutions.

the initial one.

It is very likely that the enzymic equipments of the cells are reorganized during the nitrogen-deficient growth, and their amount for photosynthesis decreases as mentioned in the previous paper¹¹⁾, and on the contrary, their amount for respiration increases. This hypothesis also receives support from the finding by Virtanen¹²⁾ that different nitrogen fractions, including enzymes, in microorganisms are affected to different extents by nitrogen starvation.

Let us make rough calculation on the balance of photosynthetic and respiratory fat formation, based upon these data.

Assuming that the mean value of the photosynthetic quotients was 1.2 during the first two days, and the photosynthetic products were carbohydrate and glycerol trioleinate ($C_{57}H_{104}O_6$)*, the following equation will be given for the photosynthetic assimilation.

This equation implies that about twice as much carbohydrate as fat is synthesized by photosynthesis.

The data, on the other hand, shows that 1.37 g of normal cells (Nitrogen: 8.50%, Lipid: 15%, Carbohydrate: 27%) grew up to 2.60 g of nitrogen-deficient cells (Nitrogen: 4.45%, Lipid: 34%, Carbohydrate: 32%) in 48 hrs, synthesizing and accumulating about 0.34 g of

carbohydrate and 0.50 g of fat per 85 mg of cell nitrogen as net products of photosynthesis and respiration. When calculation is made on fat formation by respiratory process, applying the above values and equation, on the assumption that $QO_2(N)$ was 50 throughout that period, the following equation is given for the respiration.

These equations imply that about 420 mg of fat and 830 mg of carbohydrate are synthesized by photosynthesis, and about 80 mg of fat is formed by respiration at the respiratory quotient of 1.23 at the expense of about 490 mg of carbohydrate, and consequently, about 500 mg of fat and 340 mg of carbohydrate are accumulated per 85 mg of cell nitrogen in a period of 52 hours. These values well correspond with the values obtained by the experiment, except that the period is a little longer and the respiratory quotient is a little higher than the observed value, though these deviations may be considered to be rather natural.

On the other hand, if it were not for photosynthetic fat formation at all, the equation for the respiration must be assumed as follows;

This implies that about 0.5 g of fat is synthesized by respiration in 52 hours at the respiratory quotient of 2.44, consuming about 1.63 g of carbohydrate synthesized by photosynthesis. But such a high R.Q. value as 2.44 is quite unreasonable or impossible energetically and was never observed.

11) H. Iwamoto and H. Sugimoto, This Bulletin, **22**, 410 (1958).

12) A. I. Virtanen and J. K. Miettinen, *Acta chem. scand.*, **3**, 14317 (1949).

* The fat of chlorella in an advanced stage of nitrogen deficiency is represented by glycerol trioleinate according to the author's unpublished data on the nature of Chlorella fat.

From the above calculation, therefore, it may be concluded that a considerable amount of the reducing power produced by light energy conversion may be utilized for fat synthesis, and that it is photosynthesis that is responsible for the major part of the fat accumulated at least in the earlier phase of nitrogen deficiency. About one sixth of the fat is considered to have been synthesized by respiration in the above case. And it is quite possible that TPNH_2 formed in the chloroplasts by the light energy transfer system diffuses out of the chloroplasts and is used as reducing power for fatty acid synthesis, especially in the case where the rate of carbon dioxide fixation is mainly restricted by the decrease in the amount of the related individual enzymes or enzyme systems in nitrogen deficiency as mentioned in the previous paper¹¹.

It has long been accepted that the reduction of nitrate which depends upon the photosynthetic activity occurs in illuminated *Chlorella* and green tissues, when they are supplied with nitrate, though, on the other hand, the reduction of nitrate also occurs in the dark. But reduction of other substances from carbohydrate level to fatty acid level, which depends upon the transformed light energy, has never been ascertained to occur actually. Discussion here strongly suggests its possibility.

There is also a general opinion that TPNH_2 does not diffuse out of chloroplasts. But to the authors this seems to occur in a special case such as nitrogen deficiency.

It being clarified that respiration is also responsible for fat synthesis, though to a rather small extent, it is supposed that fat synthesis will proceed by respiration when photosynthesis does not occur in the dark. And so, if the nitrogen-deficient culture is subjected to light and dark alternation, the cells are supposed to accumulate more fat than the cells grown under continuous illumination, compared at the same nitrogen content of the cells. This problem has a practical significance in the

outdoor cultivation of fat cells.

To clarify the extent of the effect of respiration in the night, some experiments were conducted, inserting 8 or 16 hours of dark period once a day in nitrogen-deficient cultivation.

The growth curves are shown Figs. 1 and 2. The algal density was plotted against the

FIGS. 1 & 2. Growth Curves in Intermittent and Continuous Illumination Cultures.

Dark time per day: 0 hr.: ○—○
8 hrs.: ×---×
16 hrs.: △...△

illumination time length. It is seen that the dark periods affected the growth rather favourably. The nitrogen and fat contents of the final crops of these cultures are shown in Table II. Marked differences are seen in fat content between the light and dark alternation culture and the continuous illumination culture, while the nitrogen contents are almost the same in each series of experiment, and the effect of fat synthesis in the dark periods is clearly observed. This is also illustrated in Fig. 3, in

TABLE II. NOCTURNAL EFFECT ON FAT ACCUMULATION

Dark period in a day (hrs.)	Total light period (hrs.)	Total dark period (hrs.)	Nitrogen content (%)	Lipid content (%)
0	168	0	2.58	50.7
8	256	128	2.50	61.2
16	128	256	2.48	60.5
0	144	0	3.11	44.9
16	144	288	3.09	52.3

which the curve represents the N-Lipid correlation usually obtained by continuous illumination cultures¹. The cells obtained by the intermittent illumination cultures appear above the curve, which indicates a more reduced state of the cells.

FIG. 3. Lipid Content of the Cells obtained by Intermittent and Continuous Illumination Cultures.

○ Intermittent illumination.
× Continuous illumination.

The effect of dark respiration on fat accumulation is not remarkable or rather negative in the later phase of nitrogen-deficient growth. When cells were put in the dark with aeration for several days, taken out of the light in the actively growing phase of nitrogen-deficient culture, the total amount of fat showed a dis-

tinguishable increase, but when cells were taken out in the stationary or slightly growing phase, it did not show any increase or rather showed a little decrease. In Table III, cells were taken in the growing phase in experiments A and A', and at the beginning of the stationary phase in experiment B. It is conceivable that active photosynthesis at the early phase of nitrogen deficiency can supply an excess of carbohydrate which is convertible into fat by dark respiration, but in the later phase of nitrogen deficiency, where photosynthesis is very faint, convertible carbohydrate is converted into fat by respiration in the light as soon as it is produced by photosynthesis. It is not clear, at present, how the accumulated carbohydrate is converted into fat in the light in the very late phase of nitrogen deficiency, as was observed at the very early stage of this series of investigation. Anyway, it is certain that the accumulated carbohydrate cannot be converted into fat in a large quantity through respiration in the dark.

SUMMARY

Participation degree of photosynthesis and respiration in fat synthesis in *Chlorella* in nitrogen-deficient growth was investigated with the medium of photosynthetic and respiratory quotients. It became clear that photosynthesis is mainly responsible for fat formation at least in the early stage of nitrogen deficiency and the reducing power resulting from light

TABLE III. EFFECT OF LONG AEROBIC INCUBATION IN THE DARK ON FAT ACCUMULATION.

Experiment	Light period (hrs.)	Dark period (hrs.)	Temp. (dark)	Algal density (g/l)	Nitrogen content (%)	Lipid content (%)
A	{	46	0	—	1.77	38.7
		46	96	8	1.55	40.1
		47	0	—	2.08	35.0
A'	{	47	96	8	1.96	37.0
		47	96	25	1.92	39.2
		163	0	—	2.30	48.6
B	{	163	96	8	2.26	48.3
		163	96	25	2.16	48.7

energy can be directly utilized for fat synthesis together with that released from carbohydrate through respiration.

The rate of respiration per unit weight of cell nitrogen showed a remarkable increase with the advance of nitrogen deficiency. This fact strongly suggests that reorganization of nitrogen compounds occurs during the nitrogen-deficient growth.

The effect of dark respiration on the accumulation degree of fat was investigated in relation to the practical nocturnal effect in

the outdoor culture for fat production. It was clarified that dark periods give a favourable effect on both growth and fat formation, especially in the growing stage. But it became clear that the amount of fat cannot be increased in a large quantity by converting the accumulated carbohydrate through respiration in a prolonged dark period.

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