EFFECTS OF STARVATION ON THE METABOLISM OF CHLORELLA

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(WITH ONE FIGURE)

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Chlorella has been widely used in studies on photosynthesis with but little attention to possible variations in its metabolic activities. The metabolism of other microorganisms has proved to be quite variable and dependent upon previous history as well as upon prevailing environmental conditions. It has been common practice, for instance, to study resting cells as opposed to actively proliferating cells. Resting cells have been variously obtained by removal of the nitrogen source from the nutrient medium, by washing the cells, or starving the cells of organic nutrients for a period of time. Assimilation of carbon may be much more efficient in resting cells than in growing cells (cf. 10) and for that reason the former have most frequently been used in studies on oxidative assimilation. In Chlorella oxidative assimilation has been studied in resting cell preparations obtained as a result of starvation, *i.e.*, aerobic incubation in the dark in the absence of organic nutrients (**6**). As a background for the larger problem of assimilation in Chlorella it becomes important to investigate the changes that take place during starvation.

Experimental

Chlorella pyrenoidosa (Emerson's strain) was grown in two units of a continuous culture apparatus in which uniform experimental material is produced day after day (MYERS and CLARK, **7**). Illumination of 90 foot-candles was provided by tungsten lumiline bulbs. The cultures were aerated with 4% carbon dioxide in air and maintained at a temperature of 25.3° C. Knops solution containing KNO_3, $MgSO_4$, and KH_2PO_4 with added iron and microelements was used as the culture medium (**4**). The population density in various culture units was 2.2 to 2.7 cmm. cells/ml.

Samples of starved or resting cells were obtained by harvesting cell suspensions in sterile flasks and placing them on a mechanical shaker in the dark at a temperature of 25–28° C. During this period the cells are provided with all requisite inorganic nutrients and become starved of reserve food supplies as a result of their own respiratory activity.

Photosynthetic and respiratory activities of the cells were determined from gas exchange measurements by the Warburg technique. Photosynthesis measurements were made in rectangular Warburg vessels in a bath with glass bottom thermostated at 25° C. and illuminated at a photosynthesis-saturating intensity of 600 f.c. by a bank of 17 sixty-watt Mazda

lamps. Respiratory gas exchange was measured at 25° C. in darkness in a second bath using the common respiration vessels. In preparation of the cell sample for experiments an aliquot was routinely centrifuged out, washed in a Knops solution of desired pH, and taken up to a definite volume in fresh Knops of the same pH.

Estimate of the volume of cells used was obtained on an aliquot of the original suspension by means of a centrifuging technique involving use of the Van Allen thrombocytocrit (**4**). Cell dry weight was obtained by drying *in vacuo* (about 4 mm.) at room temperature. A measured aliquot of suspension was centrifuged out, washed twice in distilled water, and the cells transferred in a minimum quantity of water into tared weighing flasks for drying.

The presence of starch in cells was qualitatively demonstrated by an iodine test. Cells to be tested were centrifuged out, heated to boiling, and extracted several times with hot methanol to remove the chlorophyll. Extracted cells containing starch gave a blue color upon the addition of iodine solution ($\frac{1}{3}$ Lugol's solution). The lack of such a color change was taken as evidence of absence of starch.

Results

A series of experiments was made to determine the effect of starvation on the rates of photosynthesis and respiration and on related cellular characteristics in Chlorella. Photosynthetic rates and cell volumes were determined on aliquots of a freshly harvested sample. The sample was then placed on a shaker in the dark. At intervals of one, three, and five days thereafter aliquots were withdrawn and the rate of photosynthesis measured. Respiratory activity was subsequently followed on another sample in a similar manner. The cell volume and dry weight of cells per unit volume of suspension and the pH of the suspension were followed on a third sample. Precautions were taken to keep the samples sterile throughout the period of starvation. All rate calculations were referred to the cell volumes determined on the fresh samples.

Changes in the cellular and metabolic characteristics of Chlorella as a function of time of starvation are summarized in table I.

Apparent rates of photosynthesis are given, uncorrected for respiration. Such a correction would be small; indeed, the large differences between the apparent rate of photosynthesis and endogenous respiration are particularly striking. For freshly harvested cells the apparent rate of photosynthesis measured in Knops solution at pH 4.5 is about 30 times as great as respiration; by the third day after harvesting it is about 100 times as great. This increase in differences of the two rates can be attributed mainly to the rapid decrease in endogenous respiration during and following the first day after harvesting.

Photosynthesis rates measured in Knops solution were calculated on the basis of an assimilatory quotient of 0.90; these range some 20% greater

than the rates obtained when the cells are measured in 0.1 M $KHCO_3$ buffer. The difference is consistent with data of other workers (**5, 8**). For present purposes it is sufficient to note that the behavior of the cells was qualitatively the same by the two different methods of measurement. The total decrease of only about 20% in the maximum rate of photosynthesis seemed so small that further work on this metabolic characteristic did not appear warranted within the present study.

Rates for glucose respiration were determined by adding 50 micromols

TABLE I

CELLULAR AND METABOLIC CHARACTERISTICS OF CHLORELLA AS A FUNCTION OF TIME OF STARVATION IN THE DARK AND IN THE ORIGINAL KNOPS SOLUTION IN WHICH THE CELLS HAD BEEN GROWN. RATES ARE EXPRESSED IN CMM. OXYGEN/HOUR/CMM. CELLS, REFERRED TO CELL VOLUME AT ZERO DAYS

		Days after harvesting			
		0	*1*	*3*	*5*
Apparent photosynthesis	Rate in 0.1 M $KHCO_3$ buffer	31	35	28	25
	Rate in Knops solution	40	43	34	32
Endogenous respiration	Rate	−1.26	−0.57	−0.35	−0.38
Glucose respiration	Rate	−4.7	−4.8	−3.6	−2.7
	mols O_2/mol glucose	1.46	0.99	0.91	1.02
Dry weight	mg./cc. of suspension	0.63	0.55	0.50	.....
Cell volume	cmm./cc. of suspension	2.58	2.45	2.23	.....
pH of the medium		6.35	6.50	6.56	.....

glucose to the cell suspension. After addition of substrate the rate of oxygen uptake increased continuously to a maximum, constant rate which was attained after about two hours; values cited in the table are for this period of maximum, constant rate.

The mols of oxygen required per mol of glucose oxidized were determined by adding 5 micromols of glucose after a period of endogenous respiration. There followed a period of increased rate of oxygen uptake corresponding to utilization of the substrate. On exhaustion of the substrate the rate of oxygen uptake returned to that of endogenous respira-

tion. The amount of oxygen taken up during this period of increased rate is taken as that required for oxidation of the substrate. This method is the common manometric procedure used in studies on oxidative assimilation (*e.g.*, 1) and requires the assumption that the endogenous respiration is suppressed during glucose assimilation.

The value of 1.0 mol oxygen/mol glucose for starved cells is independent of period of starvation from one to five days.[1] This confirms the value previously reported (**6**) for 30-hour starved cells and is consistent with the equation:

$$C_6H_{12}O_6 + O_2 \rightarrow 5(CH_2O) + CO_2 + H_2O.$$

For growing cells the value of 1.46 mols oxygen/mol glucose may represent a more complete oxidation and less complete assimilation; in view of the high rate of endogenous respiration, however, it is also possible that the endogenous respiration is not suppressed and that the estimated value may be too high.

The data on dry weight show the decrease which might be anticipated as a result of starvation. With starvation there is a slow increase in the pH of the suspension probably as the result of some nitrate absorption.

In many organisms starvation causes an initial depletion of storage carbohydrate. Investigations were therefore made on the time required for detectable starch to disappear from starving cells of Chlorella. Using the iodine test, starch could be demonstrated in freshly harvested cells and in starving cells for a period of about nine hours after harvesting. After about nine to ten hours no starch could be detected.

As might be expected, the most important effect of starvation appears as a decrease in the rate of endogenous respiration and as an early disappearance of one of the known storage carbohydrates. Decrease in endogenous respiration of Chlorella has previously been observed. GENEVOIS (**3**) observed a decrease in rate of from 30 to 75 per cent. in four and a half hours and noted that the extent of decrease depended upon the previous light intensity of culture of the cells. FRENCH, KOHN, and TANG (**2**) have made continuous measurements on gas exchange during starvation in studies on the temperature characteristics of Chlorella. They obtained curves for the time course of oxygen consumption which could be divided into two parts, the first portion showing the rate to be a function of time and a second portion during which the rate is independent of time. An explanation of this phenomenon was offered in the postulate that two substances, A and B, were being oxidized during the initial stage and only one, B, during the final stage. This hypothesis was strengthened by their observation that the respiratory quotient declined during the measurements from an initial value of about 0.95 to a final value of 0.65.[2] From

[1] The value of 0.91 for three days is believed to be an experimental error since comparable values from other experiments did not differ significantly from 1.0.

[2] As a matter of convenience the R.Q. is used in this paper without regard to its sign. From the conventions of sign of the gas exchange the R.Q. is actually a negative value.

the R.Q. values it was inferred that substance A is a carbohydrate-like material and that substance B is a lipid or other oxygen-poor material. Such experiments have importance which merits their repetition within the present study.

Oxygen consumption and apparent carbon dioxide production of Chlorella were followed in a series of duplicate flasks with and without potassium hydroxide. Each flask contained 2.0 ml. of a suspension containing 38.2 cmm. of freshly harvested cells which had been washed and resuspended in Knops solution at pH 4.5. The manometric experiment continued over a period of 48 hours and the flasks were occasionally flushed out with air to avoid depletion of oxygen. The results were similar to those of French, Kohn, and Tang. A period of high rate of oxygen uptake persisted for about ten hours after which the rate was much lower and the change in rate very slow. The apparent R.Q. values were found to range from 1.1 at the beginning down to 0.7 at the end of the experiment.

The R.Q. values have been labelled "apparent" from the following considerations. Carbon dioxide production in this experiment was calculated on the assumption that no retention as bicarbonate occurs in cell suspensions, an assumption valid for pH values up to about five. Although the pH of the Knops solution in which the cells were originally suspended was 4.5, this value did not remain constant over the many hours of the experiments. The pH value of the cell suspension in an experimental vessel which was sacrificed at 23 hours was 6.15 and at the end of the experiment the pH value in the other vessels had risen to 6.4. Thus pH conditions made possible the binding of carbon dioxide in the liquid suspension, with the result that less carbon dioxide existed in the gas phase, and manometric measurements of carbon dioxide evolution were too low.

In a second experiment two attempts were made to minimize the pH change and allow both oxygen and carbon dioxide exchange to be followed continuously during a two day period of starvation. In one series of flasks the phosphate concentration of the Knops solution was increased three times (3×0.0092 M KH_2PO_4) in an effort to increase the buffer capacity. Since it could be shown that the pH change occurring was largely the result of nitrate absorption, the KNO_3 of the Knops solution in another parallel series of flasks was replaced by an equivalent concentration of K_2SO_4. The cells used had been grown at a slightly higher light intensity and were known to have a somewhat higher initial rate of respiration than those of the earlier experiment. In the preparation of the cell suspension centrifuging was cut to a bare minimum and all precautions taken to avoid anaerobic conditions which might affect the subsequent gas exchange.

Representative curves for the second experiment are presented in figure 1 and the data are summarized in table II. The gas exchange in Knops solution (solid lines) showed distinct differences from that of the previous

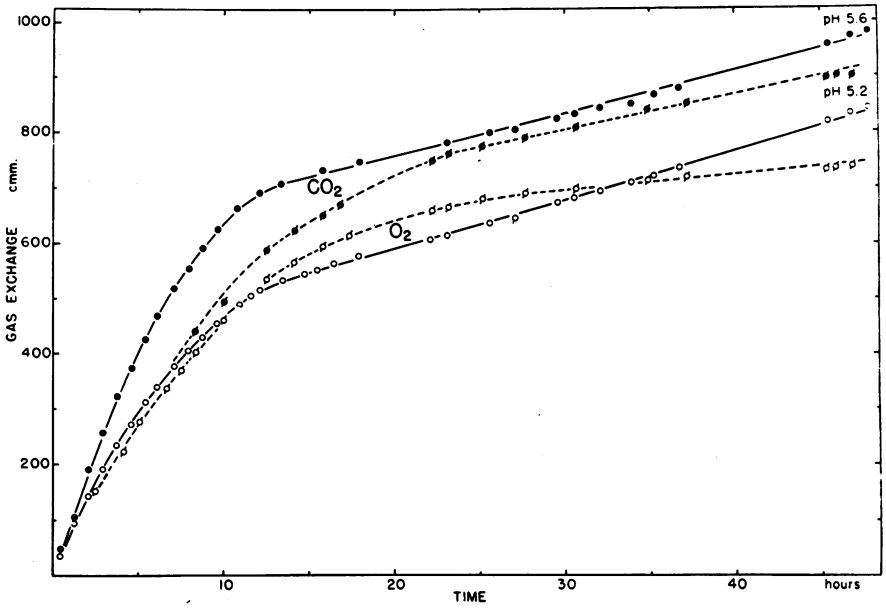

FIG. 1. Respiratory gas exchange as a function of time of starvation in Chlorella. Solid points indicate CO_2 exchange; open points indicate O_2 exchange. Solid lines indicate respiration in Knops solution with phosphate increased to 0.027 M; broken lines indicate respiration in Knops-minus-nitrate. Initial pH = 4.5. Cell quantity = 38.2 cmm. cells per flask.

experiment. Both the initial and final R.Q.'s are higher. The initial R.Q. of 1.41 has subsequently been shown to be the result of attendant nitrate reduction; it is related to the higher initial rate of respiration and perhaps also to a more careful maintenance of aerobic conditions than in the first experiment. The final R.Q. of 0.87 is also higher than in the preceding experiment. This is clearly related to the less pronounced pH increase resulting from the increased buffer capacity of the added phosphate. At the lower final pH less carbon dioxide is bound as bicarbonate and the observed carbon dioxide approaches more nearly the real value.

TABLE II

EFFECTS OF STARVATION ON RATES OF GAS EXCHANGE. RATES ARE EXPRESSED IN CMM/HOUR/CMM. CELL. INITIAL pH = 4.5

MEDIA	INITIAL (1–3) HOURS			FINAL (30–45) HOURS			FINAL pH
	O_2	CO_2	R.Q.†	O_2	CO_2	R.Q.†	
Knops with 3 × increased phosphate	−1.56	2.20	1.41	−0.23	(0.20)*	(0.87)*	5.6
Knops minus nitrate	−1.41	1.61	1.14	−0.06	(0.15)*	(2.3)*	5.2

* Values approximate only and too low due to increase in pH.

† R.Q.'s are calculated from the rates maintained during the time periods indicated and are not cumulative.

When the medium is lacking in nitrogen (broken lines of figure 1) the course of gas exchange is quite different, leading to a very low final rate of oxygen uptake. It is clear that even during starvation nitrogen deficiency has marked effects and does not provide an adequate means of study of the starvation metabolism of Chlorella. Further speculation seems premature; a more complete investigation of nitrogen deficiency is indicated.

The need for an accurate final R.Q. is evident. R.Q.'s at high pH can be determined by releasing the bound carbon dioxide with acid at the beginning and end of the experiment in duplicate vessels. Experiments using such a method were designed for cells that had been shaken in the dark for two days, and so were considered to have respiratory activity comparable to that of cells at the end of the long-time experiments. Such respiratory activity is very much less than that of fresh cells and in order

TABLE III

R.Q.S OF CELLS STARVED TWO DAYS

EXPERIMENT NO.	TIME AFTER CENTRIFUGING (MIN.)	R.Q.
1	40 – 160	0.92 0.94
2	160 – 190	0.99 0.99
3	180 – 300	0.94 0.93

to obtain measurable rates for the two-day-old cells it is necessary to increase the quantity of cell material some four times over the amount used when fresh cells are being studied.

The results of experiments in which oxygen uptake and carbon dioxide evolution were followed as a function of time in dense suspensions of two-day-old cells were unexpected. From a study of the experiment of figure 1, one would have supposed that there would be little change in the rate of gas exchange after such a period of starvation. Yet repeated experiments showed a very rapid decreasing rate of gas exchange, which persisted even as long as four hours after the cells had been placed in the experimental vessels. Under such conditions, R.Q. values were low, ranging from 0.7 to 0.8. In considering possible causes for the rapidly changing rates of gas exchange, it was noted that in the preparation of the sample for the experiment, two environmental conditions are changed: (1) the cells are taken from a pH of about 6.5 (pH of sample two days after harvesting), washed and resuspended in a Knops solution of pH 4.5; (2) the cells are packed into a small volume during centrifuging and any effects of anaerobic conditions imposed thereby might be intensified due to the density and age of the suspension used. Assuming that the effect pro-

duced was probably the result of one or a combination of these conditions, several experiments were designed to by-pass them. The cells were taken up in a Knops solution of pH 6.0 so that the effect due to pH change would be lessened and R.Q.'s were measured only after the rates had steadied off. The results of three such experiments are presented in table III. In all three cases the cells had been starved for two days and the measurements were made in a Knops solution of pH 6.0, bound carbon dioxide being released by adding acid at the beginning and end of the indicated time period.

It appears, then, that the R.Q. of starved cells is 0.9 to 1.0. It has been demonstrated (and will be reported elsewhere) that nitrate reduction with marked effect on the gas exchange is negligibly small in starved cells of Chlorella. The R.Q. of about 1.0 may be taken as evidence of a carbohydrate-like substrate for endogenous respiration even after two days of starvation.

The data of table II for the rate of oxygen uptake during the second day of starvation obtained during continuous measurement are considerably less than comparable data of table I obtained from short-time experiments on cells already starved. It is likely that the rates of endogenous respiration reported in table I are somewhat too high as a result of effects of anaerobic conditions in centrifuging and preparation of the starved cells for manometric measurements.

Discussion

The postulate of FRENCH, KOHN, and TANG (**2**) that two distinct substances are being oxidized during the endogenous respiration of Chlorella may be applied to the data obtained in this study. The times required for the disappearance of starch and for the decrease in the high rate of endogenous respiration are comparable. This suggests that starch is a substrate for the initial rapid endogenous respiration and therefore corresponds to substrate A of French, Kohn, and Tang. However, in regard to a second substrate B, which is oxidized during later periods of starvation, the present data differ from those of French, Kohn, and Tang. The demonstration of an R.Q. of 1.0 in two-day-old starved cells indicates that this substrate cannot be lipid, but must also be carbohydrate in nature. The studies of Spoehr and co-workers (**9**) have shown that lipid-like materials may accumulate in Chlorella under certain conditions of culture. At present, however, there is no reliable evidence of a lipid respiration in Chlorella.

There remains the question as to just what constitutes a truly endogenous respiration or *Eigenatmung* in Chlorella. From the standpoint of common usage all of the later data describe the endogenous respiration since no usable substrate was supplied. The time course of respiratory behavior, however, suggests a more critical examination. The initial high

rate shows a gradual decrease and then a comparatively rapid transition to the final low rate which remains constant for many hours (and probably for several days). The transition to the final rate is coincident with the disappearance of starch. During the initial period when starch is the principal substrate a reduction of nitrate may occur. All of these characteristics suggest that a truly endogenous respiration in Chlorella occurs only after prolonged starvation.

Summary

1. Photosynthetic and respiratory activity and certain cellular characteristics of *Chlorella pyrenoidosa* have been studied as a function of time of starvation (0–5 days) in darkness.

2. The rate of photosynthesis under light and carbon dioxide saturation decreases only about 20 per cent. during five days of starvation.

3. The most marked effect of starvation is the decrease in the rate of endogenous respiration. An initial high rate persists up to a time corresponding to the disappearance of starch and is followed by a low rate which approaches a constant value. It is inferred that only the final rate represents a truly endogenous respiration.

4. Over a two-day period of starvation the R.Q.'s closely approach values characteristic of carbohydrate metabolism. No evidence for an appreciable lipid metabolism could be found.

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