RESPIRATION STUDIES ON CHLORELLA. II. INFLUENCE OF VARIOUS ORGANIC ACIDS ON GAS EXCHANGE

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(WITH TWELVE FIGURES)

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Introduction

In an attempt to elucidate the respiratory mechanism of the green alga, chlorella, several of the organic acids which are known to be intermediates in the aerobic path of carbohydrate metabolism in most animals and bacteria were supplied to chlorella cells. In a previous paper (**9**), the results of growth experiments were reported and there was some evidence of utilization of the acid intermediates for growth in the dark by chlorella.

Since the organic acids supplied are assumed to enter the path of metabolism through the respiratory mechanism, the effects of the organic acids on the respiratory gas exchange (*i.e.*, oxygen uptake and carbon dioxide evolution) were investigated. The results of this investigation are reported here.

Materials and methods

The effect of the possible acid intermediates of the aerobic cycle of carbohydrate metabolism on the respiration of chlorella was studied by manometric measurements with the Warburg apparatus. All determinations were made in the dark. For this purpose, the instrument was placed in a blackened chamber equipped with an air-circulating fan to avoid rises in air and bath temperatures. The rate of shaking was adjusted to 200 two-centimeter strokes per minute. The flasks used were of the 2-side-arm, center-well, and venting-plug type. The bath temperature was regulated at 28° C. The vessel constants were calculated by the UMBREIT, BURRIS, and STAUFFER method (**17**).

The measurements of oxygen and of carbon dioxide were determined by using a pair of flasks, one of which contained KOH in the center cup to absorb the CO_2. The KOH was replaced by water in the other flask. Both flasks also contained an accordion-pleated filter paper, 2×2 centimeters square, to increase the CO_2 absorbing surface in the first flask and to equalize the volume of the gas phase in the other.

Nutrient solutions and cell suspensions added to the flasks were prepared similarly to those used for the growth experiments (**9**). The solution referred to as plain nutrient solution is composed of 1.25 gm. KNO_3, 2.46 gm. $MgSO_4 \cdot 7\,H_2O$, 1.22 gm. KH_2PO_4, 0.01 gm. $FeCl_3$, 1 ml. of Haas and Reed A–Z solution, and 1,000 ml. H_2O. The initial pH of the solution was regulated with a few drops of normal HCl to 5.4–5.6 which was found to be

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favorable for the growth of chlorella. This gives a system fairly well buffered. The buffer properties of this solution are further increased by the addition of organic acids. The cells were starved for 30 hours in the dark at 28° C before use but no attempt was made to maintain aseptic conditions in the Warburg flasks. Due to the desirability of using cells which were kept only a short period of time in the refrigerator (less than 10 days), it was not possible to use the same concentration of cells for all the experiments. Not enough cells were available from one stock suspension.

Several experiments were conducted with cell extracts replacing the cell suspensions. The extracts were prepared in several ways—by grinding the chlorella with very fine carborundum in a mortar, by using powdered glass and sand as abrasives, and also by grinding in a tissue homogenizer. The ground cells were stirred with two to three milliliters of triple-distilled water. After centrifuging, the supernatant liquid was used as the cell extract.

The system in each flask was composed of:

a) the cell suspension (1 milliliter) which was placed into the main compartment of the flask together with the necessary volume of water and nutrient solution to total five milliliters of liquid, and

b) the organic acids which were placed in the side arms and tipped into the main compartment after equilibrium had been reached.

The thermobarometer flask was set up with the same material as the other containers except for the cell suspension which was replaced by an equal volume of water. The concentrations of the cells and of the organic acids were varied to obtain results which would show most clearly the effect on respiration.

The organic acids used were the same as those used in the growth experiments. α-ketoglutarate, however, was added to the list as it was available in small quantity.

Results

Before proceeding with the experiments showing the effect of organic acids on respiration, several preliminary determinations were made to compare the value of various inorganic media. With a view toward selecting the solution which would give best results for respiration studies, the oxygen uptake and carbon dioxide evolution were measured for several systems:

a) Krebs-Ringer phosphate solution + chlorella,
b) Krebs-Ringer bicarbonate solution + chlorella,
c) phosphate buffer, pH 5.6, + chlorella,
d) the nutrient solution with various inorganic salts added + chlorella,
e) various concentrations of the plain nutrient solution + chlorella.

The first three media gave poor results in that the curves of the O_2 uptake and the CO_2 evolution were lower than those with plain nutrient solution. Varying the concentration of the nutrient solution led to the selection of $\frac{1}{3}$ normal concentration as the one giving the most desirable curve

features. Inorganic salts were added to the $\frac{1}{3}$ nutrient solution in the hope that they would produce higher respiratory rates. The curves obtained were not as regular and the respiratory quotient [2] varied more than that with the plain nutrient solution. The effect of these inorganic salts is shown in figure 1. The cell concentration for this figure was 1.4×10^8 per flask.

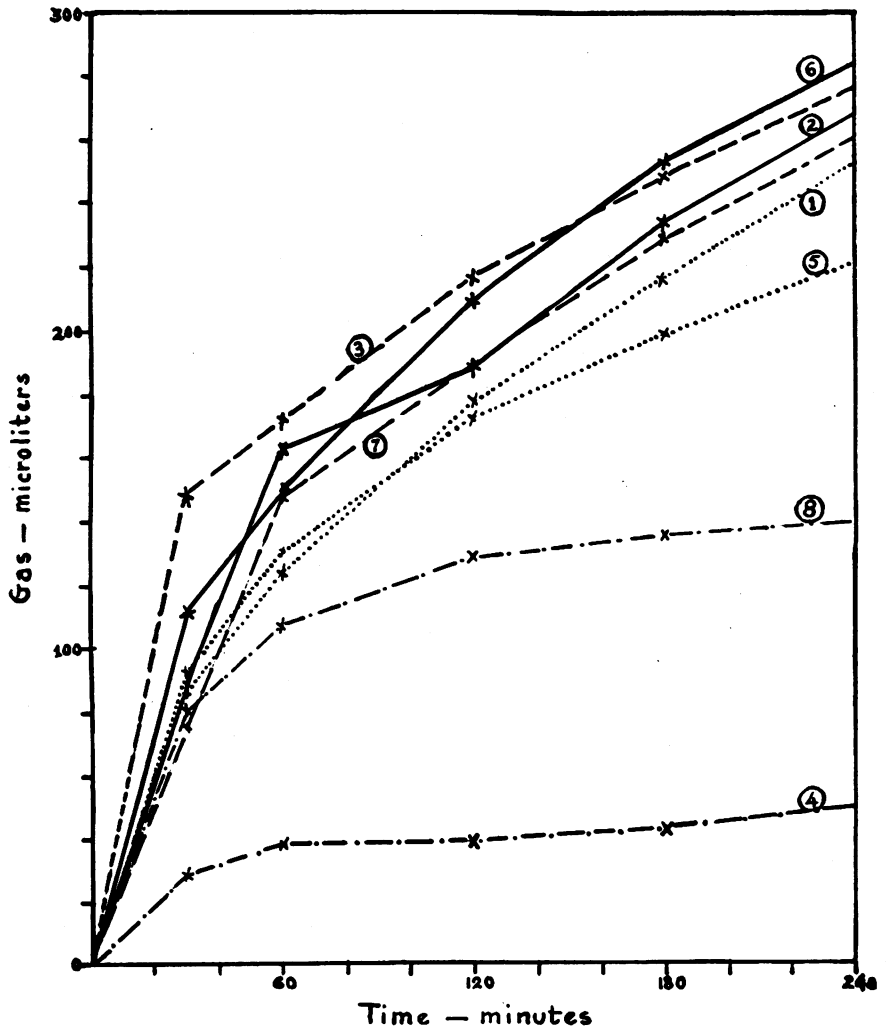

FIG. 1. Inorganic salts.

It can be seen that the QO_2 (the microliters of oxygen uptake for 10^9 cells per hour) and QCO_2 (the microliters of carbon dioxide evolution for 10^9 cells per hour) were highest when 0.006 M monosodium phosphate (curves

[2] The respiratory quotient (R.Q.) throughout does not take into account CO_2 fixation.

2 and 6) or 0.006 M monopotassium phosphate (curves 3 and 7) were added to the nutrient solution. The QO_2 and QCO_2, obtained with 0.006 M KCl, were lower than for the nutrient solution containing no added salts. The addition of 0.005 M $Ca(NO_3)_2$ gave curves very similar to those obtained with KCl. Because of the variations of R.Q. produced upon the addition of phosphate, the plain nutrient solution was used without the addition of inorganic salts in all the following experiments.

The data obtained for all the respiratory experiments are shown in table I. Values have been reported for the periods of time of 120, 130 and 240 minutes only.

Effect of glucose

Before studying the effect of the organic acids on the respiration of chlorella in the dark, it was desirable to have some basis of comparison. Respiration curves were obtained with starved cells placed in nutrient solutions containing several concentrations of glucose and also with no glucose added (as control). It was found that little variation occurred even when the concentrations ranged from 0.005 to 0.1 M, probably because even a very little glucose is sufficient to give a good respiration rate. However, as a glucose concentration of 0.08 M gave the best growth in the experiments reported in the previous section, the respiration data are shown for that particular concentration (fig. 2). The number of cells was 4.3×10^7 per flask.

The QO_2 and QCO_2 of the cells grown in glucose (curves 1 and 3) are considerably higher than those of the control (starved cells—curves 2 and 4). Although the respiration of the starved cells is appreciable, it would appear that their respiratory mechanism is slowed but not drastically affected. Even when starvation was more severe (up to three days in the dark at about 30° C), the O_2 curve was close to the same level. The CO_2 curve, however, dropped more rapidly. In the experiment reported in figure 2, it seems that the cells were not starved sufficiently. In spite of the fact that they were left the fixed length of time in the dark, the R.Q. was about unity. (The cells used in the following respiratory experiments, however, gave R.Q.'s below unity as shown by the low level of the CO_2 curves. These cells, from stock suspensions prepared at different times, were apparently more deprived of carbohydrates. In such case, the respiration rate should be lower and the relative amount of CO_2 fixation higher.)

An interesting characteristic of the cells in glucose is that they maintained a constant respiration rate for a longer period of time than the cells in plain nutrient solution. The starved cells were more readily affected by a decrease in gas pressure in the flasks. If air was admitted into the flasks by opening the manometers, the same-shaped curves were again obtained, which shows that the drop in respiration of starved cells is related to the decrease of gas pressure in the flasks.

Respiration curves of starved cells from the same stock suspension,

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON THE RESPIRATION OF CHLORELLA

Compound added and molar concentration	Figure	Curve	Microliters of O_2 Uptake: In each experiment Time in Minutes 120	180	240	For 10^9 cells per hour	Curve	Microliters of CO_2 Evolved: In each experiment Time in Minutes 120	180	240	For 10^9 cells per hour
0.00		1	178.0	215.0	254.4	450	5	171.0	199.0	221.3	400
0.006 $NaH_2PO_4 \cdot H_2O$		2	188.0	237.0	266.8	476	6	209.0	253.0	284.0	507
0.006 KH_2PO_4		3	185.4	228.0	260.0	464	7	216.0	248.0	275.4	491
0.006 KCl	1	4	38.0	43.0	49.7	89	8	128.0	134.0	140.5	253
0.08 Glucose		1	117.2	171.0	224.2	1,307	3	124.4	177.5	230.6	1,340
0.00	2	2	52.6	70.0	78.3	456	4	63.1	84.2	97.7	568
0.30 Lactate		1	23.5	38.2	52.1	930	4	31.5	57.9	84.6	1,510
0.05 "		2	12.4	21.7	39.2	700	5	26.1	40.6	58.1	1,038
0.00	3	3	18.3	26.7	31.4	561	6	9.8	16.6	19.5	348
0.20 Pyruvate		1	164.0	251.0	338.0	704	4	40.0	59.0	80.0	166
0.05 "		2	263.4	372.6	490.2	1,020	5	133.0	207.0	263.0	547
0.00	4	3	160.0	204.0	226.0	470	6	74.0	133.4	158.2	329
0.15 Acetate		1	281.6	403.3	522.7	1,537	4	146.1	223.7	300.4	884
0.05 "		2	182.4	278.3	379.4	1,115	5	126.8	177.2	238.2	701
0.00	5	3	146.8	171.7	191.3	563	6	114.5	122.1	146.8	432
0.15 Aconitate		1	55.6	66.7	69.3	1,237	4	32.2	37.4	39.5	705
0.03 "		2	39.2	42.8	45.5	812	5	15.5	25.7	30.6	546
0.00	6	3	24.2	27.6	29.6	529	6	21.6	27.0	25.7	459
0.30 Citrate		1	24.2	31.0	34.0	121	5	19.0	11.0	11.5	41
0.10 "		2	52.6	71.0	88.1	315	6	18.2	23.0	25.6	91
0.02 "		3	82.3	117.6	143.5	513	7	38.1	50.0	58.1	208
0.00	7	4	90.2	124.0	158.0	564	8	71.0	105.0	138.0	492

TABLE I—(*Continued*)

Compound added and molar concentration	Figure	Curve	Microliters of O_2 Uptake: In each experiment Time in Minutes 120	180	240	For 10^9 cells per hour	Curve	Microliters of CO_2 Evolved: In each experiment Time in Minutes 120	180	240	For 10^9 cells per hour
0.06 α-keto glutarate		1	108.0	132.0	142.0	825	4	56.1	90.2	112.0	651
0.02 " "		2	90.4	101.2	108.0	628	5	34.0	54.4	67.2	391
0.00	8	3	63.6	81.7	90.3	525	6	48.3	65.0	78.2	455
0.15 Succinate		1	25.6	41.3	54.7	595	4	22.8	37.3	51.4	559
0.03 "		2	27.8	43.5	58.6	637	5	18.3	30.2	42.9	466
0.00	9	3	21.3	34.7	46.4	504	6	16.8	28.1	38.6	420
0.15 Fumarate		1	38.8	49.5	66.7	1,191	4	10.7	17.8	38.0	678
0.03 "		2	26.1	33.5	42.4	757	5	18.3	24.0	31.4	560
0.00	10	3	23.1	27.8	30.0	536	6	14.6	19.0	22.1	395
0.15 Malate		1	26.3	38.0	50.3	898	4	24.6	35.7	47.2	843
0.03 "		2	9.2	13.8	19.6	350	5	7.8	10.9	15.7	280
0.00	11	3	16.6	23.8	28.4	507	6	8.1	15.8	20.3	363
0.15 Butyrate		1	24.1	29.3	34.2	855	4	17.8	21.6	25.8	645
0.05 "		2	13.7	18.8	23.2	580	5	12.6	17.3	21.4	535
0.00	12	3	13.0	18.2	22.0	550	6	7.8	10.7	14.3	358
0.15 Propionate		7	14.1	20.0	25.8	645	9	5.6	7.8	10.2	255
0.05 "		8	11.3	17.0	20.4	510	10	2.6	4.4	6.1	153
0.00	12	3	13.0	18.2	22.0	550	6	7.8	10.7	14.3	358

grown in the same media but at different times, were not identical. To determine the effect of the organic acids on respiration, it was then necessary to conduct, with each experiment, a determination of the cell respiration in plain nutrient solution. These curves were used as controls.

Many concentrations of acids were used, but only the most significant results have been selected for the tables.

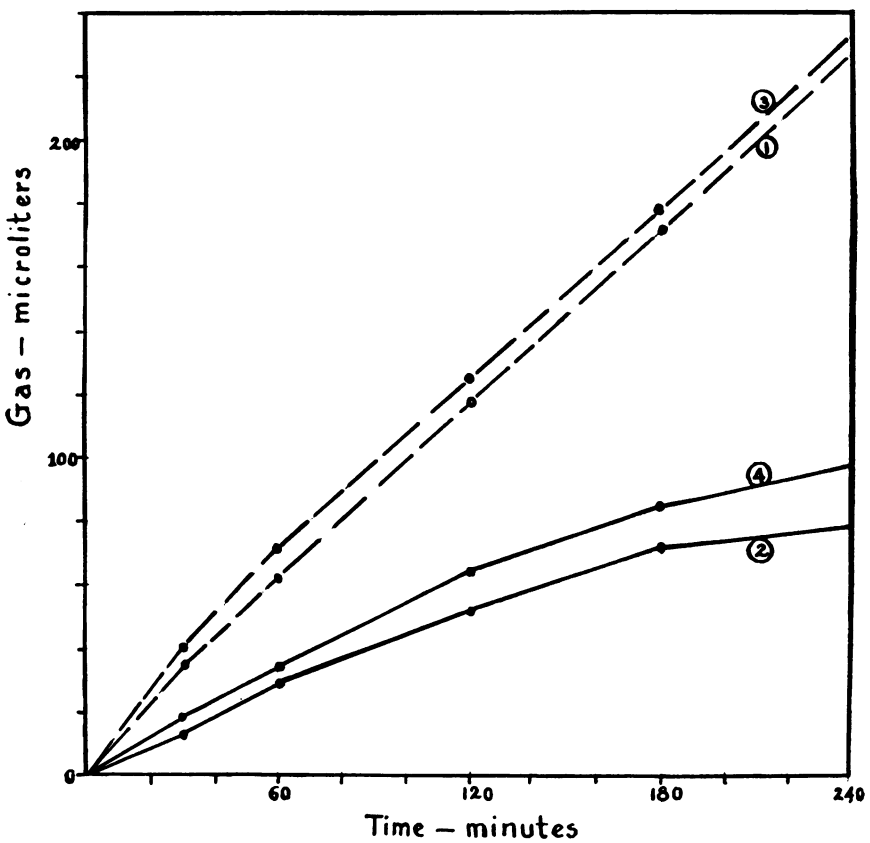

FIG. 2. Glucose.

EFFECT OF ORGANIC ACIDS

SODIUM LACTATE.—The effect of lactate on respiration is shown in figure 3. The cell concentration for this figure was 1.4×10^7 per flask. The highest respiration rate was produced at 0.3 M concentration. Above this, the respiration began to decrease rapidly and at 0.6 M, it was below the level of the control. Despite the fact that as low as 0.005 M indicated a slight amount of growth, an increase of respiration over the control became noticeable only when 0.01 M lactate was furnished. At 0.05 M, the increase was already considerable, as shown by the QO_2 of 700 microliters.

A notable feature of the respiration with lactate is that it is the only acid

studied which gave an apparent R.Q. higher than unity (*i.e.*, R.Q. at 240′ for 0.05 M = $\frac{1038}{700}$ = 1.48). Such a high R.Q. is not extraordinary. GAFFRON (10) found that the R.Q. of several species of algae (*Chlorella pyrenoidosa* and others) was abnormally high (1.2 to 2.0) when the algae were cultivated in various concentrations of glucose.

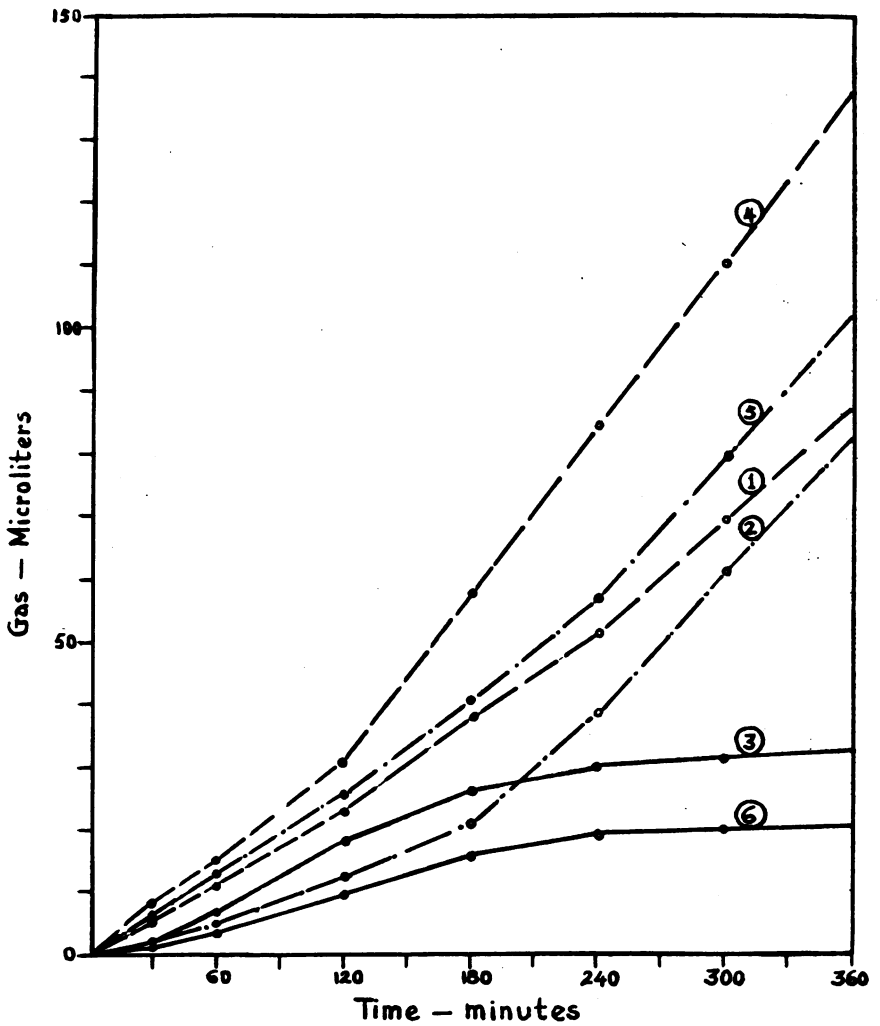

FIG. 3. Lactate.

The high R.Q. obtained with lactate may indicate that carbohydrates are being formed more rapidly with lactate than with the other acids, by the reversal of a process similar to glycolysis, and/or that in this case, there is little dark CO_2 fixation taking place.

SODIUM PYRUVATE.—The respiration of chlorella, when pyruvate was added to the nutrient solution, is shown in figure 4. The cell suspension

had a concentration of 1.2×10^8 per flask. It can be seen that the highest QO_2 was reached when the acid concentration was 0.05 M. At this concentration, the QCO_2 was higher than that of the control but it remained at a fairly low level, which denotes either an extremely low R.Q. or a large amount of CO_2 fixation, or both.

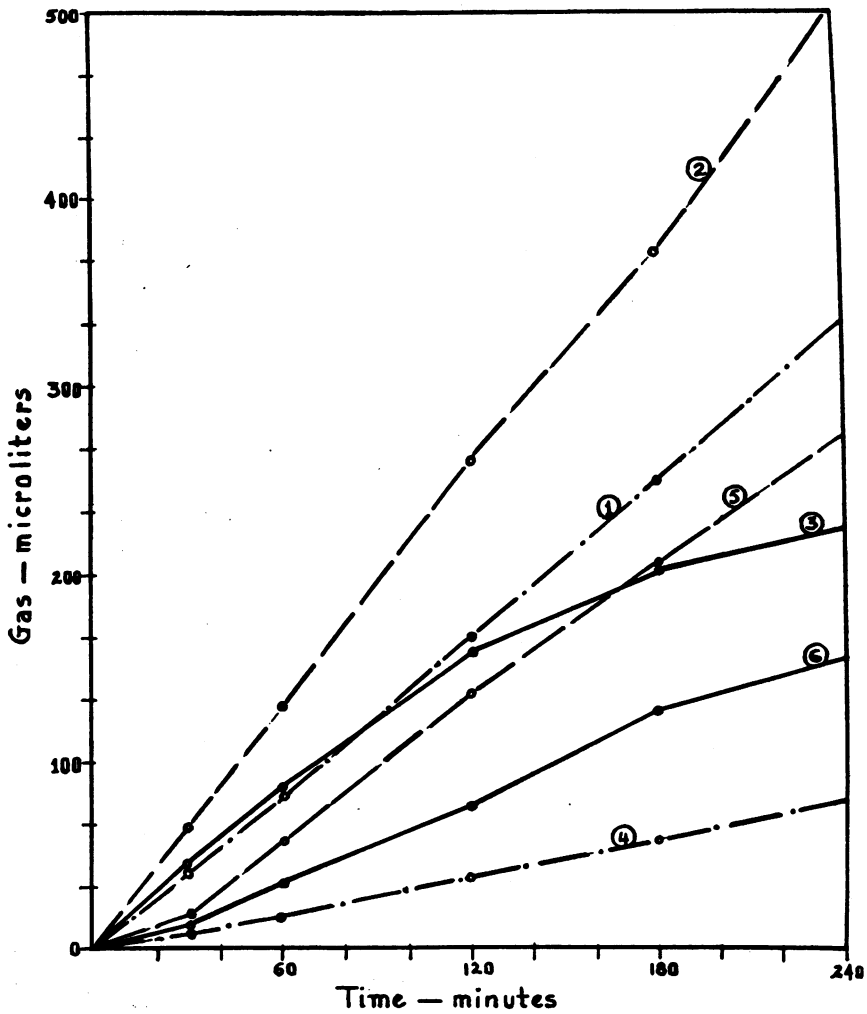

FIG. 4. Pyruvate.

Between the concentrations of 0.03 M and 0.08 M, the respiration did not change appreciably. However, below and above these concentrations, the respiration level fell. Curves 1 and 4 show this effect, obtained with 0.2 M pyruvate. The O_2 uptake remained slightly higher than for the control with as low as 0.001 M pyruvate.

Data on the respiration rate up to four hours are shown. Little significance can be obtained after this time. Between six and eight hours, the rates of respiration decreased. These effects are due mainly to decreased

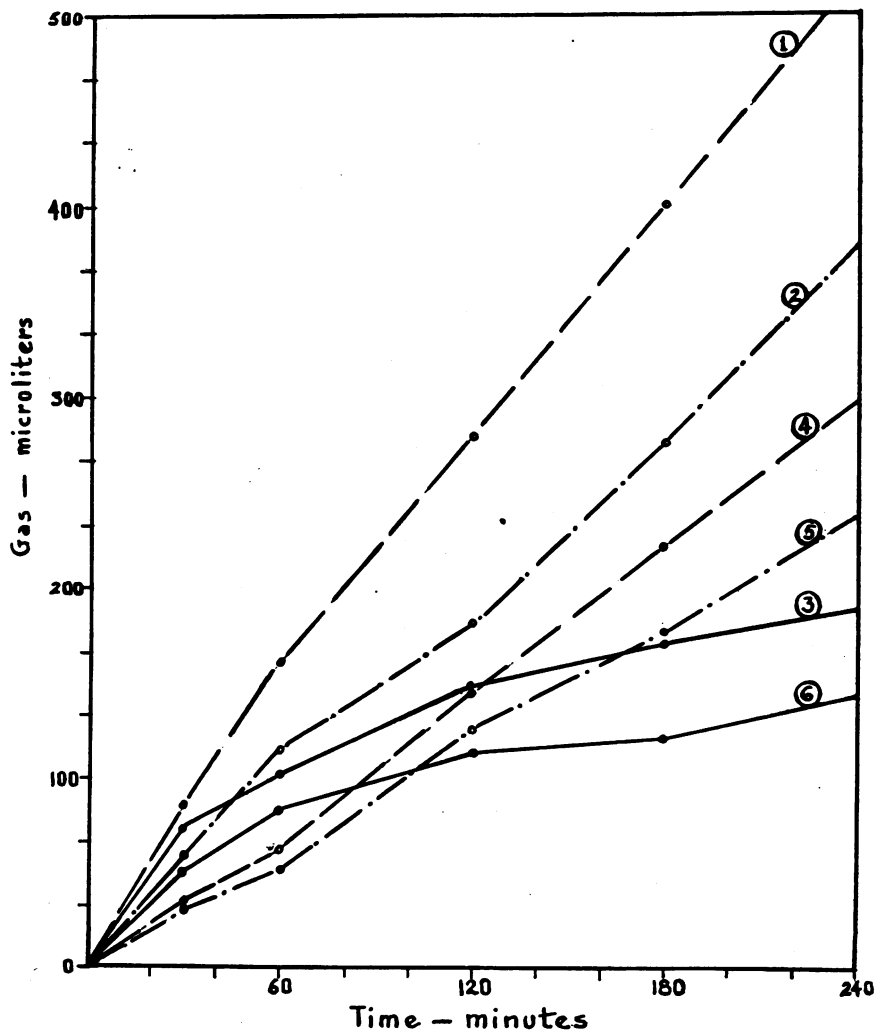

FIG. 5. Acetate.

oxygen pressure in the flasks. After 15 hours, the respiration with 0.05 M pyruvate was, however, still above that of the control.

SODIUM ACETATE.—Even at a very low concentration such as 0.005 M, acetic acid produced an appreciable amount of respiration over that of the control. As the concentration increased up to 0.6 M, respiration also increased. Above that concentration, within a 30-minute period after the acid was added, the curve of the O_2 uptake climbed rapidly and then fell

below the control. Figure 5 gives the results obtained for acetic acid concentrations of 0.15 M and 0.05 M with a suspension containing 8.5×10^7 cells. In both cases, the QO_2 was above the control.

SODIUM ACONITATE.—The respiration data for aconitic acid are shown in figure 6. The cell suspension was 1.4×10^7 per flask. The QO_2 and QCO_2

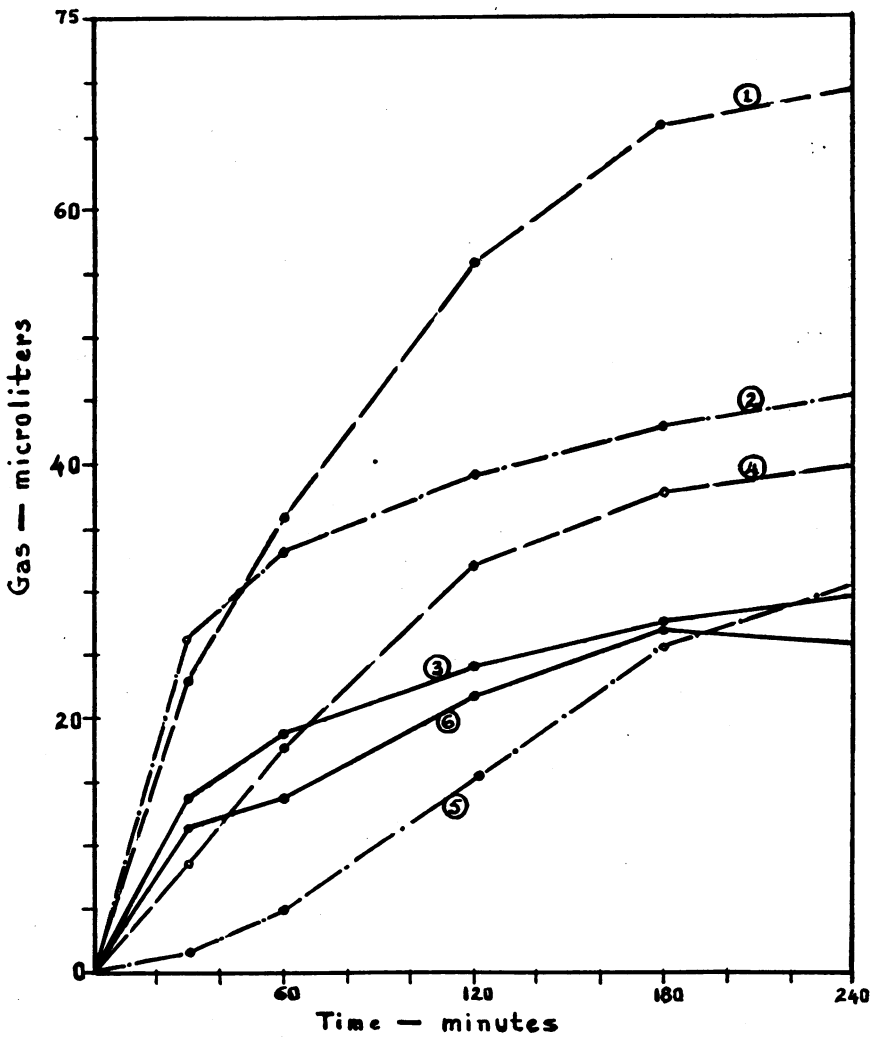

FIG. 6. Cis-Aconitate.

are higher with aconitic acid than with the control. It can also be seen that the QCO_2 is lower than the QO_2 which indicates that the R.Q. is smaller than 1 and, consequently, there must be some CO_2 fixation. This is in agreement with the growth results obtained. The rate of respiration increased with the concentration of aconitate (up to about 0.6 M), the best growth occurring with an 0.04 M concentration. A possible explanation is that after periods varying from several hours to several days, the respira-

tion rate of chlorella in high aconitate concentrations drops. This type of behavior was observed also in the case of acetate.

SODIUM CITRATE.—With all the concentrations of citrate used, the respiration curve for chlorella did not reach that of the control. The results obtained for 7×10^7 cells per flask with various levels of citrate are shown in figure 7. The maximum QO_2 was obtained for 0.02 M, when the O_2 curve

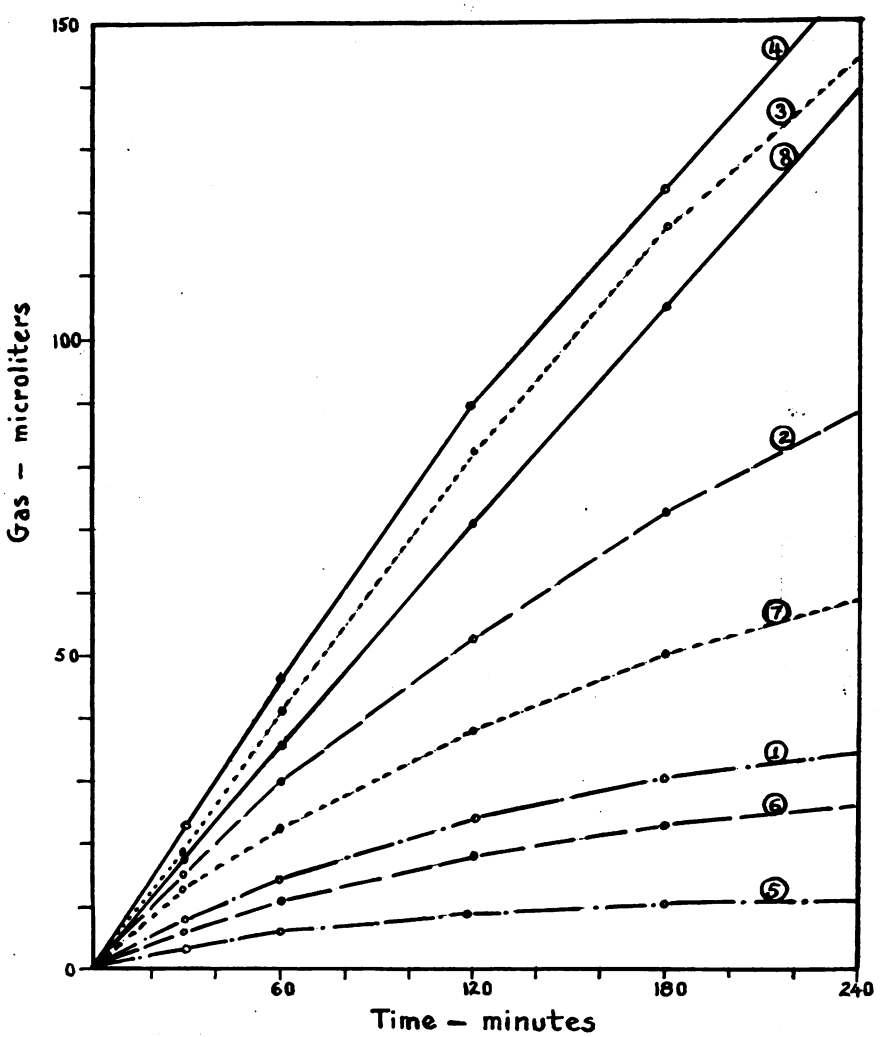

FIG. 7. Citrate.

closely approached that of the control, but remained lower nevertheless. The QCO_2 was definitely below the control. These conditions were much the same when citrate was varied from 0.01 M to 0.04 M. Above these limits the respiration rate fell more rapidly (curves 1 and 4).

With 0.02 M citrate, a small amount of growth was obtained, yet the respiration curves indicate some inhibition but to a lesser degree than with

other concentrations. It may be that a sort of adaptation takes place in the chlorella after some time and results in a change in the respiratory rate sufficient to produce assimilation of citrate and growth.

SODIUM α-KETOGLUTARATE.—Due to the small amount of α-ketoglutarate available, the respiration of chlorella was measured with only three concentrations—0.002 M, 0.04 M and 0.06 M. The number of cells per flask was 4.3×10^7. The results of the 0.04 M are omitted from figure 8. Being intermediate, they were of no special interest.

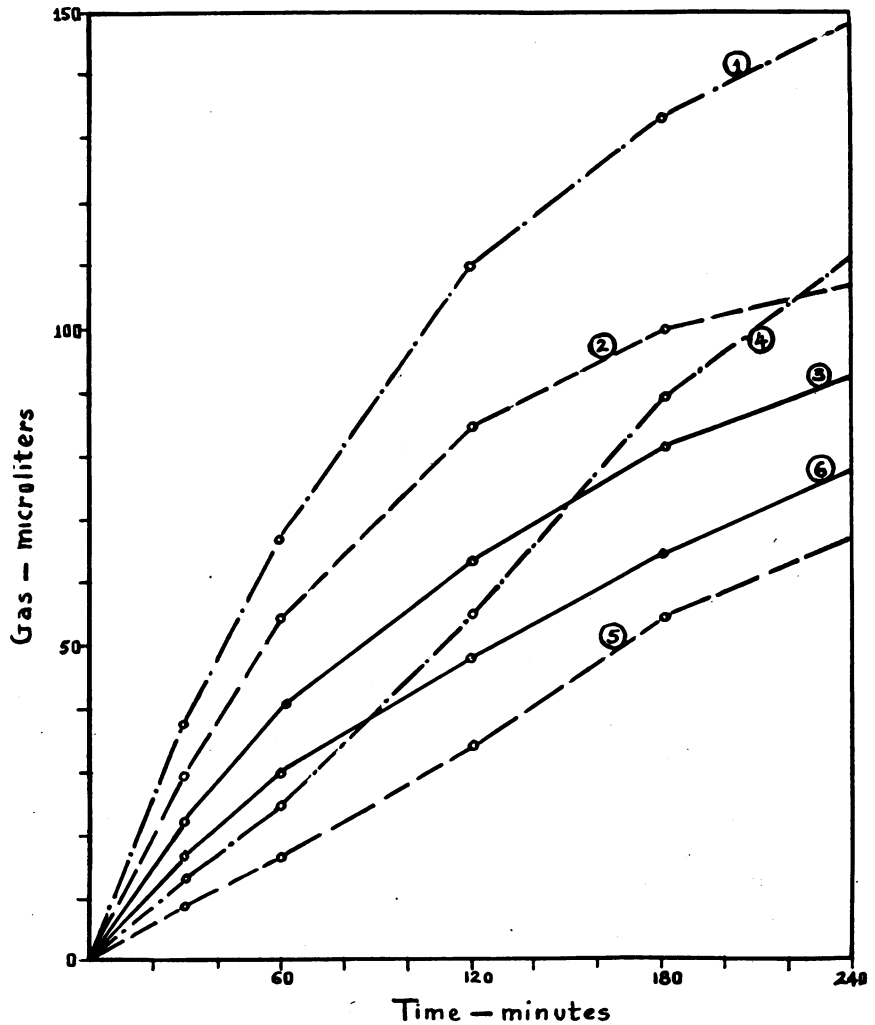

FIG. 8. α-Ketoglutarate.

It may be concluded that α-ketoglutarate was respired as the QO_2 in both cases is higher than that of the control. Oxidative assimilation is indicated by the low R.Q.

The comparison of these curves with those of the other acids signifies that α-ketoglutarate probably is a source of organic matter for chlorella growth. These respiration data, however, cannot alone be taken as final criteria for such a conclusion.

SODIUM SUCCINATE.—Succinate did not produce much rise in respiration generally. As shown in figure 9, the highest rate of respiration was ob-

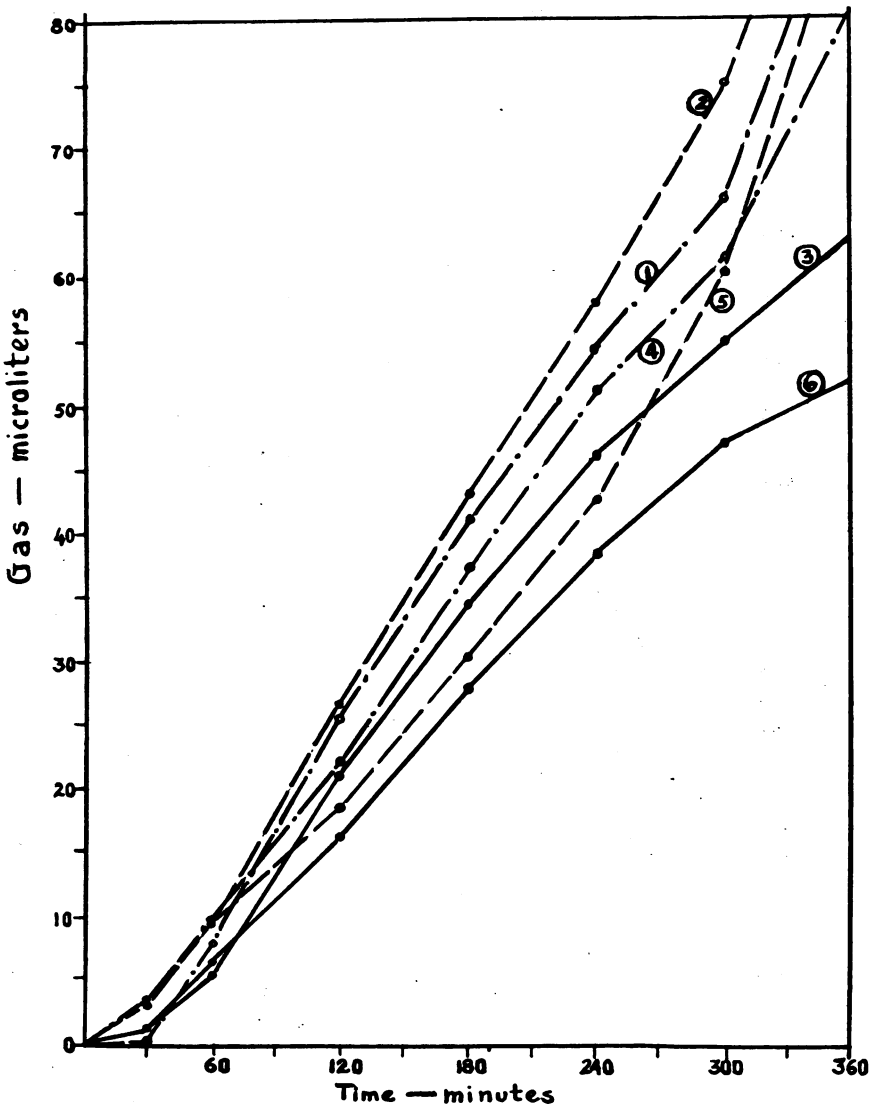

FIG. 9. Succinate.

tained for an 0.03 M concentration with 2.3×10^7 cells per flask (curves 2 and 5). In that instance, the QO_2 was 637 microliters as against 504 microliters for the control.

Up to 0.15 M, cells in succinate absorbed more oxygen than the control, but when 0.3 M succinate was used, the respiratory rate fell below the control level.

It can be seen that with succinate the O_2 uptake is higher in all cases than the CO_2 evolution, giving a R.Q. lower than 1.0.

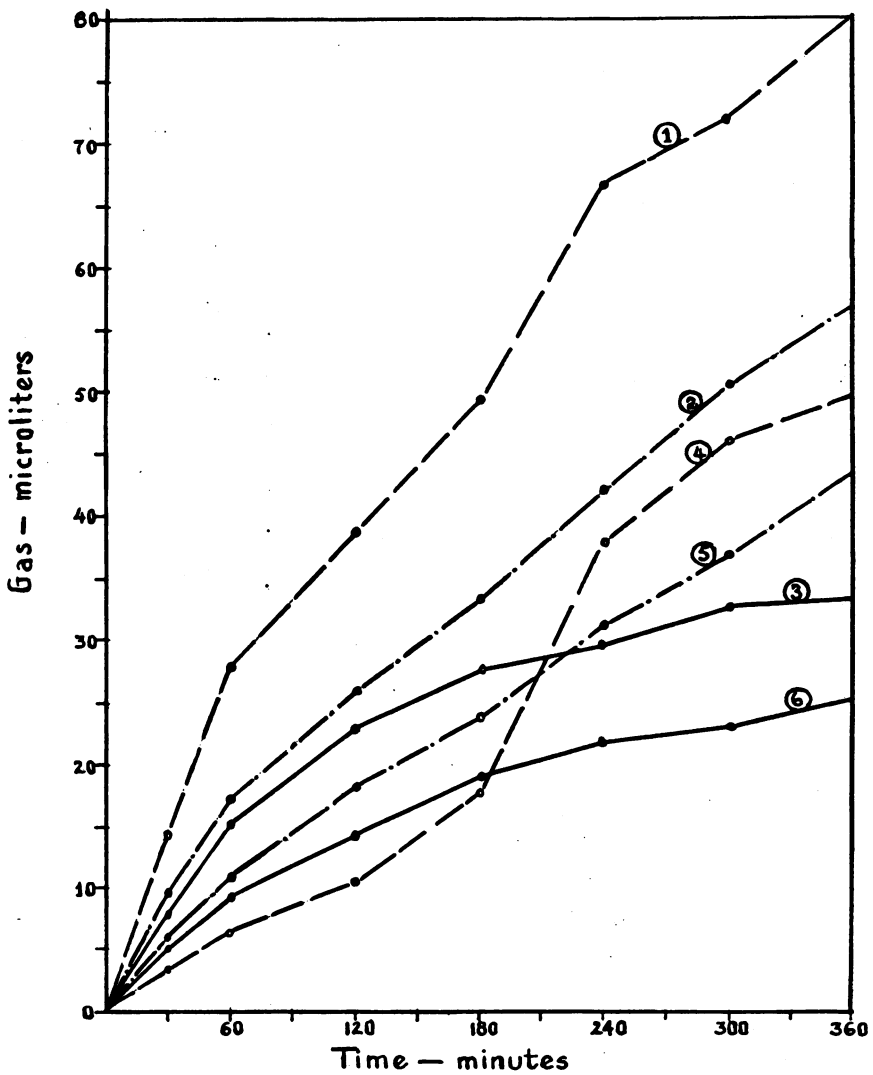

FIG. 10. Fumarate.

SODIUM FUMARATE.—The respiratory data obtained with fumarate for 1.4×10^7 cells per flask are shown in figure 10. These results are generally similar to those obtained for aconitate, the only noticeable difference being that the curves for fumarate are consistent in trend, but more irregular in shape.

SODIUM MALATE.—The data on the respiration of chlorella with malate are presented in figure 11. For the first four hours, the QO_2 of the cells

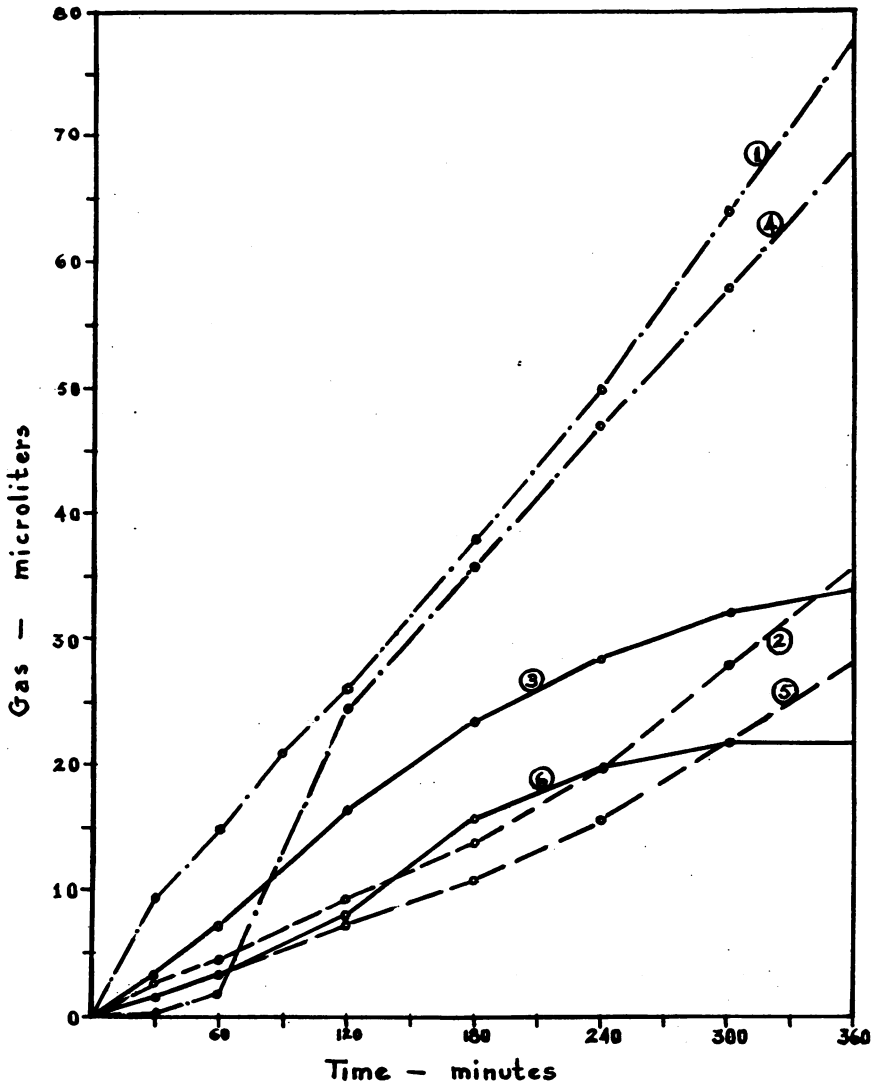

FIG. 11. Malate.

(1.4×10^7 per flask), with an 0.03 M concentration, was below that of the control. After six hours, however, the QO_2 became slightly higher and the trend continued in this manner for an indefinite period of time. In other respects these data are comparable to those for aconitate and fumarate.

SODIUM BUTYRATE.—The effect of butyrate on respiration was hardly noticeable until a concentration of about 0.02 M was supplied. For 0.05 M, the effect was negligible at first, but, after six hours and over, respiration became appreciably higher than that of the control. Figure 12 shows the

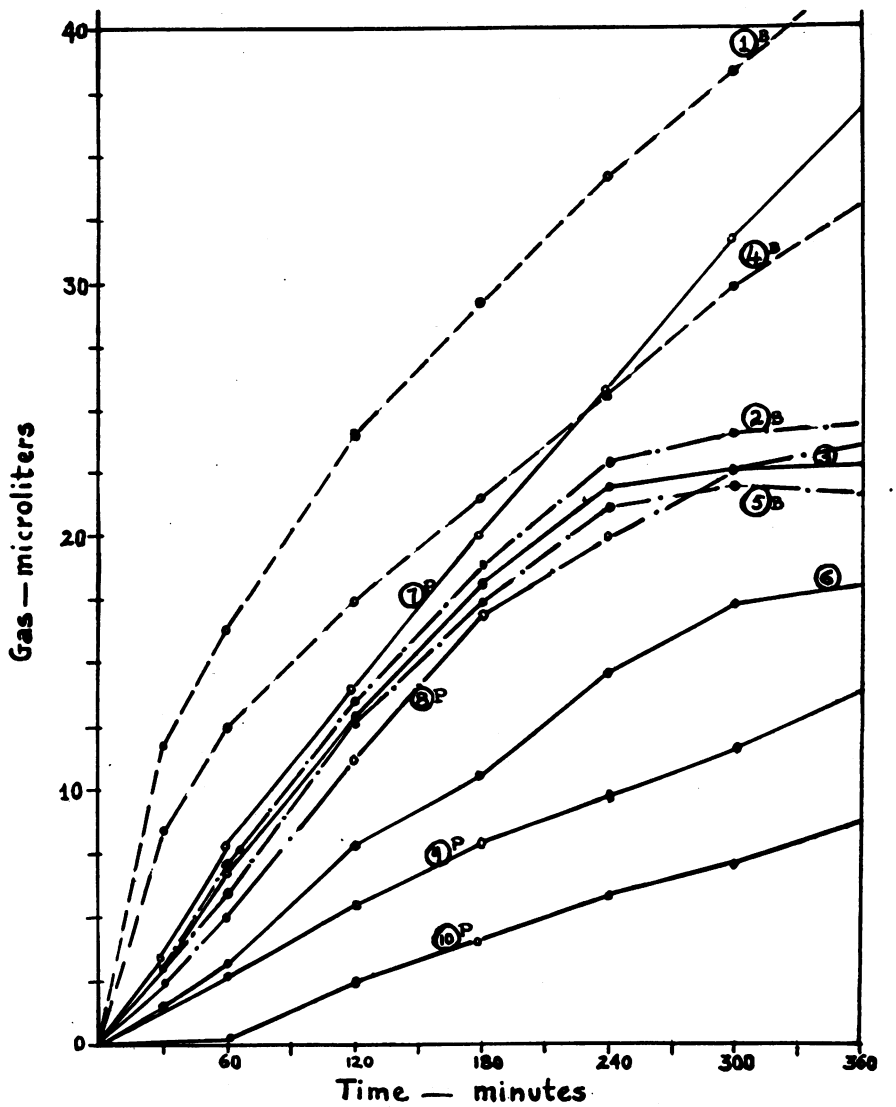

FIG. 12. Butyrate and propionate.

curves obtained for 1×10^7 cells per flask. At 0.15 M, the respiration rate was higher than at 0.05 M, as can be seen by the QO_2 and by the level of curve 1, but the growth was poorer. It is possible that, similar to lactate, the higher respiration rate does not last as long as with lower concentrations of acids or that some toxicity, due to physical or chemical causes, enters into being. It may be noted that the R.Q. is very close to unity, $(\frac{535}{580} = 0.92)$, which may indicate a low amount of CO_2 fixation and may explain the low ratio of growth obtained in the previous experiments.

SODIUM PROPIONATE.—The respiratory level obtained with propionate was generally low. The curves obtained with 1.4×10^7 cells per flask are

shown in figure 12. As much as 0.15 M propionate was necessary to raise the QO_2 to 645; at lower concentrations the oxygen uptake decreased. During the first five hours, the curves obtained for 0.05 M were lower than those of the control. After about six hours, however, they rose and remained above the control level. The R.Q. calculated at six hours was 0.38. This low value indicates that, in spite of the low respiration level, growth and the building of organic matter could have been obtained through a relatively large amount of CO_2 fixation.

Discussion

Table II gives a general picture of the results of the organic acid effects on the respiration of chlorella. The CO_2 values of the controls are shown in each case for better comparison. It can be seen that there was only a

TABLE II

SUMMARY OF RESPIRATION DATA

Organic acid salt	Concentration	Q_{O_2}	Q_{CO_2}	Order number	Growth ratio	Control Q_{O_2}	Control Q_{CO_2}
Lactate	0.05	700	1,038	6	4.10	470	329
Pyruvate	0.05	1,020	547	2	4.0	561	348
Acetate	0.05	1,115	701	1	3.97	563	432
Aconitate	0.03	812	546	4	2.97	529	459
Citrate	0.02	513	208	9	1.65	564	492
α-keto glutarate ..	0.06	825	651	3	....	525	455
Succinate	0.03	637	466	7	1.67	504	420
Fumarate	0.03	757	560	5	2.56	536	395
Malate*	0.03	350	280	11	2.23	507	363
Malate**	0.03	428	334	11	2.23	410	259
Butyrate	0.05	580	535	8	2.05	550	358
Propionate	0.05	510	153	10	1.57	550	358

*Calculated at 240 minutes.
**Calculated at 360 minutes.

small change in the QO_2 for the starved cells. The QCO_2, however, varied on a wider range which is related to the degree of starvation of the cells, the QCO_2 being smaller for the more starved cells.

The oxygen uptake of the cells varied considerably with the several organic acids. The relative order of this uptake is to be found in column 5, acetate being first with the highest value. It is interesting to observe that the order does not correlate exactly with the relative order obtained for the growth experiments. A good relationship exists nevertheless, as pyruvate, lactate, acetate and aconitate gave the highest growth and citrate, succinate and propionate, the lowest.

Beyond a certain concentration level, all of the acids studied produced a decline in respiration below that of the controls, due probably to physical or chemical toxicity.

For most of the acids (lactate, acetate, aconitate, fumarate, malate, butyrate and propionate), the oxygen uptake was greater with a concentra-

tion higher than that which gave the best growth results. It is possible that after a certain length of time, shorter than the growth period, the respiration rate falls, as in the case of acetate, or that, with a high respiration rate, the amount of CO_2 evolved is large and the relative CO_2 fixation becomes insufficient to make up for the loss of carbon and to build organic materials for growth.

It was noted that the R.Q. for all of the acids except lactate was below unity. This low R.Q. may be due to three possible causes:

1) The acids were assimilated and a large quantity of CO_2 was fixed for the possible building of carbohydrates and the accumulation of organic matter.

2) Respiration was due to the oxidation of proteins and fats as primary products. This is a possibility which may exist conjointly with the first mentioned.

3) Even without any CO_2 fixation, the amount of acid breakdown is insufficient to give a high CO_2 evolution.

The positive results obtained in the growth experiments definitely favor the oxidative assimilation and CO_2 fixation theory.

The carboxylation of 3- and 4-carbon acids and the fixation of carbon dioxide in the dark has been reported in various plant tissues. WOLF (**21**) showed that pyruvic acid added to leaf strips caused a marked decrease in the CO_2 evolution. RUBEN, HASSID, and KAMEN (**16**), using radioactive CO_2, observed the fixation of carbon dioxide in the dark in barley leaves and in chlorella suspensions.

BARKER (**3**), CLIFTON (**7**), DOUDOROFF (**8**), and ANDERSON (**2**) have demonstrated the generality of an oxidative assimilation of carbon in microorganisms. It is illustrated by the equation:

$$\text{Acetic acid} + O_2 \longrightarrow \text{Stored carbohydrates} + CO_2 + H_2O.$$

It could be assumed that the over-all reaction represents a summation of exergonic and endergonic reactions linked by the energy transfer between them in a manner similar to the mechanisms of chemosynthesis in *Thiobacillus thiooxidans* (VOGLER and UMBREIT, **19**).

Among the mechanisms suggested for the organic fixation of carbon dioxide, the carboxylation of pyruvic acid is of interest because it is directly related to carbohydrate metabolism. The carboxylation of pyruvate has been held difficult to accomplish because of the energy relations ($\Delta F = -15.2$ in aqueous solution and $\Delta F = -2.9$ in alkaline solution) involved. CARSON, RUBEN, KAMEN, and FORSTER (**6**) were unable to produce this reaction. VAN NIEL *et al.* (**18**) suggested that these carboxylations are necessary for growth and respiration because they contribute to the synthesis of 4-carbon acids (fumaric, malic, succinic, and oxalacetic), which play an important part in respiration; also, perhaps, in photosynthesis.

KRAMPITZ and WERKMAN (**12**) and KRAMPITZ, WOOD, and WERKMAN (**13**) were able to demonstrate reversible carboxylation of oxalacetic acid.

BONNER and BONNER (**4**) found that an increase of carbon dioxide concentration to bryophyllum in the dark under controlled conditions produced a definite increase in total acids. The total acids were fully accounted for by totalling the citric, isocitric and cis-aconitic, and the malic acids fractions. Bonner and Bonner state, "The other acids necessarily present in a plant respiration cycle may be present in such small quantities that available analytical methods are inadequate to detect them. They may be overshadowed by the large amount of the acids determined . . . . The fact that the increase in malate is a direct function of carbon dioxide pressure suggests that at least a portion of the oxalacetic acid formed is at once reduced to malic acid. On the other hand, isocitric accumulation with an increase of carbon dioxide would indicate that a part of the oxalacetate is removed by oxidative transformation."

KROTKOV and BARKER (**14**) using C^{14} in the carboxyl group of acetic acid found that tobacco leaves could utilize acetate in the dark. When tobacco leaves were left for three hours and five minutes in the dark, after having been placed in acetic solution for about two hours, the C^{14} was located in an unidentified water and ethanol soluble compound. When, after being placed in acetate solution for about two hours, the leaves were left in darkness for 20 hours and 45 minutes, the isotopic carbon appeared mainly as respired CO_2. In both cases, acetate was metabolized. The results strongly suggest that, once the carbohydrate supply of the leaves is exhausted, acetic acid is used directly for the formation of cis-aconitic acid in endogenous respiratory processes.

Recently MYERS, CRAMER, and JOHNSTON (**15**) reported an oxidative assimilation of acetic acid in darkness by the green algae, *Chlorella pyrenoïdosa.*

Basing their conclusions on the results of experiments with isotopic carbon dioxide, ALLEN, GEST, and KAMEN (**1**), and GAFFRON, FAGER, and BROWN (**10**) believed that starved chlorella cells were able to fix carbon dioxide by endogenous respiration. This is definitely shown in the data presented here by the good correlation existing between the utilization of the organic acids in the dark, the increase of oxygen uptake and the low respiratory quotient produced by them.

Using isotopic carbon and yeast, WEINHOUSE and MILLINGTON (**20**) observed that acetate produced an accumulation of citrate and of carbon dioxide which was distributed within the tricarboxylic acid cycle. They state, however, that another independent mechanism is available for the formation of some of the 4-carbon acids from acetic acid. KALNITSKY and WERKMAN (**11**) demonstrated that succinate is not produced in some organisms through the decarboxylation of α-ketoglutarate but by the condensation of two acetates. CALVIN and BENSON (**5**) believe that this oxidative coupling of two acetates to produce succinate also occurs in chlorella. In the present study, the low rate of respiration observed with succinate tends

to confirm the previous indications that succinate enters an independent mechanism and is not a direct intermediate in the aerobic cycle of chlorella.

Summary

The effect of various organic acids known to occur in the path of carbohydrate metabolism in animal tissues was studied on the respiration of the green alga chlorella. Manometric measurements of oxygen uptake and carbon dioxide evolution were conducted in the dark with a Warburg apparatus. The following acids were supplied: lactic, pyruvic, acetic, aconitic, citric, α-ketoglutaric, succinic, fumaric, malic, butyric, and propionic.

Respiration was stimulated in various degrees by the organic acids and their concentrations. Pyruvate and acetate produced the highest oxygen uptake and citrate and succinate the lowest, correlating well with the growth results (**9**). The apparent respiratory quotient for most of the acids was lower than unity. The relation between the oxygen uptake and carbon dioxide evolution, together with the growth results, indicate the formation of reduced compounds through oxidative assimilation and carbon dioxide fixation. The fact that the organic acids utilized for respiration and growth by chlorella are identical to those known as intermediates in the respiratory path of animal tissues indicates that the path through which the oxidation of carbohydrates is effected has much similarity with the tricarboxylic acid cycle and that it can reverse itself in the dark to build organic materials necessary for growth from organic acids and carbon dioxide.

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