OXIDATIVE ASSIMILATION IN RELATION TO PHOTOSYNTHESIS IN CHLORELLA*

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The studies of Barker (1935, 1936), Giesberger (1936), Clifton (1937), Doudoroff (1940), and others have clearly demonstrated the generality of an oxidative assimilation of carbon in microorganisms. This is a process illustrated by the over-all equation for the respiration of acetic acid by *Prototheca*,

$$CH_3COOH + O_2 \rightarrow (CH_2O) + CO_2 + H_2O,$$

in which (CH_2O) represents storage carbohydrate. Characteristically the substrate is partially oxidized to carbon dioxide and partially synthesized to storage material. From the standpoint of photosynthesis it is tempting to postulate that the over-all reaction represents a summation of exergonic and endergonic reactions linked only by the energy transfer between them in a fashion analogous to the mechanism of chemosynthesis in *Thiobacillus thiooxidans* (Vogler and Umbreit, 1942). However the very stoichiometric nature of the equation (and other characteristics) completely rules out this possibility. The over-all equation clearly represents the summation of a series of reactions meshed together by the nature of the intermediates. Thus the simplest possible analogy to chemosynthesis and photosynthesis is ruled out. On the other hand the processes of photosynthesis and oxidative assimilation have in common the synthesis of carbohydrate and may well have other interrelations.

Oxidative assimilation in the colorless alga *Prototheca* has been described in considerable detail by the work of Barker (1935, 1936) and Anderson (1945). Their studies imply that a knowledge of oxidative carbon assimilation in the colorless algae may have important bearing on the understanding of the photosynthetic process in the closely related green algae. Since the green algae also have a heterotrophic nutrition in the dark, the approach of Barker and Anderson may be applied directly. Described herein are certain characteristics of oxidative assimilation in *Chlorella* examined in relation to the photosynthetic process.

EXPERIMENTAL

Chlorella pyrenoidosa (Emerson's strain) was grown in two units of a continuous culture apparatus previously described (Myers and Clark, 1944). Culture con-

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ditions were: light intensity 90 f.-c. provided by tungsten lumiline bulbs; aeration with 4 per cent carbon dioxide; temperature 25.1°C.; population 1.8 to 2.0 c. mm. cells/ml.; medium, Knop's solution with iron and microelements (Myers, 1946). Samples of suspension harvested from the growth chambers in sterile flasks were darkened and aerated by a current of air or placed on a mechanical shaker. Manometric experiments were begun 24 to 30 hours later.

Gas exchange measurements were made by the Warburg technique using a bath with glass bottom thermostated at 25°C. The entire bath was darkened by a cloth drape but could be illuminated from below by a bank of 17 sixty watt Mazda lamps as desired. When illumination was used, control flasks were darkened by a tinfoil wrapping. Cells were prepared by centrifuging an aliquot of suspension, washing in Knop's, and taking up in an appropriate volume of Knop's solution. Most of the measurements were made in respiration flasks with KOH in the center well and ½ ml. of substrate (dissolved in Knop's) in the sidearm. A convenient rate of

TABLE I

Comparison of Freshly Harvested and Starved Cells

Rates in c.mm. O_2/hour/c.mm. cells

	Photosynthesis	Endogenous respiration	Glucose respiration	Acetic acid respiration
Freshly harvested	+43	−1.2	−2.1 ↓ (−4.2) ↓	−2.3 ↓ (−3.4) ↓
Starved (30 hrs. dark)	+40	−0.41	−2.7 (−3.5)	−2.7 (−3.5)

gas exchange was obtained with about 30 c. mm. cells in 2.0 ml. of Knop's at pH 6.8. Estimates of cell quantities in terms of cell volume were obtained on aliquots of the original suspension as previously described (Myers, 1946).

Characteristics of Starved Cells

The study of oxidative assimilation by the usual manometric techniques requires that the rate of respiration of the substrate be considerably higher than that of endogenous respiration. Freshly harvested suspensions of *Chlorella* growing in light show a fairly high rate of endogenous respiration. It has been desirable, therefore, to starve the cells for a period of time before experimental study. Such starved cells are closely analogous to the resting cells commonly used in studies of this type on other organisms. A similar procedure was followed by Genevois (1927) in his studies on the respiration of various algae.

A comparison of the behavior of freshly harvested and starved cells is presented in Table I. With starvation there is a small decrease in capacity for

photosynthesis measured under conditions of light and carbon dioxide saturation. There is no attendant change in shape in the light intensity curve, rates of photosynthesis of starved cells being proportionately lower at all light intensities. In contrast, the rate of endogenous respiration decreases by a factor of 3. Rates of respiration of glucose and acetic acid (0.02 M) are not constant but increase considerably during measurement. Rates were nearly constant in the period from about 20 to 80 minutes after substrate addition and values for these are cited in the table. Apparently constant rates are again attained after about 100 minutes and these second rates are included in parentheses. Only the first rates are to be considered in relation to the work of this paper since with small amounts of substrate the experiments are limited to a corresponding time period.

TABLE II

Substrates for Respiration

Respired (pH 6.8)	Glucose
	Acetic acid
	Proprionic acid
	Butyric acid
Respired (pH 3.8 but not at 6.8)	Glycolic acid
	Pyruvic acid
	Succinic acid
Not respired (pH 3.8 or 6.8)	Glycerol
	Ethyl alcohol
	Sucrose

Respirable Substrates

A number of possible organic substrates were examined at concentrations of 2 to 200 micromols per ml. The results are listed in Table II. The behavior of glycolic, pyruvic, and succinic acids is similar to that observed by Anderson (1945) for the respiration of pyruvic acid by *Prototheca* and is probably explainable in terms of permeability of the cell membrane to the undissociated acid which reaches appreciable concentration only at lower pH. Inability of *Chlorella* to respire ethyl alcohol, glycerol, and sucrose was not critically determined since no attempt was made to adapt the organism to these compounds. However, similar results were obtained by Genevois (1927). From the growth experiments of Bristol-Roach (1927) it is apparent that the algae differ greatly in their ability to utilize various sources of carbon. An exhaustive study of possible respirable substrates was not attempted.

From the preliminary studies glucose and acetic acid were selected for further work. With these substrates the rate of oxygen uptake was little affected by

concentration, whereas pronounced concentration effects were shown by proprionic and butyric acids.

Oxidative Assimilation

Typical curves for endogenous respiration and the oxidation of 5.0 micromols of glucose and acetic acid are presented in Fig. 1. Elaborate evidence has been presented (*cf.* Barker, 1936) that in *Prototheca* and other organisms endogenous respiration is completely suppressed during oxidation of a provided substrate. Our tests have been less exacting but lead to a like conclusion regarding respiration in *Chlorella*. Location of the exact positions of the breaks in the curves is made with some uncertainty. The amount of

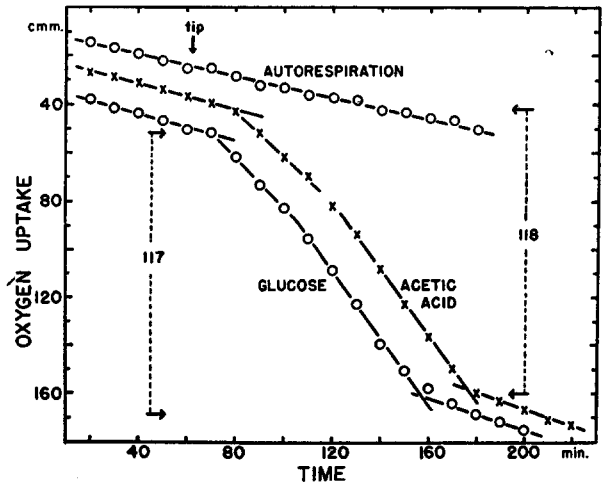

FIG. 1. Oxidative assimilation of 5.0 micromols of acetic acid and of glucose in darkness.

oxygen required by the substrate is here arbitrarily estimated as the oxygen uptake from the last point on the initial autorespiration curve to the first point on the final autorespiration curve (117 and 118 c. mm. in Fig. 1). Only small differences occur if the extrapolated intersections of the curves are used instead. While the curves for glucose and acetic acid are taken from different experiments they represent approximately equal quantities of cells. The behavior shown here of equal rates of oxygen uptake with glucose and acetic acid and equal time required for their utilization is borne out by a number of similar experiments. It suggests some common rate-limiting process in their oxidation.

The amounts of oxygen uptake required for glucose and acetic acid compiled from a number of experiments are tabulated in Table III and indicate clearly that both require one mol of oxygen per mol substrate. Measurements

of the R. Q. were attempted first at a pH of 4.5 using duplicate flasks with and without KOH. These yielded the values shown for glucose. The amount and rate of oxygen uptake by glucose were found to be independent of pH from pH 3.8 to 6.8. With acetic acid, however, no oxygen uptake could be demonstrated at low pH in flasks containing KOH although fairly high concentrations were tried.[1] The R. Q. was therefore measured at pH 6.8 and excess acid added at the end of the experiment to release bound carbon dioxide.

From the data of Table III the over-all equations for the oxidative assimilation of acetic acid and glucose may be written:

$$CH_3COOH + O_2 \rightarrow (CH_2O) + CO_2 + H_2O \quad (1)$$

and

$$C_6H_{12}O_6 + O_2 \rightarrow 5\,(CH_2O) + CO_2 + H_2O \quad (2)$$

TABLE III

Oxidative Assimilation of Acetic Acid and Glucose

Mols O_2/mol substrate determined in each case for 5 micromols of substrate. R.Q. determined in separate experiments with 50 micromols of substrate at pH 4.5 for glucose and at pH 6.8 for acetic acid.

Acetic acid		Glucose	
Mols O_2/mol	R.Q.	Mols O_2/mol	R.Q.
1.03	−1.12	1.10	−0.99
1.02	−1.14	0.90	−1.02
1.03	−1.16	1.03	−1.02
1.08	−1.09	1.05	−1.04
Average 1.04		1.02	
		1.03	
		1.02	
		0.97	
		Average 1.02	

Equations identical with (1) have been observed in several other microorganisms for acetic acid. To our knowledge equation (2) represents the most efficient assimilation of glucose yet observed in microorganisms. Here $\frac{5}{6}$ of the glucose is assimilated in comparison to the fraction of $\frac{2}{3}$ which has frequently been found in other organisms.

[1] This phenomenon might be interpreted as a distillation of acetic acid into the alkali and its removal from the cell suspension. Such an explanation seems unlikely although no check experiments have been made. It may also be that high concentrations of undissociated acetic acid block the respiratory mechanism. In this connection it has been observed that at pH 4.5 photosynthesis is completely blocked by 0.004 M acetic or 0.0012 M butyric or 0.0005 M caproic acid. The relationships between these phenomena are being investigated further.

Effect of Light on Oxidative Assimilation

The effect of light on oxidative assimilation of glucose and acetic acid has been studied by giving various doses of light and observing the resulting time course required for utilization of the substrate. A typical experiment on acetic acid assimilation is shown in Fig. 2. This was done in flasks with KOH in the center well and 5 micromols of substrate added from the sidearm. In

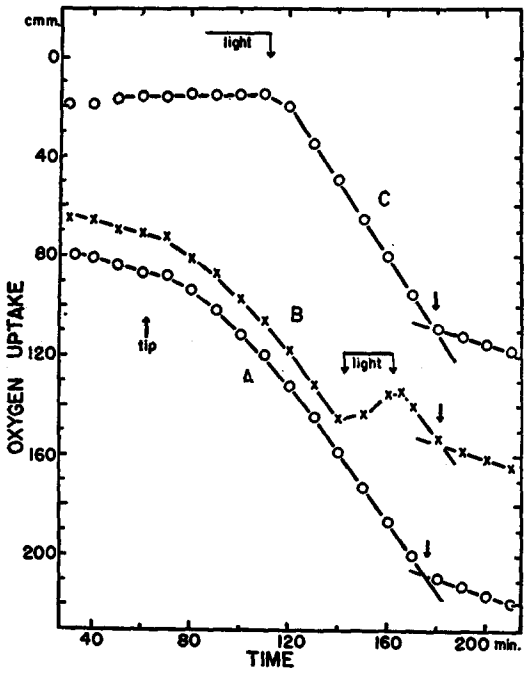

FIG. 2. Effect of light of 600 f.-c. on the oxidative assimilation of 5.0 micromols of acetic acid. A in darkness; B and C illuminated as indicated. End-points are indicated by vertical arrows. Vessels contained KOH to absorb CO_2.

the control flask A in the dark the end-point (intersection of extrapolated lines) occurs at 176 minutes. In flask B a dose of 20 minutes of light of 600 f.-c. was given during the period of assimilation. Flask C was illuminated before the substrate was added and until 50 minutes after the addition. In neither case did exposure to light significantly affect the time position of the end-point for acetic acid utilization. Even if cells are illuminated throughout the period of assimilation (indicated by a dark control) the substrate is entirely consumed. Similar experiments with glucose and with different schedules of illumination all yielded the same result: *the time course of glucose or acetic acid assimilation is not affected by illumination.*

Relation between Photosynthesis and Oxidative Assimilation

Knowing that oxidative assimilation is not directly affected by light, it becomes possible to examine its relationship to the photosynthetic process. An immediate experimental difficulty arises because of the great difference in rates of gas exchange in the two processes. The effect of superimposing the gas exchange of glucose respiration upon that of photosynthesis is so small as to be near the limit of precision of the Warburg technique. However it is feasible to superimpose a short period of photosynthesis upon oxidative assimilation. Carbon dioxide must be provided for photosynthesis and simplicity of detail requires the use of a low pH at which the carbon dioxide is not appreciably dissociated. Glucose was chosen as a substrate and the experiments carried out in small rectangular flasks of about 11 ml. volume containing 6 ml. of fluid. The vessels lacked sidearms but the substrate could be pipetted into a series of vessels in a period of a few minutes at the beginning of an experiment and closely reproducible end-points secured for the time of complete substrate utilization. Oxygen exchange was obtained from flask constants calculated on the assumption of a CO_2/O_2 exchange ratio of -0.90. Fortunately the assumption is not a critical one in the present type of experiment.

A typical experiment is illustrated in Fig. 3. Curves A and B show the time course of oxygen uptake required for 6.0 and 6.7 micromols of glucose respectively in the dark. Curves C and D show the course of oxygen exchange required for 6.0 micromols of glucose when 30 minutes of light are given as indicated. Flask C was illuminated with a photosynthesis-saturating intensity of 600 f.-c.; flask D received 40 f.-c., which is light-limiting for photosynthesis. Comparison of curve B with A shows that 0.7 micromol of glucose displaces the end-point about 14 minutes, a significant amount. But 30 minutes of illumination and attendant photosynthesis prolongs the end-point only 7 and 0 minutes in C and D respectively. It follows that the time course of glucose assimilation is not appreciably affected by simultaneous occurrence of photosynthesis.

It is of further interest to compare quantitatively curves A and C of Fig. 3. The oxidative assimilation of glucose in A required an uptake of 25 μl. of oxygen in a 30 minute period which corresponds (according to Equation 2) to a utilization of $\frac{25}{22.4} = 1.1$ micromols of glucose per 30 minutes. When illuminated for 30 minutes flask C experienced an oxygen evolution of 360 μl. in photosynthesis. The over-all equation has frequently been written in the literature (although without direct experimental verification), and almost universally written in the textbooks as

$$6\ CO_2 + 6\ H_2O \rightarrow C_6H_{12}O_6 + 6\ O_2 \qquad (3)$$

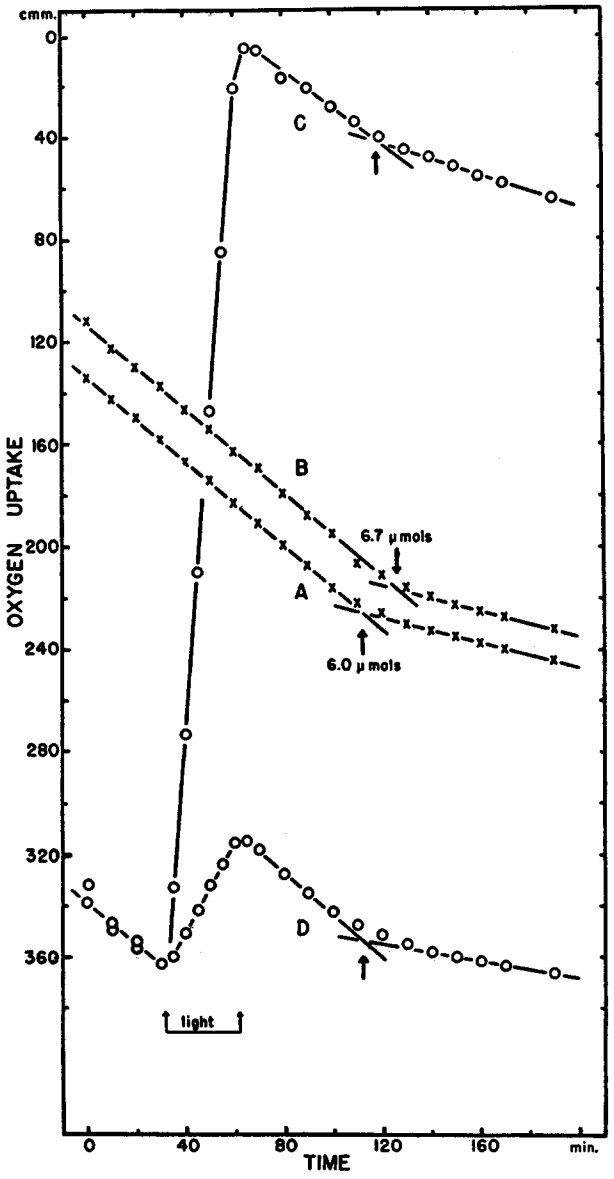

FIG. 3. Effect of photosynthesis on oxidative assimilation of glucose in vessels provided with 4 per cent CO_2. 6.0 micromols glucose in A, C, and D; 6.7 micromols in B. A and B in darkness; C illuminated with 600 f.-c. and D with 40 f.-c. as indicated. Vertical arrows indicate the end-points.

implying glucose as the first product. According to this equation $\frac{360}{6 \times 22.4}$ = 2.7 micromols of glucose[2] formed during the 30 minute period of photosynthesis. It has already been shown that the oxidative assimilation of glucose is certainly not speeded up by light or photosynthesis. The 2.7 micromols of glucose would therefore have required $\frac{2.7}{1.1} \times 30 = 72$ minutes additional for its utilization. But the end-point of glucose utilization was not significantly prolonged in curve C.

From the above argument it follows that *glucose cannot be an immediate product of photosynthesis in Chlorella.*[3] The argument may be further extended to say that the immediate product of photosynthesis cannot be any easily respirable substance; *i.e.*, any substance which will significantly increase the rate of respiration above that of the endogenous respiration.

DISCUSSION

It has been demonstrated that the accumulating products of photosynthesis in *Chlorella* must be slowly respirable (storage) materials. The same conclusion obtains from information which has long been available, *i.e.* (1) that glucose and certain other organic substrates increase the rate of oxygen uptake by algae in the dark and (2) that usually the rate of respiration is not significantly increased by a preceding period of photosynthesis.

Until carbohydrate analyses on *Chlorella* are available the nature of the (CH_2O) produced by photosynthesis and by oxidative assimilation cannot be identified. Lacking direct evidence, it is reasonable to assume that the same storage carbohydrate is formed by both processes, *i.e.* that identical material is represented by (CH_2O) in (1), (2), and in the generalized over-all equation for photosynthesis.

$$CO_2 + H_2O \rightarrow (CH_2O) + O_2. \quad (4)$$

Of the abundant literature on the products of photosynthesis (*cf.* Rabinowitch, 1945) the most interesting in the light of the present experiments is that of Smith (1944). He analyzed sections of sunflower leaves for carbohydrate after various periods of carbon dioxide uptake in photosynthesis. The relationships between formation of monosaccharides, sucrose, and starch were

[2] This value would be still larger if it should be assumed that 25 μ l. of oxygen are actually used in oxidative assimilation of glucose during this period. Without correction it is a minimum value.

[3] With increasing knowledge of the biochemistry of the process the term "product of photosynthesis" may in time become difficult of definition. It is here used, as it has been used conventionally in the past, to designate an accumulating substance formed at a rate related stoichiometrically to the rate of gas exchange and converted into other cellular constituents only at a much lower rate.

such as to suggest that sucrose and starch arise concurrently from a common precursor and that monosaccharides are subsequently produced by inversion of sucrose. The suggestion of a common precursor permits the formation of different accumulating products in different plants and allows some resolution of conflicting evidence for different final products.

Rate of photosynthesis and oxidative assimilation of glucose may be compared from the data of Table I. The rate of gas exchange in oxidative assimilation is less than $\frac{1}{10}$ of that in photosynthesis. However, in terms of (CH_2O) formation the rate of oxidative assimilation becomes about $\frac{1}{2}$ the rate of photosynthesis. And it may be that for cells grown in glucose media the rate of (CH_2O) formation in oxidative assimilation is nearly as great as in photosynthesis.

Comparison of rates of oxidative assimilation of acetic acid and glucose shows that their rates of gas exchange are about equal (in all comparable experiments) and therefore that rates of (CH_2O) formation occur in a 1:5 ration. This suggests that the final reactions leading to (CH_2O) formation are not rate-limiting and that preparatory reactions leading to some common intermediate can form this intermediate 5 times as rapidly from glucose as from acetic acid. Preliminary growth experiments have shown that *Chlorella* will grow on acetic acid in the dark but at a rate very much slower than that supported by glucose.

By no means all of the possible interrelationships between oxidative assimilation and photosynthesis have been explored. It has been shown only that light and photosynthesis do not affect the rate of utilization of glucose or acetic acid. And since it has also been inferred that the rate of their utilization is limited by preparatory reactions this behavior is not unexpected. The possibility remains that other substrates may be found whose utilization can be markedly accelerated by light. The use of starved cells and the titration of amount of substrate by the time course of its oxidative assimilation therefore offers another approach to the photosynthesis problem.

SUMMARY

1. An oxidative assimilation of acetic acid and glucose in darkness has been demonstrated in the green alga, *Chlorella pyrenoidosa*. From manometric experiments it has been shown that 1 mol (CH_2O) per mol acetic acid and 5 mols (CH_2O) per mol glucose are produced.

2. The time required for complete utilization of a limited amount of acetic acid or glucose is not affected by illumination in the absence of carbon dioxide.

3. The time required for complete utilization of a limited amount of glucose is not affected by the simultaneous occurrence of photosynthesis. It must therefore be concluded that the accumulating product of photosynthesis cannot be glucose but must be some slowly respirable (storage) material.

4. Possible interrelationships between oxidative assimilation and photosynthesis may be further studied by following, in darkness and in light, the time course of oxidative assimilation of substrates which are possible intermediates in the two processes.

BIBLIOGRAPHY

Anderson, E. H., *J. Gen. Physiol.*, 1945, **28,** 287, 297.
Barker, H. A., *J. Cell. and Comp. Physiol.*, 1935, **7,** 73.
Barker, H. A., *J. Cell. and Comp. Physiol.*, 1936, **8,** 231.
Bristol-Roach, B. M., *Ann. Bot.*, 1927, **41,** 509.
Clifton, C. E., *Enzymologia*, 1937, **4,** 246.
Doudoroff, M., *Enzymologia*, 1940, **9,** 59.
Genevois, L., *Biochem. Z.*, 1927, **186,** 461.
Giesberger, G., Beiträge zur Kenntnis der Gattung *Spirillum Ehgb.*, Dissertation, Utrecht, 1936.
Myers, J., *J. Gen. Physiol.*, 1946, **29,** 419, 429.
Myers, J., and Clark, L. B., *J. Gen. Physiol.*, 1944, **28,** 103.
Rabinowitch, E. I., Photosynthesis, New York, Interscience Publishers, Inc., 1945, **1,** 44.
Smith, J. H. C., *Plant Physiol.*, 1944, **19,** 394.
Vogler, K. G., and Umbreit, W. W., *J. Gen. Physiol.*, 1942, **26,** 157.

Note Added to Proof.—Further investigation has shown that the R. Q. of glucose respiration by freshly harvested cells may rise to a value as great as -1.6. This leads us to believe that the R. Q. values of about -1.1 cited for glucose in Table III are significantly greater than -1.0. The postulate (as in Equation 2) that storage carbohydrate is the cellular product then becomes only a first approximation.