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Fermentative Metabolism of *Chlamydomonas reinhardii*[1]

III. PHOTOASSIMILATION OF ACETATE

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ABSTRACT

The anaerobic photodissimilation of acetate by *Chlamydomonas reinhardii* F-60 adapted to a hydrogen metabolism was studied utilizing manometric and isotopic techniques. The rate of photoanaerobic (N_2) acetate uptake was approximately 20 μmoles per milligram chlorophyll per hour or one-half that of the photoaerobic (air) rate. Under N_2, cells produced 1.7 moles H_2 and 0.8 mole CO_2 per mole of acetate consumed. Gas production and acetate uptake were inhibited by monofluoroacetic acid (MFA), 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) and by H_2. Acetate uptake was inhibited about 50% by 5% H_2 (95% N_2). H_2 in the presence of MFA or DCMU stimulated acetate uptake and the result was interpreted to indicate a transition from oxidative to reductive metabolism. Carbon-14 from both [1-^{14}C]- and [2-^{14}C]acetate was incorporated under N_2 or H_2 into CO_2, lipids, and carbohydrates. The methyl carbon of acetate accumulated principally (75–80%) in the lipid and carbohydrate fractions, whereas the carboxyl carbon contributed isotope primarily to CO_2 (56%) in N_2. The presence of H_2 caused a decrease in carbon lost from the cell as CO_2 and a greater proportion of the acetate was incorporated into lipid. The results support the occurrence of anaerobic and light-dependent citric acid and glyoxylate cycles which affect the conversion of acetate to CO_2 and H_2 prior to its conversion to cellular material.

In a preceding paper (14), we reported on the stoichiometry of starch breakdown in a mutant strain (F-60) of the green alga, *Chlamydomonas reinhardii*, adapted to a hydrogen metabolism. The yields of acetate and ethanol, two major compounds of starch breakdown in the dark, were strikingly diminished during exposure to illumination, whereas CO_2 evolution was enhanced by illumination. Evidence was presented in a more recent paper (15) to substantiate a chloroplastic respiratory pathway in *C. reinhardii* whereby reducing equivalents generated during starch breakdown were eliminated as H_2 rather than coupled to the reduction of acetyl·CoA to ethanol. To account for the low yield of acetate and ethanol, we (14, 15) postulated that acetate or the intermediate acetyl·CoA formed during the anaerobic photodissimilation of starch was converted to undetermined cellular material.

Another aspect of interest here is the anaerobic stimulatory effect of acetate on the release of CO_2 and H_2 in the light by *C. reinhardii* (3) and by other green algae (18, 21) after adaptation to a hydrogen metabolism. Inasmuch as cellular assimilation of acetate requires ATP, Bamberger *et al.* (3) suggested that acetate increased gas production by consuming ATP which regulated the unspecified sequence of reactions giving rise to CO_2 and H_2. Healey (18) modifying a mechanism put forward by Jones and Myers (20) to explain the Kok effect in blue-green algae, proposed a flow of electrons from acetate via the citric acid cycle into PSI resulting in the photoevolution of H_2 from reduced Fd. The operation of an anaerobic and light-dependent citric acid cycle which affects the stoichiometric conversion of acetate to CO_2 and H_2 had been documented in the photosynthetic purple bacteria (13).

The present communication summarizes the results of a detailed investigation of the anaerobic photometabolism of acetate by *C. reinhardii* F-60, with reference to stoichiometry of gas (CO_2 and H_2) production, incorporation into cellular components, and sensitivity of the process to a variety of inhibitors. The stoichiometric relationships observed, together with the isotopic distribution following assimilation of [^{14}C]acetate, constitute strong evidence for the conclusion that anaerobic carbon oxidation occurs in part through the reactions of the glyoxylate and citric acid cycles.

MATERIALS AND METHODS

Algal Growth Conditions. *Chlamydomonas reinhardii* (Dangeard) F-60, a mutant strain with an incomplete photosynthetic carbon reduction cycle but with an intact photosynthetic electron transport chain, was obtained from R. K. Togasaki, Indiana University. Cells were grown in batch cultures on an acetate-supplemented medium (14) at 25°C on a rotary shaker under a light intensity at 1 W/m^2. Algal cells were grown for 5 d until they reached the end of the exponential phase. Some of the cells were single and motile while most were in various division stages. During the course of this investigation, we succeeded in growing the cells on the same medium in synchronous cultures with a 12 h:12 h light-dark cycle. Cells were inoculated into 1 L of culture medium through which CO_2-free air was bubbled. The temperature was maintained at 34°C and illumination (50 W/m^2) was provided by fluorescent lamps. Cells were harvested after 58 h of growth (4 h dark plus two light (12 h)-two dark (12 h) cycles followed by an additional 6 h dark period). At the time of harvesting, all the cells were in the sporangium stage of development. The two cultural procedures yielded similar data, but the rates of gas (CO_2, H_2) evolution were higher and more reproducible with the synchronized cells.

Experimental Procedure. The harvested cells were washed with 28 mM Hepes, adjusted to pH 7.5 with Tris (free base), and used directly or were resuspended in 50 mM Mes adjusted to pH 6.5 with Tris (free base). The cells were transferred to a Warburg flask attached to a constant pressure respirometer (Gilson Medical Electronics, Inc.). The temperature of the bath was 25°C. The algae were made anaerobic by four cycles of vacuum (H_2O aspirator) and flushing with O_2-free N_2 (Matheson; certified less

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than 5 μl O_2/L) and then flushing with N_2 continuously for 10 min. One or 2 h after the beginning of the incubation, acetate and inhibitors were added from the side arms of the flasks. After an additional 10 to 15 min, a light intensity of 100 W/m^2 was provided by 75 W reflector flood bulbs placed underneath the water bath.

H_2 evolution was measured manometrically in flasks containing 20% KOH with 10% pyrogallol in the center well to capture the evolved CO_2. Measurement of CO_2 was made in flasks lacking alkaline pyrogallol in the center well. Where the center well was empty or carried 1 M H_2SO_4, gas evolved was the sum of CO_2 and H_2 (13, 17). Despite the demonstration of NADP reduction by isolated fragments of F-60 (27), we have been unable to determine O_2 evolution by intact cells under the experimental conditions used here.

Chl was assayed by the method of Arnon (2). Acetate was assayed enzymically using a test combination kit (Boehringer) containing malate dehydrogenase, acetyl-CoA synthase, citrate synthase, ATP, CoA, and NAD.

Chemicals. DCMU and protosol were gifts from E. I. du Pont de Nemours & Co. and New England Nuclear, respectively. Sodium [2-^{14}C]acetate was purchased from New England Nuclear. Liquid scintillation cocktail (ACA) was from Amersham. Gasses were from Matheson.

RESULTS

Characteristics of Acetate Uptake. An average rate of anaerobic (N_2) photometabolism of acetate by *C. reinhardii* F-60 is 22 μmol/mg Chl·h. This rate is roughly doubled in the presence of air. In the dark, acetate uptake under N_2 is not detected, while in air, a rate of 36 μmol acetate/mg Chl·h is observed. For comparison, starch breakdown in F-60 in the dark is slower at 2.7 (N_2) and 1.8 (air) μmol/mg Chl·h and in contrast to acetate utilization is inhibited by light (14). Acetate utilization is known to require ATP and its absolute dependence upon illumination or O_2 indicates that ATP formed during the anaerobic dissimilation of starch is not available for the reaction. Lack of coupling between starch breakdown and acetate uptake in the dark may reflect the extremely low rate of substrate-generated ATP during anaerobic glycolysis or cellular compartmentation of the two processes. Finally, another indication that the cellular energy charge is a critical factor in these reactions is the elimination of acetate uptake but an acceleration of starch metabolism in the presence of an uncoupler (data not shown).

It is noteworthy to compare the extent of anaerobic acetate photometabolism by the green alga with the purple bacterium *Rhodospirillum rubrum* (13). On a μmol/g dry weight·h basis, the rate is 450 for the bacterium and 700 for the alga. The bacterium evolves 1800 μmol H_2/g dry weight·h that contrasts with 880 μmol/g dry weight·h for the alga. The higher rate of H_2 photoevolution for the bacterium reflects the metabolic pathways in the two organisms and will be dealt with later. Clearly the utilization of acetate coupled to the production of H_2 during photometabolism by the green alga can occur as a process of considerable magnitude.

Stoichiometry of H_2 and CO_2 Production. The two conventional pathways which could account for CO_2 and H_2 production from acetate are: (a) the citric acid cycle, and (b) the glyoxylate cycle coupled to the conversion of succinate to carbohydrate. The overall stoichiometries of the photodissimilation of acetate by these reactions is expressed by the following equations:

(a) Citric acid cycle

$$C_2H_4O_2 + 2H_2O \rightarrow 4H_2 + 2CO_2$$

(b) Glyoxylate cycle where acetate is converted to succinate ($C_4H_6O_4$) coupled to established sequences linking the dicarboxylic acid with carbohydrate ($C_6H_{12}O_6$) through pyruvate ($C_3H_4O_3$)

$$4C_2H_4O_2 \rightarrow 2\,C_4H_6O_4 + 2H_2$$
$$2C_4H_6O_4 + 2\,H_2O \rightarrow 2\,C_3H_4O_3 + 2\,CO_2 + 4\,H_2$$
$$2C_3H_4O_3 + 2\,H_2 \rightarrow C_6H_{12}O_6$$

$$4C_2H_4O_2 + 2H_2O \rightarrow C_6H_{12}O_6 + 4\,H_2 + 2CO_2.$$

A molar ratio of H_2:CO_2 of 2:1 is common to both sequences. Molar ratio values for H_2:acetate of 4 (citric acid cycle) and 1 (glyoxylate cycle) and for CO_2:acetate of 2 (citric acid cycle) and 0.5 (glyoxylate cycle) should distinguish between the two oxidative pathways. If acetate is utilized almost exclusively by either pathway, the theoretical values should be observed. If both pathways are operative, intermediate numbers will be obtained. Clearly, this formulation does not take into account competing reactions such as the direct reductive incorporation of acetate to lipid that not only will diminish the loss of carbon from the cell as CO_2 but may also diminish the yield of H_2 evolved.

A previous report from this laboratory (14) presented evidence that the amount of gas (CO_2, H_2) evolution during the anaerobic photometabolism of acetate by *C. reinhardii* was influenced by light with saturation at 70 W/m^2. We have, therefore, restricted our experiment to a light intensity of 100 W/m^2.

A series of experiments with cell suspensions of F-60 adapted to a hydrogen metabolism under N_2 were carried out to determine the stoichiometric yields of CO_2 and H_2 from acetate utilized. The results from three representative experiments are recorded in Table I. The observed H_2:CO_2 molar ratio of roughly two (2.3, 1.8, 2.1) is consistent with the proposal that the glyoxylate and citric acid cycles are of importance here. Inasmuch as the observed molar ratios, 1.87, 1.29, and 1.96 for H_2:acetate (average 1.7) and 0.82, 0.73, and 0.85 for CO_2:acetate are (average 0.8) intermediate with respect to the theoretical values expected from formulations (a) and (b), we propose that both cycles are operative in the assimilation of acetate in *Chlamydomonas* adapted to a hydrogen metabolism.

Effect of MFA[3] and DCMU on Acetate Metabolism. Healey (18) in his study with autotrophically grown *Chlamydomonas moewusii* observed complete inhibition of H_2 evolution from endogenous substances by the respiratory inhibitor, MFA, but no effect by DCMU that indicated under his conditions, the reaction was entirely dependent upon oxidative carbon metabolism. To the contrary, Bishop and Gaffron (6) reported that

Table I. *Stoichiometry of Light-Dependent H_2 and CO_2 Yield from Acetate in C. reinhardii F-60*

Cell suspensions of 3 ml in 50 mM Mes-Tris (pH 6.5) containing 104 μg Chl (experiment 1), 101 μg Chl (experiment 2), and 124 μg Chl (experiment 3) were pipetted into 15 ml Warburg flasks. The flasks were attached to manometers and were flushed with N_2. The bath temperature was 25°C. After an adaptation period of 2 h in the dark, acetate (15 μmol) was added from a side arm, a sample was taken for acetate analysis and the light (100 W/m^2) was turned on. After 4 h, the cells were spun down and the acetate (μmol) in the medium was determined: experiment 1 (4.96), experiment 2 (7.02), and experiment 3 (5.13).

Experiment	Acetate	H_2	CO_2	H_2/CO_2	H_2/Ac	CO_2/Ac
		μmol			μmol/μmol	
1	−	7.4	3.8			
	+	27.0	12.4	2.3	1.87	0.82
2	−	4.3	0.54			
	+	14.6	6.4	1.8	1.23	0.73
3	−	6.5	3.5			
	+	25.7	11.9	2.1	1.96	0.85

[3] Abbreviations: MFA, monofluoroacetic acid.

photochemical production of H_2 by *Scenedesmus obliquus* was insensitive to MFA but sensitive to DCMU and proposed H_2O as the sole source of reductant in this green alga. It was, therefore, of interest to evaluate the role of oxidative carbon metabolism in the anaerobic evolution of H_2 as well as CO_2 in *C. reinhardii* F-60 by determining the effect of the two inhibitors, MFA and DCMU, on gas production in the presence and absence of acetate. The results are presented in Figure 1 and Table II.

In cells releasing CO_2 and H_2 from endogenous reserves, 10 mM MFA has little effect but eliminated their production resulting from the addition of acetate (Fig. 1). As seen in Table II, acetate uptake is inhibited by 10 mM MFA. This concentration of MFA did not effect photosynthesis (CO_2-dependent O_2 evolution) in the wild type *Chlamydomonas* cell but did inhibit acetate-supported CO_2 uptake by 79%.

The photoevolution of H_2 from acetate by *C. reinhardii* resembles that by *C. moewusii* (18) in its sensitivity to MFA and confirms the involvement of an oxidative carbon sequence. The results cannot distinguish between the glyoxylate and citric acid cycles, since aconitase, an enzyme common to both pathways, is the ultimate site blocked competitively by fluorocitrate resulting from the condensation of fluoroacetyl-CoA and oxaloacetate. The inability of MFA to inhibit gas evolution from cells lacking acetate indicates an alternate sequence of reactions for H_2 production from endogenous sources and is consistent with the MFA results reported by Bishop and Gaffron (6) for *S. obliquus*.

DCMU at 10 μM inhibits acetate uptake and H_2 photoevolution by control and acetate-challenged cells (Table II). This concentration of DCMU has no effect on the dark respiration (O_2 uptake) of acetate by F-60, but does inhibit up to 90% photosynthesis in the wild type cell.

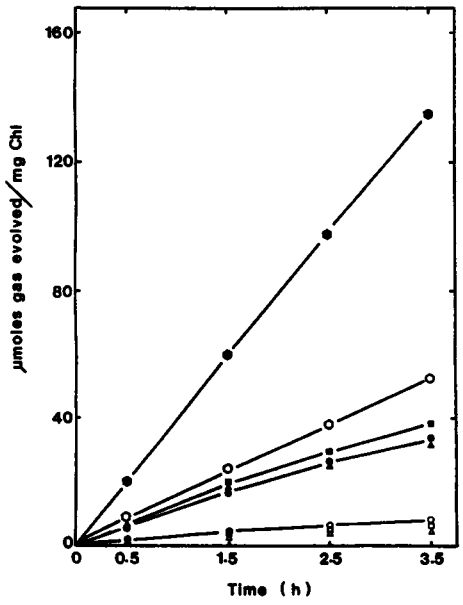


FIG. 1. Effect of MFA on acetate-supported H_2 and CO_2 photoevolution in adapted *C. reinhardii* F-60. A cell suspension in 50 mM Mes-Tris (pH 6.5) containing 105 μg Chl was placed in the main compartment of a 15 ml Warburg flask. The side arm carried acetate and MFA. When H_2 evolution was determined 0.3 ml 20% KOH with 10% pyrogallol in the center well, while the well lacked alkaline pyrogallol for CO_2 measurements. Adaptation was carried out for 2 h in the dark under N_2. Acetate (final concentration, 5 mM) and MFA (final concentration, 10 mM) both adjusted to pH 6.5 with buffer were tipped in to bring the final volume to 3 ml. The temperature of the water bath was 25°C and the light with an intensity of 100 W/m² was turned on 15 min after the side arm additions. ●, control, H_2; ●, acetate, H_2; ▲, MFA, H_2; ■, acetate, MFA, H_2; ○, control, CO_2; ○, acetate, CO_2; △, MFA, CO_2; □, acetate, MFA, CO_2

Table II. *Effect of MFA and DCMU on Acetate Uptake and H_2 Evolution in N_2 or H_2*

Cell suspensions of *C. reinhardii* containing 104 μg Chl (experiment 1) and 135 μg Chl (experiment 2) in 3 ml of 50 mM Mes-Tris (pH 6.5) were placed in the main compartment of 15 ml Warburg flasks. Acetate, MFA, and DCMU were in the side arms while the center wells carried 0.3 ml 20% KOH containing 10% pyrogallol. The flasks were attached to manometers and flushed with N_2. The cells were then adapted under N_2 in the dark for 2 h. The H_2 gas phase was achieved by flushing H_2 through the flasks for 15 min after the adaptation period. The temperature of the Warburg-type bath was 25°C. Acetate (15 μmol in 0.1 ml) and MFA (30 μmol in 0.1 ml) or DCMU (30 μmol in 0.1 ml) were tipped in 15 min before the light (100 W/m²) was turned on. After 4 h in the light, the cells were spun down with a microfuge and the supernatant fluid was assayed for acetate content. The rates of acetate uptake in experiment 1 (control) were 14.7 in N_2 and 6.2 μmol/mg Chl·h in H_2. The corresponding rates of H_2 evolution corrected for endogenous substances were 21.0 in N_2 and 1.6 μmol/mg Chl·h in H_2. In the presence of 10 mM MFA, the rates of acetate uptake were 2.6 in N_2 and 5.9 μmol/mg Chl·h in H_2. The corresponding rates of H_2 evolution corrected for endogenous substances were 3.1 and 1.3 μmol/mg Chl·h. Comparable rates were determined in the DCMU experiment.

Experiment	Reaction	Acetate Uptake	H_2 Evolution
		μmol	
1	Control N_2	6.1	8.8
	H_2 +10 mM MFA	2.6	0.7
	N_2	1.1	3.1
	H_2	2.5	0.5
2	Control N_2	11.4	17.0
	H_2 +10 μM DCMU	2.8	1.5
	N_2	1.0	3.1
	H_2	1.8	−0.65

The inhibitory effect of DCMU on algal H_2 photoevolution has been used frequently (22) to differentiate between oxidative carbon metabolism and water as the source of reductant (H_2 without DCMU-H_2 with DCMU=H_2 released from water). In the case of *C. reinhardii* F-60 the pattern (17.0 without DCMU—3.1 with DCMU) resembles more closely that by *S. obliquus (6)* than by *C. moewusii* although inhibition is not complete (Table II). Inasmuch as the presence of DCMU results in a strong inhibition of acetate uptake (Table II) and the light depresses starch breakdown, an effect not relieved by DCMU (14), the commonly applied calculation may undervalue the movement of reductant from oxidative carbon metabolism.

There is evidence that the photoevolution of H_2 resulting from oxidative carbon metabolism involves electron flow through NAD(P)H-plastoquinone oxidoreductase and PSI (4, 16). In this formulation, reduced pyridine nucleotides feed electrons into a component (plastoquinone) between the two photosystems that would account for H_2 release insensitive to DCMU. There are two reports (23, 29), however, describing inactivation of PSII activity after anaerobic incubation of intact cells and thylakoidal preparations of *C. reinhardii* wt and F-60. The inhibitory effect of anaerobiosis remains unexplained but would account for the lack of O_2 evolution by our cells. A site of electron entry located on the oxidizing side of PSII and sensitive to DCMU inhibition may result during the incubation.

Effect of H_2 on Acetate Utilization. The anaerobic conversion of acetate to CO_2 and H_2 is in net effect equivalent to an oxidation by the glyoxylate and citric acid cycles linked to additional sequences with the important difference that H_2 rather than H_2O is produced. In this anaerobic system, the H_2-releasing mechanism would function analogous to that of Cyt *c* oxidase in an O_2-consuming cell. In view of the reversibility of the H_2- uptake

and -release reaction catalyzed by *Chlamydomonas* hydrogenase (22), the effect of increasing the H_2 environment on the photometabolism of acetate was measured.

As seen in Table II, replacing N_2 with H_2 depressed both H_2 evolution and acetate consumption. The inhibition of acetate disappearance from the medium is essentially linear with increasing H_2 concentration with 50% inhibition at 5% H_2 (Fig. 2). In the presence of MFA and DCMU, H_2 increases the rate of acetate uptake with respect to the N_2 control. But H_2 evolution is depressed further on addition of MFA and when DCMU is present, H_2 is in fact consumed.

A linkage between the anaerobic photometabolism of acetate and H_2 production requires a steady supply of oxidized electron acceptors. It is understandable that blocking the turnover of these cofactors (NAD(P), FAD) by increasing the pressure of H_2 should diminish flow of carbon through the oxidative carbon cycles. The ability of H_2 to inhibit the uptake of acetate by *C. reinhardii* F-60 is taken as additional evidence of an anaerobically functioning carbon pathway resulting in the release of CO_2 and H_2. Although the light-dependent release of H_2 coupled to acetate consumption by *C. reinhardii* resembles that of *R. rubrum* (13) in being accompanied by only CO_2 (this paper and Ref. 18) and is inhibited by MFA, it differs sharply from the bacterial reaction in being inhibited rather than unaffected by external H_2.

Inhibition of acetate metabolism by MFA has been ascribed to a blocking of the oxidative carbon metabolism and by DCMU principally to an inhibition of PSII and possibly phosphorylation. The stimulation rather than inhibition of acetate uptake by H_2 shows that a transition occurs in the photometabolism of acetate when either MFA or DCMU was added (Table II). Apparently when cells are incubated with acetate in an increased reducing environment (H_2) and the oxidative carbon sequences are inhibited, the release of H_2 and presumably of CO_2 is impeded and acetate is metabolized by an alternative pathway involving reductive assimilation. Thus, depression of the oxidative carbon pathways results in a shift of acetate utilization from photodissimilation to photoassimilation. In this respect, the alga is similar to *R. rubrum* (13).

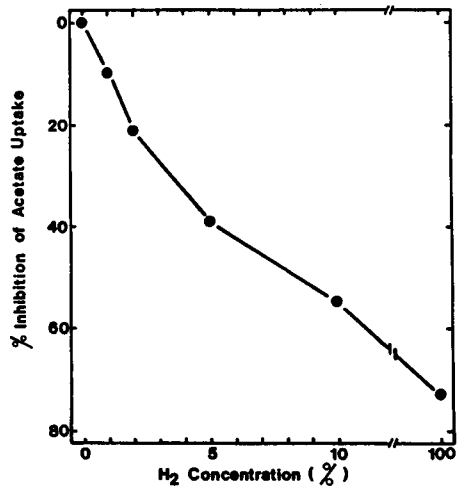


FIG. 2. Effect of H_2 concentration on acetate uptake by *C. reinhardii* F-60 in the light. A cell suspension in 50 mM Mes-Tris (pH 6.5) containing 114 μg Chl was placed in a 27 ml bottle closed with a serum cap. The cells were adapted for 2 h in the dark under N_2. Various amounts of H_2 were injected into the bottles with a syringe through the serum cap and an equivalent volume was removed to maintain constant pressure. Sodium acetate in 50 mM Mes-Tris (pH 6.5) was injected through the serum cap to bring a final volume of the cell suspension to 3 ml. The final concentration of acetate was 5 mM. Light (100 W/m^2) was turned on after the addition of the acetate. The bottles were submerged in a water bath maintained at 25°C and were constantly shaken during the experimental period. After 2 h, the cells were spun down and remaining acetate was determined in the supernatant fraction. The rate of acetate uptake under 100% N_2 was 19.9 μmol/mg Chl·h.

Experiments with [1-^{14}C]- and [2-^{14}C]Acetate. Table III shows the proportions that are found in CO_2, lipid, carbohydrate (basically starch), and pellet or insoluble material (protein, cell wall) when labeled acetate is fed to *C. reinhardii* F-60 for 1 h in the light under anaerobic (N_2, H_2) conditions and in the presence of DCMU. The incorporation of roughly 40 and 80% of the carboxyl and methyl carbons of acetate, respectively, in the lipid and carbohydrate fractions is consistent with a mechanism for the utilization of C_2 units in synthetic events. We conclude from the results of the stoichiometric (Table I) and isotopic (Table III) experiments that, in this green alga, the glyoxylate cycle is a functionally important stage in the anaerobic photoassimilation of acetate. The complete pathway from acetate units to carbohydrate involving the glyoxylate cycle and glycolysis may be summarized as follows:

Acetate → acetyl · CoA → (glyoxylate cycle) → malate → CO_2 + P-enolpyruvate → (glycolysis) → carbohydrate.

The net effect of the complete pathway would allow for a 75% conversion of acetate to carbohydrate with 25% as CO_2 released exclusively from the carboxyl carbon. In the presence of an atmosphere of N_2, 56% of the label from [1-^{14}C]acetate and 13% from [2-^{14}C]acetate was evolved as CO_2. These results cannot be explained by the proposed scheme but would be expected if a portion of the acetate were utilized by the citric acid cycle.

The recycling of [2-^{14}C]acetate in the glyoxylate cycle would yield malate with a distribution of

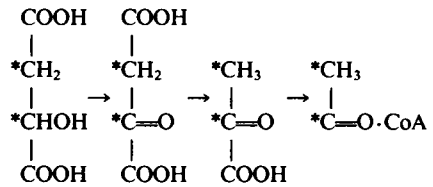
50% 50%
HOOC–CHOH–CH_2–COOH

whereas recycling in the citric acid cycle would yield malate with an isotopic distribution as

HOOC–COOH–CH_2–COOH
16.6% 33.3% 33.3% 16.6%

and $^{14}CO_2$ only where the latter cycle is operative.

Although we have assigned the release of $^{14}CO_2$ from methyl-labeled acetate to the citric acid cycle, it is of importance to note that an alternate mechanism will accomplish the same result. If center-labeled malate were metabolized by the following sequence:



uniformly labeled acetyl·CoA would be generated. The reactions of the glyoxylate cycle would produce carboxyl-labeled malate and eventually $^{14}CO_2$. In this formulation, carboxyl-labeled acetate would yield unlabeled acetyl·CoA.

In peanut and sunflower seedlings (8) and in castor bean (7), over 80% to 90% of the radioactivity derived from [2-^{14}C]acetate was located in the center carbons of malate suggesting in these tissues, the malate was derived almost exclusively by the glyoxylate cycle in spite of the fact that they contained a complete citric acid cycle. On the other hand an intermediate distribution was determined in bacteria (25). There are reports indicating that enzymes of both cycles are present in *Chlamydomonas* (9, 29) but definitive cellular distribution, which must be of importance, remains to be determined. Finally, the high yield of CO_2 and the

Table III. *Cellular Distribution of Radioactivity after Anaerobic Incubation of C. reinhardii with [1-^{14}C]- and [2-^{14}C]Acetate in the Light*

A cell suspension (2 ml) containing 150 μg Chl in 20 mM Hepes (adjusted to pH 7.5 with Tris) was placed in the main compartment of a 15 ml Warburg flask. The center well contained 50 ml KOH (20%, w/v) adsorbed onto a paper wick. After 1 h under N_2, labeled acetate (0.32 μCi) and DCMU were tipped in from the side to yield a final concentration of 1 mM and 10 μM, respectively. The bath temperature was 25°C. After 1 h in the light (100 W/m^2), the experiment was terminated by adding 0.8 ml $HClO_4$ (14% w/v) to the cell suspension through the serum stopper with a needle. The acidified cell suspension was left for 1 h at room temperature to ensure complete trapping of the evolved CO_2. The paper wick containing the KOH was transferred into a scintillation vial containing 5 ml ACS and radioactivity was determined with a Beckman LS 150 counter. One ml of the acidified cell suspension was centrifuged in a Beckman Microfuge for 1 min. The pellet was rinsed 3 times with distilled H_2O, and the supernatant fractions (unused [^{14}C]acetate, water soluble metabolites, fermentation products) were not analyzed further. The pellet was rinsed 4 times with 90% ethanol (w/v), methanol, ethanol and acetone:ether (1:1), in this order. The supernatant fractions were pooled and were dried in a scintillation vial on a hot plate (50°C). When dry, 0.75 ml benzoyl peroxide (0.5% w/v in toluene) was added and the closed vials were heated for 3 h at 50°C until the greenish color was bleached (method slightly modified from Ref. 1). ACS (5–10 ml) was added and the samples containing the lipid cellular material was counted. The pellet was suspended in 1 ml H_2SO_4 (1 M) and autoclaved 30 min in centrifuge tubes. The tubes were centrifuged and the pellet was washed twice with distilled H_2O. The supernatant fractions were pooled in a scintillation vial, ACS was added, and radioactivity was determined. This fraction (hot 1 M H_2SO_4) was assumed to consist of glucose derived from starch and sugars from the glycoproteins. Alternatively, in order to separate starch-derived glucose from other bound sugars, the pellet was treated with amyloglucosidase (13). The supernatant fracton was counted (starch fraction). The remaining pellet was autoclaved for 1 h in 2 N trifluoroacetic acid. After centrifugation, the radioactivity in the supernatant fraction was determined (assumed to be solubilized sugars mainly from the glycoproteins (1) by LSC. The pellet following removal of the carbohydrates was solubilized in 1 ml Protosol and counted by LSC. This last fraction was assumed to contain the proteins and cellular debris. Over 90% of the radioactivity added was accounted for in the various fractions. Mean values and SD are from two or more experiments or data are taken from one experiment.

Fraction	N_2		N_2 + DCMU	
	1-^{14}C-	2-^{14}C-	1-^{14}C-	2-^{14}C-
	%^{14}C assimilated			
CO_2	56.1	13.1 ± 0.8	56.4	13.6 ± 0.4
Lipids	16.9	38.1 ± 1.0	18.8	46.1 ± 2.8
Carbohydrates	25.5	40.3 ± 1.2[a]	23.4	33.5 ± 2.5
Pellet	1.4	8.4 ± 1.1	1.4	6.7 ± 0.4
	H_2		H_2 + DCMU	
CO_2	44.7	6.5 ± 1.8	36.9	4.9
Lipids	26.8	44.8 ± 3.5	34.2	49.3
Carbohydrates	26.6	38.8 ± 2.5	26.5	35.9
Pellet	1.9	10.0 ± 0.8	2.4	9.9

[a] The relative activities of the fractions solubilized by amyloglucosidase treatment (starch) and trifluoroacetic acid treatment (glycoprotein sugars) are (%^{14}C assimilated): 33.2 and 7.7, respectively, *i.e.* a total of 40.9, which compares favorably with 40.3 ± 1.2% ^{14}C assimilated, found in the hot 1 M H_2SO_4 fraction.

absence of the C_2 compounds (acetate and ethanol) during the photoanaerobic, but not during the breakdown of starch by *C. reinhardii* in the dark, is additional evidence indicating that both oxidative carbon cycles are operative and that both cycles require light in order to function.

Much more isotope is recovered in the lipid and carbohydrate fractions from the methyl carbon than from the carboxyl carbon of acetate. This result is not unexpected in view of acetate metabolism by the oxidative carbon cycles prior to its conversion into these cellular components. We interpreted the data in Table II to indicate that H_2 depresses the oxidative carbon sequences resulting in an increased reductive assimilation of acetate. Consistent with this suggestion is not only the increase in isotopic labeling of the lipids but also a shift in position dependence (^{14}methyl *versus* ^{14}carboxyl) ratio of the lipids from 2.3 to 1.3 when N_2 is replaced by H_2. Even though the precise mechanism remains to be determined, the presence of H_2 causes a decrease in carbon lost from the cell as CO_2 and a greater proportion of the acetate incorporated into lipid. Inasmuch as the amount of label detected in the carbohydrate fraction when compared to that in the CO_2 and lipid is less affected by substituting H_2 for N_2, we conclude that the citric acid cycle rather than the glyoxylate cycle is the more sensitive to control by a reducing environment.

The ^{14}C in the pellet is found mostly in a fraction soluble in 1 N NaOH, and is therefore attributed to incorporation of acetate in proteinaceous materials. Since it represents a small percentage of the total distribution, it was not investigated further. Protein synthesis in algae adapted to an anaerobic metabolism has been demonstrated by Klein and Betz (24).

DCMU like H_2 inhibits the assimilation of acetate (Table II). In contrast to H_2, the PSII inhibitor did not affect the incorporation of isotope into CO_2 but did modify inversely the isotopic distribution between lipid and carbohydrate (Table III). The differential effect of these agents can be rationalized on the assumption that only H_2 interrupts the oxidative carbon sequences.

In confirmation of an earlier report (10), we could not detect poly-β-hydroxybutyrate utilizing the spectrophotometric procedure of Ward and Dawes (33) following extraction of a cell

suspension with ethanol, methanol, acetone, acetone-ether and finally hot chloroform. The polymer is insoluble in the first group of solvents but readily soluble in hot chloroform. No radioactivity was detected in the chloroform fraction. These results are taken as evidence that this bacterial polymer is not a product of photoanaerobic acetate assimilation in *Chlamydomonas*.

DISCUSSION

Two mechanisms have been described to account for the photoevolution of H_2 among the algae (22). One involves the participation of PSII and implicates water as the source of electrons. It is characterized by the simultaneous photoproduction of both H_2 and O_2, prevention by inhibitors of the Hill reaction and insensitivity to inhibitors of respiration. The other depends on oxidative carbon metabolism for reducing power and involves energy-dependent reverse electron flow by PSI to the redox level equivalent to that of Fd. In the second mechanism, there is a simultaneous release of H_2 and CO_2 and an insensitivity to inhibitors of the Hill reaction but sensitivity to respiratory poisons. Linkage of reduced pyridine nucleotides generated by the operation of oxidative carbon metabolism and the photosynthetic electron transport chain is provided by NAD(P)H-plastoquinone oxidoreductase.

The glycolytic pathway has been suggested to account for NAD(P)H production by the glyceraldehyde 3-P dehydrogenase reaction from exogenous glucose and from starch. Inasmuch as the glycolytic pathway is located within the chloroplast, chloroplast respiration (5, 15) represents a reasonable model for the final steps in photohydrogen production. Cytoplasmic carbohydrate dissimilation can be accommodated in this model by the Pi translocator whereby chloroplastic Pi is exchanged for cytoplasmic triose-P(glyceraldehyde 3-P or dihydroxyacetone P).

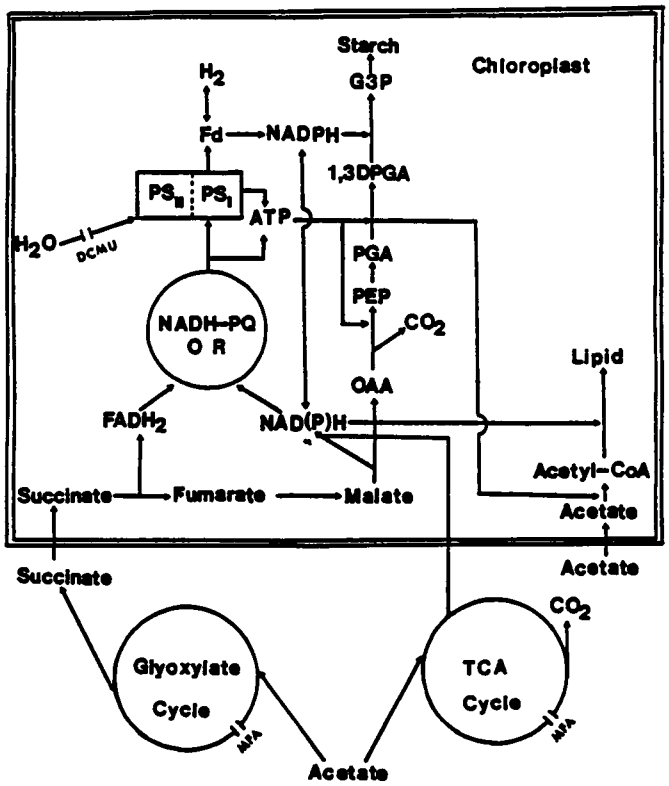


FIG. 3. Proposed scheme for the anaerobic photoassimilation of acetate into carbohydrate and lipid coupled to the evolution of CO_2 and H_2. PGA, glycerate 3-phosphate; PEP, phosphoenol pyruvate; 1,3DPGA, glycerate 1,3-diphosphate; G3P, glyceraldehyde 3-P; NADH-PQ OR, NADH-plastoquinone oxidoreductase; OAA, oxaloacetate; $FADH_2$, reduced flavin adeninedinucleotide.

The present study has shown that the photoevolution of H_2 from acetate by algae is more complex than previously considered. The mechanism proposed by Healey (18) is based on the flow of reductant generated by the action of the citric acid cycle dehydrogenases into PSI. We have now presented evidence that the anaerobic photodissimilation of acetate is additionally dependent upon the reactions of the glyoxylate cycle.

In algae the enzymes of the citric acid and glyoxylate cycles are located in the mitochondria (17) and in the cytoplasm (32), respectively. The stimulation, rather than inhibition of H_2 photoevolution by uncouplers shows that ATP produced by the chloroplasts or possibly by the mitochondria does not promote reverse electron flow from NAD(P)H to a potential capable of reducing H^+. Based on the known impermeability of the chloroplast envelope to the pyridine nucleotides (19), we suggest that a dicarboxylic acid shuttle functioning between the mitochondria, cytoplasm, and the chloroplast provides a reasonable mechanism for disposal of reducing power produced by the pyridine nucleotide-linked dehydrogenases in terms of H_2 evolution.

The mechanism proposed in Figure 3 suggests that the enzymic oxidation of succinate to fumarate occurs within the chloroplast. If this were so, reduced flavin adeninedinucleotide ($FADH_2$) generated during the reaction is a potential substrate for NAD(P)H-plastoquinone reductase. An alternate hypothesis is based on the observation of Frenkel (12) who demonstrated a light-dependent reduction of NAD with succinate as substrate by chromatophores of *R. rubrum*. Finally, there is experimental evidence (11) that mammalian mitochondria can reverse electron flow with formation of NADH, dependent on both ATP and succinate. Inasmuch as photophosphorylation is the principal source of ATP, this reaction presumably does not operate extensively during anaerobiosis.

The site(s) of the enzymic activation of acetate by acetate kinase (acetate + ATP → acetyl·CoA + PPi + AMP) is complex in the anaerobic eukaryotic cell since acetyl·CoA as well as ATP have been reported (28) unable to penetrate the chloroplast envelope. The enzyme is distributed between the chloroplast, cytoplasm, and mitochondria of the algal cell (26). The routes of acetate metabolism involving both reductive and oxidative carbon pathways (Fig. 3) would be understandable if ATP could traverse the *Chlamydomonas* chloroplast either complexed to Mg^{2+} (30) or by an adenylate transporter (31). In the latter mechanism, adenine nucleotides are exchanged for PPi, a product of the acetate thiokinase reaction.

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