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Fermentative Metabolism of *Chlamydomonas reinhardtii*[1]

I. ANALYSIS OF FERMENTATIVE PRODUCTS FROM STARCH IN DARK AND LIGHT

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ABSTRACT

The anaerobic starch breakdown into end-products in the green alga *Chlamydomonas reinhardtii* F-60 has been investigated in the dark and in the light. The effects of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP) on the fermentation in the light have also been investigated.

Anaerobic starch breakdown rate (13.1 ± 3.5 micromoles C per milligram chlorophyll per hour) is increased 2-fold by FCCP in the dark. Light (100 watts per square meter) decreases up to 4-fold the dark rate, an inhibition reversed by FCCP. Stimulation of starch breakdown by the proton ionophore FCCP points to a pH-controlled rate-limiting step in the dark, while inhibition by light, and its reversal by FCCP, indicates a control by energy charge in the light.

In the dark, formate, acetate, and ethanol are formed in the ratios of 2.07:1.07:0.91, and account for roughly 100% of the C from the starch. H_2 production is 0.43 mole per mole glucose in the starch. Glycerol, D-lactate, and CO_2 have been detected in minor amounts.

In the light, with DCMU and FCCP present, acetate is produced in a 1:1 ratio to formate, and H_2 evolution is 2.13 moles per mole glucose. When FCCP only is present, acetate production is lower, and CO_2 and H_2 evolution is 1.60 and 4.73 moles per mole glucose, respectively.

When DCMU alone is present, CO_2 and H_2 photoevolution is higher than in the dark. Without DCMU, CO_2 and H_2 evolution is about 100% higher than in its presence. In both conditions, acetate is not formed. In all conditions in the light, ethanol is a minor product. Formate production is least affected by light.

The stoichiometry in the dark indicates that starch is degraded via the glycolytic pathway, and pyruvate is broken down into acetyl-CoA and formate. Acetyl-CoA is further dissimilated into acetate and ethanol. In the light, acetate is produced only in the presence of FCCP and, when photophosphorylation is possible, it is used in unidentified reactions. Ethanol formation is inhibited by the light in all conditions.

Most of the recent work connected with anaerobic metabolism in algae has focused on the assimilation and formation of H_2 (4, 18). As pointed out by Klein *et al.* (20), since H_2 is only one of several end-products of fermentation in green algae, it is necessary to place the H_2 production in the broader context of the fermentative metabolism in order to gain a deeper understanding of the mechanisms that account for its evolution. In addition to H_2, one or more of the following end-products of algal carbohydrate (endogenous starch or exogenous glucose) fermentation have been detected: CO_2, formate, acetate, ethanol, lactate, glycerol, and butanediol (3, 19, 20, 28).

Classical glycolysis followed by subsequent metabolism of the pyruvate to the various end-products has been proposed to account for the anaerobic catabolism of starch or glucose in the green algae (17, 19, 27). Except for H_2 evolution, the effect of light on fermentative carbon flow has received little attention. This is principally due to the problems involved with photosynthetic fixation of the CO_2 that might be evolved fermentatively. The first to attempt to resolve this question were Klein and Betz (20) who reported that light had no effect on the rate of starch breakdown or the pattern of fermentation in *Chlamydomonas moewusii*. But they used extremely low levels of light (160 lux) in order not to eliminate the H_2 metabolism and to prevent a refixation of the CO_2 evolved. On the other hand, Hirt *et al.* (15) demonstrated that light can inhibit severely the rate of anaerobic glycolysis in *Scenedesmus obliquus* as monitored by D-lactic acid formation. Bamberger *et al.* (2) made use of *C. reinhardtii* F-60, a mutant characterized by an incomplete photosynthetic carbon reduction pathway but an intact photosynthetic electron transport chain, to monitor 'true' CO_2 and H_2 evolution. To account for their results, they proposed an involvement between anaerobic carbohydrate metabolism and the photosynthetic electron transport chain, implying that carbohydrate degradation is entirely or partially localized in the chloroplast. The purpose of our study was to establish for the first time a complete fermentative balance in *C. reinhardtii* F-60 between starch and the end-products in the dark, and subsequently to determine whether light would modify this pattern. Since it had been shown that H_2 evolution was stimulated by light, it was of interest to check whether this increased H_2 production would occur at the expense of a reduced end-product of fermentation. This point has some significance in relation to the controversial question of the source of the H_2 evolved in light. Experimental conditions also included the presence of FCCP[3], a weak acid proton ionophore that uncouples photophosphorylation but has no effect on substrate level phosphorylation, and the presence of DCMU, an inhibitor of electron transport from PSII to plastoquinone. These inhibitors were used in an attempt to minimize the complexity arising from the interactions between starch dissimilation and the light-induced electron transport and a possible reutilization of the fermentative end-product. Preliminary reports of this work have appeared elsewhere (8, 9).

MATERIALS AND METHODS

Algae and Growth Conditions. *Chlamydomonas reinhardtii* (Dangeard), mutant strain F-60, obtained from R. K. Togasaki, Indiana University, was grown on an acetate-supplemented me-

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[3] Abbreviations: FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone; O/R, oxidation/reduction; PCV, packed cell volume; RQ, respiratory quotient.

dium (24, modified from 11) at 25°C on a rotary shaker in 2-L flasks under a low light intensity (0.6 w/m^2). Stock cultures were maintained on agar plates of the same medium. Cells from one plate were used to innoculate one flask. 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, with two or four, sometimes more, daughter cells enclosed in the mother cell wall. Cell density was 1×10^7 to 1.5×10^7 cells/ml, Chl content was 13.9 ± 4 μg/10^7 cells, PCV was 7.2 ± 1.5 μl/10^7 cells, dry weight is 0.5 ± 0.2 mg/10^7 cells, and starch content was 184 ± 28 μmol C/mg Chl. No bacterial contamination was observed upon microscopic examination.

Experimental Procedures. Algal cells (1 L) were harvested by centrifugation (1500*g*, 8 min), washed with 50 to 100 ml of 28 mM Hepes (free acid) adjusted to pH 7.5 with Tris (free base), and resuspended in the same buffer at a final density of 70 to 140 μg Chl/ml. Cells were kept on ice until onset of the experiment. A cell suspension of 3 ml was placed in 20 ml Warburg flasks attached to a constant pressure respirometer (Gilson Medical Electronics, Inc.). Inhibitors were added in the side arm of the Warburg flask as a 5 or 10 mM solution in 100% methanol in order to insure that the final concentration of methanol would not be over 0.2% v/v. Methanol was added at the same final concentration in control flasks. KOH at 20%, when present, was placed in the center well of the flask as a CO_2 trapping agent. The algal suspension was incubated in a water bath maintained at 28°C, with shaking speed at 80 cycles/min. Light was provided by 75-w reflector flood bulbs placed underneath the water bath. The algae were made anaerobic by 4 cycles of vacuum (water aspirator) and flushing with O_2-free N_2 (Matheson; certified less than 5 μl O_2/L), and then flushing with the same N_2 continuously for 10 min. Thirty min after the beginning of incubation, inhibitors were added, a sample was taken from two control flasks, light was turned on, and the first reading was made. Three and one-half h later, the incubation was terminated by placing the flasks on ice. H_2 evolution was measured manometrically in flasks where KOH was present in the center well. HCO_3^- initially bound (to the cells or to the buffer) or produced during the adaptation period was measured as the gas evolved by tipping 0.6 ml 2 M H_2SO_4 from a side arm into the cell suspension just after the adaptation period in two control flasks containing no KOH. Acid was also tipped into flasks containing no KOH at the end of incubation. The total gas evolved in those flasks was assumed to be the sum of H_2 and CO_2 evolved during incubation and CO_2 evolved from HCO_3^- initially present, and CO_2 evolution was calculated accordingly.

For time course experiments, 38-ml bottles sealed with a serum cap were used, and samples were taken anaerobically with a syringe. In experiments where substrates were fed to the cells, they were added before the incubation at a final concentration of 1 mM.

Analysis of Nongaseous Metabolites. Starch was determined by a method modified from Klein and Betz (19): 1 ml cell suspension was spun down (6000*g*, 5 min), and the supernatant fraction was set aside for analysis of soluble products. The pellet was resuspended in 0.1 ml methanol, washed with 2 ml methanol, and then with 2 ml 100 mM Na-acetate (pH 4.5), and resuspended in 1.7 ml of the acetate buffer. The samples were then sonicated for 10 s (Branson Sonifier, microprobe, energy level 6). After autoclaving the samples for 10 min to solubilize the starch, 2.2 units of amyloglucosidase were added, and the samples were incubated 14 h in a water bath at 55°C. After the samples were adjusted to 2.0 ml with H_2O and centrifuged to get a clear supernatant fraction, glucose was assayed with the glucose oxidase–peroxidase method (Sigma 510).

Soluble metabolites were measured in the incubation medium after spinning the cells down for starch analysis. After ethanol and lactate had been measured, samples were kept frozen for subsequent analyses. When metabolites inside the cells were measured, 1 ml of cell suspension was pelleted, washed with incubation buffer, and 0.5 ml of 0.3 M $HClO_4$ was added. Samples were sonicated 10 s in the cold, centrifuged (6000*g*, 5 min), and the supernatant fractions saved. The pellets were rinsed with the same amount of $HClO_4$, and the two supernatant fractions were pooled. To neutralize the acid and remove the perchlorate, 0.4 ml of 0.75 M $KHCO_3$ was added, the samples were centrifuged (6000*g*, 5 min), and the supernatant fractions were adjusted to 2 ml with incubation buffer.

Ethanol was assayed with alcohol dehydrogenase (6), formate with formate dehydrogenase (16), D-lactate with D-lactate dehydrogenase, using the same method as with L-lactate dehydrogenase (17), glycerol with glycerokinase and glycerol-P dehydrogenase (30), and acetate with acetyl-CoA synthetase, citrate synthase, and malate dehydrogenase (test-combination for acetic acid, Boehringer-Mannheim, 148261).

Other Analyses. Chl was assayed by the method of Arnon (1). For dry weight measurements, 5-ml cell suspensions were washed with distilled H_2O, resuspended in 1 to 2 ml distilled H_2O, and dried on preweighed filter papers in an oven at 95°C for 24 h. PCV was measured after centrifuging 1 ml cell suspension for 10 min at full speed in a clinical table-top centrifuge in a micro-tip graduated centrifuge tube. Cell counts were performed with a hemocytometer. Light intensities were measured with an International Light radiometer, model 700A.

Chemicals. Biochemicals and buffers were from Sigma, except the acetic acid determination kit. Other chemicals were from Fisher Scientific Co. DCMU and FCCP were a gift from E. I. DuPont de Nemours and Co. Hepes (free acid) was recrystallized in methanol and dried over vacuum before use, since it was found that it contained approximately 1 mol % ethanol.

Calculations. Carbon recovery is defined as the ratio of mol of C in the fermentative end-products to the mol of C in the starch metabolized. Hydrogen recovery is defined as the ratio of mol of available H in the fermentative end-products to the mol of available H in the starch metabolized, where the mol of available H in a compound of general formula $C_aH_nO_z$ is calculated as $4a + n - 2z$. O/R index is defined as the ratio of the weighed sum of the O/R values of the oxidized end-products to the weighed sum of the O/R values of the reduced end-products, where the O/R value of a compound of general formula $C_aH_nO_z$ is calculated as $z - n/2$. Average balances are calculated by averaging individual balances of single experiments.

RESULTS

Starch Breakdown and Light. Our study on the effects of anaerobicity in *C. reinhardtii* F-60 focuses on the metabolism of starch, the principal endogenous reserve carbohydrate of this alga, and which is totally confined to the chloroplast. Compared to aerobic starch breakdown, the anaerobic rate in the first h is essentially similar but increased thereafter over the aerobic value by approximately 50% (Fig. 1). Also depicted in this figure is the observation that 10 μM FCCP stimulates starch degradation under both atmospheres, and eliminates the Pasteur effect (as defined by a ratio of starch consumed in N_2/starch consumed in air higher than 1).

Light decreases anaerobic starch breakdown by a factor of approximately 2 and 4 at 40 w/m^2 and 100 w/m^2, respectively (Fig. 2). This decrease cannot be attributed to photosynthetically produced O_2 since a similar inhibition is observed in the presence of 10 μM DCMU. In sharp contrast to the inhibition by light of starch breakdown, H_2 evolution is increased in light and nearly saturated at 40 w/m^2. While 10 μM DCMU is ineffective with respect to starch breakdown, this concentration of DCMU decreases H_2 production at 100 w/m^2 to 55% of the control level.

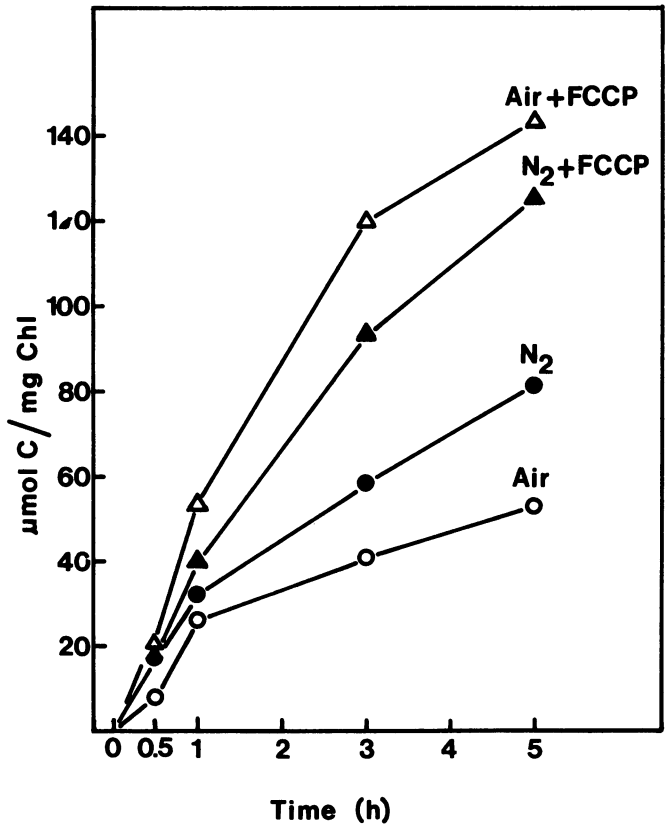

FIG. 1. Time course of starch breakdown under aerobic and anaerobic conditions in dark. Cells which were under anaerobic conditions were flushed with N_2 for 10 min, and the first sample was taken 20 min thereafter. FCCP (10 µM) was added after adaptation.

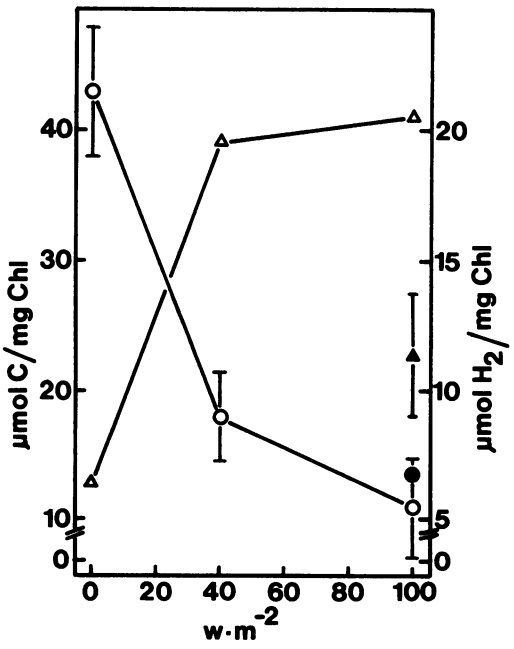

FIG. 2. Anaerobic starch breakdown and H_2 evolution at various light intensities. Cells were adapted in dark under N_2 for 30 min prior to incubation (for 3.5 h) either in dark or in light. DCMU (10 µM) was added after adaptation. (○), µmol C in the starch consumed; (●), µmol C in the starch consumed in presence of DCMU; (△), µmol H_2 evolved; (▲), µmol H_2 evolved in presence of DCMU.

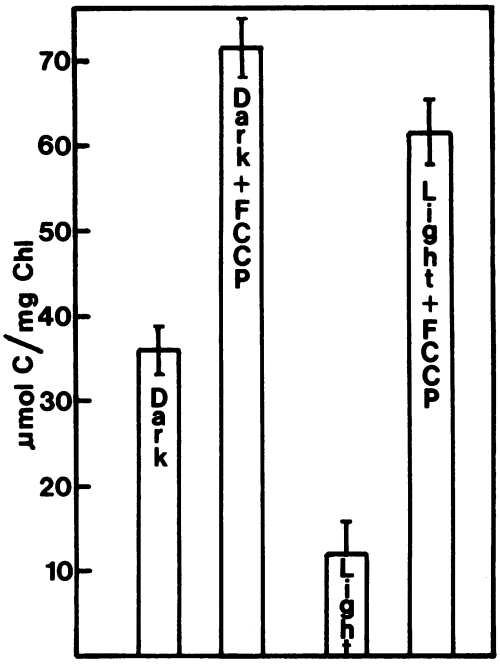

FIG. 3. Effect of light and FCCP on anaerobic starch breakdown. Cells were adapted for 30 min under N_2 prior to 3.5 h incubation in dark or light. FCCP (5 µM) was added after adaptation.

Table I. *Fermentative Products from Starch by C. reinhardtii F-60: Carbon Recovery, Recovery of Available Hydrogen, and O/R Balance*

Cells were adapted for 30 min under N_2 in the dark, and incubated in the dark or in the light (100 w/m²) for 3.5 h. Data are taken from duplicates in eight or more experiments (dark), or in three or more experiments (light). Starch breakdown rates (µmol C/mg Chl·h) in the same experiments as in Table II are the following: dark, 13.1 ± 3.5; light, 4.95 ± 1.35.

Fermentative Product	Dark	Light
	mol product/mol Glc[a]	
Formate	2.07 ± 0.27	0.90 ± 0.23
Acetate	1.07 ± 0.18	none
Ethanol	0.91 ± 0.11	0.12 ± 0.07
Glycerol	0.04 ± 0.01	0.02 ± 0.01
CO_2	0.02 ± 0.05	2.48 ± 0.94
H_2	0.43 ± 0.07	6.92 ± 1.10
Balances		
C recovery (%)	103 ± 10	59 ± 9
H recovery (%)	102 ± 7	76 ± 9
O/R index	0.94 ± 0.06	0.79 ± 0.23

[a] mol product/mol Glc refers to the glucose units in the starch consumed.

In the presence of FCCP, the light-induced inhibition of starch breakdown is offset, resulting in similar rates for both light and dark (Fig. 3).

Fermentative Balance in Dark and Light. Fermentative products yielded from the anaerobic breakdown of starch are listed in Table I. In dark, formate, acetate, and ethanol are produced in approximate ratios of 2:1:1, and account for more than 90% of the C in the starch consumed. Glycerol is synthesized in minor amounts. Traces of D-lactate, but no L-lactate, have been detected in some experiments (data not shown). Glycerol and lactate have been monitored both in the medium and inside the cells. CO_2, when evolved, is produced in minor amounts. H_2 evolution in dark serves as a minor sink for the reducing power generated during starch metabolism, accounting for no more than 20% of

the reduced equivalents, while ethanol formation serves as the major sink. C and H recoveries are close to 100%, which is taken as evidence that no major fermentative product in the dark has been overlooked, and no source of C other than starch is used to any significant extent. The fact that free NH_3 is not found in amounts greater than 1 to 3% (mol/mol C in starch consumed, data not shown) is an indication that protein and amino acid metabolism is minimal under these conditions. In the dark, FCCP has no effect on the stoichiometry of the fermentation, except for decreasing H_2 production to 33% ± 12% (relative to starch consumed) of the control. The accuracy of the method used does not allow us to document whether this decreased H_2 production is accompanied by an increased ethanol formation. The inhibition of dark H_2 evolution by uncouplers has been first documented by Gaffron and Rubin (7).

Light (100 w/m^2) affects the stoichiometry (Table I) in addition to inhibiting the rate of starch breakdown. While the large increase in CO_2 evolution can be partially attributed to cellular respiration sustained by the photosynthetically generated O_2 which is coupled to H_2 photoevolution, some other mechanism must be invoked to account for CO_2 evolution in the presence of DCMU (Table II) which is significantly greater than in the dark (Table I). When compared to the dark, H_2 evolution, in the absence or presence of DCMU, is dramatically stimulated in the light, both in relative terms (Table I) and in absolute terms (Fig. 2). If the H_2 evolved in the presence of DCMU reflects the same amount of H_2 coming from the oxidation of starch in the absence of DCMU, a calculation can be made to estimate the fraction of CO_2 derived from cellular respiration in the absence of DCMU: (H_2 in light [7 mol] − H_2 in light and DCMU [3 mol]) · 0.5 = 2 mol O_2 evolved photosynthetically, and an equivalent amount of respiratory CO_2 if the RQ is 1. This leaves 2.5 − 2 = 0.5 mol CO_2 produced in the light independently of respiration. The amount of CO_2 (1 mol) produced in the presence of DCMU is twice higher and is unaccounted for.

Ethanol production in light is inhibited, whether in control (light alone) samples (Table I) or in the presence of either DCMU or FCCP (Table II). This inhibition cannot be ascribed to a consumption of the ethanol produced, since ethanol is not used aerobically or anaerobically, in dark or in light, by *C. reinhardtii* F-60 (data not shown). Similarly, acetate production is totally prevented in light (Table I) or in light with DCMU (Table II). In the presence of FCCP (Table II), however, the ratio of acetate to formate exceeds the ratio of 1:2 found in the dark. Inhibition of both acetate and ethanol production is effected already at a light intensity of 40 w/m^2 (data not shown). In light, without FCCP, incomplete recoveries of C and H indicate that some of the metabolites derived from starch are diverted either to an undetermined fermentative product, or to a build-up of reserve material other than starch. No pyruvate or D-lactate is found either in the medium or inside the cells. In the presence of FCCP, both C and H recoveries are much higher, and in some experiments higher than 100%, suggesting that possibly some other source in addition to starch is utilized. Less than 1 mol % (relative to C in starch consumed) NH_3 is formed in these experiments, which is an indication that it is not likely that amino acids are used as another C source. In the light, with or without DCMU, exogenous acetate is taken up anaerobically, but this does not occur in the presence of FCCP (data not shown).

DISCUSSION

The presence of a Pasteur effect in algae has been postulated to originate from the energy required for the cell to survive under anaerobic stress until normal aerobic conditions are restored (26). The larger effect of FCCP in the dark on aerobic relative to anaerobic starch breakdown is indicative that the rate limiting step differs under the two conditions. Aerobically, coupling of oxidative phosphorylation is presumably pace setting, while under anaerobiosis, a pH-dependent enzymic step regulates the rate. This is postulated because in the dark and in N_2, FCCP, a weak acid proton translocator, can mediate its effects on the glycolytic flux only through pH-sensitive enzymic reactions, since under these conditions phosphorylation is not coupled to electron flow. It is assumed that FCCP will equalize the pH of the various cellular compartments with the pH of the buffer in the incubation medium. It is known that proton ionophores do not affect substrate level phosphorylation (25). The fact that there is no inhibition of starch breakdown by light in the presence of FCCP (Fig. 3) is taken as evidence that this inhibition is mediated through the energy charge. When photophosphorylation is uncoupled from electron transport, a 'dark' situation is reestablished, at least as far as the factors affecting the rate of starch catabolism are concerned. Fructose-6-P kinase, a key control enzyme of the glycolytic pathway, has been documented to be affected both by the energy charge and pH in a variety of organisms (26, 29). It is possible that the site of control of

Table II. *Fermentative Products from Starch by C. reinhardtii F-60: Carbon Recovery, Recovery of Available Hydrogen, and O/R balance in the Light under Various Conditions*

Cells were adapted for 30 min under N_2 in the dark, and DCMU and/or FCCP (in 100% methanol) were added. Cells were incubated for 3.5 h in the light (100 w/m^2). Data are taken from duplicates in two or more experiments. Starch breakdown rates (μmol C/mg Chl·h) are the following: DCMU, 5.4 ± 2.9; FCCP, 20.4 ± 2.7; DCMU + FCCP, 23.1 ± 4.3.

Fermentative Product	10 μM DCMU	5 μM FCCP	10 μM DCMU + 5 μM FCCP
		mol product/mol Glc[a]	
Formate	1.44 ± 0.06	2.13 ± 0.55	1.71 ± 0.38
Acetate	none	1.29 ± 0.08	2.01 ± 0.17
Ethanol	0.10 ± 0.08	0.12 ± 0.04	0.08 ± 0.02
Glycerol	0.02 ± 0.01	0.02[b]	0.04[b]
CO_2	1.06 ± 0.23	1.60 ± 0.28	0.24 ± 0.01
H_2	2.92 ± 0.65	4.73 ± 0.72	2.13 ± 0.13
Balances			
C recovery (%)	43 ± 4	117 ± 13	103 ± 10
H recovery (%)	42 ± 7	106 ± 10	104 ± 7
O/R index	1.07 ± 0.19	1.11 ± 0.02	0.94 ± 0.16

[a] mol product/mol Glc refers to the glucose units in the starch consumed.
[b] Duplicates from one experiment only.

anaerobic glycolysis in green algae is also located at this step. Consistent with our postulate are the results of a study of the effects of light on algal fermentative rates by Hirt *et al.* (15), who reported that light inhibited anaerobic glycolysis (monitored by D-lactate formation) in wild-type *Scenedesmus*, but increased it in a mutant unable to carry out photophosphorylation. These results were taken as an indication that the light-induced inhibition of glycolysis is due to the state of the energy charge, with the implication of a similar mechanism to the one mediating the Pasteur effect. On the other hand, a control of the amylolytic step by pH alone may play a role, inasmuch as it was shown that the pH optimum of *C. reinhardtii* amylase is close to 7.5 (Levi C, M Gibbs 1984 Plant Physiol 74: 459–463).

Fermentative Balance in the Dark. Figure 4 summarizes the pathways accounting for the fermentative balance of product formation from starch in the dark by *C. reinhardtii*. The only route for starch dissimilation consistent with the stoichiometry recorded in Table I is the Embden-Meyerhof pathway resulting in the formation of two pyruvates per glucose. Pyruvate may be subsequently converted to formate and acetyl-CoA, a reaction catalyzed by pyruvate-formate lyase. This enzyme has been described only in bacteria where it functions solely in fermentative metabolism (12).

In the dark, two pathways can account for the conversion of acetyl-CoA to acetate and ethanol. In one, half of the acetyl-CoA is converted to acetate by a deacylase while the remainder is reduced to ethanol with acetaldehyde as intermediate. In the alternate pathway, acetyl-CoA is reduced to acetaldehyde which undergoes a dismutation sequence resulting in the formation of acetate and ethanol. Key to either pathway is the presence of acetaldehyde dehydrogenase. Clearly, enzymic analysis will be required to sort out the most likely route of acetyl-CoA metabolism.

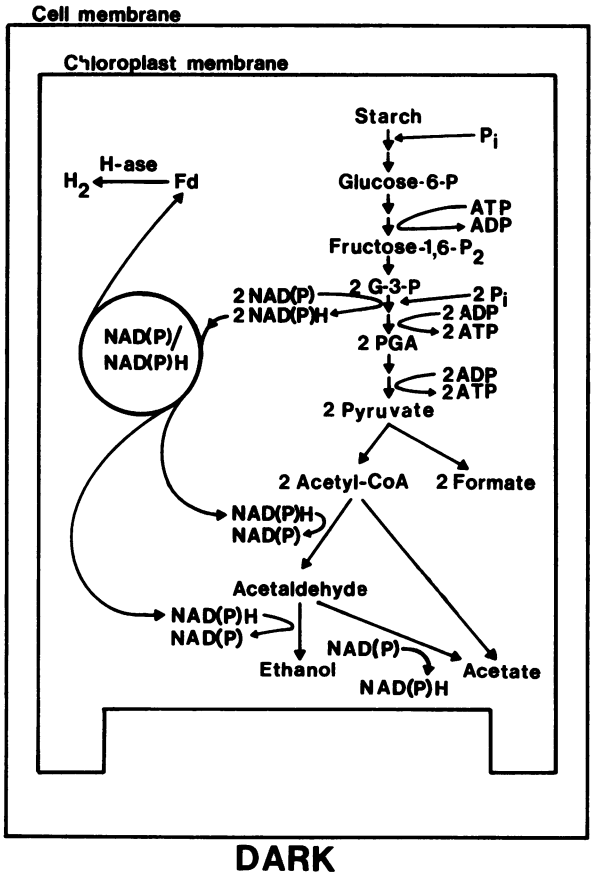

FIG. 4. Proposed pathways of C and H in dark under anaerobiosis in *C. reinhardtii* F-60. The ratio of ethanol to acetate is somewhat lower than 1, and this accounts for NAD(P)H used in H_2 production. The stoichiometry of H_2 production in relation to starch breakdown is not shown. Two possible pathways for acetate formation are indicated. Glycerol has been omitted (minor amounts produced only).

In addition to a C balance, one important feature of fermentation is an even H balance (Fig. 4). In the dark, H_2 can arise only from the NAD(P)H → Fd → H_2 sequence, since H_2 evolution from formate is ruled out by stoichiometric analysis and by our inability to detect both formate utilization by the intact cells, and formate dehydrogenase in crude extracts (data not shown). The major H sink in this complex fermentation is ethanol, and an apparent ratio of slightly less than one ethanol per glucose satisfies the turnover of most of the pyridine nucleotides (and H), and allows for the evolution of H_2.

Fermentative Balance with Illuminated Cells. A fermentative balance between starch and end-products as affected by light, DCMU, and FCCP was carried out under four conditions: (a) in the presence of DCMU and FCCP; (b) in the presence of FCCP; (c) in the presence of DCMU; (d) light only. The results are presented in Tables I and II. Inasmuch as each condition resulted in a stoichiometry differing in some way from that observed in the darkened cells, it is convenient to discuss first the data obtained in the presence of FCCP and DCMU (Table II), since they bring about a balance most closely resembling that obtained in the absence of light. Thus, FCCP offsets the inhibitory effects of light on the rate of starch breakdown and, by eliminating photosynthetic O_2 evolution coupled to H_2 evolution, DCMU maintains anaerobicity, thereby preventing the formation of respiratory CO_2. The ratio of formate produced to glucose consumed remains at 2, and the ratio of 1 of formate to acetate plus ethanol is conserved at the expense of a doubling of acetate to offset the lack of ethanol formation. Since light eliminates ethanol production under all conditions, this aspect of the fermentative balance is dealt with in a separate section. The fact that H_2 is evolved at a rate of approximately 50% of the rate in light without DCMU (Tables I and II) shows that H_2 is evolved from the oxidation of an endogenous source, presumably starch. It is assumed that reducing equivalents formed during the oxidation of glyceraldehyde 3-P to glycerate 3-P can feed their electrons at a site in the photosynthetic electron transport chain beyond the DCMU block (Fig. 5). That NADH can donate electrons to the chain and can give rise to H_2 by this route has been documented in cell free preparations (5, 10). Finally, an O/R index of roughly 1 and reasonable C and H recoveries are indicative of an even balance.

In the presence of FCCP only (Table II), C and H recoveries are high, the formate yield remains 2 mol/mol glucose consumed, and less acetate is produced, but more gas (CO_2 and H_2) is evolved than in the conditions when DCMU is included in the reaction mixture. Ethanol is a minor product. Inasmuch as the ratio of acetate plus ethanol to formate is less than one, we assume that some acetyl-CoA is respired through the mitochondrial citric acid cycle with photosynthetically generated O_2 acting as the terminal electron acceptor, resulting in the high yield of CO_2 (Fig. 5). On the basis of 2 mol formate/mol glucose, we assume an equivalent ratio for acetyl-CoA to glucose. Considering that 1.3 mol acetate and 0.1 mol ethanol are measured, then 0.6 mol acetyl-CoA can be assumed to be oxidized to 1.2 mol CO_2 (against 1.6 mol CO_2 found experimentally) and thus 4.8 mol reducing equivalents would be generated. Subtracting the H_2 evolved in the presence of DCMU (4.7 − 2.1), 2.6 mol H_2 must have been derived from H_2O, resulting in the evolution of 1.3 mol O_2, an amount sufficient to satisfy the 4.8 mol reducing equivalents presumably formed in the citric acid cycle. The H balance is even if, after accounting for the reduced equivalents

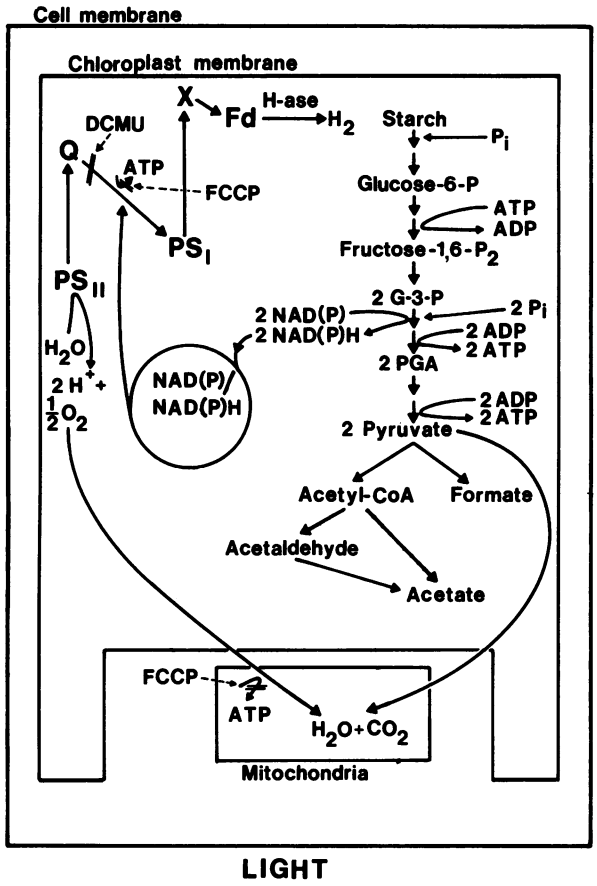

FIG. 5. Proposed pathways of C and H in light under a N_2 atmosphere in *C. reinhardtii* F-60. Stoichiometry of photosynthetic water splitting, H_2 production, and CO_2 production is not shown. DCMU block and inhibition of coupled phosphorylations by FCCP are shown. In absence of DCMU, photosynthetically produced O_2 is thought to allow mitochondrial respiration. In presence of DCMU (true anaerobiosis), the source of CO_2 produced is not shown. Acetate production in light occurs in presence of FCCP only. Two possible pathways for acetate formation are indicated; for simplification, the NAD(P)H turnover involved in one of those pathways is not shown. Glycerol and ethanol have been omitted (minor amounts produced only).

in the 0.1 mol ethanol, 1.8 × 2 mol reducing equivalents from glycolysis remain to account for H_2 not arising from H_2O (Fig. 5). The calculated total H_2 evolution is 2.6 + 1.8 = 4.4 mol, which compares favorably with the 4.7 mol found experimentally.

In the presence of DCMU alone (Table II), the fermentative pattern is characterized by a poor C and H recovery, the absence of both ethanol and acetate, and a formate production lower than 2 mol/mol glucose consumed, which is taken as an indication that not all the C flow proceeds through pyruvate-formate lyase. This condition may reveal the decarboxylation of pyruvate to CO_2 and acetaldehyde catalyzed by pyruvate decarboxylase, as proposed for *C. moewusii* (19). The absence of acetate when the uncoupler is removed from the reaction medium indicates the requirement of ATP for the reutilization of the two carbon acid. It is this reutilization of the acetate into unidentified compounds which may account for the low (43%) recoveries of C and H.

When the algae are illuminated in the absence of FCCP and DCMU (Table I), acetate is not found in the reaction medium, and the ratio of formate to glucose drops from 2 to 1, indicating alternate metabolism of pyruvate, while both CO_2 and H_2 evolutions are strikingly increased. Once again, the high CO_2 yield is apparently the result of an activation of the citric acid cycle by photosynthetically generated O_2, and the H_2 evolution apparently represents the combined yields from two sources, namely H_2O and starch. As in the case when DCMU was included, the C and H recoveries are low, indicating the formation of unaccounted for compounds.

Inhibition of Ethanol Production in the Light. Illumination prevents the formation of ethanol (except for minor amounts) under all conditions tested. Assuming acetaldehyde and alcohol dehydrogenases remain active, it seems that the inability of the cell to produce ethanol is due to the absence of available reduced pyridine nucleotides during illumination. It may well be that in the light the electron transport chain is oxidized since the electrons can be bled off as H_2. This oxidized state would favor the transfer of reducing equivalents generated during the oxidation of glyceraldehyde 3-P to glycerate 3-P from the reduced pyridine nucleotides to the chain at the plastoquinone site, preventing the reduction of acetyl-CoA to acetaldehyde and ethanol. The flow of reducing equivalents preferentially into the photosynthetic electron transport chain would account for the increased H_2 evolution (Tables I and II). This would occur at the expense of ethanol, the major metabolic sink for H in the dark.

Cellular Compartmentation. The question of the cellular compartmentation of the enzymic reactions of the fermentative degradation of starch into its end-products is worthy of comment and has been dealt with in Figures 4 and 5 by assigning the complete pathway (with the exception of the mitochondrially located citric acid cycle) to one compartment, the chloroplast. We have done this primarily because it is difficult to envisage an even H balance both in the dark and in the light if the oxidative (glyceraldehyde 3-P dehydrogenase) and the reductive (acetaldehyde and ethanol dehydrogenases) steps of the fermentative pathway are assigned to different cellular locations. We have come to this conclusion on the basis of the well documented reports that the inner membrane of the higher plant chloroplast (14) and of the algal chloroplast (22) is impermeable to the pyridine nucleotides. We recognize the fact that triose-P can efflux to the cytoplasm, but we have discounted this occurrence because the cytoplasmically generated ATP would not be available to the chloroplastic fructose-6-phosphokinase (23). The cellular site of the hydrogenase remains unknown but presumably is in the chloroplast to account for light-induced H_2 metabolism. Finally, this laboratory has recently reported the isolation of a photosynthetically competent chloroplast from *Chlamydomonas* (21). The validity of cellular assignation of the fermentative events, depicted in Figures 4 and 5, now can be tested.

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