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# Fermentative Metabolism of Hydrogen-evolving *Chlamydomonas moewusii*[1]

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## ABSTRACT

**The anaerobic metabolism of *Chlamydomonas moewusii* under both light (160 lux) and dark conditions has been examined using manometric and enzymic techniques. During anaerobiosis starch is broken down to glycerol, acetate, ethanol, $CO_2$, and $H_2$. The release of $CO_2$ and $H_2$ comes to an end when the starch pool is depleted.**

**There are only slight differences in the ratio of the end products of fermentation between light and dark metabolism. In the light, glycerol production is diminished and $H_2$ evolution is enhanced, whereas the production rate of all other end products generally does not change.**

*Chlamydomonas moewusii* belongs to a group of unicellular green algae which are capable of utilizing or evolving molecular hydrogen (6, 9, 14). The evolution of $H_2$ arises in darkness or in light, if the cells are exposed to anaerobiosis in an atmosphere of $N_2$ or inert gas.

There have been several suggestions to explain this $H_2$ evolution as part of the fermentative metabolism of green algae. In their first paper about $H_2$ production in *Scenedesmus* Gaffron and Rubin (7) suggested that the evolution of $H_2$ depends at least in the dark on the degradation of a reserve substance. Exogenous glucose, which was fermented to lactic acid, increased the rate of $H_2$ evolution. Damaschke (5) got similar results with *Chlorella pyrenoidosa,* which also produced lactic acid during fermentation and which could be stimulated by glucose too.

For *Chlamydomonas* Healey (9) proposed a fermentative process including the citric acid cycle as source for $H_2$ evolution in the dark. He assumed that the surplus NADH could either reduce some unknown acceptor or bring its electrons to a higher redox level at the expense of ATP from where $H_2$ would be released. Under light conditions NADH should give its electrons by a light-driven reaction through the electron transport chain of photosynthesis to hydrogenase. This idea of a light-driven electron transport from HADH to hydrogenase has been supported by Ben-Amotz and Gibbs (1) and King *et al.* (13), who assumed plastoquinone to be the site of NADH oxidation. A similar mechanism for the light-dependent evolution of $H_2$ by *Scenedesmus* has been proposed by Kaltwasser *et al.* (11), who also started to elucidate the fermentation of *Scenedesmus.*

To evaluate the validity of all of these suggested mechanisms for the evolution of $H_2$ it seems necessary to know first the anaerobic metabolism of green algae. This includes the measurement of substrates and end products of fermentation in algae. This paper presents a first approach to the metabolism of *C. moewusii* under conditions of $H_2$ evolution.

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## MATERIALS AND METHODS

*C. moewusii,* strain 11-5/10, from the Pflanzenphysiologisches Institut der Universität Göttingen was grown and harvested as described elsewhere (14). Concentrated cell suspensions ($6 \times 10^7$ cells/ml) were used throughout all experiments. Illumination was provided by two fluorescent lamps (Philips TL 40W-1/32) where necessary; the light intensity at the surface of the Warburg vials was 160 lux. The vessels were routinely gassed before the start of an experiment with $O_2$-free $N_2$ to provide for anaerobic conditions. $O_2$ and $CO_2$ were absorbed by alkaline pyrogallol (20).

Polyphosphate has been extracted following the method of Harold (8) and determined after hydrolysis in 1 N HCL according to Lowry and Lopez (15).

Glycerides have been extracted according to the method of Kates (12). A 1-ml sample of the extract was dried with a stream of air. To the remaining dry material 1 ml of 0.41 N KOH in alcohol was added and the glycerides were saponified in a water bath (70 C) for 90 min. After cooling in ice the solution was adjusted to pH 5 with 15% (w/v) $HClO_4$. Thereafter 0.5 ml of distilled $H_2O$ and 1 ml of $CHCl_3$ were added and the mixture was well mixed in order to separate Chl and glycerol. After centrifugation (12,000*g*, 10 min) samples were taken from the upper phase in which glycerol could be determined according to Wieland (19).

Starch has been determined as reducing sugars after enzymic hydrolysis according to the following procedure: 2 ml of the cell suspension were centrifuged and the sediment extracted twice with 5 ml of acetone (100%) to remove Chl. The residue was washed once with 2 ml of K-phosphate (20 mM, pH 6.7) and suspended in 2 ml of acetate buffer (0.1 M, pH 4). Then the suspension was sonicated for 15 sec using a Branson Sonifier, model S 75, and subsequently heated in a boiling water bath for 5 min to solubilize the starch. Enzymic hydrolysis of the starch was initiated by mixing 0.5 ml of the sample and 1.5 ml of acetate buffer (0.1 M, pH 4) with 0.01 ml of amyloglucosidase (1.4 units). The samples were shaken in a water bath at 40 C for 2 hr after which time reducing sugars could be determined according to Rick and Stegbauer (16).

The polysaccharides analyzed by this procedure contain $\alpha$-(1, 4) glycosidic linkages as well as $\alpha$-(1, 6) glycosidic linkages. This could be shown by differential enzymic hydrolysis using $\alpha$-amylase, $\beta$-amylase, pullulanase, and $\alpha$-(1–1, 6) amyloglucosidase. Although we did not investigate further its structure we can state that the bulk of starch in *C. moewusii* consists of amylopectins.

Glycerol, acetate, and ethanol have been determined according to Wieland (19), Bergmeyer and Möllering (2), and Bernt and Gutmann (3), respectively.

Chl (*a* + *b*) has been determined according to Jeffrey and Humphrey (10).

The enzymes used in this study have been purchased from Boehringer Mannheim GmbH, Biochemica. All other chemicals used were of the highest purity available.

## RESULTS

**Storage Products.** Growing cells of *C. moewusii* accumulate reserve substances which can be easily seen microscopically. We found polyphosphates, glycerides, and starch. Under anaerobic conditions the amount of polyphosphates and glycerides does not change significantly. Only the depletion of starch is constant and considerable (Fig. 1). This indicates that starch is the main substrate which is broken down anaerobically by *C. moewusii*. The disappearance of starch is accompanied by the release of $CO_2$ and $H_2$ (Fig. 1; *cf.* refs. 6, 9, 14) which ceases when the starch pool is depleted (Fig. 1).

**End Products.** The anaerobic fermentation of *C. moewusii* leads to the following five end products: glycerol, acetate, ethanol, $CO_2$, and $H_2$. It may be classified as a heterofermentation. Figure 2 shows the time course of anaerobic starch breakdown and the release of all five end products of fermentation. Glycerol, acetate, and ethanol are not bound in the cells but appear in the suspending medium. This could be checked by comparing their concentrations in an acidic extract with the medium from which the cells had been removed.

**Fermentation in the Dark.** Although considerable efforts have been made to get equal cell material with respect to growth phase and cell number the ratios between the end products were not constant for all experiments. The following interpretation of Table I refers therefore to the mean value of 10 experiments.

Table I presents the fermentation products of *C. moewusii* for a time interval of 130 min in the dark after the cells had been adapted to anaerobiosis. About 92% of the starch degraded could be found in the end products. Besides smaller and similar amounts of ethanol and acetate, glycerol is predominant, a product more reduced than starch. Its accumulation together with the evolution of $H_2$ reflects the metabolic state of the cells: in the conversion of carbohydrate to ethanol and acetate more reducing equivalents are produced than they are able to consume anaerobically. The $CO_2$ evolved corresponds well with the decarboxylation of pyruvate to yield ethanol and acetate. $H_2$ evolution is poor in the dark. It might be explained by the over-all balance of reducing equivalents (see under "Discussion").

**Fermentation in the Light.** In Figure 2 the time courses of fermentation under light and dark conditions are compared. There are no drastic differences between the two conditions. The breakdown of starch is unaffected as are the release of $CO_2$ and ethanol. Acetate is variable to some extent, so its higher production in the light, as it is shown in Figure 2, is not reproducible. The differences in the production rates of $H_2$ and glycerol, however, seem to be real. $H_2$ evolution is enhanced in the light whereas glycerol production is diminished. The smaller rate of glycerol production, however, can only partially account for the more vigorous $H_2$

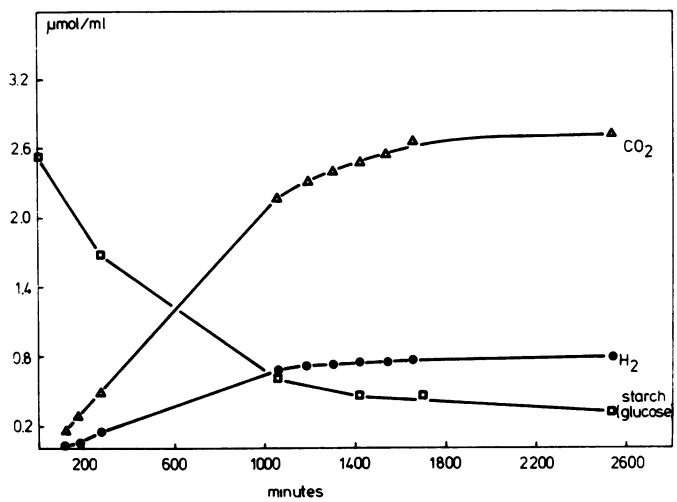


FIG. 1. Relationship between starch breakdown, $H_2$ evolution, and $CO_2$ release during fermentation in the dark. Chl ($a$ + $b$) = 88 µg/ml.

Table I. Fermentation products of Chlamydomonas moewusii in the dark

The data represent mean values of ten experiments. The values refer to a time interval of 130 minutes, during which the production rate of all end products was constant.

| Product | Breakdown or accumulation respectively [1] µmol/ml | % of starch (related to $C_3$-units) | | |
|---|---|---|---|---|
| Starch (in $C_3$-units) | 1.37 | 100 | | |
| Glycerol | 0.62 | 45.2 | | |
| Acetate | 0.36 | 26.2 | 46.6 | 91.8 |
| Ethanol | 0.28 | 20.4 | | |
| $CO_2$ | 0.58 | 42.3 | | |
| $H_2$ | 0.14 | - | | |

[1] In all experiments the cell concentration was about 6 x $10^7$ cells/ml. This corresponds to approximately 0.12 mg chlorophyll/ml.

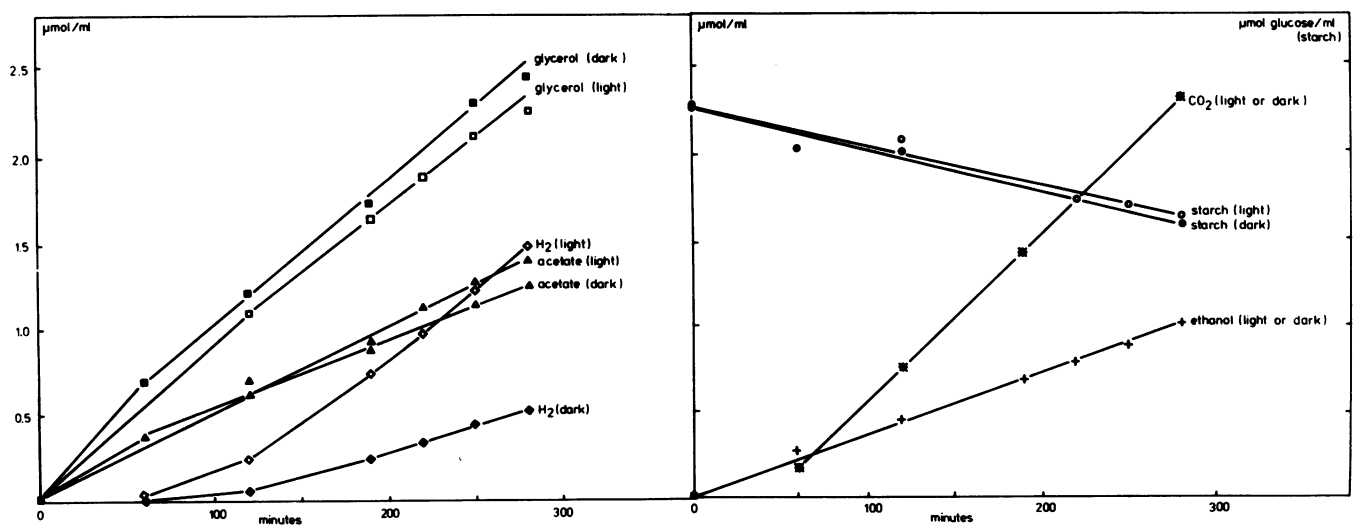


FIG. 2. Time course of starch breakdown coupled to the release of end products during a period of anaerobiosis under light and dark conditions. Both illustrations refer to the same experiment. They have been separated for convenience. Light intensity = 160 lux. Chl ($a$ + $b$) = 173 µg/ml.

evolution. In contrast to dark conditions the evolution of $H_2$ in the light is not completely explained by fermentation.

## DISCUSSION

*C. moewusii* does not seem to be exceptional among green algae with respect to storage products and end products of fermentation. However, as far as we know, the combination of these end products, as we found it in this species, has never been reported for green algae. Weis and Mukerjee (18) found a similar fermentation for *Chlorella vulgaris*; their list of end products was completed later by Syrett and Wong (17).

Our results on *Chlamydomonas* demonstrate that in the dark $H_2$ is derived from starch (Fig. 1). This finding confirms experiments from Kaltwasser *et al.* (11) who supposed that the breakdown of starch in *Scenedesmus* could account for all of the $H_2$ evolved, even in the light.

From experiments with radioactive labeled glucose Kaltwasser *et al.* (11) concluded that *Scenedesmus* breaks down starch via the Embden-Meyerhof pathway. We did not use exogenous substrates, because *C. moewusii* takes up these substrates at a very slow rate and under aerobic conditions only (unpublished). In our experiments, however, the amount of $CO_2$ evolved (Table I) indicates that *Chlamydomonas* like *Scenedesmus* uses the Embden-Meyerhof pathway during fermentation.

Figure 3 and Table II summarize our recent conception of the fermentation of *C. moewusii* in the dark. Starch is broken down primarily to pyruvate. From the viewpoint of energy conservation the formation of ethanol would be most favorable. In this case the generation and consumption of reducing equivalents would be well balanced. However, there seems to be a block between pyruvate and ethanol so that pyruvate accumulates. We have observed a continuous rise in the concentration of pyruvate during the first 4 hr of anaerobiosis (unpublished). Consequently, pyruvate is decarboxylated oxidatively to acetyl-CoA, which is converted to acetate. The production of glycerol and the evolution of $H_2$ besides ethanol and acetate are consequences of the accumulation of reducing equivalents. Since during ethanol formation the same amount of NADH is consumed as it is produced the NAD/NADH ratio for this pathway is balanced (Table II). During acetate formation, however, reducing equivalents are generated but not consumed (equal to 2 mol of NADH/mol of acetate). Their accumulation would suppress any net flux in glycolysis. For the organism in the absence of $O_2$ the reoxidation of reducing equivalents is essential to keep glycolysis with its energy-yielding processes running. In *C. moewusii* most of the surplus reducing equivalents seem to be used for the reduction of dihydroxyacetone-P to glycerol. Obviously, the capacity for this pathway is limited and a small portion of these reducing equivalents is released via hydrogenase in the form of molecular hydrogen.

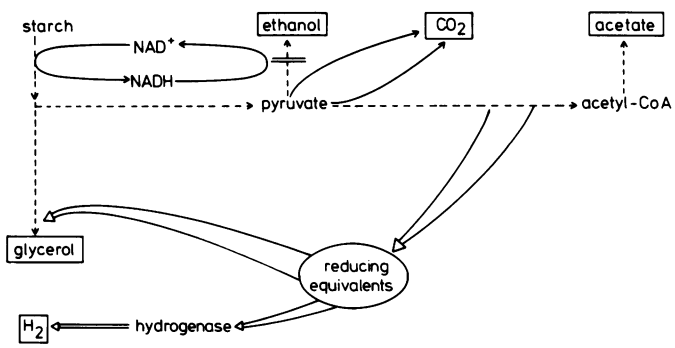


FIG. 3. Hypothetical scheme for the fermentation of *C. moewusii* in the dark. Bars between pyruvate and ethanol indicate that this is the rate-limiting step in the formation of ethanol. A detailed explanation of the scheme is presented under "Discussion."

Table II. Production and consumption of reducing equivalents ($RH_2$) during fermentation of Chlamydomonas moewusii.

The values are derived from the data presented in Table I. It is assumed that the Embden-Meyerhof pathway is used and that pyruvate is decarboxylated oxidatively to acetate.

| Product | Enzymatic reaction: Oxidation | Enzymatic reaction: Reduction | $RH_2$ (μmol/ml): produced | $RH_2$ (μmol/ml): consumed |
|---|---|---|---|---|
| Ethanol | [1]GAP → 1,3-PG | Acetal. → Eth. | 0.28 | 0.28 |
| Acetate | GAP → 1,3-PG | - | 0.36 | - |
| Acetate | Pyr. → Ac.-CoA | - | 0.36 | - |
| Glycerol | - | DAP → α-G-P | - | 0.62 |
| $H_2$ | - | $XH_2$ → $H_2$ | - | 0.14 |
| Sum | | | 1.00 | 1.04 |

[1]Abbreviations: GAP (glyceraldehyde-3-P); 1,3-PG (1,3-diP-glycerate); Pyr. (pyruvate); Ac.-CoA (acetyl-CoA); Acetal. (acetaldehyde); Eth. (ethanol); DAP (dihydroxyacetone-P); α-G-P (α- glycerol-P); $XH_2$ (unknown reductant).

The above considerations imply that in *C. moewusii* $H_2$ evolution in the dark depends on the ratio of acetate to glycerol. There will be a release of $H_2$ only if this ratio is greater than 0.5. This is in agreement with our experimental results.

Our experiments give no indications on the distinct reactions yielding reducing equivalents from pyruvate and their consumption at the hydrogenase. The same holds for the chemical nature of these reducing equivalents produced in the oxidative decarboxylation of pyruvate, although the presence of pyruvate dehydrogenase in *Chlamydomonas* (4) favors the idea that they might be NADH.

The evolution of $H_2$ in the light seems to be more complicated. Although the same metabolic pathways are used and the rate of end product release is not significantly changed, the evolution of $H_2$ is about twice as much as in the dark. This corresponds well with results from Kaltwasser *et al.* (11) who also found no pronounced changes in *Scenedesmus* between the metabolic pathways in the light and in the dark.

Besides enhanced $H_2$ production the only difference between light and dark metabolism in *C. moewusii* is the diminished glycerol production in the light. This difference, however, could only partially account for the higher rate of $H_2$ evolution in the light. The discrepancy between $H_2$ evolution in the light and fermentation could be explained either by photosynthetic splitting of water, which is in contrast to the results of Healey (9) and Kaltwasser *et al.* (11), or some other unknown light-dependent process. Until now we cannot decide between these assumptions on the basis of our results.

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