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Effect of Molecular Hydrogen and Carbon Dioxide on Chemo-Organotrophic Growth of *Acetobacterium woodii* and *Clostridium aceticum*

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Abstract. During growth of *Acetobacterium woodii* on fructose, glucose or lactate in a medium containing less than 0.04% bicarbonate, molecular hydrogen was evolved up to 0.1 mol per mol of substrate. Under an H_2-atmosphere growth of *A. woodii* with organic substrates was completely inhibited whereas under an H_2/CO_2-atmosphere rapid growth occurred. Under these conditions $H_2 + CO_2$ and the organic substrate were utilized simultaneously indicating that *A. woodii* was able to grow mixotrophically.

Clostridium aceticum differed from *A. woodii* in that H_2 was only evolved in the stationary phase, that the inhibition by H_2 was observed at pH 8.5 but not at pH 7.5, anf that in the presence of fructose and $H_2 + CO_2$ only fructose was utilized.

The hydrogenase activity of fructose-grown cells of *C. aceticum* amounted to only 12% of that of $H_2 + CO_2$-grown cells. With *A. woodii* a corresponding decrease of the activity of this enzyme was not observed.

Key words: *Acetobacterium woodii* – *Clostridium aceticum* – Mixotrophic growth – H_2 utilization – CO_2 fixation – H_2 inhibition of chemoorganotrophic growth.

Acetate is an important product in the anaerobic degradation of organic material. It is formed in a number of fermentative processes from precursors such as pyruvate, fructose-6-phosphate, xylulose-5-phosphate, glycine or citrate (Gottschalk and Andreesen 1979). A few organisms, e.g. *Clostridium thermoaceticum* and *C. formicoaceticum* are able to use CO_2 as an additional hydrogen acceptor and to produce acetate thereof (Fontaine et al. 1942; Andreesen et al. 1970). These organisms are, however, unable to utilize H_2 for the reduction of CO_2 to acetate, a conversion that can be accomplished by *Clostridium aceticum* (Wieringa 1936) and by *Acetobacterium woodii* (Balch et al. 1977). Both species are able to grow at the expence of the formation of acetate from H_2 and CO_2:

$$4\,H_2 + 2\,CO_2 \rightarrow CH_3\text{-}COO^- + H^+ + 2\,H_2O \quad \Delta G'^0 = -95\,\text{kJ}.$$

Since these species grow also with a number of organic substrates it was of interest to determine whether they could utilize these substrates and $H_2 + CO_2$ simultaneously.

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Materials and Methods

Bacteria. Acetobacterium woodii (DSM 1030) was obtained from S. Schoberth (Jülich, Germany), and *Clostridium aceticum* strain Wieringa (DSM 1496) from H. A. Barker (Berkeley, USA).

Media and Cultivation. A. woodii was grown in a medium which contained per 900 ml distilled water: NH_4Cl, 1 g; $MgSO_4 \cdot 7\,H_2O$, 100 mg; mineral and vitamin solutions according to Wolin et al. (1964), 20 ml each (in the vitamin solution the cyanocobalamin content was increased to 2 mg/l); resazurine, 1 mg; Difco yeast extract, 2 g; $NaHCO_3$, 10 g and 1 M potassium phosphate buffer, pH 7.0, 5 ml. For the preparation of medium low in $NaHCO_3$, this compound was omitted and the amount of phosphate buffer was increased to 50 ml. The media were brought to a boil for a few seconds while they were continuously gassed with N_2/CO_2 (80:20, v/v) or N_2 (medium low in bicarbonate). Aliquots of 45 ml were distributed to 500 ml glass bottles which were tightly sealed with rubber stoppers and sterilized. Depending on the experiment to be performed the atmosphere was N_2 or was changed to H_2 or H_2/CO_2 (67:33, v/v). Traces of oxygen were removed from gas streams by passage over a hot copper catalyst column heated to 225°C (Schoberth and Gotschalk 1969).

5 ml of a separately sterilized 0.1 M fructose solution and 0.5 ml of a sterile reducing agent (NaOH, 2.9 g; cysteine hydrochloride $\cdot H_2O$, 4.5 g; $Na_2S \cdot 9\,H_2O$, 4.5 g; distilled water, 160 ml) were added using sterile syringes. The medium was inoculated with 4% of cell suspension which had been grown on H_2/CO_2. The cultures were incubated at 30°C.

Media containing other organic substrates were prepared in the same way. Final concentration of substrates: glucose, 10 mM and sodium L-lactate, 20 mM. These concentrations were chosen in order to allow the formation of approximately similar amounts of acetate.

C. aceticum was cultured in essentially the same way except that buffers given above were replaced by 5 ml 1 M potassium phosphate buffer, pH 8.0 plus 8.4 g Na_2CO_3, or by 160 ml 1 M K_2HPO_4 in the case of medium low in bicarbonate. The final pH of the media was 8.5 which is the optimum pH for chemolithotrophic growth of *C. aceticum* (Wieringa 1940; Braun et al. 1981). For growth at pH 7.5, 5 ml of 1 M potassium phosphate buffer, pH 7.0 and 10 g $NaHCO_3$ were added (160 ml of 1 M potassium phosphate buffer, pH 7.0 in the case of medium low in bicarbonate).

Growth of *A. woodii* and *C. aceticum* was followed by measuring the optical density at 600 nm in a Zeiss PM 4 spectrophotometer. Samples were reduced with 20 µl 2% (w/v) sodium dithionite to decolorize the resazurine indicator. Readings were corrected for the optical density of the freshly inoculated medium.

Analytical Methods. Acetate was determined with acetyl-CoA synthetase according to Dorn et al. (1978), fructose according to Bernt and Bergmeyer (1974) and L-lactate according to Gutmann and Wahlefeld (1974). For the determination of glucose the procedure given by the Merck AG (No 3389) was followed.

H_2/CO_2 consumption of *A. woodii* and of *C. aceticum* was determined as ml gas uptake using glass syringes. H_2 production was measured using a gas chromatographical method. The gas chromatograph (Perkin Elmer

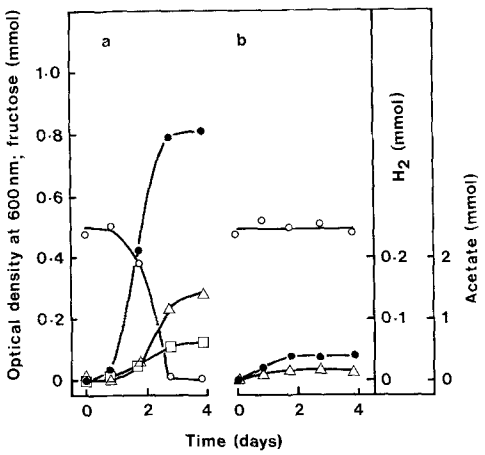


Fig. 1. Growth of *Acetobacterium woodii* on fructose in a medium low in bicarbonate (0.04%) under an N_2-atmosphere (**a**) and under an H_2-atmosphere (**b**). Experiments were carried out in 500 ml-bottles containing 50 ml medium (see Methods). Symbols: ●, optical density; ○, fructose; △, acetate; □, H_2

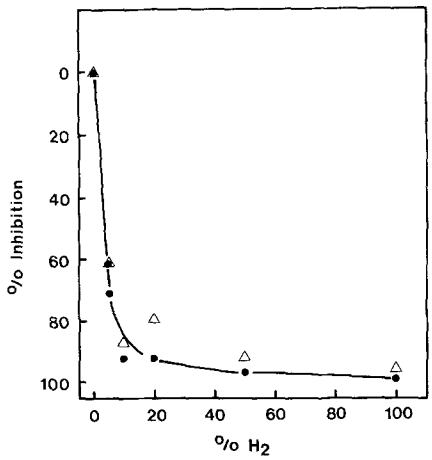


Fig. 2. Inhibition of chemoorganotrophic growth of *Acetobacterium woodii* by molecular hydrogen in a medium low in bicarbonate (0.04%). The preparation of the culture media was as described in Materials and Methods except that glucose was used instead of fructose. H_2 and N_2 were mixed with a gas mixing pump (Wösthoff, Bochum, Germany). Values were taken from a 13 day old culture. 0% inhibition corresponded to an optical density of 0.96 and to 1.37 mmol acetate produced, respectively. Symbols: ●, optical density; △, acetate produced

F 20 H) was equipped with a 6 m silica gel column or a 2 m molecular sieve 5 Å steel column and a hot wire detector. The injector and the oven temperature was 100° C, the detector temperature was 230° C. N_2 was used as carrier gas. Carrier gas pressure, 7.8 N · cm^{-2}; bridge voltage, 2; attenuation, 1. Evaluation of the peaks was done with a Hewlett-Packard 3370 B integrator.

Preparation of Cell Extracts. Cells were harvested under an H_2- or N_2-atmosphere and either used immediately for extract preparation or stored at −20° C under H_2 until needed. Cell disruption was done with a French pressure cell at 138 MPa under H_2. Following centrifugation at 15,000 × *g* and 4° C for 30 min, the supernatant was used for determining hydrogenase activity. Protein was determined according to Bradford (1976) with bovine serum albumin as a standard. A comparison with the Amidoschwarz-method according to Schaffner and Weissmann (1973) showed no significant difference.

Determination of Hydrogenase Activity. Hydrogenase was assayed by the manometric measurement of H_2-uptake using benzylviologen as hydrogen acceptor at 30° C (van der Westen et al. 1978) or methylene blue. In the latter case the main compartment of a Warburg vessel contained in a volume of 2 ml: 1 M potassium phosphate buffer, pH 8.0, 0.1 ml; bovine serum albumin, 1 mg; and 0.06 M methylene blue, 0.2 ml. The center well contained: 5 N KOH or NaOH, 0.1 ml. The side arm contained varying amounts of cell extract (0.1 − 1 units). Gas phase: H_2. The reaction was started by tipping the cell extract into the main compartment.

Chemicals. Chemicals were reagent grade. Fructose and glucose were purchased from Merck AG (Darmstadt, FRG), lactate from Sigma (St. Louis, USA), Coomassie Brilliant Blue and bovine serum albumin from Serva (Heidelberg, FRG). Enzymes and enzyme substrates were purchased from Boehringer (Mannheim, FRG) or Merck (Darmstadt, FRG).

Results

Evolution of H_2 During Chemoorganotrophic Growth of Acetobacterium woodii and Inhibition of Growth by H_2

When *Acetobacterium woodii* was grown on fructose in a medium low in bicarbonate under an N_2-atmosphere, the production of some molecular hydrogen was observed (Fig. 1 a). This indicated that the hydrogenase of this organism was functional also under chemoorganotrophic growth conditions. When a similar growth experiment was performed under an H_2-atmosphere the cells did not grow significantly (Fig. 1 b). Molecular hydrogen, therefore, inhibited growth of *A. woodii* on fructose in a medium low in bicarbonate. The dependence of the growth inhibition on the partial pressure of H_2 is shown in Fig. 2. From a double reciprocal plot a K_1 value of $P_{H_2} = 0.02$ atmospheres was calculated.

Table 1. Evolution of H_2 and production of acetate during chemoorganotrophic growth of *Acetobacterium woodii* under an N_2-atmosphere

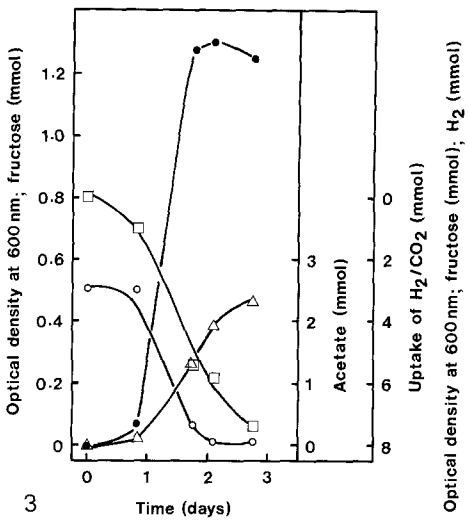
Substrate	Final $OD_{600\,nm}$	Substrate consumed (mmol/culture)	Products formed (mmol/culture)	
			Acetate	H_2
D-Fructose	0.80	0.50	1.30	0.063
D-Glucose	0.95	0.50	1.14	0.052
L-Lactate	0.47	0.96	1.20	0.100

Experiments were carried out in 500 ml-bottles containing 50 ml CO_2-free medium under an N_2-atmosphere. The inoculum was 2 ml of a chemolithotrophic culture per bottle and resulted in a final concentration of bicarbonate of 0.04%

The same phenomenon, formation of some H_2 in an N_2-atmosphere and inhibition of growth by H_2 was observed when *A. woodii* was grown with glucose or with L-lactate. Growth on DL-glycerate was also inhibited by H_2 but only little H_2 was produced when *A. woodii* was allowed to grown on this substrate in an N_2-atmosphere. The amounts of acetate and of H_2 produced from fructose, glucose and lactate are summarized in Table 1. It can be seen that per mol of fructose, glucose or lactate approximately 0.1 mol of H_2 was evolved. Carbon dioxide was formed only in very small quantities.

Simultaneous Utilization of $H_2 + CO_2$ and Organic Substrate by A. woodii (Mixotrophy)

The inhibition of chemoorganotrophic growth by H_2 indicated that a hydrogenase was present in cells growing under such conditions, and it was of interest to study the effect of CO_2. Replacement of the H_2-atmosphere by an atmosphere contain-



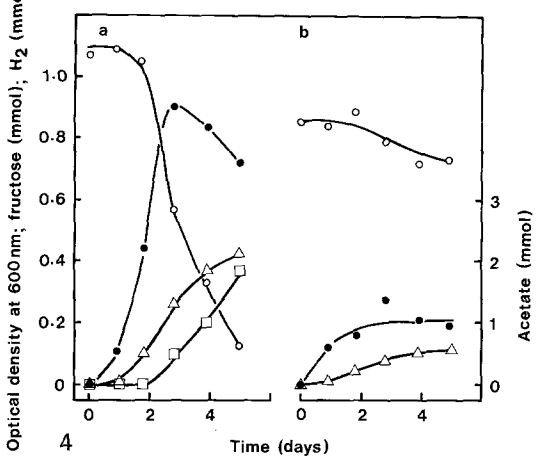


Fig. 3
Growth of *Acetobacterium woodii* on fructose and $H_2 + CO_2$ in medium containing bicarbonate (1%). The experiment was carried out in a 500 ml-bottle containing 50 ml medium (see Methods). Symbols: ●, optical density; ○, fructose; △, acetate; □, $H_2 + CO_2$ (67:33 v/v)

Fig. 4
Growth of *Clostridium aceticum* on fructose in a medium low in bicarbonate (0.03%) under an N_2-atmosphere (**a**) and under an H_2-atmosphere (**b**). Experiments were carried out in 500 ml-bottles containing 50 ml medium (see Methods). Symbols: ●, optical density; ○, fructose; △, acetate; □, H_2

Table 2. Mixotrophic and chemolithotrophic growth of *Acetobacterium woodii*

Substrate	Final $OD_{600\,nm}$	Substrates consumed (mmol/culture)		Acetate formed (mmol/culture)
		Organic compound	$H_2 + CO_2$	
D-Fructose	1.30	0.50	7.37	2.32
D-Glucose	1.30	0.51	13.30	3.04
L-Lactate	0.87	0.93	12.00	2.96
$H_2 + CO_2$	0.80	–	29.9	4.36

Experiments were carried out in 500 ml-bottles containing 50 ml medium with 1% bicarbonate under an H_2/CO_2-atmosphere (67:33, v/v). The inoculum was 2 ml of a chemolithotrophic culture per bottle

Table 3. Effect of pH on the inhibition of chemoorganotrophic growth of *Clostridium aceticum* by H_2

Atmosphere	pH	Final $OD_{600\,nm}$	Fructose consumed (mmol/culture)	Acetate produced (mmol/culture)	H_2 produced (mmol/culture)
N_2	8.5	0.88	0.97	2.1	0.37[a]
H_2	8.5	0.21	n.d.	n.d.	–
N_2	7.5	0.77	0.48	1.5	0.028[a]
H_2	7.5	0.70	0.44	1.45	–

n.d., not determined
[a] H_2 was produced only in the stationary phase
Experiments were carried out as described in Table 1 and in Methods

ing 67% H_2 and 33% CO_2 resulted in fast and reproducible growth of *A. woodii*. The growth curve as obtained with fructose as chemoorganotrophic substrate is given in Fig. 3. It is apparent that in comparison to Fig. 1 the final optical density of the culture was considerably higher and that more acetate was produced. This was apparently a consequence of the simultaneous utilization of fructose and $H_2 + CO_2$. In Table 2 the data for growth on fructose, glucose or L-lactate in the presence of $H_2 + CO_2$ and for chemolithotrophic growth are summarized. It can be seen that with all three substrates the amounts of acetate exceeded those which theoretically could have been formed from the organic substrates. The consumption of H_2 and CO_2 accounted for the difference. The final optical densities were higher than in growth experiments under N_2 (see Table 1) or under chemolithotrophic growth conditions. In no case diauxic growth was observed.

Effect of H_2 and $H_2 + CO_2$ on Chemoorganotrophic Growth of Clostridium aceticum

When analogous experiments were carried out with *C. aceticum* the results turned out to be quite different. During growth of this organism on fructose, H_2 was not evolved before the cells had entered the stationary phase (Fig. 4a). Inhibition of growth by H_2 was pH dependent. At pH 8.5 it was almost complete (Fig. 4b) but at pH 7.5 a significant inhibition could not be observed, and the growth curve resembled the one depicted in Fig. 4a. The data obtained are summarized in Table 3. The most interesting finding in this connection was, however, that mixotrophic growth did not occur to a significant extent. Fructose was rapidly consumed, while $H_2 + CO_2$ was taken up only in minute amounts (Fig. 5). Per mol of fructose 2.2 mol of acetate were produced.

Hydrogenase Activities in A. woodii and C. aceticum

Because of the differences between *A. woodii* and *C. aceticum* as to mixotrophic growth it was of interest to compare the levels of hydrogenase in these organisms. It is apparent from Table 4 that differences were noticable. *A. woodii* exhibited a rather high hydrogenase activity irrespective whether the cells were grown with $H_2 + CO_2$ or with fructose. A marked decrease in activity, however, was found when *C. aceticum* was transferred from chemolithotrophic growth to growth on fructose.

Effect of CO_2 on Chemoorganotrophic Growth of A. woodii and C. aceticum

Although CO_2-free media were prepared for the corresponding chemoorganotrophic growth experiments (see Materials and Methods), some bicarbonate was introduced with the inoculum, which always was taken from chemolithotrophic cultures. This resulted in initial bicarbonate concentrations in the growth medium of 0.04% (*A. woodii*) and 0.03% (*C.*

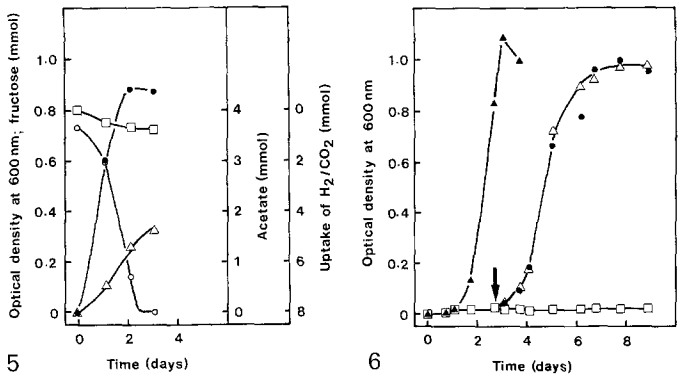


Fig. 5. Growth of *Clostridium aceticum* on fructose at pH 8.5 in the presence of $H_2 + CO_2$ in a medium containing 1 % bicarbonate. Comparable results were obtained at pH 7.5. Experiments were carried out in 500 ml-bottles containing 50 ml medium (see Methods). Symbols: ●, optical density; ○, fructose; △, $H_2 + CO_2$ (67:33 v/v)

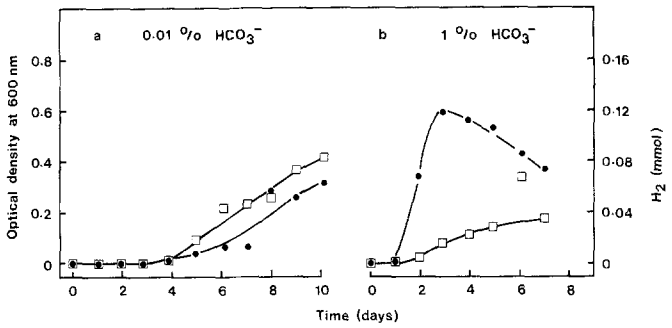


Fig. 7. Growth of *Clostridium aceticum* on fructose under N_2 in a medium low in bicarbonate (0.01 %) (**a**) and rich in bicarbonate (1 %) (**b**) at pH 8.5. Experiments were carried out in 500 ml-bottles containing 50 ml medium (see Methods). Symbols: ●, optical density; □, production of H_2

Fig. 6. Effect of CO_2 on growth of *Acetobacterium woodii* with fructose in a medium low in bicarbonate (0.01 %). Experiments were carried out in 500 ml-bottles containing 50 ml medium (see Methods). The atmosphere of the two control cultures was N_2 (▲——▲) and H_2 (□——□), respectively. At the time indicated by an arrow the atmosphere of two cultures was changed from H_2 to N_2 (△——△) and from H_2 to $N_2 + 0.01\%$ HCO_3^- (●——●), respectively

Table 4. Specific activity of hydrogenase (H_2-uptake) in $H_2 + CO_2$- and fructose-grown cells of *Acetobacterium woodii* and *Clostridium aceticum*

Organism		Specific activity (U/mg)	
		$H_2 + CO_2$	Fructose
A. woodii		2.0	2.7
C. aceticum	pH 8.5	2.2	0.26
	pH 7.5	–[a]	0.35

[a] No growth with $H_2 + CO_2$ at pH 7.5. Assay conditions are given in Methods

aceticum), respectively. In order to show that the presence of H_2 and not the absence of bicarbonate caused the observed H_2 inhibition of chemoorganotrophic growth, *A. woodii* was grown under an H_2 atmosphere until no further growth occurred (Fig. 6; 0.01 % bicarbonate had been added with the inoculum). At this point residual bicarbonate should have been consumed by the cultures through the formation of acetic acid from H_2 and CO_2. A change of the atmosphere from H_2 to N_2 (arrow) resulted in an immediate resumption of growth. No difference in the growth rate could be seen between this culture, and a second one supplemented with 60 µmol bicarbonate to give again the starting concentration of 0.01 %.

This experiment indicated that the inhibition observed was primarily due to the presence of H_2 rather than to an absolute requirement for CO_2. However, CO_2 is necessary for the relief of the H_2-inhibition of chemoorganotrophic growth.

Whereas growth of *A. woodii* on fructose seemed not to be dependent on the presence of bicarbonate, *C. aceticum* showed such a dependence. With 0.01 % bicarbonate a considerable lag-phase was observed, and growth occurred only together with some H_2 evolution (Fig. 7). 0.03 % bicarbonate was already sufficient to allow reproducible growth (see Fig. 4). At this and at higher bicarbonate concentrations (1 %), H_2 evolution took only place after the cultures had entered the stationary phase.

Discussion

Methanogenic bacteria as well as the acetogenic bacteria *Acetobacterium woodii* and *Clostridium aceticum* have in common that they are able to grow at the expence of the reduction of CO_2 with H_2. Both groups of microorganisms differ, however, in many aspects. Whereas the methanogens contain wall components, coenzymes and lipids which are not found in other anaerobes (see Wolfe 1979) the acetogens seem to be closely related to other fermentative organisms. *A. woodii* contains murein of the crosslinkage type B with ornithinyl residues as interpeptide bridges like *Eubacterium limosum* (Kandler and Schoberth 1979) and *C. aceticum* of the type A with a direct crosslinkage as in *C. formicoaceticum* (Kandler personal communication). Both organisms are devoid of coenzyme F_{420} which is typical for methanogens. Another difference concerns the substrates utilized. Compounds which serve as substrates for methanogenic bacteria are $H_2 + CO_2$, carbon monoxide, formate, methanol, methylamines and acetate (Wolfe 1979; Hippe et al. 1979; Thauer and Fuchs 1979). The acetogens, on the other hand, are typical facultative chemolithotrophs capable of growing with $H_2 + CO_2$ but also with sugars and acids such as L-lactate and glycerate (Balch et al. 1977; Braun et al. 1981). In the case of the acetogens, therefore, the question arises whether or not the organic substrates and $H_2 + CO_2$ can be utilized together. The results of this study show that this is true for *A. woodii*. Apparently the synthesis of hydrogenase is not repressed by *A. woodii* when growing on organic compounds, and $H_2 + CO_2$ are used as substrate simultaneously with fructose, glucose or lactate. *C. aceticum* seems to be unable to take advantage of the simultaneous presence of an organic substrate and of $H_2 + CO_2$, only the organic substrate being utilized under such conditions.

The simultaneous utilization of organic and inorganic substrates for energy conservation by facultative chemolithotrophs growing in batch culture has not been observed frequently. However, as Matin (1978) has pointed out, this kind of mixotrophy may be much more important under conditions of nutrient limitation. In batch culture the rate of ATP formation from either one substrate may be high enough to cover the organism's needs, and appropriate regulatory mechanisms may determine whether the organic or the inorganic energy source is utilized preferentially. Chemolithotrophic energy conservation connected with an assimilation of organic compounds is more common among facultative chemolithotrophs and is found among hydrogen-oxidizing bacteria

(Gottschalk 1965; Schlegel and Eberhard 1972) and thiobacilli (Rittenberg 1969; Matin 1978) and also in *Pseudomonas oxalaticus* (Dijkhuizen et al. 1978), *Methanobacterium ruminantium* (Bryant et al. 1971) and *Desulfovibrio desulfuricans* growing with sulfate, H_2, CO_2 and acetate (Badziong et al. 1978).

That growth of saccharolytic anaerobes is inhibited by low partial pressures of molecular hydrogen is also not very common. It has been reported for *Clostridium cellobioparum* (Chung 1976). For that organism and for *A. woodii* and *C. aceticum* this is probably not a disadvantage because partial pressures of H_2 in their habitats are low (Hungate 1967; Kasper and Wuhrmann 1978). Furthermore, in *A. woodii* and *C. aceticum* this inhibition is relieved by CO_2, probably because CO_2 lowers the redox level of some cellular hydrogen carriers. In the case of *C. aceticum* H_2 causes some inhibition at pH 8.5 but not at pH 7.5. It is somewhat in accordance with this that chemolithotrophic growth of *C. aceticum* is restricted to pH values of around 8.5 and does not occur at pH 7.5 (Wieringa 1940; Braun et al. 1981).

The production of H_2 during growth of *A. woodii* in an N_2 atmosphere amounts to approximately 0.1 mol H_2 per mol of fructose. This value could probably be increased considerably if H_2 was continuously removed. In accordance with this are the findings of Winter and Wolfe (1980) that a mixed culture of *A. woodii* and a methanogenic bacterium converted fructose into two mol acetate and 1 mol each of CO_2 and methane.

The tendency to evolve H_2 during heterotrophic growth is much lower with *C. aceticum* than with *A. woodii*, and H_2 is produced by the former organism only in the stationary growth phase. The requirement of bicarbonate for growth may be connected to this; *C. formicoaceticum* which does not evolve H_2 under any conditions shows also such a requirement (Andreesen et al. 1970).

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