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Hydrogen Metabolism in *Clostridium acetobutylicum* Fermentation

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The initial growth of *Clostridium acetobutylicum* was not inhibited by 1 atm of H_2 while H_2 reduced glucose consumption in a solventogenic culture of a phosphate limited 2-stage chemostat. Under 1 atm of H_2, a solventogenic culture consumed hydrogen, but an acidogenic culture produced hydrogen. H_2 consumption by the solventogenic culture was enhanced by the addition of 5 mM neutral red, an artificial electron carrier with a redox potential of −325 mV. Hydrogenase activity, measured in both directions of production and consumption, showed that activity coupled with methyl viologen is higher in an acidogenic culture than in a solventogenic culture, and that the two cultures have similar activities for methylene blue reduction. The solventogenic culture showed a higher activity coupled with neutral red than the acidogenic culture. From these results, it is hypothesized that hydrogen producing hydrogenase activity is high during the acidogenic phase, and decreases as solventogenesis starts, and that the solventogenic culture produces a second hydrogenase which uses an electron carrier other than ferredoxin. This hypothesis was supported by the fact that enzyme activities involved in electron flow can be coupled to neutral red, indepedent of ferredoxin, and that neutral red addition to the fermentation system increased butanol yield, with a decrease in production of less reduced fermentation products, and H_2.

Acetone-butanol-ethanol(ABE) fermentation by *Clostridium acetobutylicum* is characterized by early acidogenesis, followed by solventogenesis. During the acidogenic stage the bacterium produces acetate and butyrate with gaseous products, and the electron flow is separated from the carbon flow, producing hydrogen. On the other hand, the organism uses electrons to produce alcohols from acids during the solventogenic phase.

Hydrogen metabolism by *Clostridium acetobutylicum* is changed during the initiation of solvent production (10). Hydrogen production, and *in vivo* hydrogenase as measured by the tritium exchange method, decreased as solvent production started. The solventogenic culture showed lower *in vitro* hydrogenase activity coupled with methyl viologen than the acidogenic culture (7). On the other hand, hydrogenase activity coupled with methylene blue did not change during ABE fermentation (1). The reversibility of hydrogenase activities in this bacterium was reported by Yarushalmi *et al.*, who showed that the yields of more reduced products (butanol and ethanol) increased with a decrease in acetone yield at a higher hydrogen partial pressure (20).

Modulators for electron flow can change hydrogen metabolism during ABE fermentation. Hongo (8) observed an increase in butanol yield by the addition of the artificial electron carrier, neutral red, into the ABE fermentation system. The redox potential of the dye is −325 mV. Other dyes of higher potential showed little or no effect on butanol yield. Higher butanol yields were observed in fermentation with added methyl viologen (18, 19), whose redox potential (−440 mV) is lower than that of neutral red.

Lactate is metabolized by *Clostridium acetobutylicum* through pyruvate in the presence of fermentable sugar. Lactate added fermentation produced trace amount of acetone with an increased butanol yield. This modulation is believed to be due to the high redox potential of the lactate reduction reaction to pyruvate (12). ABE fermentation under carbon monoxide, a hydrogenase inhibitor, also increased the butanol yield (9).

Mortenson *et al.* (16) purified and characterized two

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functionally different hydrogenases from *Clostridium pasteurinum*. One showed activities in both directions of production and consumption, and the other showed activity only in the direction of consumption.

Hydrogenase activitiy is the most important factor to be considered in the regulations and modulation of hydrogen metabolism. Studies were made on the relationship bewteen hydrogen partial pressure and growth of *C. acetobutylicum*, on hydrogeanse activities with different electron carriers, and on other oxidoreductases using a two-stage chemostat, an acidogenic first stage and a solventogenic second stage.

MATERIALS AND METHODS

Chemicals

All chemicals were reagent grades. They were purchased from either Sigma Chemical Co. (St Louis, Mo) or Mallinckrodts (Paris, Ky). Radioactive chemicals were supplied by New England Neuclear Co., (Boston, Mass). Gases were purchased from Matheson Scientific, Inc.(Joliet, Ill).

Bacterial Strain and Its Maintenance

Clostridium acetobutylicum ATCC 4259 was used throughout the study. Preserved soil cultures were prepared and maintained as described by Kim *et al.* (9).

Medium and Culture Conditions

Strictly anaerobic procedures were employed throughout medium preparation and cell cultivation processes (9). Inoculum cultures and batch cultures were made using CAB medium (9). For the maintenance of acidogenic and solventogenic cultures a 2-stage phosphate-limited chemostat was operated. This chemostat used 0.1 $g \cdot l^{-1}$ of KH_2PO_4 in place of 0.7 $g \cdot l^{-1}$ of KH_2PO_4 and 0.7 $g \cdot l^{-1}$ of K_2HPO_4 of the CAB medium. The first growth vessel used a 1 *l* Bioflow fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) with a 460 m*l* working volume. The pH of the acidogenic culture was maintained at 5.5 ± 0.1 by using an automatic pH controller and 3 N NaOH solution. The second vessel was a 2 *l* Bioflow fermentor with a 125 m*l* working volume. The pH was controlled at 4.5 ± 0.1 by automatic addition of 3 N H_2SO_4 or 3 N NaOH. The culture vessels were kept anaerobic by a continuous flow of O_2-free N_2 at a rate of 50 $ml \cdot min^{-1}$, and the medium reservoir was kept under a positive pressure of about 5 psig of N_2. The cultures were stirred at 100 rpm. The dilution rate was regulated to 0.27 hr^{-1} for the first vessel and 0.1 hr^{-1} for the second vessel, by inflow of fresh medium into the first vessel using a peristaltic pump. Overflow from the first vessel to the Second vessel, and from the second vessel to the culture receiver, were at the same rate as the inflow of fresh medium into the first vessel. Two bubble traps were used between the medium reservoir and the first vessel to prevent bacterial contamination of the reservoir from the growth vessel.

Quantification of Substrate, Fermentation Products and Growth

Glucose was determined by the glucose oxidase method and growth was measured by optical density at 660 nm (9). Soluble fermentation products were analyzed by gas chromatographic methods using column packed with a Chromosorb 101 for acetate, butyrate and butanol, and a Super Q column for ethanol and acetone (9). H_2 was also determined by gas chromatographic method (9).

Cell-free Extract Preparation

Enzyme activities were measured using cell-free extracts or cell suspensions prepared under anaerobic conditions (14). Cell-free extracts of acidogenic cells were prepared by passing cell suspensions through a French press cell at a pressure of 1400 kg/cm^2 after the cells were suspended in anaerobic distilled water containing 3 mM dithiothreitol and 2 $g \cdot ml^{-1}$ DNase. The solventogenic cell suspension (about 20 mg dry $cell \cdot ml^{-1}$) was treated with 50 $unit \cdot ml^{-1}$ Mutanolysin (Sigma Chem. Co.) at 37℃ for 1 hr using 0.1 M phosphate buffer (pH 6.8) before being frozen, thawed and passed through a French press cell under the same conditions as above. The protein content was determined by Lowry's method (15). Cell suspensions, as an enzyme source, were prepared by suspending cells in the same buffer as in the enzyme assay.

Enzyme Assays

Anaerobic conditions were employed in all enzyme assays (14). Hydrogenase activities were measured in both directions of H_2 consumption and production using cell suspensions.

H_2 consumption activities were assayed spectrophotometrically with an Eppendorf recording spectrophotometer (Brinkmann Instruments, Inc., Westbury, N.Y.). Activity was measured by recording the reduction of the artificial electron acceptors, methyl viologen (redox potential -440 mV), neutral red (-325 mV), and methylene blue ($+11$ mV). The assay was performed at 37℃ in a 1.6 m*l* cuvette. The anaerobic reaction mixture (1 ml) contained an electron acceptor (2 mM methyl viologen, 0.12 mM neutral red or 50 M methylene blue), 2 mM dithiothreitol, and 0.1 M Tris-HCl buffer (pH 7.5) or 0.1 M phosphate buffer (pH 6.5) under 1 atm H_2 pressure in the headspace. The reaction was started by adding cell-free extract or cell suspension to the anaerobic prewarmed cuvette. Extinction coefficients of the dyes were; methyl viologen, $\varepsilon_{578\,nm} = 9.18$ $mM^{-1} \cdot cm^{-1}$; neutral red, $\varepsilon_{540\,nm} = 7.12$ $mM^{-1} \cdot cm^{-1}$; methylene blue, $\varepsilon_{590\,nm} = 30.90$ $mM^{-1} \cdot cm^{-1}$ at pH 7.5,

and methyl viologen, $\varepsilon_{578\,nm}=8.46\ mM^{-1}\cdot cm^{-1}$; neutral red, $\varepsilon_{540\,nm}=16.68\ mM^{-1}\cdot cm^{-1}$; methylene blue, $\varepsilon_{590\,nm}=37.34\ mM^{-1}\cdot cm^{-1}$ at pH 6.5.

Hydrogen producing hydrogenase activities were determined by measuring H_2 evolution from the artificial electron carriers, methyl viologen and neutral red, reduced by sodium dithionite. The reaction mixture consisted of 0.1 M Tris-Hcl buffer (pH 7.5), or 0.1 M phosphate buffer (pH 6.5) containing 10 mM sodium dithionite and the electron carrier (2 mM methyl viologen or 0.12 mM neutral red). Vials (25 *ml*), each containing 2 *ml* of reaction mixture, were prewarmed at 37℃ using a shaking water bath (200 stroke$\cdot$min^{-1}) before the reaction was started by adding the cell suspension. Samples from the head space were taken every 3 min for 60 min to analyze H_2 production by gas chromatographic method. The H_2 accumulation was linear up to 30 min in all assays. Blanks without electron carriers produced no detectable H_2.

Pyruvate dehydrogenase (Co-A acetylating, E.C.1.2.7.1) activity was determined by monitoring the reduction of methyl viologen or neutral red, as electron acceptors (12). The reaction mixture contained 5 mM pyruvate, 0.1 mM coenzyme A, 7 mM sodium arsenate and either 2 mM methyl viologen or 0.1 mM neutral red in 1 *ml* Tris-HCl buffer (pH 7.8). Assay was performed under the same conditions as hydrogen consuming hydrogenase assays.

NAD(P)-ferredoxin oxidoreductase activities were measured by the spectrophotometric methods described by Blusson *et al.* (4) and Petitdemange *et al.* (17). Ferredoxin-NAD(P) reductase activities were determined by monitoring the reduction of NAD or NADP at 334 nm ($\varepsilon_{334\,nm}=6.2\ mM^{-1}\cdot cm^{-1}$) with pyruvate as the reductant of ferredoxin. The reaction mixture for ferredoxin-NAD reductase contained 0.1 M Tris-HCl buffer (pH 7.6), 2.5 mM NAD, 0.15 mM coenzyme A, 25 mM FAD, 12 mM pyruvate, and 12 mM $KH_2A_3O_4$. The anaerobic cuvette containing 1 *ml* of reaction mixture was prewarmed at 37℃ after the headspace gas was replaced with carbon monoxide. The reaction was started by adding pyruvate. For the ferredoxin-NADP reductase assay, NAD was replaced by an equal quantity of NADP.

NAD(P)H-ferredoxin reductase activities were determined by monitoring the reduction of metronidazole at 320 nm ($\varepsilon_{320\,nm}=9.31\ mM^{-1}\cdot cm^{-1}$). The reaction mixture for NADH-ferredoxin reductase contained 0.1 M Tris-HCl buffer (pH 7.6), an NADH generating system, an acetyl-CoA generating system, 10 μM FAD, 24 μg$\cdot$ml^{-1} of ferredoxin and 0.1 mM metronidazole. For the NADPH-ferredoxin reductase assays, NADPH generating system was used in place of an NADH generating system. The NADH generating system consisted of 0.25 mM NADH, 30 μ*l* of ethanol and 45 units of yeast alcohol dehydrogenase. The NADPH generating system contained 0.5 units of porcine heart isocitrate dehydrogenase, and 2.5 mM trisodium DL isocitrate. Acetyl-CoA was generated using 2 units of phosphotransacetylase with 20 mM acetylphosphate and 1 mM coenzyme A in the presence of 30 mM $(NH_4)_2SO_4$ and 2 mM reduced glutathione.

NAD(P)-neutral red oxidoreductase activities were measured at 540 nm ($\varepsilon_{540\,nm}=7.12\ mM^{-1}\cdot cm^{-1}$). The reaction mixture for the neutral red-NAD(P) reductase assay contained 0.1 M Tris-HCl buffer (pH 7.6), either 0.4 mM NAD or NADP, 0.15 mM neutral red and 2 mM dithiothreitol. About 90% of the dye was reduced by sodium dithionite before the reaction was started by the addition of cell-free extract. For the NAD(P)H-neutral red reductase assay either NADH or NADPH was used in place of NAD or NADP with chemical reduction of the dye.

RESULTS

Initial Growth under H_2 Gas Phase

The CAB medium was made using 30 g$\cdot$*l*$^{-1}$ glucose with varying concentrations of 0 to 100% H_2 in the headspace of anaerobic pressure tubes. The initial growth rate was determined by measuring OD660nm changes during 12 hrs static culture at 35℃ after the tubes were inoculated with 24 hr old 5% inoculum culture (Table 1). Growth was not inhibited by H_2 gas at the initial growth phase. Due to autolysis, the changes in cell concentration were not consistent at later stages of growth.

Table 1. Initial growth rate of *Clostridium acetobutylicum* in the presence of hydrogen in the headspace.

Initial H_2 Partial Pressure in Headspace (%)	Growth Rate (hr^{-1})
0	0.52
2.5	0.47
5.0	0.44
10.0	0.50
25.0	0.48
50.0	0.52
75.0	0.55
100.0	0.52

H_2 was added to anaerobic pressure tubes containing 10 *ml* of CAB medium with 30 g$\cdot$*l*$^{-1}$ glucose before autoclave. Turbidity was monitored up to 10 hr after 24 hr old 5% culture was inoculated. The initial pH was 5.4.

H_2 Consumption by Acidogenic and Solventogenic Cultures

Samples taken from the 2-stage chemostat were used to measure H_2 consumption. Using syringes 10 *ml* samples were placed into anaerobic pressure tubes, filled with H_2 from the chemostat, then the tubes were centrifuged at room temperature. The cell pellets were resuspended in fresh medium at pH 5.5 for the acidogenic culture, and at pH 4.5 for the solventogenic culture with and without 5 mM neutral red, and incubated at 34℃. Changes in H_2 content were monitored by gas chromatographic methods (Fig. 1). The acidogenic culture produced H_2 without a lag time under a 100% H2 atmosphere, while H_2 was consumed by the solventogenic culture before a rapid increase in H_2 production. Neutral red did not have any effects on the H_2 metabolism of acidogenic culture, but the dye increased the H_2 consumption of the solventogenic culture.

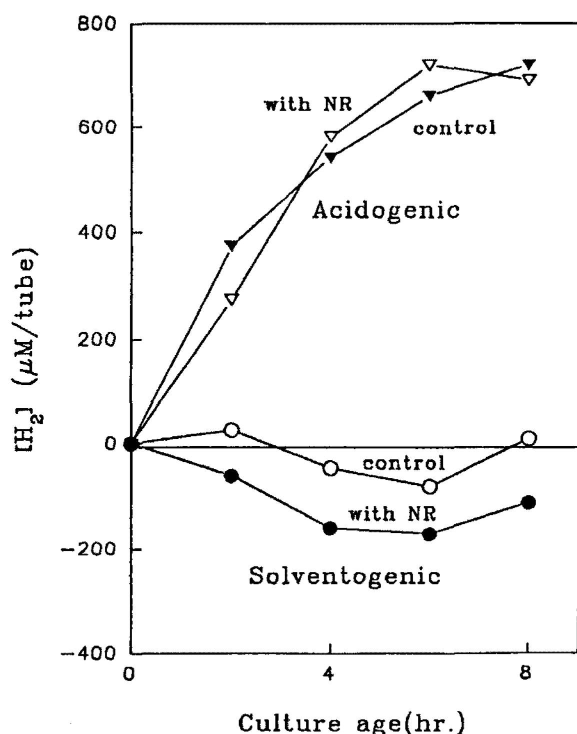


Fig. 1. Hydrogen production and consumption by acidogenic and solventogenic cultures.

Samples (10 *ml*) from a phosphate limited two-stage chemostat were placed into anaerobic pressure tubes, filled with H_2, then the tubes were centrifuged. The cells were resuspended in fresh CAB medium at pH 5.5 for the acidogenic culture, and at pH 4.5 for the solventogenic culture before being incubated in a water bath (34℃). Samples were taken from the headspace to analyze H_2 content. The initial value of H_2 (about 830 $\mu M \cdot tube^{-1}$) was substracted from the measured value of H_2.

Changes in Glucose Consumption by H_2

A 2-stage chemostat was gassed by N_2 for 6 days to obtain a steady-state before the gas was changed to hydrogen. Remaining glucose concentrations were determined in the samples taken from both growth vessels gassed by N_2 and H_2 (Table 2). No difference in glucose concetrations was observed between H_2 and N_2 gassed conditions in the acidogenic first growth vessel, while less glucose was consumed in the solventogenic second growth vessel when the culture was gassed with H_2 than when gassed with N_2. The reduced consumption of glucose probably resulted from H_2 reduction of the pyridine nucleotide pool of the solventogenic culture.

Hydrogenase Activities Coupled with Different Artificial Electron Carriers

Samples were taken from a 2-stage chemostat to prepare cell suspensions for the determination of hydrogenase activities coupled with artificial electron carriers (Table 3). Significant differences in activities were measured between the two cultures using methyl viologen and neutral red, but similar activities were measured using methylene blue. The acidogenic culture showed much higher activities, both in production and consumption, than the solventogenic culture when the reactions were coupled with methyl viologen. At pH 7.5 the activities were higher than at pH 6.5 in all assays using methyl viologen. On the other hand, the solventogenic culture showed a higher activity coupled with neutral red than the acidogenic culture, and consumption activities were higher at pH 6.5 than at pH 7.5.

Enzyme Activities of Oxidation-Reduction

Cell-free extracts were made from the acidogenic culture grown at pH 5.5 for 24 hr and from the solventogenic culture grown at pH 4.5 for 24 hr. They were used to measure enzyme activities involved in electron flow

Table 2. Effects of hydrogen bubbling on glucose consumptions in acidogenic and solventogenic cultures of two-stage chemostat.

Culture	Dilution Rate (hr^{-1})	Gas	Glucose Concentration in Culture (mM)
Acidogenic	0.27	N_2	178.3
	H_2	176.0	
Solventogenic	0.1	N_2	48.5
	H_2	69.5	

A phosphate-limited two-stage chemostat was maintained using CAB medium with 45 $g \cdot l^{-1}$ glucose at pH 5.5 in the first vessel, and at pH 4.5 in the second vessel. Under these conditions, the first vessel maintained acidogenesis, and the second solventogenesis. With each gas, the culture was run for 5 days in order to obtain steady state before taking samples for glucose measurement. The gassing rate was 0.5 volume.volume $culture^{-1} \cdot min^{-1}$.

Table 3. Hydrogen activity coupled to different electron carriers.

e^- Carrier	pH	Activity (μM H_2 mg $cell^{-1}min^{-1}$)			
		Acidogenic Culture		Solventogenic Culture	
		H_2 Production	H_2 Uptake	H_2 Production	H_2 Uptake
Methyl Viologen	6.5	3.31	1.15	0.65	0.61
(E=−440 mV)	7.5	4.31	7.13	1.01	1.76
Neutral Red	6.5	0.18	0.40	0.31	1.04
(E=−325 mV)	7.5	0.33	0.25	0.54	0.81
Methylene Blue	6.5	NA	1.43	NA	1.66
(E=+11 mV)	7.5	NA	10.03	NA	7.28

NA: not assayed.
Activities were determined using cell suspensions in 0.1 M Tris-HCl buffer at pH 7.5, and 0.1 M phoshpate buffer at pH 6.5. H_2 production activities were measured by monitoring the increase in H_2 concentration after the electron carriers were reduced using dithionite. Methylene blue was not used to measure H_2 production activities because of the diference in redox potentials. H_2 uptake activities were measured spectrophotometrically at 578 nm for methyl viologen ($\varepsilon_{pH\ 7.5}=9.18\ mM^{-1}\cdot cm^{-1}$, $\varepsilon_{pH\ 6.5}=8.46\ mM^{-1}\cdot cm^{-1}$), at 540 nm for neutral red ($\varepsilon_{pH\ 7.5}=7.12\ mM^{-1}\cdot cm^{-1}$, $\varepsilon_{pH\ 6.5}=16.68\ mM^{-1}\cdot cm^{-1}$), and at 590 nm for methylene blue ($\varepsilon_{pH\ 7.5}=30.90\ mM^{-1}\cdot cm^{-1}$, $\varepsilon_{pH\ 6.5}=37.34\ mM^{-1}\cdot cm^{-1}$).

Table 4. Activities of oxidation-reduction enzymes coupled with neutral red (NR).

Enzyme	Activity (μM·mg $protein^{-1}\cdot min^{-1}$)	
	Acidogenic	Solventogenic
Hydrogenase	2.80	3.01
DEAE treated	2.50	2.97
+24 $\mu g\cdot ml^{-1}$ Fd	2.33	3.05
Pyruvate dehydrogenase	0.49	0.23
DEAE treated	0.48	0.23
NR-NAD reductase	0.33	8.8×10^{-3}
MR-NADP reductase	0.10	7.6×10^{-3}
NADH-NR reductase	0.08	0.14

Activities were determined spectrophotometrically at 540 nm ($\varepsilon=7.12\ mM^{-1}\cdot cm^{-1}$) using cell-free extracts from cultures grown at pH 5.5 (acidogenic culture), and pH 4.5 (solventogenic culture) for 24 hr. Ferredoxin was removed from cell-free extracts by treatment with DEAE cellulose.

using neutral red as an electron carrier (Table 4). It was found that hydrogenase and pyruvate dehydrogenase activities, coupled with neutral red, were independent of ferredoxin in both cultures. Though the activities were low, electrons were transferred between neutral red and pyridine nucleotides.

NAD(P)-ferredoxin Oxidoreductase Activities

NAD(P)-ferredoxin oxidoreductase activities were measured using cell-free extracts (Table 5). Generally, the activities were low, compared to the other enzyme activities in electron flow. Activities of NADP-ferredoxin oxidoreductase were higher than NAD-ferredoxin in the acidogenic culture. These results indicate that NAD-ferredoxin oxidoreductase operates to oxidize NADH for a catabolic purpose, and NADP-ferredoxin oxidoreductase operates to reduce NADP for an anabolic purpose. This is in agreement with Petitdemange (17).

Table 5. Ferredoxin-NAD(P) oxidoreductase.

Culture	Activity (nM·mg $protein^{-1}\cdot min^{-1}$)			
	Fd Oxidation by		Fd Reduction by	
	NAD	NADP	NADH	NADPH
Acidogenic	4.85	8.92	2.49	5.78
Solventogenic	2.33	11.82	3.58	4.86

Fd: ferredoxin
The methods of Blusson *et al.* (4) and Petitdemange *et al.* (17) were followed to measure the activities using cell-free extract similar to Table 4. Pyruvate was used as the reductant of ferredoxin in ferredoxin-NAD(P) reductase assays. In NAD(P)H-ferredoxin reductase assays, NADH was regenerated by ethanol and yeast alcohol dehydrogenase, and NADPH was regenerated by isocitrate and isocitrate dehydrogenase from porcine heart.

Shift of Carbon and Electron Flow by Neutral Red

CAB medium with 30 $g\cdot l^{-1}$ glucose was prepared in anaerobic pressure tubes with and without 5 mM neutral red, before being inoculated with 5 % inoculum culture grown at 34℃ for 24 hr. The tubes were incubated at 34℃ for 72 hr before soluble fermentation products and H_2 were analyzed by gas chromatographic methods (Table 6). H_2 production was reduced by half in the presence of 5 mM neutral red, and excess electrons were used to produce more butanol, at the expense of less reduced products, including acetate, acetone and

Table 6. Shift of carbon and electron flows due to neutral red.

Treatment	H_2 Production	Fermentation Products (mM)				
	(μM/tube)	Ethanol	Acetone	Acetate	Butanol	Butyrate
Control	1498.7	5.9	18.2	26.3	32.7	17.1
NR (5 mM)	789.9	6.0	4.3	17.4	53.2	8.9

5 mM of Neutral red was added to an anaerobic pressure tube containing 30 $g \cdot l^{-1}$ of glucose CAB medium after autoclave. The tubes were inoculated by 24 hr old culture. The initial pH was 5.4.

butyrate.

DISCUSSION

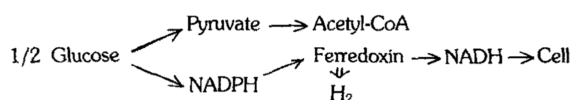
Previous studies (9, 10) showed that hydrogenase activity decreased as the *C. acetobutylicum* culture became solventogenic, producing less H_2. The acidogenic culture showed higher hydrogenase activities than the solventogenic culture when the activities were measured either by tritium exchange method or by the method of oxidation or reduction of methyl viologen. In this study, we found that high hydrogen partial pressure does not reduce the initial growth rate of the bacterium, and that the acidogenic culture produces H_2, which was consumed by the solventogenic under a 100% H_2 atmosphere. Less glucose was consumed by the solventogenic culture when gassed by H_2 than N_2, but glucose consumption by the acidogenic culture was not affected by gassing with either N_2 or H_2. These results suggest that different hydrogenases operate in the acidogenic and solventogenic cultures. The hydrogenase activity active in the acidogenic stage decreases in the solventogenic phase, when a second hydrogenase activity appears.

This hypothesis was supported by hydrogenase activities measured using different electron carriers. The activity coupled with methyl viologen was higher in the acidogenic culture than in the solventogenic culture, but the solventogenic culture showed higher hydrogenase activity coupled with neutral red than the acidogenic culture. This result suggests that the hydrogenase active in the solventogenic culture uses an electron carrier other than ferredoxin.

Several enzymes involved in electron flow showed activities using neutral red as an electron carrier. These include oxidoreductases of pyruvate, and pyridine nucleotides.

Based on the experimental results, the following electron metabolism is proposed for *C. acetobutylicum* (Fig. 2). During the acidogenic phase of fermentation the first hydrogenase oxidizes reduced ferredoxin to produce H_2, and NADH was reoxidized by ferredoxin. When the first hydrogenase activity decreases at the onset of solventogenesis, the electron flow is diverted from H_2 formation

ACIDOGENIC CULTURE



SOLVENTOGENIC CULTURE

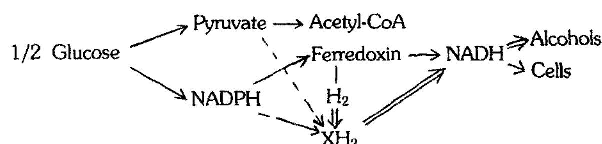


Fig. 2. Model of electron flow in acetone-butanol fermentation.
Bold lines show the major flux, and dotted lines show probable reactions.

to reduction of NADP, which is the electron carrier for the major butanol dehydrogenase (7, 11). This second hydrogenase consumes H_2 to reduce an unknown electron carrier, which in turn reduces NADP. The second hydrogenase has activities in both H_2 consumption and production using an electron carrier other than ferredoxin with similar characteristcs to neutral red.

Lactate is fermented by *C. acetobutylicum* in the presence of fermentable sugar (12). The NAD-dependent lactate dehydrogenase of the bacterium is active only in the direction of pyruvate reduction (6). The possiblity exists that there is another lactate dehydrogenase which catalyzes the oxidation of lactate, to reduce the unknown electron carrier.

In *C. acetobutylicum*, hydrogenase activity measured using methylene blue was found not to change during fermentation, though activity coupled with methyl viologen and with neutral red, as well as the tritium exchange activity (10), changed as the culture became solventogenic. This might be the result of methylene blue being a non-specific electron carrier for both of the enzymes operating during the fermentation, while methyl viologen and neutral red are specific for individual enzymes.

The butanol yield was increased with a decreased pro-

duction of H_2 and less reduced carbon compounds in the presence of neutral red. Neutral red seems to be able to mediate the electron flow in *C. acetobutylicum*, but further studies are needeed to clarify the role of neutral red in *C. acetobutylicum* fermentation.

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