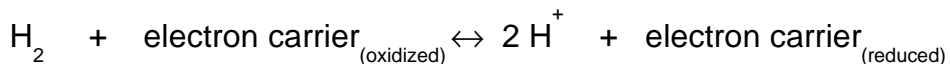


INTRODUCTION

Hydrogenase catalyzes the reaction involving the simplest molecule, H₂ :



Different types of hydrogenase have been purified and characterized. They differ in their structural and functional properties, including electron carrier specificity, cofactor content, sensitivity to inactivation by molecular oxygen and the physiological direction of the reaction they catalyze.

The physiological direction of the reaction depends upon the role of hydrogenase in an organism. The reaction can be in both directions when catalyzed by a bidirectional hydrogenase or it can be predominantly in one direction when catalyzed by a unidirectional hydrogenase (Chen and Blanchard, 1978, 1984). The enzyme enables an organism either to use H₂ as a source of reducing power and energy or to use protons as a terminal electron acceptor for the disposal of excess reducing power.

The distribution of hydrogenase is widespread among microorganisms. Besides the presence of hydrogenase in many bacteria, it is also present in some eukaryotes including algae, fungi and protozoa (Mortenson and Chen, 1974; Wu and Mandrand, 1993). Hydrogenase activity was reported in some higher organisms such as higher plants and frog eggs, but further research revealed that these observations were due to bacterial contamination (Schlegel and Schneider, 1978).

With one exception (Zirngibl et al., 1992), all hydrogenases have been reported to be iron-sulfur proteins. Besides iron, many of them also contain nickel or nickel and selenium. Therefore, based on their metal content, they are divided into three categories; iron-only hydrogenases (Adams, 1990), nickel-containing hydrogenases, and nickel-selenium containing hydrogenases (Przybyla et al., 1992). After the development of molecular cloning techniques, the genes encoding many hydrogenases have been cloned and sequenced. Based on their metal content, physiological function, and particularly amino acid sequence homologies, thirty sequenced hydrogenases have been classified into six classes and they appear to have evolved from at least three different ancestors (Wu and Mandrand., 1993). The six classes are:

Class I: Hydrogen-uptake membrane bound Ni-Fe hydrogenases from aerobic, facultative anaerobic and anaerobic bacteria.

Class II: Periplasmic and membrane-bound hydrogen-uptake Ni-Fe (Se) hydrogenase from sulfate-reducing bacteria.

Class III: Fe-only hydrogenase from strictly anaerobic bacteria.

Class IV: Soluble Ni-Fe (Se) hydrogenase from methanogenic bacteria and aerobic hydrogen-oxidizing bacteria.

Class V: Labile hydrogen-producing hydrogenase of *Escherichia coli*

Class VI: Soluble hydrogenases of cyanobacteria.

Classes III and VI are related to neither each other nor the other classes.

The results of homology analysis revealed that almost all hydrogenases have some common motifs probably participating in the formation of hydrogen and the transfer of electrons.

Current research on hydrogenase has an emphasis on understanding the structure-function relationships within this heterogeneous group of enzymes and on the regulatory properties that pertain to the physiological roles of the enzyme and possible applications of metabolic engineering for improving the performance of H_2 -metabolizing fermentative organisms.

LITERATURE REVIEW

Historical background

The first report of hydrogen gas production by microorganisms was made in 1887 by Hoppe-Seyler. However, until 1931 there was no report on the existence of an enzyme that catalyzed the hydrogen evolution reaction. Stephenson and Stickland (1931) were the first to describe the existence of an enzyme that catalyzes the hydrogen activation reaction in *E. coli* and named it hydrogenase. After the discovery of hydrogenase in *E. coli*, there were many attempts to isolate and characterize the enzyme. Starting from Bovarnick (1941), the first person who reported an active cell-free extract of hydrogenase from *E. coli*, the early work was not very successful due to the difficulty of working with membrane-bound type of hydrogenase. The discovery of a cytoplasmic hydrogenase in clostridia made purification of hydrogenase more feasible. In 1971, Nakos and Mortenson reported apparently homogeneous preparations (38-fold purified) of hydrogenase of *Clostridium pasteurianum* W5. Three years later, Chen and Mortenson (1974) reported further purification of the enzyme from *C. pasteurianum* W5 and it required a 320-fold purification to reach homogeneity; this hydrogenase was shown to be an iron-sulfur protein. With improvements in purification methods and the use of strictly anaerobic techniques since the 1970s, a tremendous amount of research on hydrogen-metabolizing organisms and on hydrogenase has ensued. This increase in interest of hydrogenase research is due in part to the fact that hydrogen gas produced from biomass and waste organic compounds is a promising alternative energy for the future. In the 1980s, developments in the metalloenzyme field changed our understanding of hydrogenase. The discovery of 3Fe-cluster and nickel in hydrogenase was made during this period (Przybyla et al., 1992). In 1995, the first crystal structure of the nickel-iron hydrogenase from *Desulfovibrio gigas* was reported and based on this crystal structure a plausible electron and proton transfer pathway was suggested (Volbeda et al., 1995).

Structural and functional properties of hydrogenases

a) Structural and functional properties of nickel-containing hydrogenases

Nickel-containing hydrogenases have been isolated and characterized from many different organisms, e.g., methanogens, sulfate-reducing bacteria and some nitrogen fixing bacteria. They are considered as the major and the most widely present hydrogenases. They are generally oxygen-stable and form a heterogeneous group. Their iron contents ranges from 4 to 40 g atom/mole, and their molecular mass ranges from 50,000 to 750,000 Dal, and they contain one to four subunits (Graf and Thauer., 1981 ; Voordouw et al., 1989). Viologen dyes and methylene blue are the artificial electron carriers, and NAD^+ , ferredoxin, flavodoxin, the deazaflavin Factor 420 (F_{420}), and cytochrome c_3 are the natural electron carriers for nickel-containing hydrogenases. There is no unique cellular location for nickel-containing hydrogenases. They can be cytoplasmic (Schneider et al., 1979), periplasmic (Hatchikian et al., 1978) or membrane-bound (Adams and Hall, 1978). Some of the

nickel-containing hydrogenases also contain selenium atom (Yamazaki, 1982). The role of selenium is not exactly known, but it was proposed that selenium has a role in the regulation of enzymatic activity (Teixeira et al., 1987). Based on their subunit structure, nickel-containing hydrogenases are divided into two groups: monomeric ones having a single nickel-ligating polypeptide and multimeric ones having two dissimilar subunits, one of which contains nickel. The most extensively characterized heterodimeric nickel-containing hydrogenase is the hydrogenase of *Desulfovibrio gigas* (Volbeda et al., 1995). This periplasmic enzyme has two dissimilar subunits with molecular weights of 28,000 and 60,000. Nickel and an unknown metal are associated with the large subunit. The small subunit has two [4Fe-4S] clusters and one [3Fe-4S] cluster. The proposed role of the [4Fe-4S] clusters is in electron transfer from nickel to an external electron carrier which in this case is cytochrome c_3 . There is no definitive evidence about the role of the [3Fe-4S] cluster, but it may be involved in oxygen stability or electron transfer to or from an unidentified electron donor or acceptor. The hydrogenase of *Chromatium vinosum* is a good representative for monomeric nickel-containing hydrogenases (Albracht et al., 1983). It has a molecular weight of 60,000 and contains a single nickel and a non-heme iron center. The iron-sulfur center is organized as a [3Fe-4S] cluster but under appropriate conditions it may be present as a [4Fe-4S] cluster. Another example for monomeric nickel-containing hydrogenases is the hydrogenase I of *E. coli* (Francis et al., 1990). It is a membrane-bound hydrogenase, and it is homologous to the large subunits of multimeric nickel-containing hydrogenases. Based on this finding, they proposed that the large subunit of multimeric, nickel-containing hydrogenase has the ability to catalyze hydrogen activation in the absence of the small subunit.

b) Structural and functional properties of iron-only hydrogenases

In 1984, Peck, LeGall and co-workers showed that the periplasmic *Desulfovibrio vulgaris* hydrogenase does not contain nickel (Huynh et al., 1984). After this important discovery, it has been demonstrated in several laboratories that hydrogenases from several strictly anaerobic fermentative bacteria, e.g., *Clostridium pasteurianum* and *Megasphaera elsdenii* do not contain nickel (Adams and Mortenson, 1984). *C. pasteurianum*, a mesophilic anaerobe, is unique in that it possesses two different iron-only hydrogenases (Chen and Blanchard, 1978, 1984). Whereas hydrogenase I is a bidirectional hydrogenase, hydrogenase II preferentially catalyzes hydrogen oxidation. In contrast to nickel-containing hydrogenases, iron only hydrogenases have not been widely found. All iron-only hydrogenases contain two or more [4Fe-4S] clusters (the F clusters) and a unique type of [Fe-S] center (the H cluster). The H cluster appears to be the site of hydrogen oxidation or hydrogen production (Adams, 1990). The F clusters function in electron transport between the H cluster and external electron carriers. The iron-only hydrogenases of *C. pasteurianum* and *M. elsdenii* are monomeric, cytoplasmic enzymes with a molecular weight of approximately 60,000, and they are very sensitive to oxygen and requires strictly anaerobic conditions for purification.

The physiological roles of hydrogenase

Metabolism consists of catabolic and anabolic reactions that together transform a variety of raw material into cellular content and excreted end products. During catabolism, oxidation reactions occur and the electrons that are released from these oxidation reactions are used for the synthesis of new cellular material (reductive anabolic reactions) or for energy conservation, but there is always an excess of released electrons in the form of reduced electron carriers. To balance the oxidation-reduction reaction (and to regenerate the oxidized electron carriers), a final electron sink is always necessary for the disposal of electrons that are no longer needed. The electron sink is provided by substances such as molecular oxygen, which are known as terminal electron acceptors.

While aerobic organisms use molecular oxygen as the terminal electron acceptor, anaerobic organisms use a variety of substances as the terminal electron acceptors. Hydrogen ions (protons) are used as a terminal electron acceptor by many anaerobes, such as the sugar-fermenting clostridia. In these organisms the use of omnipresent hydrogen ions as a terminal electron acceptor allows them to release the excess reducing power in the form of hydrogen gas. The enzyme hydrogenase catalyzes the formation of molecular hydrogen and thus plays a crucial role in maintaining the necessary redox balance in these organisms.

Fermentation of glucose by the strict anaerobe *Ruminococcus albus* is a good example of biological hydrogen production (Thauer, 1977). In this bacterium, glucose is converted to two pyruvate molecules by the glycolytic pathway, and two NADHs are produced along with a gain of two ATPs. Pyruvate is then metabolized to acetate and CO_2 for additional ATP production along with the reduction of the electron carrier protein ferredoxin (Fd). This organism is able to dispose of all the excess reducing power, resulting from pyruvate oxidation as well as the electrons primarily transferred to pyridine nucleotides in the form of hydrogen gas, if H_2 can be effectively removed during growth, and hence obtains up to three or four moles of ATP per mole of glucose.

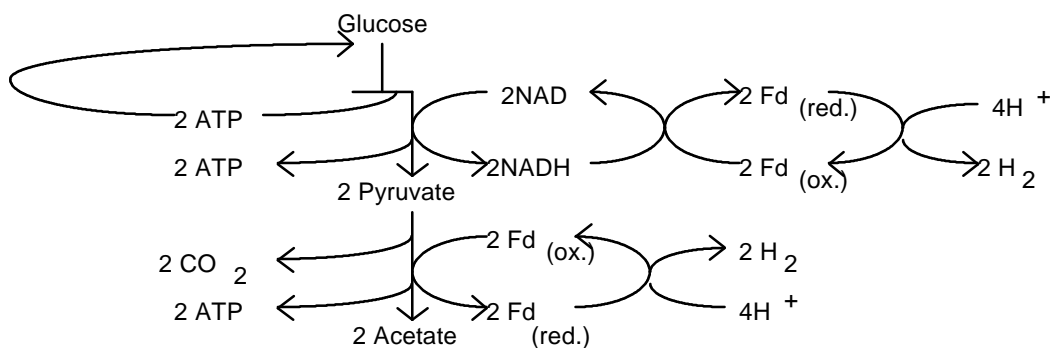


Figure 1. Glucose fermentation of *R. albus*.

In some organisms, hydrogenase catalyzes the H_2 -oxidation reaction (hydrogen uptake) and the reducing power released by this oxidation reaction is then used for reductive biosynthesis and energy conservation. *Alcaligenes eutrophus* H16 is an aerobic hydrogen-oxidizing bacterium (Bowien and Schlegel, 1981). In this organism, two structurally different hydrogenases have been purified. One of them is soluble, contains FMN as a coenzyme and reduces NAD^+ . The other one is membrane bound, flavin-free, and unable to react with pyridine nucleotides. The significance of these two hydrogenases lies in the fact that their physiological functions are different. Under autotrophic conditions, the membrane-bound enzyme provides the electrons to be used in the energy conserving process. The soluble enzyme is not involved in energy conservation; it reduces NAD^+ to $NADH$, which is then used in carbon dioxide fixation to form cellular material.

Methanobacterium thermoautotrophicum is a methane-producing bacterium and contains an active uptake hydrogenase which catalyzes the reduction of factor 420 (Graf and Thauer, 1981). This type of hydrogenase is significant since the major amount of hydrogen produced in the nature is trapped by methane-producing bacteria by the action of its uptake hydrogenase (Schlegel and Schneider, 1978). Reduced factor 420 then acts as the central electron donor for CO_2 reduction to form methane.

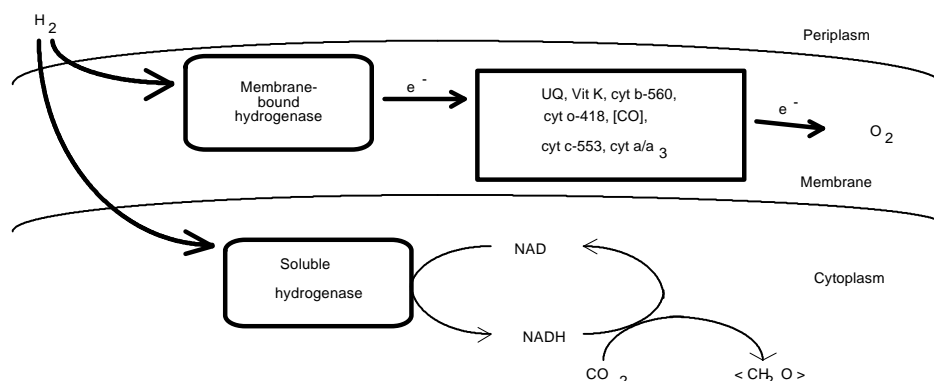


Figure 2. Hydrogenases in *Alcaligenes eutrophus* H 16

The importance of solvent production and the role of hydrogenase in solvent-producing clostridia

The production of acetone and n-butanol by bacterial fermentation was one of the most important industrial processes during the first half of this century. The organisms used for this purpose were members of the genus *Clostridium* (Prescott and Dunn, 1959). After the 1950s, the high costs of raw material and product recovery coupled with the success in obtaining these chemicals synthetically from petrochemicals caused a decrease in the use of this fermentation process. However, the supply of petroleum is limited, whereas uses for acetone and n-butanol have expanded. Acetone and n-butanol are now important feedstock chemicals and potential fuel additives, besides their traditional uses as solvents. The use of

renewable biomass as the raw material for the production of these chemicals will decrease the pressure placed on the fossil resources and the environment. These considerations have renewed interests in the acetone-butanol fermentation. There is now active research worldwide to improve this fermentation, and one area of new research is to use metabolic engineering to improve the yield or the rate of this fermentation.

In batch cultures of the solvent-producing clostridia, two metabolic phases are present (Chen, 1993). In the early acidogenic phase, a typical butyric acid fermentation is carried out with the production of acetic acid, butyric acid, hydrogen and carbon dioxide (Fig. 3). After the acidogenic phase, the strictly anaerobic, spore-forming bacteria start to produce mainly n-butanol and acetone. Therefore, the late phase is known as the solventogenic phase. Following the glycolytic pathway, the acid and solvent producing pathways share a sequence of reactions from pyruvate to butyryl~CoA (Figure 3, reactions 8, 9, 10, 11 following pyruvate oxidation). The reason for solvent production to occur has been proposed as a method of detoxification (Gottschal and Morris, 1981; Terracciano and Kashket, 1986; and references therein). It was suggested that in the early exponential phase of growth, continuous production of acetate and butyrate causes an accumulation of these undissociated acids in the environment. Since these acids are membrane permeable and are able to disturb the transmembrane proton gradient, they are harmful to the organism if their concentrations reach a certain level. In order to prevent the harm that can be caused by these undissociated acids, the cell starts converting them into less toxic neutral solvents. When the cell switches from acid production to solvent production, the sporulation process also starts. Therefore, another proposed reason for solventogenesis to occur is that it provides more time for the bacteria to form mature spores (Durre et al., 1995). However, the butyric acid-producing species, such as *Clostridium butyricum* and *C. pasteurianum*, do not require butanol formation to facilitate sporulation.

In the acidogenic phase, the oxidation of glucose by glycolysis results in the production of two NADHs. The conversion of pyruvate to acetyl~CoA and CO₂ produces reduced ferredoxin. In the ensuing acetate-producing pathway, there is no available reaction to oxidize the reduced NADH and Fd to maintain the cell's redox balance. The production of butyric acid also leaves an excess of reduced electron carriers, although it is only half of what is left during acetate production. Normally, 20% of acetyl~CoA is used in acetate production when ATP is formed via phosphotransacetylase and acetate kinase. Therefore, additional exit for the excess reducing power is necessary for the cell. Through the hydrogen evolution reaction catalyzed by hydrogenase, the clostridia are able to dispose of the excess reducing power without the need for other terminal electron acceptors than protons.

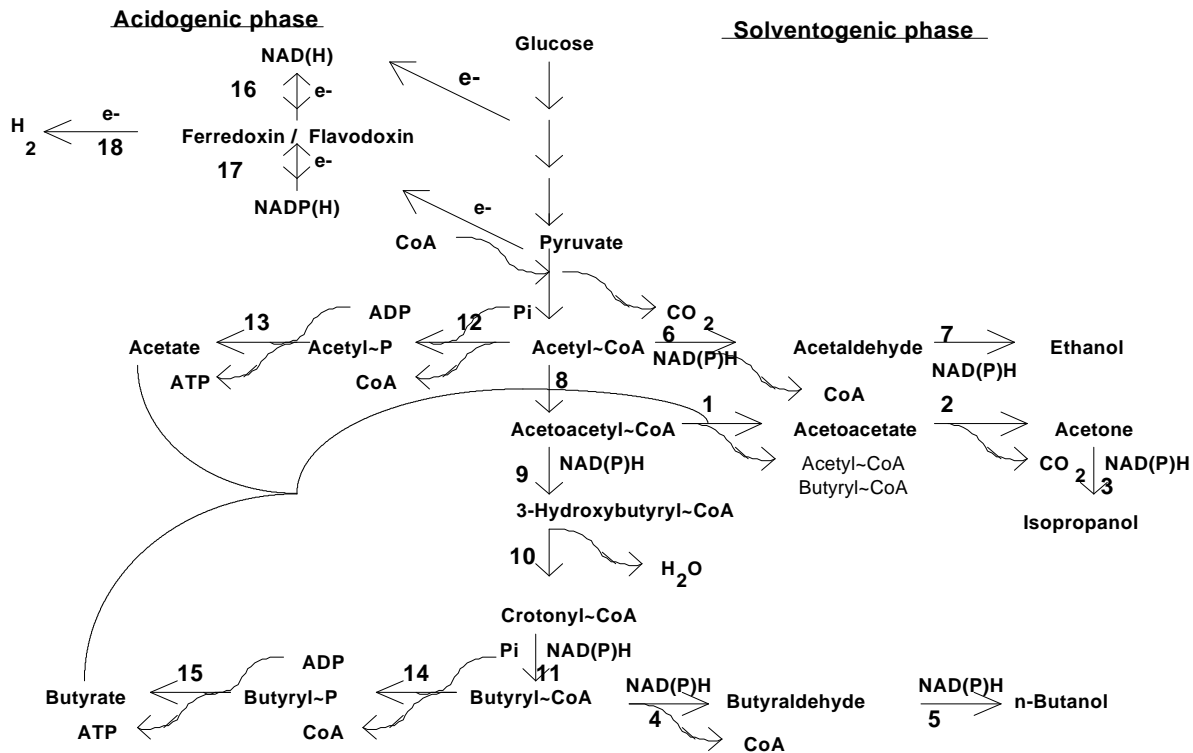


Figure 3. Metabolic pathway of acid and solvent production in clostridia. *C. beijerinckii* NRRL B593 carries out all the reactions depicted in this figure. Other solvent-producing species may carry out all or some of the reactions. Enzymes catalyzing the key reactions are (1) acetoacetate:butyrate/acetate CoA transferase; (2) acetoacetate decarboxylase; (3) alcohol (isopropanol) dehydrogenase; (4) aldehyde (butyraldehyde) dehydrogenase; (5) alcohol (butanol) dehydrogenase; (6) aldehyde (acetaldehyde) dehydrogenase; (7) alcohol (ethanol) dehydrogenase; (8) acetoacetyl~CoA thiolase; (9) 3-hydroxybutyryl~CoA dehydrogenase; (10) crotonase; (11) butyryl~CoA dehydrogenase; (12) phosphotransacetylase; (13) acetate kinase; (14) phosphotransbutyrylase; (15) butyrate kinase; (16) Fd:NAD⁺ oxidoreductase; (17) Fd:NADP⁺ oxidoreductase; (18) hydrogenase.

In the solventogenic phase, formation of n-butanol or ethanol will consume additional reducing power but the amount of excess in reducing power varies and is dependent on the use of alternative solvent-producing pathways. If no preformed acids were reutilized during solventogenesis, all the reducing power produced by glycolysis and by oxidation of pyruvate to acetyl~CoA would be used for concurrent production of n-butanol and ethanol. If metabolism of acetyl~CoA proceeds to acetone formation, which does not involve a reduction reaction, then all the reducing power produced by concurrent metabolism will have to be released by hydrogen evolution. Production of acetone is mechanistically coupled to the reutilization of preformed acids (Reaction 1 in Fig.3; Hartmantis et al., 1984), and the reutilized acetic and butyric acids become acetyl~CoA and butyryl~CoA, which are then used in the production of acetone, ethanol, or butanol, with the consumption of different amounts of reducing power as explained below.

The formation of an acetoacetyl~CoA from glucose is accompanied by the release of 8 electrons. The formation of acetyl~CoA or butyryl~CoA from preformed acids via the CoA transferase reaction (Figure 3, reaction 1) does not involve oxidation or reduction. When butanol is produced from preformed butyric acid, only four electrons or two moles of NAD(P)H are consumed for each mole of n-butanol formed, which leaves an excess of 4 electrons. When acetate is reutilized (forming acetyl~CoA), it can lead to the production of ethanol, acetone or n-butanol and in each case some of the reducing power is left unused. When ethanol is produced from this acetyl~CoA, only 4 electrons or two moles of NAD(P)H are used. The formation of an acetoacetyl~CoA from two acetyl~CoA that are produced via acetate will leave 16 electrons or 8 moles of NADH unused. When n-butanol is produced from this acetoacetyl~CoA only four moles of NAD(P)H are utilized. When acetone is produced from this acetoacetyl~CoA, no NAD(P)H is used. Therefore, in solventogenesis there is always excess reducing power to be disposed of in the form of hydrogen gas, but the amount is less than that from acidogenesis (Peterson and Fred, 1932; Kim and Zeikus, 1985)

Hydrogen production and hydrogenase level in acidogenic and solventogenic cells

Peterson and Fred (1932) reported the amount and the composition of gases produced at different stages of growth of *C. acetobutylicum* strain 105. They showed that at the early stage of fermentation, more hydrogen is produced than carbon dioxide, and the ratio reverses during solvent production. At the acidogenic stage, 80% of the total gas is hydrogen. At the solventogenic stage, even though the percentage of hydrogen decreases from 80% to 40%, there is a continuous net production of hydrogen. Kim and Zeikus (1985) compared the rate of hydrogen production and *in vivo* hydrogenase activity between acid-producing and solvent-producing cells of *C. acetobutylicum* ATCC 4259. A tritium exchange method was used in the measurement of *in vivo* hydrogenase activities. They found that in acid-producing cells, the specific activity of hydrogenase remained constant, but the shift from acidogenesis to

solventogenesis was accompanied by a decrease of about 50% in the specific activity of hydrogenase. When they measured the rate of hydrogen production, they observed three distinct metabolic phases. In the initial fast growth phase, the rate of hydrogen production was the highest. Between the fast growth phase and the solventogenic phase, the rate of hydrogen production decreases by about 50%. In the solventogenic phase, the rate of hydrogen production decreased by about 50% again, reaching a level which is 25% of that displayed by the fast growing acid-producing cells. Junelles et al. (1988) measured the hydrogen-uptake activity (methyl-viologen linked) in whole cells of *C. acetobutylicum* ATCC 824 at different stages of growth, and they reported a continuous decrease in hydrogenase activity after the peak of n-butanol production. These findings are in contrast to those reported by Andersch et al. (1983). They reported that the hydrogenase activity in the acidogenic cells (grown at pH 5.8) of *C. acetobutylicum* DSM 1732 is at the same level as in the solventogenic cells (grown at pH 4.3) when it was measured by the reduction of methylene blue. However, the hydrogenase activity of solventogenic cells could only be detected in the assay after a lag period of 10 to 15 minutes. The conclusion they made from this observation was that the hydrogenase from solvent-producing cells was present in an inactive form and hydrogenase was activated after a lag period when it was flushed 15 minutes with hydrogen gas. More recently, Gorwa et al. (1996) found that the hydrogenase activity in solventogenic cells of *C. acetobutylicum* ATCC 824 was lower than in the acidogenic cells when the activity was measured in the hydrogen evolution direction by gas chromatography and it cannot be activated by hydrogen flushing. In a study carried out with extracts of *C. beijerinckii*, George and Chen (1983) reported that the specific activities of hydrogenase, measured manometrically by using methyl viologen as the electron acceptor or donor, were lower in solvent-producing cells than in acid-producing cells, but the ratio of the hydrogen-uptake activity over the hydrogen-evolution activity did not change, suggesting the presence of one hydrogenase.

The growth of *C. acetobutylicum* ATCC 824 at neutral pH on mixtures of glycerol and glucose was studied by Vasconcelos et al. (1994). Their expectation was that glycerol would generate twice as much NADH as glucose and hence more NADH could be used for producing more hydrogen. However, the experimental results were quite surprising because a very low amount of hydrogen was produced, and most of the NADH was used for n-butanol and ethanol production. When they measured the activities of hydrogenase in extracts by using methyl viologen as the electron acceptor or donor, they could not detect any significant differences in activity when compared with the activity of hydrogenase in glucose grown cells. A similar study was performed by Girbal et al. (1995). When they added neutral red to a culture of *C. acetobutylicum* ATCC 824, they observed a decrease in hydrogen production and a sharp increase in alcohol production. However, when they measured the activities of hydrogenase in cell extracts they could not detect any activity loss when neutral red-treated cells were compared with cells not treated with neutral red. On the other hand, they found that the addition of neutral red increased the activity of Fd:NAD⁺ oxidoreductase and caused

the formation of a larger NADH pool. In iron limited cultures of *C. acetobutylicum* ATCC824, it was reported that the amount of solvents produced doubled, whereas the level of hydrogenase activity decreased 20-40% when methyl viologen was used as the electron acceptor (Junelles et al., 1988). These results are qualitatively in agreement with those from studies in which hydrogenase activity was inhibited by CO (Kim et al., 1984; Datta and Zeikus, 1985) and in that a decrease in hydrogenase activity is accompanied by an increase in n-butanol production. It suggests that in the solventogenic phase, hydrogen production through hydrogenase actually consumes more reducing power than what can be considered as excess and hence diminishes the potential for n-butanol production.

Modulation of electron flow in solvent-producing clostridia

The activity of hydrogenase can be modulated in several ways. For instance, flushing a culture with carbon monoxide [CO inhibits hydrogenase and forces the transfer of electrons from reduced ferredoxin to NAD(P)^+ resulting in an increase in availability of NAD(P)H (Datta and Zeikus, 1985) or increasing the partial pressure of hydrogen gas (Yerushalmi and Volesky, 1985; Doremus et al., 1985) resulted in a decrease in hydrogen production and caused an increase in n-butanol production. Another strategy to modulate electron flow in clostridia involved the use of artificial electron carriers such as methyl viologen (Rao and Mutharasan, 1986 ; Peguin et al., 1994). The redox potential of methyl viologen is close to that of clostridial ferredoxin (- 400mV). Therefore, methyl viologen is able to replace ferredoxin in ferredoxin-dependent reactions. Addition of methyl viologen to a culture increased the activity of ferredoxin- NADP^+ oxidoreductase 60-fold (reaction number 17 in Figure 3) and created an artificial electron transport chain (Peguin et al., 1994). Electrons flow through pyruvate:ferredoxin oxidoreductase to methyl viologen then from methyl viologen to ferredoxin: NAD^+ oxidoreductase (reaction number 16 in Figure 3). This altered electron transport pathway probably made less ferredoxin available for hydrogen production but more NADH available for ethanol and n-butanol formation (Rao and Mutharasan, 1986). The increase in the yield of alcohols by artificial modulation does not increase the yield of acetone because no reducing power is necessary for acetone formation.

Molecular characterization of hydrogenase genes of clostridia

Based on partial amino acid sequences determined from purified hydrogenase I of *C. pasteurianum*, oligonucleotide probes were designed for the cloning of the structural gene for this hydrogenase (Meyer and Gagnon, 1991). The gene has been cloned and sequenced, and it has a high A+T content (68 %) and shows a biased codon usage. The sequence of *C. pasteurianum* hydrogenase I is consistent with the assertion that the sequences of iron hydrogenases and nickel-iron hydrogenases are not related (Voordouw, et al., 1989; Meyer and Gagnon, 1991). *C. pasteurianum* hydrogenase I has been proposed to contain a H cluster and two F clusters (Adams, 1990). The deduced amino acid sequence of *C. pasteurianum* hydrogenase I showed that 22 cysteines are present in the protein with a calculated molecular weight of a

63,836. The presence of 8 cysteine residues in the N terminal region can accommodate two [4Fe-4S] clusters (the F clusters). Five cysteine residues in the C terminal region of the protein are proposed as the ligands for the H cluster. The amino acid sequence of hydrogenase I allows a comparison with sequences of other hydrogenases. Based on these comparisons, it was suggested that the H cluster, the center of hydrogen activation is conserved among hydrogenases regardless of the type and iron-sulfur content (Meyer and Gagnon, 1991). Using the cloned hydrogenase I gene as a probe, homologous hydrogenase genes have been cloned and sequenced from *C. acetobutylicum* ATCC 824 (Gorwa et al., 1996) and *C. acetobutylicum* P262 (Santangelo et al., 1995). The hydrogenase gene of *C. acetobutylicum* ATCC 824 encodes a 64,415-Da protein which shows a strong similarity to the hydrogenase I of *C. pasteurianum*. An analysis of the level of mRNA for hydrogenase in acidogenic and solventogenic cells showed an agreement with the level of activity in both types of cells. In solventogenic cell, the amount of hydrogenase mRNA and protein was lower than that in acidogenic cell. This indicates that expression of this hydrogenase is regulated at the transcriptional level. The *C. acetobutylicum* hydrogenase also appears to contain a H cluster and two F clusters as found in *C. pasteurianum*. The protein encoded by the *C. acetobutylicum* P262 hydrogenase gene has an 82% similarity and 67% identity with *C. pasteurianum* hydrogenase I. When RNA was isolated from different morphological and physiological stages of a batch culture of *C. acetobutylicum* P262, no difference was found in the expression of the gene during acidogenesis as opposed to solventogenesis (Santangelo et al., 1995). The organization of genes flanking the hydrogenase gene of *C. acetobutylicum* P262 differs from that in *C. acetobutylicum* strains DSM 792, DSM 1731 and ATCC 824 (Santangelo et al., 1995).

“*C. acetobutylicum* P262” has now been found to belong to another species whereas the other three strains of *C. acetobutylicum* are equivalent (Johnson et al., 1997), which suggests differences in the organization and regulation of the hydrogenase gene among different solvent-producing clostridia.

Purpose of study

When the sugar-fermenting clostridia produce acetic and butyric acids, the excess reducing power is disposed of as hydrogen gas through the action of hydrogenase. The quantitative aspect of the need to produce hydrogen gas for balancing the oxidation-reduction reactions is more complex in solvent-producing clostridia. In solvent-producing clostridia, the production of n-butanol or ethanol from concurrently metabolized glucose does not leave an excess of reducing power. However, the solvent-producing clostridia also produce acetone, and the production of acetone is mechanistically dependent on the reutilization of preformed acetic or butyric acids. When acetone and additional n-butanol and ethanol are produced from preformed acetic and butyric acids, an excess in reducing power occurs and the degree of excess in reducing power depends on the ratio of these products formed. The amount of hydrogen gas produced during the solventogenic phase thus reflects the

flow of carbon through alternative pathways. However, it is not certain whether or not hydrogenase only plays a passive role in removing the excessive reducing power, or hydrogenase may compete against the alcohol-producing enzymes for reducing power and hence has an active role in determining the amount of reducing power that is available for the alcohol-producing pathways.

The effect of carbon monoxide, an inhibitor for hydrogenase, on increasing n-butanol production by *C. acetobutylicum* (Kim et al., 1984) suggests that the routing of carbon through the alternative solvent-producing pathways is affected by the availability of reducing power in the solvent-producing cells. Previous studies showed a decrease in the rate of hydrogen production (Peterson and Fred, 1932; Kim and Zeikus, 1985) and in hydrogenase activities (George and Chen, 1983; Kim and Zeikus, 1985; Gorwa et al., 1996) when *C. acetobutylicum* or *C. beijerinckii* cells enter the solventogenic phase. However, other studies did not show a change in hydrogenase activities or the level of its mRNA between acid-producing and solvent-producing cells (Andersch et al., 1983; Santangelo et al., 1995). Because hydrogenase has not been purified from any solvent-producing species, some of the conflicting results may be partly caused by limitations associated with the use of whole cells or cell extracts in the measurements of hydrogenase activities. For instance, the activity measurements with whole cells may be more susceptible to inequilibrium of hydrogen gas between the gas and liquid phases, and hydrogenase may be more susceptible to oxygen inactivation in the cell extracts than in a more purified state. Furthermore, multiple hydrogenases may be present in solvent-producing clostridia, and their relative levels may change with the growth stage. The purpose of this study was therefore to measure hydrogenase activities in both the hydrogen-production and hydrogen-oxidation reactions using properly calibrated assays under strictly anaerobic conditions and using both acid-producing and solvent-producing cells as the source of hydrogenase. The objectives were to search for clues for the presence of multiple hydrogenases in *C. acetobutylicum* and to obtain stability information of the enzyme to facilitate its purification in an ensuing study. Properties of the hydrogenase(s) of the solvent-producing clostridia may be used in a rational approach to improve this fermentation.

MATERIALS AND METHODS

Materials

Tryptone, dextrose, yeast extract and 2,3,5-Triphenyl tetrazolium chloride were obtained from Fisher scientific Co. (Fair Lawn, N.J). Trizma base (Tris[hydroxymethyl]aminomethane), DNase I, lysozyme, Sephacryl S-300, glucose 6-phosphate dehydrogenase, acetoacetic acid (lithium salt), sodium dithionite, methylene blue, methyl viologen and antifoam C were obtained from Sigma Chemical Co. (St. Louis, Mo.); DE-52 was obtained from Whatman; the dye binding protein assay kit, acrylamide and bis were obtained from Bio-Rad laboratories (Richmond, Calif.).

Cultures of *C. acetobutylicum* ATCC 824

Stock cultures of *C. acetobutylicum* strain ATCC 824 (American Type Culture Collection) were stored as frozen spore suspensions at -70°C . To grow batch cultures, 4 ml of spore suspension was thawed at room temperature and heat shocked in boiling water for 2 minutes. The heat shocked spore suspension was transferred to 50 ml of a potato medium prepared under nitrogen (George and Chen, 1983) and incubated at 35°C . After the observation of foaming indicating active growth and a microscopic examination for a high density of motile cells, the potato culture was transferred to 1 L of tryptone-yeast extract-sucrose or glucose (TYS or TYG) medium (George and Chen, 1983) or 1 L of clostridial medium (Weisenborn et al., 1989) depending upon desired growth conditions. After 5 to 7 hours of incubation, samples were taken and growth was followed by optical density measurements at 600nm by using a Beckman DU 7400 spectrophotometer. At each sampling, the morphology of the growing cells was examined under a microscope. When the culture reached an O.D of 2-3, it was ready for inoculation into 9 L of a desired medium. Inoculation was made under an atmosphere of mixed gas ($80\%\text{N}_2$, $10\%\text{CO}_2$ and $10\%\text{H}_2$), and the culture was incubated at 35°C . Based on the optical density of the culture, cells were harvested by centrifugation. The cell paste was washed with anaerobic 50mM Tris.Cl buffer at pH 7.5, recentrifuged, and frozen in liquid nitrogen in small chunks and then kept at -70°C until use.

Media

Clostridial medium

10 L clostridial medium contained 16.7 g K_2HPO_4 ; 16.7 g KH_2PO_4 ; 4 g MgSO_4 ; 0.1 g MnSO_4 ; 0.1 g FeSO_4 ; 10 g NaCl; 20 g asparagine; 50 g yeast extract; 20 g $(\text{NH}_4)_2\text{SO}_4$; 2 ml (1:1 diluted) antifoam C; 10 ml (0.1g/ 100ml distilled water) resazurin, 500 g sucrose, and distilled water to make up 10L.

Tryptone-yeast extract-glucose (TYG-60 or TYG-20) medium

10 L of medium contained 10 g tryptone; 50 g yeast extract; 0.1 g biotin; 1.7 g Na_2SO_4 ; 0.1 g *p*-aminobenzoic acid; 34 g K_2HPO_4 ; 2 ml (1:1 diluted) antifoam C; 10 ml

(0.1 g / 100 ml distilled water) resazurin, 10 ml mineral I solution (George et al., 1983), desired amount of glucose (600g for TYG-60 or 200g for TYG-20) and distilled water to make up 10 L.

Tryptone -yeast extract-sucrose (TYS-60 or TYS-20) medium

The composition is similar to that of the TYG medium, except that sucrose replaced glucose as the carbon source.

Preparation of medium

During the preparation, all the medium ingredients except glucose were dissolved in 5 L of distilled water in a 10 L Bellco-fermentor. Glucose was dissolved in 5 L of distilled water and autoclaved separately and added to the medium after autoclaving at 121 °C for 35 minutes. While the medium was cooling down to 35 °C slowly, a mixed gas (80%N₂, 10%CO₂ and 10%H₂) purged the headspace to maintain anaerobic conditions.

Preparation of cell-extracts

Frozen cells were thawed and incubated with stirring at room temperature under argon in Tris.Cl (pH 8.5, 50 mM) containing lysozyme (2 mg/ml) and DNase I (0.1 mg/ml) for 2 hrs. Three ml of buffer was used per g of cell paste (Chen and Blanchard, 1984). Cell-free extract was the supernatant after centrifugation at 37,000 x g for 30 min. All operations were performed under argon or hydrogen and all anaerobic buffers contained 1 mM of Na₂S₂O₄.

Column chromatography

Column chromatography was performed under anaerobic conditions. The flow rate was controlled by a peristaltic pump, and fractions were collected in anaerobic, stoppered vials. Active fractions were kept as frozen droplets in liquid nitrogen.

Protein determination

The dye binding assay (Bradford, 1976) was used with bovine serum albumin as standard.

Assays for hydrogenase

H₂ production assay

The H₂ production activity of hydrogenase was measured manometrically by using dithionite-reduced methyl viologen as the electron donor (Peck and Gest, 1956; Chen and Mortenson, 1974). The reaction was carried out in Warburg flasks (about 15 ml capacity) under H₂ at 30 °C, and the Gilson submarine respirometer was used in manometric measurements. In a total volume of 2 ml, the reaction mixture contains 1 mM methyl viologen (2 micromoles) and 15 mM Na₂S₂O₄ (30 micromoles) in 50 mM Tris.Cl, pH 8. One unit of enzyme activity is defined as the production of 1 micromole of H₂ per minute.

H₂ oxidation assay

Reduction of methylene blue or methyl viologen under H₂ can be visually observed as a qualitative test for hydrogenase activity. Hydrogen oxidation was measured manometrically in a setup similar to that for measuring H₂ production. The reaction was carried out in 50 mM Tris.Cl, pH 8, under 1 atm hydrogen with 5 mM methylene blue or methyl viologen as the electron acceptor.

Assay for acetoacetate decarboxylase

Acetoacetate decarboxylase catalyzes the production of acetone and CO₂ from acetoacetate. The production of CO₂ was measured manometrically. The reaction mixture contained 83 mM acetoacetic acid (lithium salt) in 0.2 M sodium acetate buffer at pH 5 (Davies, 1943).

Assay for phosphoglucosomerase

The production of glucose-6-phosphate from fructose-6-phosphate was measured by monitoring the increase in absorbance at 340 nm from production of NADPH in a coupled enzyme system with glucose 6-phosphate dehydrogenase as the indicator enzyme (Bergmeyer, 1974). The reaction mixture (1ml) had the following compositions: 44 mM Tris.Cl at pH 7.4; 25 mM Fructose 6-phosphate; 25 mM NADP⁺; 0.5 unit (10 microliter) glucose-6-phosphate dehydrogenase.

Polyacrylamide gel electrophoresis and activity staining

Electrophoresis was performed in 7.5% polyacrylamide gels with acrylamide/bis = 38 and with a 2.5% stacking gel; the electrode buffer used was at half strength of the stock solution (Davis, 1964). Methyl viologen linked hydrogenase activity in gels was located by activity stains of extruded tube gels that were evacuated and gassed with hydrogen several times in 13 x 100 mm stoppered glass tubes. Methyl viologen-reducing activity was located by immersing the anaerobic gels in 10 mM methyl viologen in 50 mM Tris.Cl pH 8.0 under H₂, to which 3 microliter of a 0.1 mM sodium dithionite solution was added to remove residual oxygen. After the blue band(s) of hydrogenase(s) appeared, an equal volume of a degassed 2.5% (w/v) solution of 2,3,5,-triphenyl tetrazolium chloride was added. The reduction of tetrazolium produced red band(s) which preserved the electrophoretic pattern of hydrogenase. The gels were then rinsed and kept in distilled water.

Determination of molecular weights

The molecular weight of hydrogenase was determined by gel filtration on a Sephacryl S-300 column (2.5 cm x 44 cm) with the following protein standards (with molecular weights in parentheses): thyroglobulin (669,000), yeast alcohol dehydrogenase (150,000), conalbumin (77,000), chymotrypsinogen (25,000) and ribonuclease A (13,700).

Determination of oxygen sensitivity

Half ml of a crude extract was placed in an anaerobic vial (2 ml) and then opened to air and gently bubbled with air for one minute. To control the rate of

bubbling, air was introduced into the liquid through a polyethylene tubing attached to an 18 gauge hypodermic needle, and 60 bubbles were delivered from a syringe (Chen and Blanchard, 1984). Samples were removed after a specified time of exposure at room temperature and immediately subjected to five cycles of evacuation and equilibration with hydrogen to remove oxygen. The remaining hydrogenase activities were measured.

Determination of thermostability

Half ml of a crude extract was transferred into a degassed Eppendorf vial (1.5 ml) then heated at 55 °C, 60 °C or 65 °C for 10 min, 30 min or 60 min. After the heat treatment, the vials were transferred onto an ice-water bath and assayed for hydrogenase activities.

RESULTS AND DISCUSSION

Preparation of solventogenic and acidogenic cells of *C. acetobutylicum* ATCC 824

C. acetobutylicum requires a pH below 5 to switch from acidogenesis to solventogenesis (Bahadur and Saraj, 1960; Bahl et al., 1982; Husemann and Papoutsakis, 1986). A culture pH of 4.5 is widely used for the generation of solventogenic cells, whereas at neutral pH, cells remain in the acidogenic phase. Besides an acidic pH, a high sugar concentration is also necessary for solvent production to occur. In this study, cultures of *C. acetobutylicum* ATCC 824 were grown at pH 4.5 and 6.5, respectively, in media containing different levels of sugars for the generation of solventogenic and acidogenic cells. An example of growth and solvent production was shown in figure 4. To produce solventogenic cells, the culture pH was allowed to fall from 6.8 to 4.5 during the first 7-9 hours of growth, and the pH was then maintained at 4.5 by the addition of KOH using a pH controller. Solvents became detectable by gas chromatography after 12 to 13 hours of incubation. In earlier experiments, the clostridial medium was used to grow *C. acetobutylicum* ATCC 824 at pH 4.5. After 23 hours of incubation, n-butanol and acetone concentrations reached 7.2 and 5.6 mM, respectively. This level of solvents is lower than what is normally produced in media containing tryptone. For this study, cells displaying a high solvent-producing activity are desired because such cells are expected to have a more distinctive hydrogenase content, if the hydrogenase content differs between acidogenic and solventogenic cells. To improve solvent production, cells were grown on a tryptone-yeast extract-sucrose medium (TYS-60) at pH 4.5. This medium, however, allowed only a small increase in solvent production compared with the cultures grown on the clostridial medium. After 23 hours of incubation, the concentrations of n-butanol and acetone reached 12 and 5 mM, respectively. The final optical density (8.4) of the culture in TYS-60 was lower than that (14) of the culture grown on the clostridial medium. When the carbon source for the tryptone-yeast extract-based medium was changed from sucrose to glucose, a 3.3-fold increase in n-butanol concentration (24 mM) and a 2.3-fold increase in acetone concentration (13 mM) were observed in TYG-60 than in the clostridial medium. After 23 hours of incubation, a final O.D of 10 was reached. Therefore, all the solventogenic cells for this study were grown on TYG-60 at pH 4.5.

To obtain acidogenic cells the culture pH was controlled at 6.5, and the following media were tested: TYG-60, TYG-20 and TYS-20. In the TYG-60 medium at pH 6.5, 20 mM of n-butanol and 5 mM of ethanol were produced but no acetone was formed after a 21-hour incubation period (Table 1; Fig.7). These cultures are neither acidogenic nor solventogenic in the usual sense, and hence the cells were not suitable for this study. [This physiological state will be the subject of a future study on the regulation of expression of solvent-producing enzymes.] When the culture was grown on a TYG-20 medium at pH 6.5, the n-butanol and ethanol concentrations were 7.8 mM and 5mM, respectively, without any acetone production. When the culture was grown

on TYS-20 at pH 6.5, n-butanol was accumulated to 2.3 mM without any acetone production (Table 1; Fig. 9). Thus, all the acidogenic cells for this study were grown on TYS-20 at pH 6.5.

Activities of hydrogenase in extracts of solventogenic and acidogenic cells

The linear range of hydrogenase assays with respect to the amount of enzyme present in the reaction mixture was established in both directions of reactions before the assays were used in the comparative study of acidogenic and solventogenic cells. For the hydrogen-uptake assay with methylene blue as the electron acceptor, the linear range was between 5 and 55 microliters of hydrogen oxidized per minute. For methyl viologen-linked hydrogen-uptake activity, the linear range was between 2.5 and 26 microliters of hydrogen oxidized per minute. The midpoint potential of methylene blue is more positive than that of methyl viologen, and the methylene blue-linked hydrogenase activity is generally higher than the methyl viologen-linked hydrogenase activity. Therefore, the routine H_2 -uptake assay was performed with methylene blue.

The hydrogen-evolution activity was measured by using dithionite reduced methyl viologen as the electron donor. The linear range for the hydrogen-evolution assay was between 5 and 24 microliters hydrogen evolved per minute.

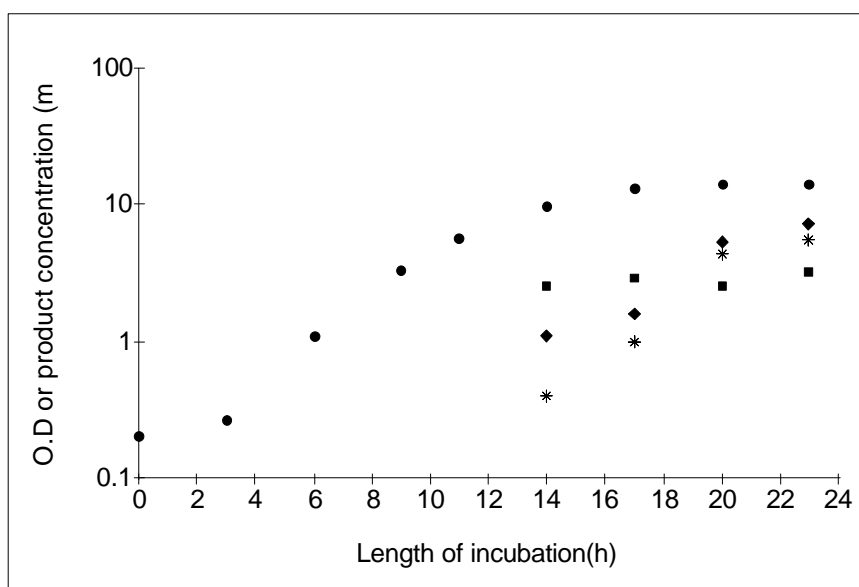


Fig 4. An example of growth and solvent production of *C. acetobutylicum* ATCC 824 in 10 L of the clostridial medium at pH 4.5. Symbols : •, O.D at 600 nm; ■, ethanol; ◆, n-butanol; *, acetone.

The ratio of the hydrogen-uptake activity over the hydrogen-evolution activity was determined in extracts of solvent and acid producing cells. Acetoacetate decarboxylase is only expressed during solventogenesis, and its activity was used to distinguish solventogenic cells from acidogenic cells. Phosphogluco- isomerase is expressed constitutively (Yan et al., 1987). Therefore, phosphoglucoisomerase activity

was also measured in cell-extracts to serve as a control. In solventogenic cell-extracts, the specific activity of acetoacetate decarboxylase increased from 2.0 U/mg to 3.95 U/mg between 13 and 21 hours of incubation when active solvent production occurred. In acidogenic cells, no acetoacetate decarboxylase activity was detected. Acidogenic cells had a higher hydrogenase activity level than solventogenic cells in both directions of reaction. However, the ratio of the hydrogen-uptake activity over the hydrogen-evolution activity was comparable in both cell types. This would suggest that in both metabolic phases, there is only one type of hydrogenase present. The difference in hydrogenase activity level in the two cell types indicates that the level of hydrogenase is regulated during solventogenesis. A decrease in the hydrogen evolution activity when cells enter solventogenesis is consistent with an increased consumption of reducing power by the cell for n-butanol formation. If the hydrogenase activities observed in this study reflect the amount of hydrogenase protein in the cells, there must be an effective mechanism for the cell to shut down hydrogenase synthesis and/or to inactivate hydrogenase during the solventogenic switch. The results also indicate that the physiological reaction for the hydrogenase is hydrogen production.

Table 1. Enzyme activities in solventogenic cells of *C.acetobutylicum* ATCC 824

<u>Length of incubation</u>	<u>Hydrogen uptake activity (U/mg)</u>	<u>Hydrogen evolution activity (U/mg)</u>	<u>Acetoacetate decarboxylase activity</u>	<u>Phosphoglucose isomerase activity (U/mg)</u>	<u>Ratio of uptake activity over evolution activity</u>
13	12.5±0.4	1.20±0.10	2.00±0.07	0.29	10.4
17	13.9±0.3	1.10±0.08	2.99±0.14	0.33	12.6
19	12.7±0.5	0.86±0.05	3.68±0.15	0.33	14.7
21	12.7±0.5	0.90±0.07	3.95±0.12	0.34	14.1

Table 2. Enzyme activities in acidogenic cells of *C. acetobutylicum* ATCC 824.

<u>Length of incubation</u>	<u>Hydrogen uptake activity (U/mg)</u>	<u>Hydrogen evolution activity (U/mg)</u>	<u>Acetoacetate decarboxylase activity</u>	<u>Phosphoglucose isomerase activity (U/mg)</u>	<u>Ratio of uptake activity over evolution activity</u>
15	37.4±2.0	2.24±0.25	N.D*	0.25	16.7
17	37.3±1.3	2.90±0.14	N.D	0.29	12.8
19	40.6±2.5	2.44±0.27	N.D	0.34	16.6
21	34.5±1.5	3.10±0.20	N.D	0.35	11.1

* Not detectable

Molecular weight of hydrogenase

The molecular weight of hydrogenase in acidogenic and solventogenic cells was determined by gel filtration on a Sephacryl S-300 column. The elution volume for hydrogenase was determined by measuring both the hydrogen-uptake and the hydrogen-evolution activities in fractions. The main activity peak for both reactions was located in the same fraction with both cell types. The molecular weight of hydrogenase was found to be 65,000 for both cell types. The result suggests that

either there is only one type of hydrogenase present in both cell types or the multiple hydrogenases, if present, have very similar molecular weights.

Oxygen sensitivity of hydrogenase in solventogenic and acidogenic cell-extracts

Chen and Blanchard (1978) reported a significant difference in oxygen sensitivity between the unidirectional and the bidirectional hydrogenases of *C. pasteurianum* W5. They observed 50% inactivation in 5 minutes after the bidirectional hydrogenase was exposed to air. Unlike the bidirectional hydrogenase, 50% inactivation by oxygen occurred in 30 minutes for the unidirectional hydrogenase. This finding inspired us to compare oxygen sensitivity of hydrogenase in solventogenic and acidogenic cell-extracts of *C. acetobutylicum* to see if there are multiple hydrogenases in this organism that show different oxygen sensitivities. Our experimental result showed an increase up to 1.9-fold in the ratio of the hydrogen-uptake activity over the hydrogen- evolution activity in solventogenic cell-extract after an exposure to air.

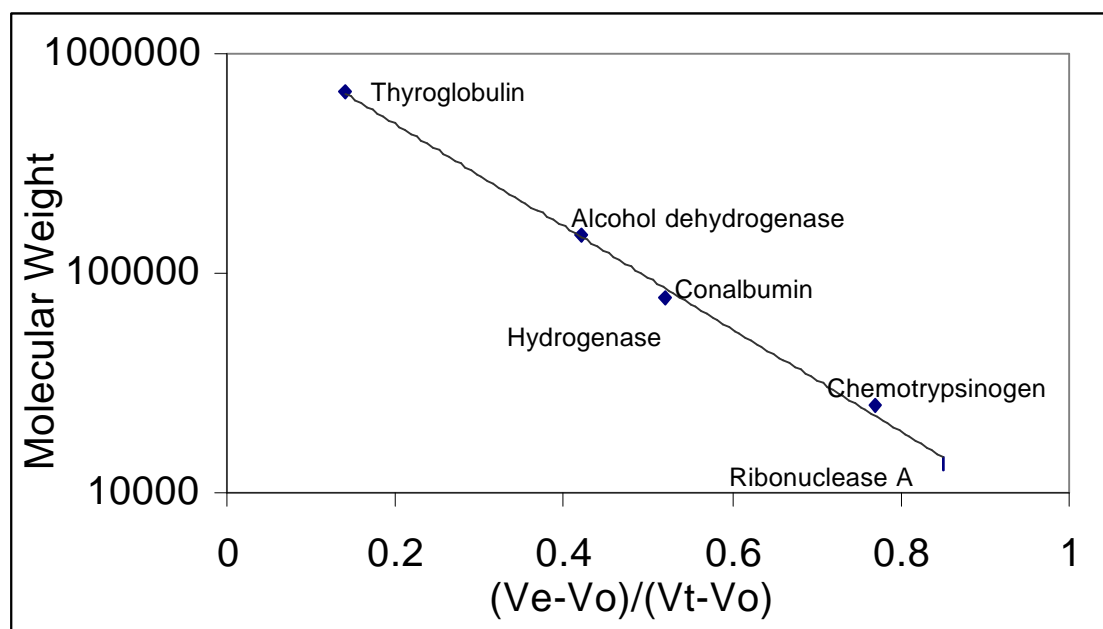


Fig 5. Molecular weight of hydrogenase as determined by gel filtration on a Sephacryl S-300 column (2.5 cm x 44 cm) eluted by 50 mM Tris.Cl buffer (0.1 M in KCl), pH 8.0. Anaerobic conditions were maintained by equilibrating the column with deaerated anaerobic buffer containing 1 mM $\text{Na}_2\text{S}_2\text{O}_4$. The flow rate was 50 ml/h and fractions of 2.5 ml were collected. Thyroglobulin (669,000), alcohol dehydrogenase (150,000), conalbumin (77,000), chemotrypsinogen (25,000) and ribonuclease A (13,700) were used as molecular weight standards. The void volume (V_o) was determined with blue dextran 2000. V_e is the elution volume, and V_t is the total volume of the gel bed.

This may be interpreted as the presence of a more oxygen stable hydrogen-uptake hydrogenase in solvent-producing cells. There was no such increases in the ratio of the hydrogen-uptake activity over the hydrogen-evolution activity when acidogenic cell-extract was exposed to air. The parallel loss in the hydrogen-uptake and evolution activities in acidogenic cell-extracts after exposure to air suggests the presence of a single hydrogenase in acidogenic cells. However, it is unclear why the rate of inactivation of the hydrogen-evolution activity differed significantly between the acidogenic and solventogenic cell-extracts, if this activity resides in the same hydrogenase.

Thermostability of hydrogenase in solventogenic and acidogenic cell-extracts

Solventogenic and acidogenic cell-extracts of *C. acetobutylicum* were heat treated at 55 °C, 60 °C and 65 °C for 10 min., 30 min. and 60 min., respectively. In solventogenic cell-extracts, significant increases were observed in the ratio of the hydrogen-uptake activity over the hydrogen-evolution activity when the extract was treated at a higher temperature for a longer period. For example, when treated at 55 °C, 60 °C or 65 °C for 60 min., a 1.5, 1.7 or 2.8-fold of increase in the ratio of the hydrogen-uptake activity over the hydrogen-evolution activity was observed for the solventogenic cell-extracts. In acidogenic cell-extracts, the increase in the ratio of the hydrogen-uptake activity over the hydrogen-evolution activity ranged from 1.2 to 1.9 when the extract was treated at 55 °C, 60 °C and 65 °C for 60 min. These increases may suggest the presence of a hydrogen-uptake hydrogenase which is more heat stable than the bidirectional hydrogenase in *C. acetobutylicum*.

Isolation of hydrogenase by ion-exchange chromatography

DEAE-cellulose (DE-52) was successfully used in the separation of the unidirectional hydrogen-oxidizing hydrogenase from the bidirectional hydrogenase of *C. pasteurianum* W5 (Chen and Blanchard, 1984). The unidirectional hydrogen-uptake hydrogenase was eluted from a DE-52 column at a lower salt concentration than the bidirectional hydrogenase.

In this study, a DE-52 column was also used to examine the chromatographic behavior of hydrogenase to see if multiple forms of hydrogenase are present in *C. acetobutylicum*. A significant difference in the ratio of the hydrogen-uptake activity over the hydrogen-evolution activity was detected when the active fractions of DE-52 column were compared with the cell-extract. The ratio of the hydrogen-uptake activity over the hydrogen-evolution activity also varied among the fractions. This change in the ratio of hydrogenase activities can be attributed to a significant decrease in the hydrogen evolution activity in the partially purified fractions. The reason for the decrease in hydrogen evolution activity is not clear. It may be caused by an inactivation during chromatography of a bidirectional hydrogenase as suggested by the results of oxygen and heat-induced inactivation, or a bidirectional hydrogenase was bound to DE-52 strongly and was not eluted under the conditions used. A third possibility is that the hydrogen-uptake and hydrogen-evolution activities of a

hydrogenase can be differentially altered. These possibilities will need to be further pursued in an ensuing study.

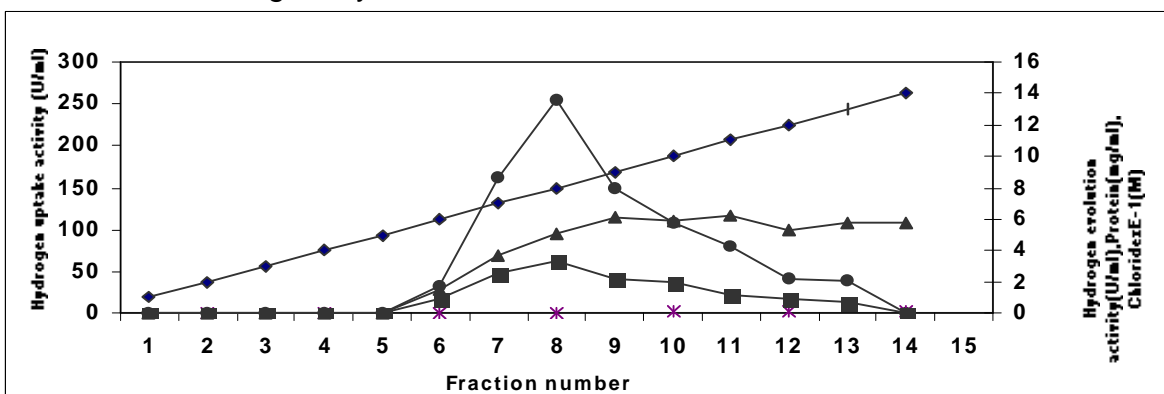


Figure 6. Elution of hydrogenase activities from a DE-52 column. (2.5 x 4.5cm). The column was equilibrated with 25 mM Tris.Cl at pH 8.0, and hydrogenase from a DE-52 treated solventogenic cell-extract was eluted with a KCl gradient (0-0.4M , total volume 150 ml).

Symbols: ■, hydrogen evolution activity; • , hydrogen-uptake activity; ▲ , protein (mg/ml), ◆ $\text{Cl}^- \times 10^{-1}$ (M).

Isolation of hydrogenase by gel filtration

The two hydrogenases of *C. pasteurianum* W5 were partially separated from each other by gel filtration on a Sephadex G-150 column.(Chen and Blanchard, 1978). When a cell-extract of *C. pasteurianum* W5 was fractionated on a Sephadex G-150 column, two hydrogen-uptake peaks were observed. The first hydrogen-uptake peak coincided with the hydrogen-evolution activity peak (the bidirectional hydrogenase), whereas the second hydrogen-uptake peak was eluted in fractions containing the trail of the hydrogen-evolution activity peak.

We examined cell-extracts of *C. acetobutylicum* by gel filtration to see if multiple forms of hydrogenase are present in this species. The results are summarized in Figure 14 and Table 9 for the solventogenic and in Figure 15 and Table 10 for the acidogenic cell. Only one activity peak for both directions of the hydrogenase-catalyzed reactions for both cell types was observed. The ratio of the hydrogen-uptake activity over the hydrogen-evolution activity varied among fractions, but it was never lower than the ratio found in the cell-extracts. The increase in the activity ratio was not, however, as much as that observed in solventogenic cell-extracts following ion-exchange chromatography. This suggests that either there is only one kind of hydrogenase present in both solventogenic and acidogenic cells or the different kinds of hydrogenase have very similar molecular weights and thus cannot be separated by gel filtration.

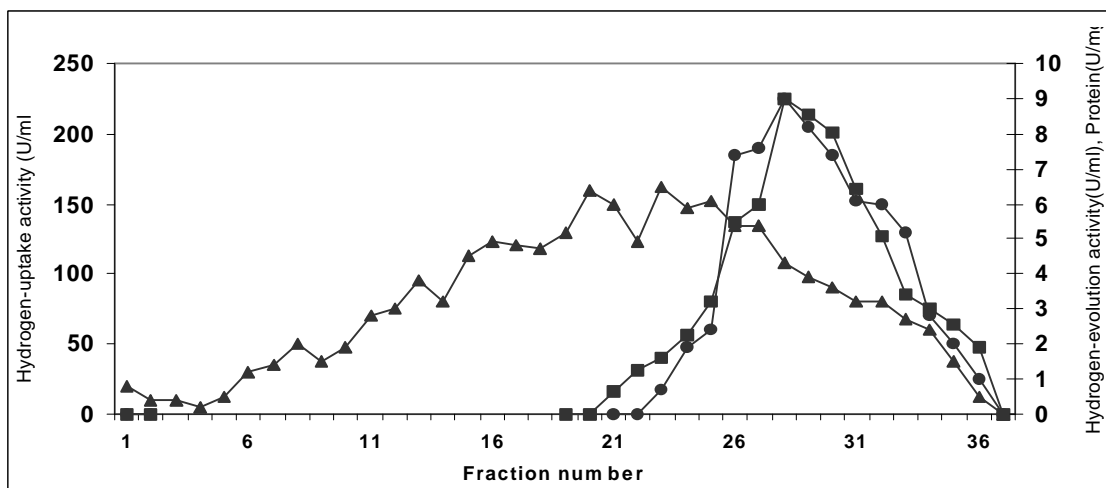


Figure 7. Elution of hydrogenase activities from a Sephacryl S-300 column (2.5 x 44 cm). A concentrated solventogenic cell-extract (5 ml) was applied to the column. The column was equilibrated with 50 mM Tris.Cl (pH 8.0) containing 0.1 M KCl, and 2.5-ml fractions were collected. Symbols: ■, hydrogen-evolution activity; •, hydrogen-uptake activity; ▲, protein (mg/ml).

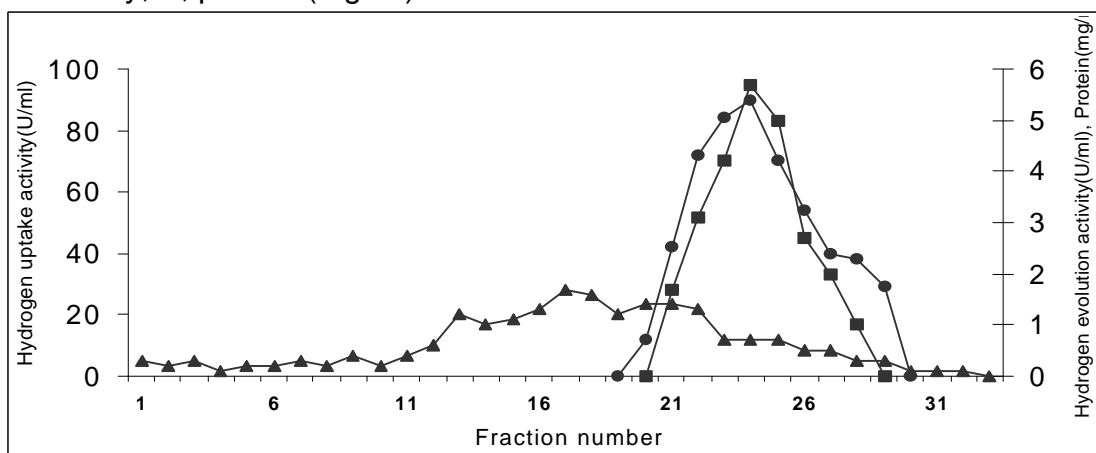


Figure 8. Elution of hydrogenase activities from a Sephacryl S-300 column (2.5 cm x 44 cm). A concentrated acidogenic cell-extract (2.5 ml) was applied to the column. The column was equilibrated with 50 mM Tris.Cl (pH 8.0) containing 0.1 M KCl, and 2.5-ml fractions were collected. Symbols: ■, hydrogen-evolution activity; •, hydrogen-uptake activity; ▲, protein (mg/ml).

Resolution of hydrogenase by polyacrylamide gel electrophoresis

The electrophoretic patterns of hydrogenase from solventogenic and acidogenic cells before and after chromatographic separations were examined. The position of hydrogenase bands was determined by an activity stain as described in Materials and Methods. A major band ($R_f = 0.35$) and four minor bands (R_f 's = 0.22, 0.37, 0.40 and 0.48) were observed with extracts of both acidogenic and solventogenic cells. The

minor bands were absent in samples eluted from the DE-52 or Sephacryl S-300 column. The results suggest that either multiple forms of hydrogenase are present in the solvent-producing clostridia or the hydrogenase forms complexes with other cellular constituents to give the minor bands. Isolation of hydrogenase by ion exchange chromatography or gel filtration may cause the dissociation of hydrogenase from other molecules in the complexes and result in the disappearance of the minor bands.

Before the purification of hydrogenase I and II from *C. pasteurianum*, multiple hydrogenase bands were also observed when extracts of *C. pasteurianum* were examined by polyacrylamide gel electrophoresis, and the presence of six hydrogenase isoenzymes was claimed (Ackrell and Mower, 1965; Ackrell et al., 1966; Kidman et al., 1968). Because there is no further evidence to suggest the presence of more than two hydrogenases in *C. pasteurianum*, the electrophoretic pattern of hydrogenase in extracts of *C. acetobutylicum* will be more accurately interpreted after hydrogenase(s) has been purified from this organism.

Concluding Remarks and Future Work

The present data show that the level of hydrogenase was higher in acidogenic cells than in solventogenic cells of *C. acetobutylicum*. This may be the result of a combination of transcriptional and posttranscriptional regulation of hydrogenase activity during the metabolic shift from acid production to solvent production to prevent the over consumption of reducing power by hydrogenase during solventogenesis. The increase in the ratio of the hydrogen-uptake over the hydrogen-evolution activities after the isolation of hydrogenase by DEAE-cellulose and Sephacryl S-300 column chromatography suggests the presence of multiple forms of hydrogenase in both cell types. However, a clear separation of these multiple forms could not be achieved during this study.

In future work, the purification of hydrogenase from acidogenic and solventogenic cells will be performed, and an emphasis will be placed on monitoring the ratio of the hydrogen-uptake activity over the hydrogen-evolution activity. By this approach, it may be possible to locate the isoenzymes in different fractions and to purify and characterize them separately. This purification may also provide a definitive answer to the questions as to how hydrogenase activity decreases during solventogenesis and what might be the physiological role for each hydrogenase, if multiple forms indeed exist. Finally, this study may help the ultimate goals which are to understand the triggering mechanism and the associated metabolic changes during the switch from acidogenesis to solventogenesis in solvent-producing clostridia and the regulation of hydrogen metabolism as a possible way of improving the yield of butanol.

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