# Elements of

# the Electron Transport Pathway for

# *Methanosphaera stadtmanae.*

By

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

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Winnipeg, Manitoba

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**Elements of the Electron Transport Pathway for *Methanosphaera stadtmanae***

**BY**

**Denny Wong**

**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of**

**Doctor of Philosophy**

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## Abstract

In *Methanosphaera stadtmanae*, $H_2$ is used to provide electrons to $CH_3OH$ to $CH_4$. The enzyme used to convert $H_2$ into the usable form for the cell is the hydrogenase. The hydrogenase found in *Msph. stadtmanae* can only be assayed in the presence of an artificial electron acceptor, methyl viologen and cannot directly couple $H_2$ to the reduction of the methanogenic electron carrier, cofactor $F_{420}$. The enzyme has been purified and was found to be composed of two different subunits, with molecular masses of 68,750 (α-subunit) and 63,500 (β-subunit). The molecular mass, determined using native gradient gel electrophoresis, was estimated tentatively as 49,500. This was smaller than either of the subunits. The pH optimum for the purified enzyme is 9.5. The $K_m$ for the artificial electron acceptor is 7.9 mM MV. The enzyme is an iron-sulfur protein, with 11.2 ± 0.7 moles of iron and 18.5 ± 3.1 moles of acid-labile sulphide per mole of hydrogenase.

There must, however, be a mechanism of reducing the electron carrier, $F_{420}$, which is present at low levels, for the purposes of biosynthesis in the absence of an $F_{420}$-reducing hydrogenase activity. Such an activity was detected, in the form of the $F_{420}H_2$ dehydrogenase. This activity is extremely $O_2$-labile. The activity was also found to be stimulated (up to 30-fold) in the presence of salts. The pH optimum is 7.0. The $K_m$ for $F_{420}H_2$ was determined to be 21.5 μM. The dehydrogenase coupled the oxidation of $F_{420}H_2$ to phenazine at a slightly lower rate than with artificial acceptors (metronidazole plus methyl viologen) and the $K_m$ for this alternate electron acceptor was found to be 142.9 μM. Phenazine was found to serve as an alternative to the natural electron acceptor (methanophenazine) for the enzyme purified from *Methanosarcina mazei* strain Gö1.

Using optimal conditions for the dehydrogenase, $H_2$ was found to be linked to $F_{420}$ through the addition of phenazine, with reduction occurring at a low rate (0.35 nmoles $F_{420}$/min/mg) in extracts.

The discovery of this $F_{420}H_2$ dehydrogenase activity in a methanogen outside of the *Methanosarcinaceae* suggested that there may be other methanogenic species that may carry this activity and a survey of methanogenic species encompassing all three

Orders of the methanogens determined that all of the tested species carry this activity. Though many of these also carry an $F_{420}$ hydrogenase, an $F_{420}H_2$ oxidation activity for many of these was shown to be coupled to phenazine.

**Acknowledgments**

I would like to thank the National Sciences and Engineering Research Council (NSERC) of Canada for funding this research. Also I would like to thank my thesis supervisor, Dr. Richard Sparling, for his guidance and patience with the work and all of the members of the advisory committee, Drs. Anne Adkins, Ron Lyric (retired), Peter Maeba, and Isamu Suzuki. Of special consideration, past and present members of the Department of Microbiology Graduate Students, including Lateef Adewoye, Doug Barker, Norman Barnabe, John Buchko, Peter Chan, Ishita Chatterjee, Lesia Harahuc, Rosemarie Masau, Jeanna Strutinsky (the X-phile), Travis Takeuchi, Lorelee Tschetter and John Wiley. I would like to thank also the past and present members of the anaerobic lab-rats, Patrick Chong, Tamiko Hisanaga, Dwayne Elias, Zhaosheng Lin, and George Meichel for their levity and advice. Of course, I would like to thank my family, who have supported me throughout this journey.

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## List of Abbreviations

ACS.....acetyl-CoA synthase
BV.....benzyl viologen
CoB.....coenzyme B
CODH.....carbon monoxide dehydrogenase
CoM.....2-mercaptoethanesulfonic acid
DEAE.....diethylaminoethyl
DNase.....deoxyribonuclease
DTT.....dithiothreitol
EDTA.....ethylenediaminetetraacetic acid
ETP.....electron transport phosphorylation
$F_{420}$.....8-hydroxy-5-deazaflavin
$F_{420}H_2$.....reduced cofactor $F_{420}$
FAD.....flavin adenine dinucleotide
FMN.....flavin mononucleotide
$H_4$MPT.....tetrahydromethanopterin
$H_4$SPT.....tetrahydrosarcinopterin
HA.....hydroxylapatite
HEPES.....N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HI.....hydrophobic interaction
HS-HTP.....7-mercaptoheptanoylthreoninephosphate
IE.....ion exchange
kDa.....kilodalton
*Mb*.....*Methanobacterium*
*Mc*.....*Methanococcus*
MCAC.....metal chelate affinity chromatography
MES.....2-(morpholino)ethanosulfonic acid
MFR.....methanofuran
*Ml*.....*Methanolobus*
MRF.....methyl-reducing factor
*Ms*.....*Methanosarcina*
*Msp*.....*Methanospirillum*
*Msph*.....*Methanosphaera*
*Mst*.....*Methanosaeta*
MTZ.....metronidazole
MV.....methyl viologen
MWCO.....molecular weight cut-off
$NAD^+$.....nicotinamide adenine dinucleotide
$NADP^+$.....NAD phosphate
PAGE.....polyacrylamide gel electrophoresis
PIPES.....piperazine-N,N'-bis-(2-ethanesulfonic acid)
PMSF.....phenylmethylsulfonyl fluoride

## List of Abbreviations (Cont'd)

SDS-PAGE.....sodium dodecyl sulfate PAGE
SLP.....substrate-level phosphorylation
THF.....tetrahydrofolic acid
Tris.....trihydroxymethylaminomethane
Tris-Cl.....tris hydrochloride

# 1. Literature Review

## 1.1. Methane and methanogenesis, ecology and biology.

Methane gas production has been known to humankind for many centuries. Long before the process was understood, people knew of the occasional incidents in which a highly volatile (ie., explosive) gas bubbled out of marshlands, rice paddies, waste dumps, or landfills. The first recorded scientific study into the combustible air coming out of marshland areas was performed in 1776 by Alessandro Volta in Europe (Wolfe, 1993).

The mystery combustible gas was officially named methane by the International Congress on Chemical Nomenclature in 1892. Methane, the smallest and simplest of the volatile organic compounds, is made up of a single C atom and has the chemical formula $CH_4$ (MW= 16.0426 g/mole). Methane, along with other atmospheric trace gases carbon dioxide, nitrous oxide, and the chlorofluorocarbons (CFCs), are collectively known as the "Greenhouse gases" (Dickinson and Cicerone, 1986). These gases absorb reflected solar radiation in the infrared wavelengths (ie. heat) and can cause temperatures to elevate (Wang et al, 1976), resulting in the so-called "greenhouse effect" (Ramanathan, 1975). Consequently, they are important molecules from a ecological perspective.

Methane is a colourless and odourless gas that is found naturally deep within the Earth's crust as a component of natural gas and thus has economic value. An important source of methane is biological (Daniels, 1984; Pearce, 1989) and estimates in the early 1990's have placed the biological output of methane at around a billion tonnes per year (Blaut et al, 1992).

A common factor of the methane-producing locales, including marshlands, rice paddies, and landfills, is the presence of decaying organic matter. The biological production of methane is a process at the terminal end of the anaerobic decomposition of organic matter. At this point a complex mix of different bacterial species combine to convert dead organic matter into its smallest constituent molecules. This community of microorganisms work cooperatively as the end products of metabolism of one organism are used by other organisms in the community. The process of the microbial digestion or

anaerobic microbial food chain (Figure 1) has been described (Daniels, 1984; Ferry, 1992).

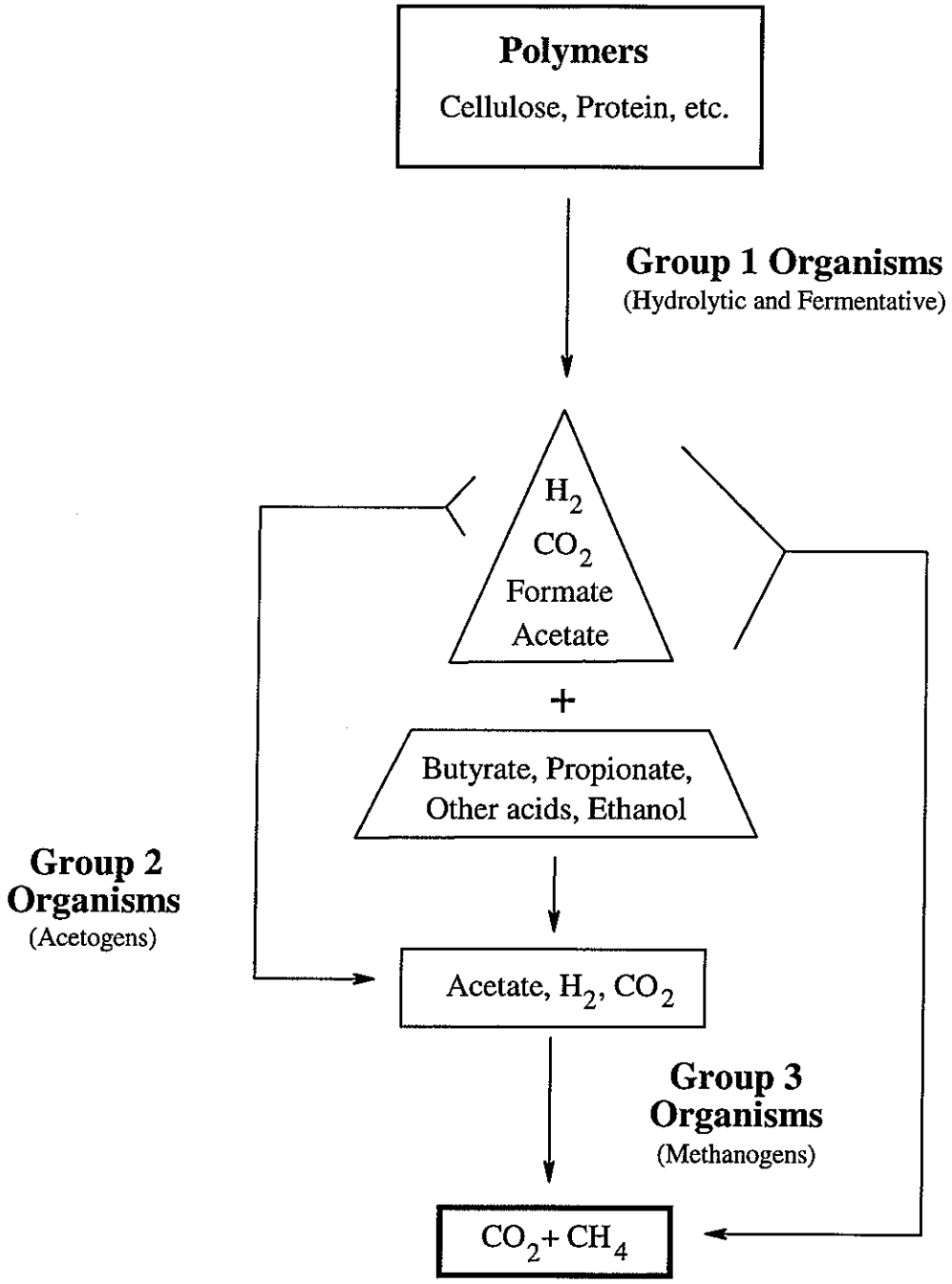
Other important microbial processes have been studied, in particular, interspecies hydrogen transfer (Iannotti et al, 1973) and interspecies formate transfer (Boone et al, 1989), the organisms involved are mainly anaerobes. The conversion of metabolic end products of one organism (eg. Group1) may enhance the process of the degradation of organic substrates as the accumulation of these end products may be inhibitory to the (Group 1) organism producing them. One group (Group 1) within the community of microorganisms converts the complex organic matter (eg., polysaccharides, long chain fatty acids in lipids and the amino acids in proteins) to simple molecules like $CO_2$ and $CH_4$ (produced by the Group 2 and 3 organisms). Depending on the composition of the microbial community and the presence of other nutrient factors, incomplete oxidation of substrate may also yield short chain fatty acids, eg. acetic and formic acid. These short chain fatty acids, along with methanol, methylamines, and $CO_2$ are the substrates for the biological process of methanogenesis.

Methane is produced by a group of microorganisms that are termed the methanogens, or the methane-producing bacteria. They constitute a major segment of the Archaea, a Domain, or a classification of organisms, in the modern phylogenetic classification called the "Tree of Life". The other Domains of this Tree of Life are the Eukarya (the eukaryotes, including humans) and the Bacteria (comprising the bulk of the common prokaryotic organisms, Woese et al., 1990). The Archaea are a unique and distinct group of prokaryotes, different in many ways from the morphologically similar members of the Bacteria.

### 1.2. The Archaea.

The Archaea or archaebacteria (Woese, 1981) were given that name because many species of this Domain were isolated from rather harsh or hostile environments, such as undersea hydrothermal vents, eg., *Methanopyrus kandleri* (Burggraf et al, 1991) where temperatures can range from near freezing to 200°C, hot springs, and high salt pools, eg.,

**Figure 1** - Anaerobic process of the digestion of organic matter to methane. (Figure modified from Daniels, 1984)


Polymers
Cellulose, Protein, etc.
Group 1 Organisms
(Hydrolytic and Fermentative)
$H_2$
$CO_2$
Formate
Acetate
+
Butyrate, Propionate,
Other acids, Ethanol
Group 2
Organisms
(Acetogens)
Acetate, $H_2$, $CO_2$
Group 3
Organisms
(Methanogens)
$CO_2$+ $CH_4$

*Methanococcoides burtonii* isolated from a hypersaline lake in Antarctica (Franzmann et al,1992). In addition, many of the Archaea are also strictly anaerobic organisms ie. intolerant to the presence of $O_2$. These environments are likened to what the environment was like on Earth approximately 3 to 4 billion years ago. Hence the term archaebacteria, the "archae" prefix referring to their resembling forms of bacteria that thrived when extreme environments existed.

The Archaea are considered a throwback to the early days of life on Earth and comprise a separate Domain of organisms from the Bacteria that includes the familiar *Escherichia coli* and the Eukarya (the higher, more complex organisms ranging from the microscopic, single-cell forms like the protozoa, up to the multicellular forms, including the mammals). Despite their physiological differences from the Bacteria and Eukarya, the Archaea share some interesting similarities to both of these Domains.

First of all, morphologically they are prokaryotic with no sub-cellular membrane-bound organelles such as the nucleus, Golgi apparatus, endoplasmic reticulum, mitochondria, and chloroplasts that are found in the eukaryotic cells. The Archaeal ribosomes are the same size (70S) but are less complex than those found in the Bacteria (Matheson, 1992). However, the Archaea do not use a formylated-methionyl tRNA to initiate protein synthesis as do the Bacteria (Woese, 1981).

A significant difference that distinguishes the Archaea from both the Bacteria and the Eukarya is their lipid structure. The archaeal lipids are ether-linked whereas the lipids of the other groups are ester-linked (Makula and Singer, 1978; Woese, 1981). The ether linkage is more resistant to chemical nucleophilic attack than the ester linkage (which has a C=O that forms a resonance state that is more susceptible to attack). The ether-linked form provides stability to the lipid structure under the extreme conditions in which many of these organisms grow (Sprott, 1992). Minor amounts of ether-linked lipids have been found in a few members of the Eukarya, eg. in the brown alga, *Dilophus fasciola* (Amico et al, 1977). The side-chain groups of the archaebacterial lipids are an area of interest for study, since they come in a variety of unique forms and configurations depending on the group of Archaea (Kates, 1992). An unusual core lipid structure in the

Archaea are the macrocyclic diether lipids and a tetraether lipid, ie., structurally 2 archaeol (*2,3*-di-o-phytanyl-*sn*-glycerol) lipid molecules linked head-to-head to form caldarchaeol lipids (2,2',3,3'-tetra-o-dibiphytanyl-*sn*-diglycerol) that are found in some members of the methanogenic bacteria (Sprott, 1992). The stereochemistry (sn-2,3 versus the sn-1,2 of ester-linked glycerolipids), the regularly branched phytanyl chains, the constant length of these chains, and the usually saturated nature of these lipids are distinguishing features compared to lipids from the Bacteria and Eukarya (Sprott, 1992).

Recently, it was discovered that the transcriptional systems of the Archaea had elements that were not unlike that found in the Eukarya. In particular, the RNA polymerase in members of the Archaea was found to be very similar in structure to RNA polymerase II in the Eukarya (Baumann et al, 1995; Langer et al, 1995).

Several properties of members of each of the Domains are shown on Table 1.1. There are a number of properties that are shared between the Domains and properties which are specific to a single Domain.

The Archaea is divided into two groups (Kingdoms), the Crenarchaeota and the

**Table 1.1 - Some Properties of the Archaea, Bacteria, and Eukarya**

| Property | Archaea | Bacteria | Eukarya |
|---|---|---|---|
| **Cell Type** | Prokaryotic | Prokaryotic | Eukaryotic |
| **Lipids** | Ether-linked | Ester-linked | Ester-linked (some Ether-linked) |
| **Ribosome Size** | 70S | 70S | 80S |
| **Formyl-Met as Initiator tRNA** | No | Yes | No |
| **Subcellular Organelles** | No | No | Yes |
| **Introns** | Present in t-RNA | No | Yes |
| **Number of Chromosomes** | 1 | 1 | >1 |
| **Sensitive to Kanamycin?** | No | Yes | No |

* Table modified from: Woese, 1981.

Euryarchaeota (Winkler and Woese, 1991; Woese et al, 1990). However, the distinction between these two kingdoms of the Archaea has in recent years become less strict as more isolates have been discovered that physiologically fall under one classification but genetically (on the 16S rRNA level) belongs in the other Kingdom. The Crenarchaeota are typically represented by the thermoacidophilic Archaea which includes *Sulfolobus solfataricus*, *Pyrococcus furiosus*, and *Pyrodictium brockii*. The Euryarchaeota are typically represented by the halophilic Archaea like *Halococcus* spp. and *Halobacterium* spp., and the most widespread group of the Archaea, the methanogens.

**1.3. The Methanogens and anaerobic culture, a historical perspective.**

In terms of the number of species the methanogens are a major component of the Archaea with more than 60 species isolated as of 1990 (Garcia, 1990) and many more since that report. The methanogens, as a whole, utilize a rather narrow range of substrates to produce methane. The most commonly used substrate is the combination of $H_2$ plus $CO_2$. In addition, methanol, $H_2$ plus methanol, methylamines (methylamine, dimethylamine, and trimethylamine), acetate, formate, and, to a small degree, CO are also used as substrates for methanogenesis (Blaut, 1994; Daniels et al, 1984). Although most methanogens use $H_2$ plus $CO_2$ as methanogenic substrate, acetate is a significant contributor for global methane production. Acetate may be found in many environments, including sludge and sewage digestors, or naturally occurring marine and freshwater environments. It is interesting that with the preponderance of acetate in the environment, only members of the two genera, *Methanosarcina* and *Methanothrix* (*Methanosaeta*) are known to utilize this substrate (Jetten et al, 1992). Many methanogenic substrates are also used for the synthesis of cell carbon.

Methanogenic activity and specific methanogenic species have been isolated from a variety of environments, including soil sediments (*Methanosarcina barkeri* Strain Fusaro, Kandler and Hippe, 1977; *Methanolobus tindarius*, König and Stetter, 1982), marine environments (*Methanococcus thermolithotrophicus*, Huber et al, 1982) anaerobic sewage sludge and industrial sludge digesters (*Methanobacterium formicicum*, Bryant and

Boone, 1987; *Mb. thermoautotrophicum*, Fuchs et al, 1978), the intestinal tract of mammals (*Methanosphaera stadtmanae*, Miller and Wolin, 1983; *Methanosphaera cuniculi*, Biavati et al, 1988; *Methanobrevibacter smithii*, Miller et al, 1982), from a hypersaline lake in Antarctica (*Methanococcoides burtonii*, Franzmann et al, 1992), and from undersea hydrothermal vents (*Methanopyrus kandleri*, Huber et al, 1989).

The study of the methanogenic bacteria goes back more than a half century since the discovery of the first methanogens in the 1930's. In the 1940's, H.A. Barker isolated an organism that grew in and produced methane from ethanol in a $CaCO_3$-buffered medium. This organism was given the name of *Methanobacillus omelianskii*, but has also been referred to as *Methanobacterium omelianskii* in the literature (Barker, 1939). It would become the most studied methanogenic culture and its biochemistry would form the basis of our early understanding of methanogenesis. This culture would later turn out to be a coculture of a methanogen, later named *Methanobacterium bryantii* strain M.o.H., a $H_2$:$CO_2$-utilizing methanogen, and a syntrophic ethanol-utilizing organism which was referred to as the S-organism (Bryant et al, 1967). In a symbiotic relationship of these two organisms, the S-organism metabolized ethanol into $CH_3CO_2H$ (acetic acid) and $H_2$. The $H_2$ product was subsequently utilized by the methanogen along with the $CO_2$ already in the medium in the form of $CaCO_3$. Their relationship was a truly symbiotic one since under the early culturing conditions, they could not grow without the other. The $H_2$ that was produced by the S-organism was inhibitory towards its own metabolism when it accumulated while *Mb. bryantii* required the $H_2$ to produce methane from $CO_2$. This, as it turned out, was the first recorded instance of interspecies hydrogen transfer. In fact, many of the early "pure" cultures of methanogens turned out to be cocultures since, at that time, methanogenesis from alcohols higher than methanol (Barker, 1939) and the use of fatty acids higher than acetic were reported (Stadtman and Barker, 1951). We currently know of no methanogens in pure culture that can use these compounds directly as methanogenic substrates.

In the early days, it was very difficult to culture methane-producing bacteria, since their survival required strict anaerobic conditions and the culture methods were based

mainly on those for the more easily culturable aerobic or facultatively anaerobic bacteria. *Mb. omelianskii* required 5 days under these growing conditions before the maximum level of methane was detected, with "fermentation" being complete by 8 days (Barker, 1939). With a revolutionary technique designed by Hungate (Hungate, 1950), more effective methods were developed in order to culture and study these fascinating organisms. This allowed researchers to better understand and exploit these bacteria.

One of the first methods devised for the culture of anaerobic microorganisms was the Hungate roll tube (Hungate, 1947) which allowed researchers to study these anaerobes on solid media under a closed, strictly anaerobic system. The roll tube had a layer of agar on the inside surface of the tube on which the anaerobes would grow. A variation on this system used liquid medium instead of the solid agar. The tubes were sealed at the open end with a rubber stopper in order to close the tube and prevent the entry of $O_2$. This method also introduced the first pre-reduced medium, and later, a standard addition of cysteine-sulfide was used to prepare a consistently reduced medium (Hungate, 1969). This pre-reduction was important since this reduced the lag time (and hence the overall growth time) by eliminating the adaptation period during which the methanogens reduced the medium until it had reached the ideal redox potential of below -330 mV (Hungate, 1969).

Gassing manifold systems were developed to prepare media conveniently under anaerobic conditions (Balch et al, 1979). The basic principle of their operation involved the alternating evacuation of the closed vessel to remove the headspace gas, and replacing it with an $O_2$-free gas phase like $N_2$ or a gas mixture of $H_2$:$CO_2$. Repeated evacuation and gassing removes the $O_2$ from both the headspace and the medium by essentially a dilution effect. Prior to this method, the principle method of making anaerobic media anaerobic was by boiling the heat stable medium to drive $O_2$ out of solution and then cooling the medium under an $O_2$-free gas like $N_2$ (Hungate, 1969).

Larger scale systems required growth in a liquid medium which was achieved with the Balch-Wolfe tube (Balch et al, 1976) and variants of the Balch-Wolfe system including the serum bottle modification (Miller and Wolin, 1974) which was a modern

modification of the Hungate tube system. The serum bottle has been adapted for use with larger vessels (1 litre and greater up to and including 60 to 300 litre size fermenters that have been used in several laboratories) so that larger amounts of culture could be collected for study of the enzymology of anaerobic bacteria.

A significant discovery was that growth of hydrogenotrophic ($H_2$-utilizing) methanogenic bacteria was enhanced under a pressurized atmosphere (Balch and Wolfe, 1976). Since then, all of the methanogenic bacteria have been grown under a gas phase headspace of 1 to 2 atmospheres over standard atmospheric pressure. However overpressurized culture bottles sometimes exploded during or just after autoclaving, which has led to the development of some safety devices (Daniels, 1985).

Special methods were also devised to perform experiments or studies on enzymes under anaerobic or near-anaerobic conditions. In particular, column chromatography, an important procedure in the isolation of proteins, may need to be run in strictly anaerobic conditions if the biomolecules were inherently unstable under aerobic conditions. A method for conducting anaerobic column chromatography has been designed (Repaske, 1971). Anaerobic ultrafiltration of samples was used in the purification of an $O_2$-sensitive enzyme, the uptake hydrogenase of *Clostridium pasteurianum*, an anaerobic bacterial $N_2$-fixer (Adams and Mortenson, 1984).

Enzyme assays are important for the study of specific reactions that occur within the cell. Activities of $O_2$-sensitive enzymes or reactions that are inhibited by the presence of $O_2$ cannot be monitored by conventional assay methods. Therefore a method was developed by which extracts and other solutions were transferred anaerobically by syringes to stoppered reaction tubes (Daniels and Wessels, 1984).

It was one thing to be able to grow the organisms under strict anaerobic conditions, it was another to perform experiments and manual manipulations under the same strict anaerobic conditions that the methanogens and other strict anaerobes require. It may not be feasible, nor economical to run or incubate these apparatus under a continuous stream of an $O_2$-free gas like $N_2$ continuously during the course of the experiment. Anaerobic chambers, a modification of medical glove boxes were made

allowing one to perform manipulations anaerobically (Anraki and Freter, 1972). The use of such a device allowed the researcher to run experiments that were until then, only possible under an anaerobic or a near-anaerobic environment. The chamber is filled with any $O_2$-free gas or gas mixture and equipment is brought into the glove box via an airlock which minimized the amount of $O_2$ entering the chamber. The use of an anaerobic glove box for the method of column chromatography has been described (Gunsalus et al, 1980) where the closed column was set outside of the chamber since most chromatography columns were too long to place inside the glove box. The inlet and outlet tubes from the column were fed directly into the glove box through stoppers fitted on the chamber where the buffers were stored and the eluate collected under strict anaerobic conditions. This however limited the use of the chamber to ambient or laboratory temperature conditions unless one decides to operate the chamber in a cold room.

Taken to the extreme, a laboratory was constructed in the National Institute of Health in Bethesda, Maryland, U.S.A. that was an actual anaerobic laboratory. It was designed by a leader in the field of anaerobe research, Thressa Stadtman (Poston et al, 1971) to conduct research on anaerobic microorganisms without the hassle of using an anaerobic chamber and to allow the researcher to use instruments that would normally not be able to enter an anaerobic chamber/glove box.

As more methanogens and other anaerobic prokaryotes were discovered, the amount of research being performed on anaerobic bacteria (and even anaerobic eukaryotes) began to mount. Thus the age of anaerobic, and in particular, methanogenic research was beginning with the ability to study the specific elements of methanogenesis, including the certainly novel enzymes that are involved, and as we shall soon see, the unique cofactors of methanogenesis.

### 1.4. Methanogenesis and the methanogenic pathways, a look into some unique cofactors.

Studies on the methanogens began with trying to understand the physiological processes at the protein/enzyme level and to define the elements involved in the process

of methanogenesis from different substrates. The most common methanogenic substrate used is the combination of $H_2$:$CO_2$. Approximately 65% of all the known methanogens use these two gases in combination to produce methane (Garcia, 1990).

The complete pathway of methanogenesis from $CO_2$ was the first of the three major methanogenic pathways to be determined. Other pathways of methanogenesis using either methanol or acetate have since been elucidated and have been found to use a part of the $CO_2$ pathway as well as some pathway specific reactions and enzyme systems.

### 1.4.1. Pathway of methanogenesis from $CO_2$

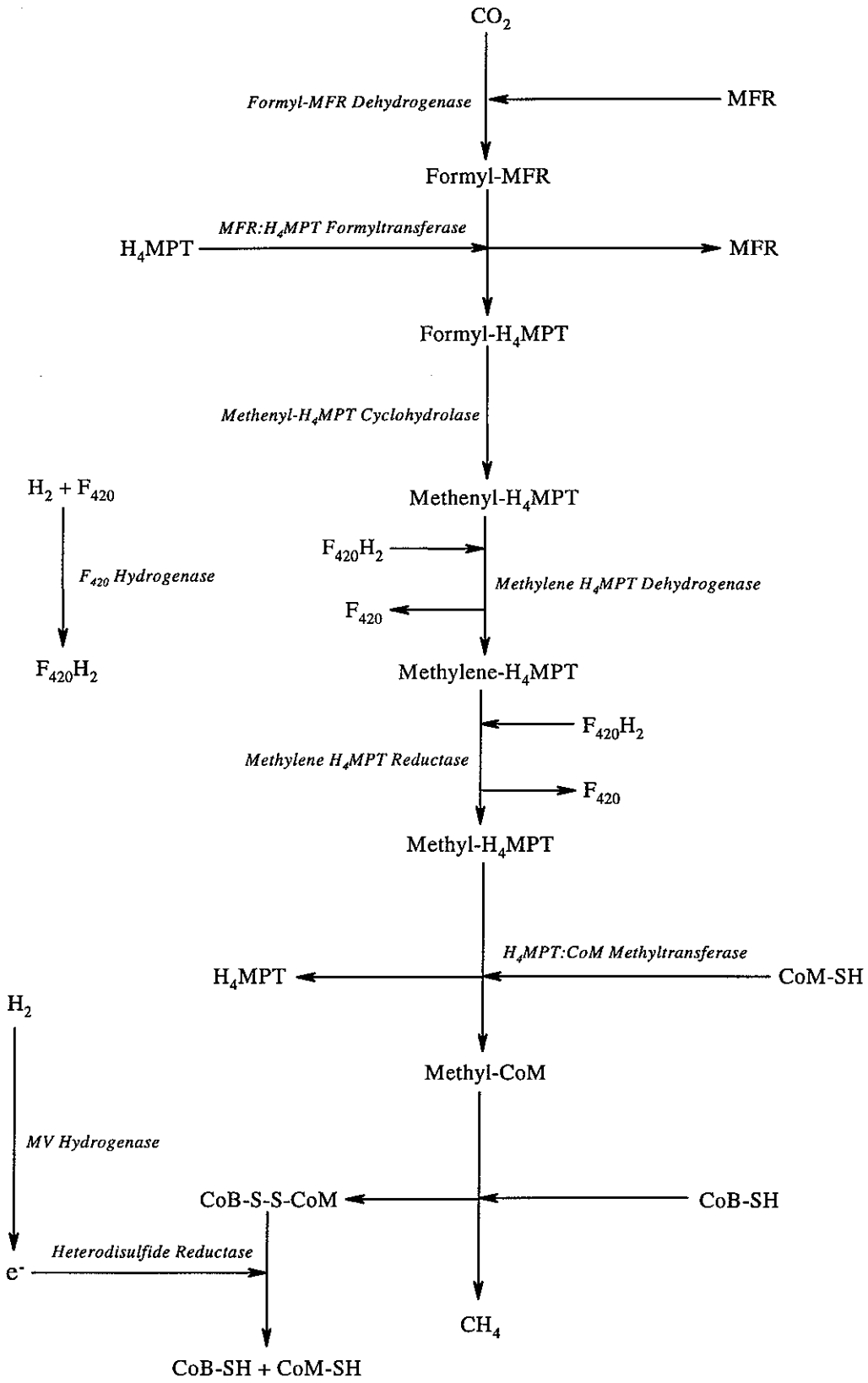
The process of methanogenesis occurs via a complex set of reactions involving interesting enzymes and a unique set of coenzymes, or cofactors. The basic pathway for $CO_2$ reduction (Figure 2) to methane has been described in the last decade (Daniels et al, 1984; Rouviere and Wolfe, 1988; Deppenmeier et al, 1996) based on the presence of these enzymes and coenzymes.

Methane production from $CO_2$ occurs via the overall reaction:

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O \quad (1)$$

The Gibbs free energy of this reaction is -138.8 kJ/mole $CH_4$ (Daniels et al, 1984; Thauer et al, 1977), more than enough free energy to form ATP ($\Delta G^{\circ\prime}$ = 31.8 kJ/mole ATP). These values are at standard conditions (ie. 1.0M concentration of solutes, gas pressures at 1.0 atmosphere, temperature of 25°C, and pH 7.0 ) which are never encountered *in situ*. The actual *in situ* concentrations of the substrate gases vary from location to location. Partial pressures of $4.8 \times 10^{-5}$ atm (approximately 36 nM) for $H_2$ has been recorded in the sediments of Lake Mendota (Conrad et al, 1986), $2.7 \times 10^{-5}$ atm (28 nM) in a rice paddy (Conrad et al, 1987), and $2.7 \times 10^{-4}$ atm (203 nM) in sewage sludge (Smolenski and Robinson, 1988). At a partial pressure of $10^{-3}$ atm, the dissolved $H_2$ concentration is 1 μM and the $\Delta G^{\circ\prime}$ for methanogenesis is -62.8 kJ/mole $CH_4$ which is sufficient to produce no more than 1 mole ATP per mole $CH_4$ (Thauer et al, 1977).

**Figure 2 -** Pathway of methanogenesis from $CO_2$ as determined from *Methanobacterium thermoautotrophicum* strain ΔH (Rouviere and Wolfe, 1988). Enzymes found to be involved in specific reactions are *italicized.*


$CO_2$
*Formyl-MFR Dehydrogenase*
MFR
Formyl-MFR
*MFR:$H_4$MPT Formyltransferase*
$H_4$MPT
MFR
Formyl-$H_4$MPT
*Methenyl-$H_4$MPT Cyclohydrolase*
Methenyl-$H_4$MPT
$H_2 + F_{420}$
*$F_{420}$ Hydrogenase*
$F_{420}H_2$
$F_{420}H_2$
$F_{420}$
*Methylene $H_4$MPT Dehydrogenase*
Methylene-$H_4$MPT
$F_{420}H_2$
*Methylene $H_4$MPT Reductase*
$F_{420}$
Methyl-$H_4$MPT
*$H_4$MPT:CoM Methyltransferase*
$H_4$MPT
CoM-SH
$H_2$
*MV Hydrogenase*
Methyl-CoM
CoB-S-S-CoM
CoB-SH
$e^-$
*Heterodisulfide Reductase*
$CH_4$
CoB-SH + CoM-SH

At the initial step of methanogenesis from $CO_2$ (top of figure 2), an unusual cofactor, methanofuran (MFR), also known as the carbon dioxide reducing (CDR) factor is used. This molecule, first isolated in 1982 (Romesser and Wolfe, 1982), combines with $CO_2$ in an energy requiring process ($\Delta G°' = +16$ kJ/mol, Blaut, 1994) to produce formylmethanofuran. The structure of methanofuran (Figure 3) has been determined (Leigh et al, 1984) to be a rather long linear organic molecule, with a 5-member furanose ring at the active end of the chain. This molecule donates the $C_1$-formyl group to the primary $C_1$ carrier for methanogenesis, for further conversion into the ultimate end product, methane.

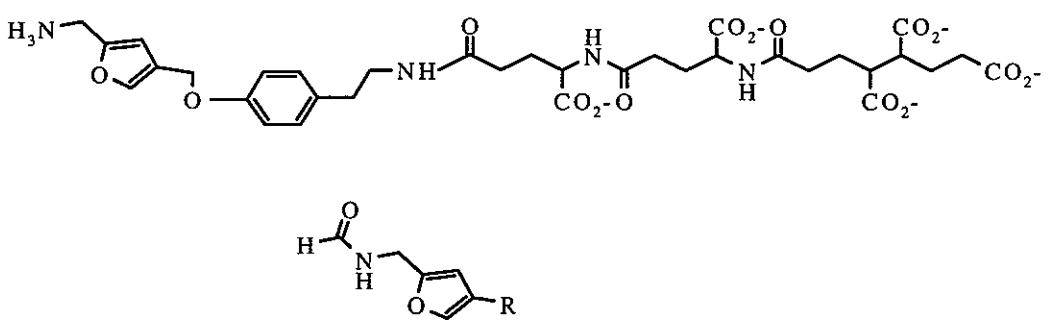


**Figure 3** - Structure of methanofuran, carbon dioxide reduction factor (CDR), and the formylated form of the coenzyme (Leigh et al, 1984).

An important cofactor involved in methanogenesis from $CO_2$ is a novel pterin called methanopterin (MPT), also known as cofactor $F_{342}$ or the formaldehyde activation factor (FAF) (Escalante-Semerena et al, 1984a). This compound, initially described in 1978 (Daniels and Zeikus, 1978), was determined to be the primary carbon carrier (see figure 2) for the process of methanogenesis at the formyl, methenyl, methylene, and methyl-level (DiMarco et al, 1990; Escalante-Semerena et al, 1984b). Its chemical structure (Figure 4) has been determined (van Beelen et al, 1984).

Initially, the carbon carrier of methanogenesis was thought to be tetrahydrofolate (THF), based on studies of the methanogenic coculture of *Mb. omelianskii* (Wood and Wolfe, 1965). The active form of MPT, is a highly reduced form, tetrahydromethanopterin ($H_4$MPT) which is both light and $O_2$-sensitive. It is a structural analog to the THF

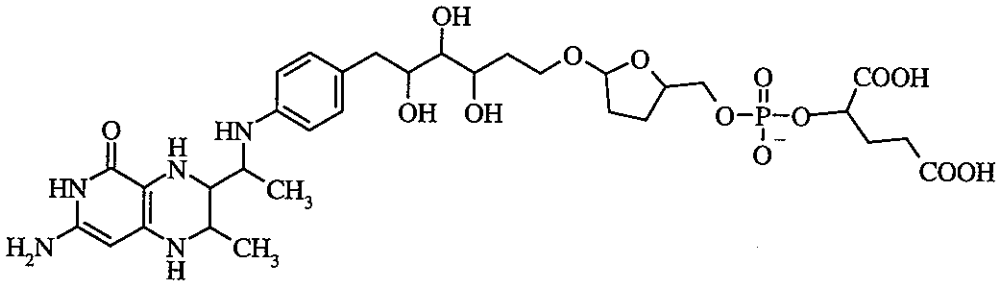


**Figure 4 -** Structure of tetrahydromethanopterin (van Beelen et al, 1984).

coenzyme (Figure 5) used in the purine salvaging pathway of the Bacteria and is also used in the acetate pathway of the acetogenic bacteria.

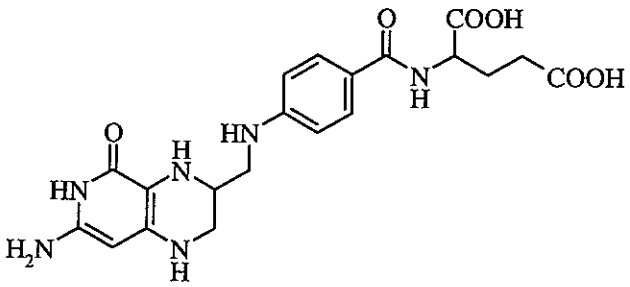
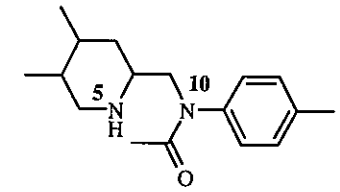


**Figure 5 -** Structure of tetrahydrofolic acid, a structural analogue of tetrahydromethanopterin.

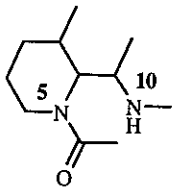
Elements of the pathway involving $H_4$MPT were found to be similar to the THF pathway (Figure 6) as analogous reduction steps occur in both pathways. The most significant difference between the two pathways is the initial binding of the $C_1$-group to the carrier. In the folate pathway, the initial intermediate is $N^{10}$-formyl-tetrahydrofolate, while the first intermediate in the methanogenic pathway is $N^5$-formyl-tetrahydromethanopterin (DiMarco et al, 1990).

Each of the reductive steps catalyzed by specific enzymes (see later), are energetically favourable (save one). The conversion of formyl-$H_4$MPT (CHO-$H_4$MPT) to methenyl-$H_4$MPT (CH≡$H_4$MPT) is only slightly thermodynamically favourable ($\Delta G°'$=

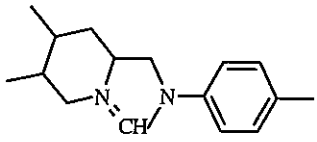
**Figure 6 -** Comparison of the two pathways, the tetrahydrofolate and the tetrahydromethanopterin $C_1$-carrier pathways used for purine salvage (THF) and methanogenesis ($H_4$MPT).



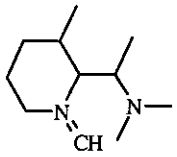

$N^{10}$-formyl THF



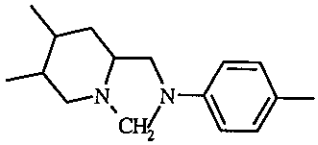

$N^5$-formyl $H_4$MPT



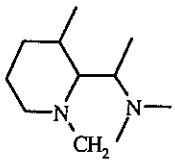

$N^5$,$N^{10}$-methenyl THF



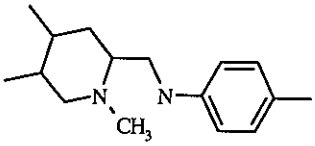

$N^5$,$N^{10}$-methenyl $H_4$MPT



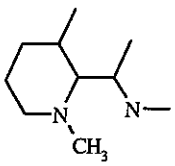

$N^5$,$N^{10}$-methylene THF




$N^5$,$N^{10}$-methylene $H_4$MPT




$N^5$-methyl THF




$N^5$-methyl $H_4$MPT

-2 kJ/mol). The next reduction step, to methylene-$H_4$MPT ($CH_2$=$H_4$MPT), requires a small input of free energy ($\Delta G°'$= +6.5 kJ/mol). The final reduction step to methyl-$H_4$MPT ($CH_3$-$H_4$MPT) also releases a small amount of energy ($\Delta G°'$= -5 kJ/mol).

In the last stage of the methanogenic pathway (bottom of figure 2), the methyl group of $CH_3$-$H_4$MPT is converted to methane through the use of two more important cofactors. One has been named coenzyme M (CoM). This molecule accepts the methyl-group from $H_4$MPT and is required for methanogenesis, regardless of which one of the various substrates are used. The transfer reaction from $H_4$MPT to CoM has a significant free energy release and is thus very favourable thermodynamically (see section 1.4.4.). This molecule was the first of the unique methanogenic cofactors to be discovered (McBride and Wolfe, 1971). It is a simple molecule (Figure 7), 2-mercaptoethane-sulfonic acid being its IUPAC standard chemical name.




**Figure 7** - Structure of coenzyme M (CoM) and methyl-CoM (McBride and Wolfe, 1971).

The other coenzyme involved at the terminal step of methanogenesis (bottom of figure 2) is 7-mercaptoheptanoylthreonine phosphate (HS-HTP). This coenzyme, whose structure is shown in Figure 8, provides the electrons via the methylreductase system in order to reduce the methyl group attached to CoM releasing it as methane (Noll and Wolfe, 1987). The two oxidized coenzymes combine into a heterodisulfide, HTP-S-S-CoM (Bobik et al, 1987; Ellerman et al, 1988), which must then be reduced to recycle the two coenzymes for another round of methanogenesis. The source of electrons for this coenzyme appears to be derived from methanophenazine (Abken and Deppenmeier, 1998). This has only been isolated from one methanogen growing on methanol, but can grow on $H_2$:$CO_2$.

The HS-HTP is only the minimum active portion of the coenzyme. The actual physiological cofactor was found to be an even more complicated molecule, uridine 5'-

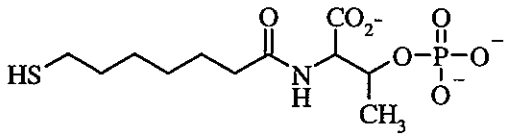


**Figure 8 -** Structure of 7-mercaptoheptanoylthreonine phosphate (HS-HTP).
(figure modified from DiMarco et al, 1990)

[N-(7-mercaptoheptanoyl)-O-3-phosphothreonine-P-yl(2-acetamido-2-deoxy-β-mannopyranuronsyl)(acid anhydride)]-(1→4)-O-2-acetamido-2-deoxy-α-glucopyranosyl diphosphate (see figure 9), also known as the methyl-reducing factor (MRF), not to be confused with methanofuran MFR, the carbon dioxide reducing factor described earlier. The current suggestion is to use CoB as the short form for MRF (in which case the heterodisulphide would be represented as CoB-S-S-CoM). CoB was described in association with the methanoreductosome (Mayer et al, 1988) which was reported to be a membrane-associated body that is composed of the methylreductase system, though, there is a suggestion and some evidence for this being an artifact of the drying process in negative staining (Sprott and Beveridge, 1993) due to the properties of the methylreductase enzyme.

Other ancillary cofactors were found, one being coenzyme $F_{430}$, not to be confused with coenzyme $F_{420}$. Coenzyme $F_{430}$ is a nickel tetrapyrrole (see Figure 10) (Deikert et al, 1980) whereas the latter is a flavin derivative. This coenzyme is involved in, and is

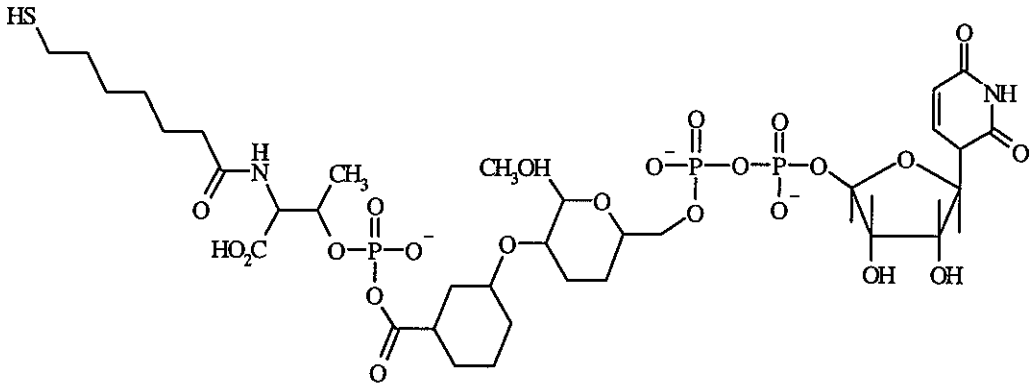


**Figure 9 -** Proposed structure of coenzyme B (Sauer et al, 1987).

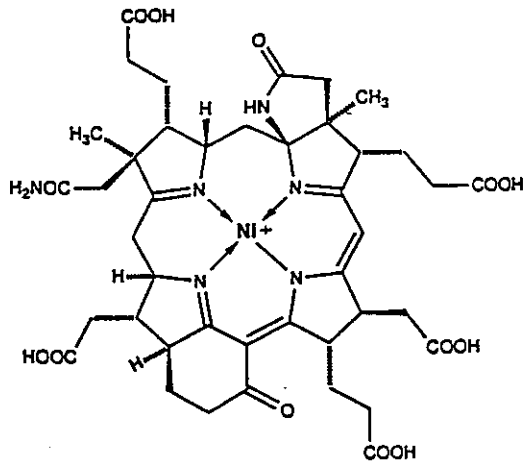



**Figure 10** - Structure of coenzyme $F_{430}$ (Diekert et al, 1980).

required for, the terminal step of methane production which is the reduction of the $CH_3$-CoM, catalyzed by methylreductase (Hartzell and Wolfe, 1986). The $F_{430}$ is bound, though not covalently, to the methylreductase enzyme and is likely involved in the reduction reaction to convert the methyl group into methane (Thauer et al, 1993).

An important methanogenic coenzyme, coenzyme $F_{420}$ (based on its absorption spectrum maximum), is involved in many of the electron transfer (reduction) steps in methanogenesis. This compound was discovered in 1972 (Cheeseman et al, 1972). The structure of the cofactor (Figure 11) has been determined (Eirich et al, 1978), to be 8-hydroxy-5-deazaflavin, and is a derivative of known electron carrying compounds, the flavins.

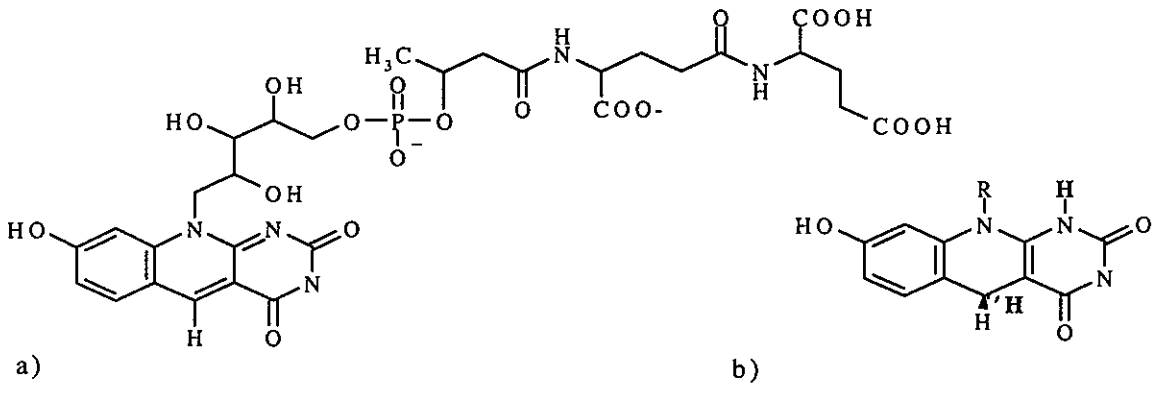



**Figure 11** - Structure of coenzyme $F_{420}$. The key electron carrier in the methanogens (Eirich et al, 1978). (a) Structure of oxidized $F_{420}$. (b) Showing reduced $F_{420}$. R = rest of the molecule.

Typically the electron donor for $CO_2$ methanogenesis is $H_2$. The electrons extracted from this gas by the enzyme, hydrogenase (see section 1.7.1.) will usually reduce the methanogenic cofactor, $F_{420}$ directly. However, other electron sources that may be used include primary alcohols like ethanol and secondary alcohols like 2-propanol and 2-butanol (Widdell, 1986; Zellner and Winter, 1987), eg., *Methanobacterium palustre* uses secondary alcohols as electron donors in $CO_2$ methanogenesis (Zellner et al, 1989).

The pathways for methanogenesis from other substrates have been described and involve certain portions of the pathway of methanogenesis from $CO_2$. They all share the methylreductase and heterodisulfide system.

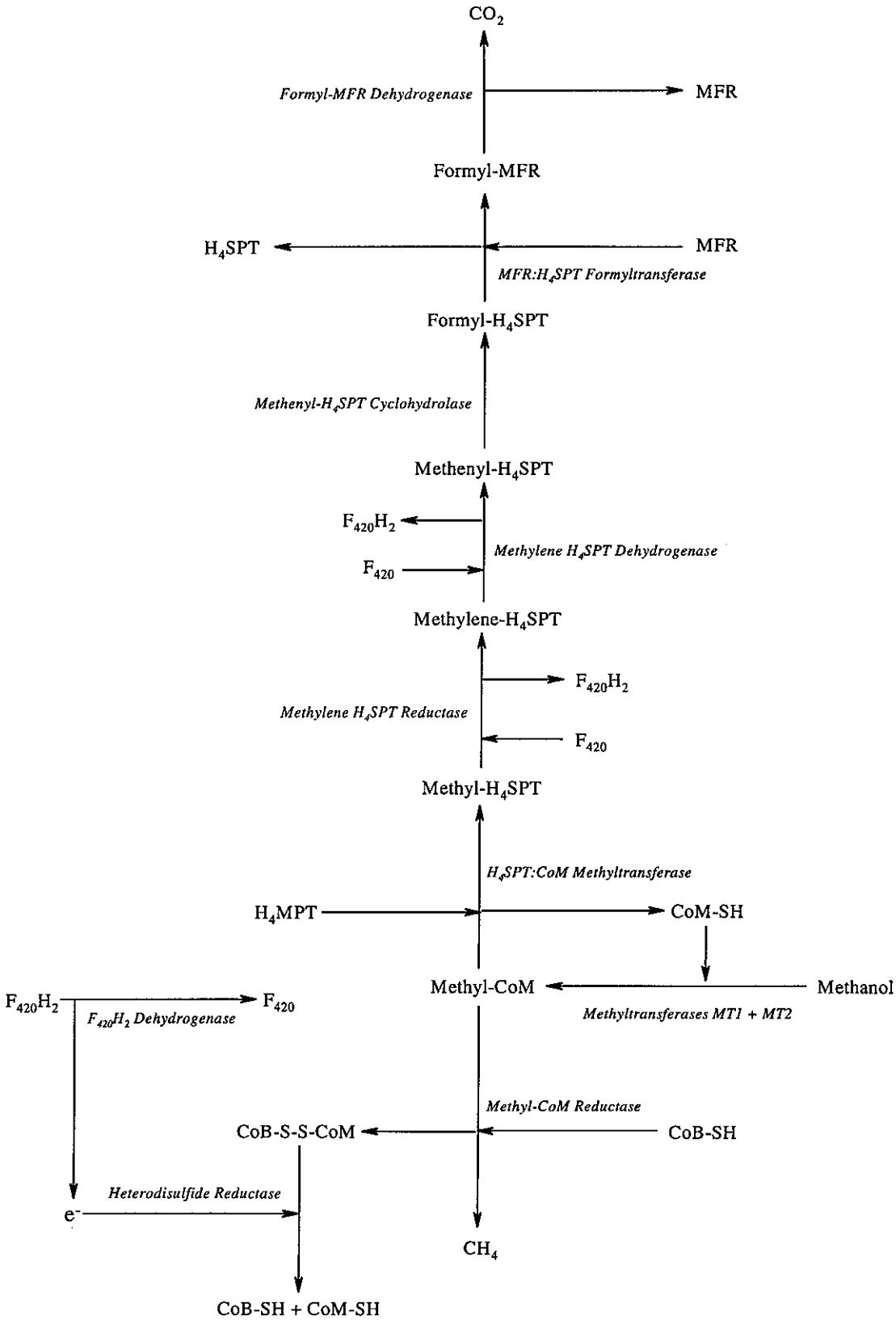
### 1.4.2. Methanogenesis from methanol

Methanogenesis from methanol can occur in either of two ways; (1) without a separate electron donor (methanol disproportionation) or (2) with $H_2$ (or another electron source) as the electron donor (figure 12). The first form of methanol methanogenesis follows the overall reaction:

$$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O \quad (2)$$

The process is energetically favourable ($\Delta G^{\circ\prime} = -103.5$ kJ/mol $CH_4$, Daniels et al, 1984) under standard conditions. This form of methanogenesis involves the oxidation of one mole of methanol to $CO_2$ to provide the electrons for the reduction of three moles of methanol to methane. This is termed disproportionation since a fixed proportion of the substrate, here methanol, is converted to an alternate end product, here $CO_2$, to allow for the conversion of methanol, into methane. This method of methanogenesis requires the complete pathway of methanogenesis from $CO_2$ although it uses much of the $CO_2$ pathway in the reverse direction to produce the necessary reducing equivalents to form methane (see Figure 12), note that the main carbon carrier is a $H_4$MPT derivative, tetrahydrosarcinopterin ($H_4$SPT) which differs by the addition of a glutamyl residue (van

**Figure 12 -** Pathway of methanogenesis from methanol (+/- $H_2$). Enzymes found to be involved in specific reactions are *italicized*. (Figure modified from Keltjens and Vogels, 1993)


$CO_2$
Formyl-MFR Dehydrogenase
MFR
Formyl-MFR
$H_4SPT$
MFR
MFR:$H_4$SPT Formyltransferase
Formyl-$H_4$SPT
Methenyl-$H_4$SPT Cyclohydrolase
Methenyl-$H_4$SPT
$F_{420}H_2$
$F_{420}$
Methylene $H_4$SPT Dehydrogenase
Methylene-$H_4$SPT
$F_{420}H_2$
Methylene $H_4$SPT Reductase
$F_{420}$
Methyl-$H_4$SPT
$H_4$SPT:CoM Methyltransferase
$H_4$MPT
CoM-SH
Methyl-CoM
Methanol
Methyltransferases MT1 + MT2
$F_{420}H_2$
$F_{420}$
$F_{420}H_2$ Dehydrogenase
Methyl-CoM Reductase
CoB-S-S-CoM
CoB-SH
Heterodisulfide Reductase
$e^-$
$CH_4$
CoB-SH + CoM-SH

Beelen et al, 1984). In the conversion of methanol, the methyl group of the methanol is transferred directly to cofactor CoM through the action of a pair of methyltransferases, and undergoes direct conversion to methane as described above.

Based on the process described above, methanogenesis from methanol alone requires the same cofactors as methanogenesis from $CO_2$. As well the enzymes required are the same with the exception of an additional pair of interesting methyltransferases that are specifically involved in methanol activation (see section 1.5.).

The other method of methanogenesis from methanol utilizes an external electron donor like $H_2$. The overall reaction for this method of methanogenesis is:

$$CH_3OH + H_2 \rightarrow CH_4 + H_2O \quad (3)$$

The overall reaction is energetically more favourable than methanol disproportionation ($\Delta G^{\circ\prime} = -112.5$ kJ/mol $CH_4$, Thauer et al, 1977). It does not require the complete pathway used for methanogenesis from $CO_2$, and, in fact, requires just the terminal steps, ie., uses the steps of methyl group transfer and conversion to methane described above for the methanol-alone conversion (Figure 12).

Two species of the genus *Methanosphaera* strictly grow on methanol plus $H_2$ as sole methanogenic substrates (Biavati et al, 1988; Miller and Wolin, 1985). The methanogenic pathway for *Msph. stadtmanae* has been described (Van der Wijngaard et al, 1991).

### 1.4.3. Methanogenesis from acetate

The energy released by methanogenesis from acetate is the lowest of all the methanogenic substrates ($\Delta G^{\circ\prime} = -27.6$ kJ/mol $CH_4$, Daniels et al, 1984). This may explain the slow growth of methanogens that use acetate as the sole methanogenic substrate, eg., *Methanothrix concilii*, renamed *Methanosaeta concilii*, grows to a maximum density in 2 1/2 to 3 weeks (Patel, 1984). The overall reaction is stated as follows:

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (4)$$

The pathway of methanogenesis from acetate (Figure 13) has been elucidated and involves only the terminal end of the methanogenic pathway from $CO_2$. The acetic acid is first activated with ATP to acetyl-phosphate, followed by its conversion into acetyl-CoA. Acetyl-CoA is then cleaved into "CO" and the methyl group is transferred to $H_4SPT$ to form $CH_3$-$H_4SPT$ (Fischer and Thauer, 1989), which proceeds as it would in methanogenesis from $CO_2$ (Ferry, 1992). The overall reaction from acetyl-CoA is as follows:

$$\text{Acetyl-CoA} + \text{CoM} \rightleftharpoons CH_3\text{-CoM} + CO_2 + \text{CoA-SH} + 2\,e^- \quad (5)$$

The "CO" is oxidized to $CO_2$, by the carbon monoxide dehydrogenase (CODH, see section 1.7.3.) with the electrons produced being used to reduce the methyl group to $CH_4$.

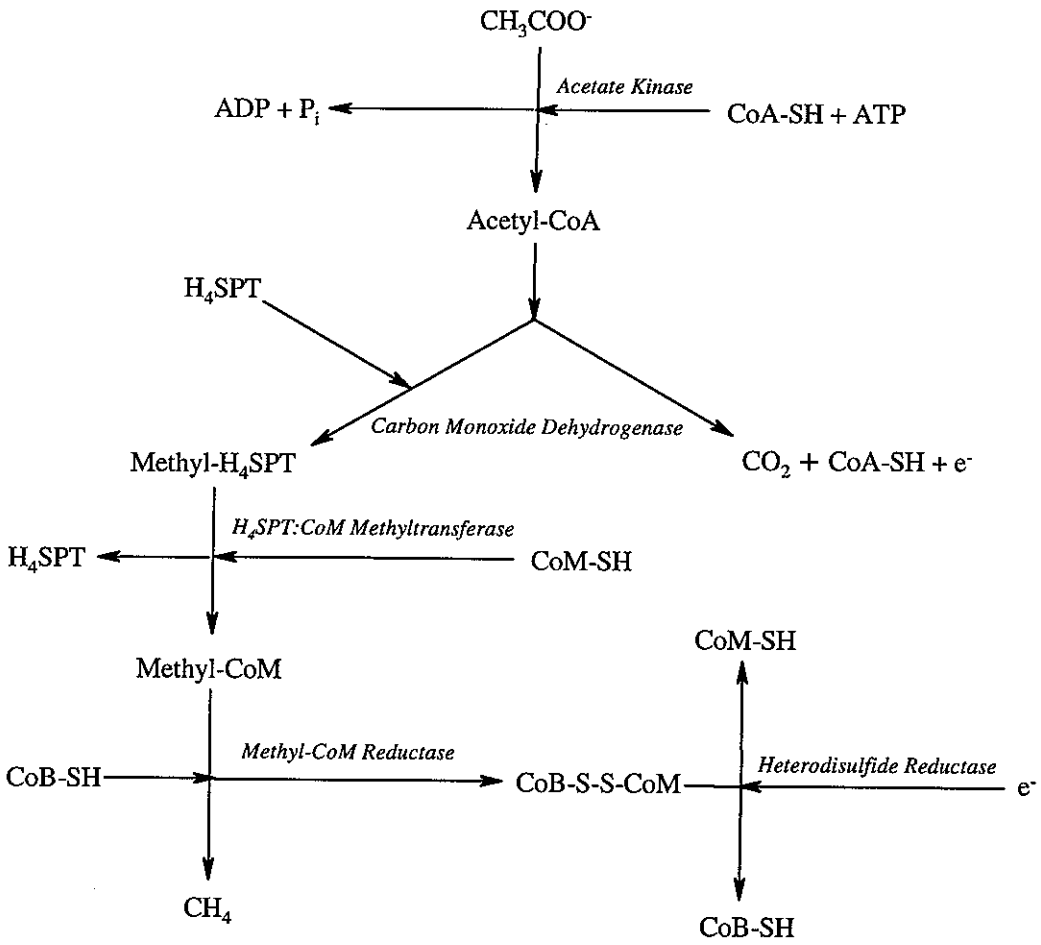
Therefore, acetate methanogenic pathway shares the terminal end of the methanogenesis by the $CO_2$ pathway with the $C_1$-group entering as the methyl-$H_4MPT$ one step prior to where the $C_1$-group of methanol enters.

### 1.4.4. Energy conservation in the Methanogens

Since methanogenesis is the method by which these cells obtain energy, it is important to see where and how energy is conserved in the form of the energy-rich phosphate bond in ATP (Lipmann, 1941), the primary energetic molecule in all organisms (Thauer et al, 1977). The formation of ATP from ADP requires a minimum of 31.8 kJ/mole (7.6 kcal/mole) to be released from a biological reaction (Thauer et al, 1977). The common modes of ATP formation are via substrate-level phosphorylation (SLP) and electron transport phosphorylation (ETP).

SLP occurs during the well known Embden-Meyerhoff and Entner-Doudoroff pathways in which the intermediates of the pathways are phosphorylated and dephosphorylated, the latter reaction being coupled to the production of ATP. ETP has a

**Figure 13 -** Pathway of methanogenesis from acetate in *Methanosarcina* spp. Enzymes found to be involved in specific reactions are *italicized.* (Figure modified from Ferry, 1993)


$CH_3COO^-$
Acetate Kinase
ADP + $P_i$
CoA-SH + ATP
Acetyl-CoA
$H_4SPT$
Carbon Monoxide Dehydrogenase
Methyl-$H_4SPT$
$CO_2$ + CoA-SH + $e^-$
$H_4SPT$:CoM Methyltransferase
$H_4SPT$
CoM-SH
Methyl-CoM
CoM-SH
Methyl-CoM Reductase
CoB-SH
CoB-S-S-CoM
Heterodisulfide Reductase
$e^-$
$CH_4$
CoB-SH

strict requirement for membranes and occurs in the electron transport chain, or respiratory chain (Anraku, 1988) as well as in photosynthesis (Thauer, 1988) and generates a proton motive force (pmf). Electrons are transported from one electron carrier to another electron carrier with a higher or more positive redox potential to the terminal electron acceptor with the concomitant formation of pmf. ATP is formed by a membrane-bound ATP synthase or ATPase as protons (or other ions) are translocated across the membrane at the same time (Pederson and Carafoli, 1987). In anaerobic microorganisms, electrons are used to reduce a variety of terminal electron acceptors including $CO_2$ to methane, $NO_3^-$ to $NO_2^-$, fumarate to succinate, and $SO_4^{2-}$ to $S^{2-}$. Experiments with uncouplers indicate the most likely method of energy generation is the formation of a proton or ion gradient (proton-motive or ion-motive force) across a membrane (Thauer et al, 1977).

The bioenergetics of methanogenesis have been reviewed extensively (Blaut et al, 1992; Daniels et al, 1984; Deppenmeier et al, 1996; Thauer, 1990; van Verseveld and Thauer, 1987). In the methanogenic pathway there are only a few sites where sufficient free energy is released to form ATP. The Gibb's free energy associated with each of the methanogenic reactions are summarized on Table 2. Only three of these reactions are sufficiently energetic enough to form ATP, ie., methyl transfer from $H_4$MPT to CoM-SH (-29.7 kJ/mole), the release of $CH_4$ from CoM-SH (-43 kJ/mole), and the reduction of the heterodisulfide of CoM-SH and HS-HTP (-42 kJ/mole).

SLP does not occur in the methanogens at any point in the methanogenic pathway. There is no phosphorylation of intermediates at these steps nor dephosphorylation at the $CO_2$-activation step involving formylmethanofuran dehydrogenase where an input of energy is required. Instead, ATP is formed via a chemiosmotic mechanism, involving the generation of transmembrane potentials of protons and sodium ions which are converted to ATP via an $H^+$-ATPase, and sodium ions, via an $Na^+$-ATPase respectively. Both enzymes have been isolated from methanogenic bacteria (Carper and Lancaster, 1986; Dharmavaram and Konisky, 1987; Inatomi, 1986; Scheel and Schäfer, 1990). Studies with membrane vesicles have determined that both a proton motive and a sodium motive force drives the synthesis of ATP in *Methanosarcina mazei* strain Gö1 (Becher and

**Table 1.2 - Standard Gibbs Free Energy ($\Delta G°'$) for Methanogenic Reactions**

| Methanogenic Reaction | $\Delta G°'$ (kJ/mole)* |
|---|---|
| $CO_2 + H_2 + MF \rightarrow CHO\text{-}MF + H_2O$ | +16.0 |
| $CHO\text{-}MF + H_4MPT \rightarrow CHO\text{-}H_4MPT + MF$ | -5.0 |
| $CHO\text{-}H_4MPT + H^+ \rightarrow CH{\equiv}H_4MPT + H_2O$ | -2.0 |
| $CH{\equiv}H_4MPT + F_{420}H_2 \rightarrow CH_2{=}H_4MPT + F_{420} + H^+$ | +6.5 |
| $CH_2{=}H_4MPT + F_{420}H_2 \rightarrow CH_3\text{-}H_4MPT + F_{420}$ | -5.0 |
| $CH_2{=}H_4MPT + H_2 \rightarrow CH_3\text{-}H_4MPT + H^+$ | -5.5 |
| $CH_3\text{-}H_4MPT + HS\text{-}CoM \rightarrow CH_3\text{-}S\text{-}CoM + H_4MPT$ | -29.0 |
| $CH_3\text{-}S\text{-}CoM + HS\text{-}HTP \rightarrow CH_4 + CoM\text{-}S\text{-}S\text{-}HTP$ | -43.0 |
| $CoM\text{-}S\text{-}S\text{-}HTP + H_2 \rightarrow CoM\text{-}SH + HTP\text{-}SH$ | -42.0 |
| $F_{420} + H_2 \rightarrow F_{420}H_2$ | -13.5 |

* - values from Blaut, 1994.

Müller, 1994).

The reactions of $CH_3$-$H_4$MPT:CoM methyltransferase, $CH_3$-CoM methylreductase, and heterodisulfide reductase have been experimentally linked to the production of ion gradients. The methyltransferase is linked with the generation of a sodium gradient (Becher et al, 1992) and the methylreductase system and specifically, the heterodisulfide reductase reaction are linked with the generation of a proton gradient (Blaut et al, 1987; Deppenmeier et al, 1991). Therefore these reactions generate a transmembrane ion gradient which allows the cell to synthesize ATP through either the $H^+$-ATPase or the $Na^+$-ATPase. In acetate conversion to methane the oxidation of the CO to $CO_2$ is also coupled to proton translocation (Bott et al, 1986). This is important since acetate activation requires ATP hydrolysis to form acetyl-CoA.

Some unique, interesting enzymes are involved in the specific steps of methanogenesis from the different substrates. The enzymology of methanogenesis from the three major methanogenic substrates, $H_2$ plus $CO_2$, methanol, and acetate will be discussed in further detail.

## 1.5. The Enzymology of methanogenesis.

The enzymology of the methanogens is interesting especially when one considers all of the different substrates that are used for growth and methanogenesis. The enzymology of the methanogens has been reviewed recently (Blaut, 1994; Deppenmeier et al, 1996; Ferry, 1992; Rouviere and Wolfe, 1988). Most, if not all of the enzymes involved in $CO_2$ methanogenesis, have been purified from several methanogens including the hydrogenotrophic species *Methanobacterium thermautotrophicum* strains ΔH and Marburg and various strains of methylotrophic species, *Methanosarcina barkeri*. *Methanosarcina* uses part of the $CO_2$ pathway in methanogenesis from methanol and from acetate, as well as using the complete $CO_2$ pathway when it grows on $H_2$:$CO_2$. In fact, the whole genus *Methanosarcina* can utilize essentially all known methanogenic substrates, with the exception of formate, to produce methane (Garcia, 1990).

### 1.5.1. Enzymology of $CO_2$ methanogenesis.

The first enzyme, formyl-MF dehydrogenase, which catalyzes the activation of the $CO_2$ to formylmethanofuran (figure 2), has been isolated from several methanogens including *Mb. thermoautotrophicum* strain Marburg (Börner et al, 1991) and *Ms. barkeri* strain Fusaro (Karrasch et al, 1990) which uses the enzyme in the final step of methanol oxidation to $CO_2$. This enzyme is a molybdenum-iron-sulfur protein with the molybdenum bound in the form of a molybdopterin. It is a very large protein in *Ms. barkeri* with a molecular mass of 220 kDa and is composed of 6 subunits with masses of 65, 50, 37, 34, 29, and 17 kDa. The enzyme from *Mb. thermoautotrophicum* is smaller and less complex with a native molecular mass of 100 kDa and subunits of 60 and 45 kDa. Interestingly, in *Methanobacterium wolfei* there is a second form of the enzyme, an isozyme, which replaces the molybdenum with tungsten (Schmitz et al, 1992).

The second enzyme in the methanogenic pathway is formylmethanofuran:$H_4$MPT formyltransferase which transfers the formyl group from methanofuran to $H_4$MPT, the primary $C_1$ carrier in methanogens. This enzyme has been purified from a number of methanogens including *Mb. thermoautotrophicum* (Donnelly and Wolfe, 1986), *Ms.*

*barkeri* (Breitung and Thauer, 1990), and *Mpy. kandleri* (Breitung et al, 1993). The enzyme from *Mb. thermoautotrophicum* strain ΔH is composed of a single subunit of 41 kDa while the enzyme from *Ms. barkeri* is a monomer of 32 kDa and that from *Mpy. kandleri* is a monomer of 35 kDa.

The first enzyme involved in the conversion of the formyl group is the $N^5$,$N^{10}$-methenyltetrahydromethanopterin cyclohydrolase which converts the formyl group into the methenyl group (which does not change the oxidation level of the carbon). The enzyme has been purified from various methanogens including *Ms. barkeri* (te Brömmelstroet et al, 1990), *Mb. thermoautotrophicum* strain Marburg (Mukhopadhyay and Daniels, 1989), and *Mpy. kandleri* (Breitung et al, 1991). The enzyme, purified aerobically from *Mb. thermoautotrophicum* strain Marburg, is a dimer with a subunit of 41 kDa. The enzyme from *Ms. barkeri* is similar with a dimer of 41 kDa subunits. The enzyme found in *Mpy. kandleri* is different in that it is a monomer of a 41.5 kDa polypeptide.

The next enzyme in the methanogenic pathway is $N^5$,$N^{10}$-methylenetetrahydro-methanopterin dehydrogenase which catalyzes the conversion of the methenyl into a methylene group. The enzyme has also been purified in *Mb. thermoautotrophicum* strain Marburg (Mukhopadhyay and Daniels, 1989) and *Mpy. kandleri* (Ma et al, 1991). The enzyme in *Mb. thermoautotrophicum* is composed of a single subunit of 32 kDa in size and that in *Mpy. kandleri* is a tetramer of 44 kDa subunits (170 kDa total native molecular weight).

An interesting side note is the observation that in *Mb. thermoautotrophicum* there are two forms of this dehydrogenase, one is coenzyme $F_{420}$-dependent and one is $H_2$-dependent, ie. it has hydrogenase activity (Zirngibl et al, 1990). These two dehydrogenases have been determined to be distinct, separate enzymes (von Bünau et al, 1991). The combination of these two forms of the enzyme have been found to work together in *Mb. thermoautotrophicum* when grown under $Ni^{2+}$-limited conditions (ie., no $F_{420}$-hydrogenase) and the combination provides reduced $F_{420}$ for methanogenesis (Afting and Thauer, 1998). The $H_2$-forming dehydrogenase has been purified from 2 other

methanogens, *Mpy. kandleri* (Ma et al, 1991) and *Methanococcus thermolithotrophicus* (Hartmann et al, 1996). However, in *Ms. barkeri*, the $F_{420}$-dependent form is the only form that is present in this species (Blaut et al, 1992).

The enzyme involved in the conversion of the methylene-$H_4$MPT to the methyl-$H_4$MPT is the $N^5$,$N^{10}$-methylenetetrahydromethanopterin reductase. This enzyme has been isolated from *Mb. thermoautotrophicum* strain ΔH (te Brömmelstroet et al, 1990) and *Mpy. kandleri* (Ma et al, 1991). This enzyme is cofactor $F_{420}$-dependent and is a rather large protein of some 300 kDa (*Mpy. kandleri*), but is only made up of a single type of subunit with a molecular mass of 38 kDa.

The enzyme involved in the transfer of the methyl group from $H_4$MPT to CoM is methyltetrahydromethanopterin:CoM methyltransferase which was first identified in *Mb. thermoautotrophicum* (Sauer, 1986). In the first report, the enzyme was found to be unstable. However, in a second study using *Mb. thermoautotrophicum* strain ΔH, a reductive activation was found to be required for activity. Catalytic amounts of ATP activated the system, but several strong reducing systems could replace the ATP, eg., titanium citrate, an oxygen scavenging anaerobic buffering system (Zehnder and Wuhrmann, 1976). A method of directly assaying this enzyme was devised only recently (Fischer et al, 1992). Prior to this, the enzyme could only be assayed indirectly, requiring an enzyme system to be present, thus hindering the purification of this enzyme.

The methyltransferase was first purified from *Mb. thermoautotrophicum* strain Marburg. It is a corrinoid (a cobalt atom containing tetrapyrrole) protein with a molecular mass of approximately 100 kDa and is a heterotrimer composed of subunits of 31, 33, and 35 kDa (Kengen et al, 1992). Corrinoids have been found in a number of other bacteria as $C_1$ carriers (Stupperich et al, 1990). The enzyme in *Methanosarcina* strain Gö1 is a membrane protein that acts as a primary sodium pump (Becher et al, 1992) and thus, is active in the bioenergetics of the cell.

The enzyme system that catalyzes the reduction of the methyl group of the $CH_3$-CoM is comprised of two enzymes and a cofactor, HS-HTP (or CoB). The methyl-CoM reductase reduces the methyl group on $CH_3$-CoM, with electrons provided by HS-HTP,

releasing $CH_4$ and forming a heterodisulfide of CoM and HTP (CoM-S-S-HTP or CoM-S-S-CoB). This enzyme has also been purified from a number of methanogens including the well-studied *Mb. thermoautotrophicum* strain Marburg (Ellerman et al, 1989). This enzyme is very large (300 kDa) with a pair of tightly bound, though not covalently, molecules of coenzyme $F_{430}$ (Ellefson and Wolfe, 1981). This represents another methanogenic enzyme that exists in multiple, genetically-distinct forms (isozymes) within the same cell (Rospert et al, 1990).

The second enzyme in the methylreductase system is heterodisulfide reductase. In the methyl-CoM reductase reaction, methane is released with the concomitant formation of a heterodisulfide of the two coenzymes, CoM and CoB. In order to recycle these coenzymes, the heterodisulfide reductase reduces the disulfide bond with $H_2$ (*Mb. thermoautotrophicum*, Hedderich and Thauer, 1988) or $F_{420}H_2$ (*M. tindarius* and *Ms. mazei* strain Gö1, Deppenmeier et al, 1990) depending on the methanogenic substrate. This enzyme from *Mb. thermoautotrophicum* strain Marburg (Hedderich et al, 1990) is extremely large (550 kDa) and made up of 3 different subunits in a $\alpha_4\beta_4\gamma_4$ arrangement with molecular masses of 80, 36, and 21 kDa. Heterodisulfide reductase is an iron-sulfur protein with 4 molecules of bound FAD per enzyme. It is important to note that the pure enzyme does not have a dependence on either of $H_2$ or $F_{420}$ as electron donors. The assay for this enzyme is performed using only methyl viologen as the electron donor, however using membrane vesicles along with methyl viologen hydrogenase or $F_{420}H_2$ dehydrogenase and an electron carrier in the form of a cytochrome (Blaut et al, 1990) or a novel molecule which acts like a quinone, methanophenazine (Abken et al, 1998), $H_2$ or $F_{420}H_2$ can act as electron donors for the heterodisulfide reductase reaction.

### 1.5.2. Enzymology of methanol methanogenesis.

Methanogenesis from methanol alone requires most of the enzymes described above, judging by the number of those enzymes being purified from *Ms. barkeri*, as described in the previous section. In addition, there are several enzymes specifically required for the activation of the substrate, methanol (van der Meijden et al, 1983).

The enzyme involved in methyl group transfer from methanol, methanol:5-hydroxybenzimidazolyl-cobamide methyltransferase (or $MT_1$) was purified from methanol-grown *Ms. barkeri* (van der Meijden et al, 1984). This enzyme is an $O_2$-stable corrinoid enzyme. The active site contains a Co atom that is directly involved in the binding and release of the methyl group. The enzyme has a molecular mass of 122 kDa in the native form with subunit masses of 34 and 53 kDa in a $\alpha_2\beta$ arrangement.

The second enzyme involved in methyl group transfer is the Co-methyl-5-hydroxybenzimidazolylcobamide:CoM methyltransferase (or $MT_2$) and has also been purified from *Ms. barkeri* (van der Meijden et al, 1983). This enzyme is also $O_2$-stable with an approximate molecular mass of 40 kDa and is made up of a single subunit.

The enzymes catalyzing the alternative form of methanol methanogenesis with $H_2$ as the electron donor are similar to those in methanol disproportionation. In both *Methanosarcina* spp. and *Methanosphaera stadtmanae* (van de Wijngaard et al, 1991), both methyltransferases, $MT_1$ and $MT_2$, have been detected under these growth conditions. In addition, in *Msph. stadtmanae*, the enzymes for the rest of the $CO_2$ pathway are present in low levels except for the complete absence of methanofuran (van de Wijngaard et al, 1991).

### 1.5.3. Enzymology of acetate methanogenesis.

Methanogenesis from acetate involves the cleavage of the acetate into the carbonyl and methyl groups (figure 13). The carbonyl portion is oxidized to $CO_2$ while the methyl group is transferred to a corrinoid containing methyltransferase associated with the carbon monoxide dehydrogenase (CODH) complex. The complex transfers the methyl group to coenzyme CoM, which functions as described in the previous sections on $CO_2$ and methanol methanogenesis.

In the two genera that perform methanogenesis from acetate, activation of acetate is performed by different enzyme systems. In *Methanosarcina* spp., acetate kinase, in combination with phosphotransacetylase, is used, whereas in *Methanothrix* (*Methanosaeta*) spp., this step is performed by the acetyl-CoA synthetase (ACS) (Jetten et

al, 1992). ACS forms a complex with another enzyme, the CODH which is involved in electron transfer reactions in acetate methanogenesis (see next section).

The enzymes of $CO_2$ methanogenesis have been also detected in the cell extracts from *Methanosarcina* spp. when grown on acetate (Jablonski et al, 1990) but are only present at low levels. This supports their role in the steps of the oxidation of the carboxyl-group of the acetate to produce electrons for the reduction of the methyl-group. Another enzyme of note, carbonic anhydrase, has also been detected, but its role in acetate methanogenesis in the oxidation of the carboxyl-group is only speculative at this point (Ferry, 1993).

As alluded to previously and shown above, the methanogenic cofactor $F_{420}$ is the important carrier of electrons in the production of methane from the various substrates and for other processes in the cell. The electron transport system in the methanogens, centering on this cofactor, will be described in the next section.

## 1.6. Electron transport and redox reactions in the Methanogens.

### 1.6.1. Electron carriers in the Methanogens.

Cofactor $F_{420}$, a 5-deazaflavin, is the primary electron carrier in methanogenic bacteria for the reduction of the various methanogenic substrates to methane (as had been described in section 1.4.1.). It is an obligate two electron carrier, like the nicotinamides, whereas flavins are either one or two electron acceptors. A flavin derivative is also present in a number of bacterial species, including species of *Streptomyces*, *Mycobacterium*, and *Nocardia* (Daniels et al, 1985). The cofactor is involved in several important biological functions including DNA photorepair and antibiotic (chlortetracycline) synthesis in *Streptomyces* spp. (Eker et al, 1981; McCormick and Morton, 1982), DNA photorepair in *Anacystis nidulans* (Malhotra et al, 1992), and glycolysis, as the cofactor for the glucose-6-phosphate dehydrogenase enzyme in *Mycobacterium* spp. (Purwantini and Daniels, 1996) and *Nocardia* spp. (Purwantini et al, 1997).

Besides the methanogens, other members of the Archaea possess a 5-deazaflavin,

these include *Archaeoglobus*, a genus of sulfate-reducers (Stetter, 1988), *Ferroglobus* (Hafenbradl et al, 1996), a genus of iron oxidizing, nitrate-reducing Archaea, and *Halobacterium*, a genus of extreme halophiles (Larsen, 1984). The first two genera not only have this methanogenic cofactor but also carry most of the methanogenic enzymes which they use to fix $CO_2$ (Burgraff et al, 1990; Vorholt et al, 1995; Vorholt et al, 1997). In *Halobacterium*, the cofactor is involved in DNA photorepair (Iwasa et al, 1988) in addition to electron transport (de Wit and Eker, 1987). As well, a eukaryotic microorganism carries this deazaflavin, eg., the green alga *Scenedesmus acutus* (Eker et al, 1988) used in DNA photoreactivation.

Methanogens also possess other electron carriers that are not devoted to the production of methane, these include the nicotinamides ($NAD^+$ and $NADP^+$), flavins (which are non-covalently bound prosthetic groups on enzymes), b and c-type cytochromes (limited to a few members of the Order *Methanomicrobiales*), and some unusual quinone derivatives (α-tocopheroquinone in *Mb. thermoautotrophicum*) (Daniels et al, 1984). The electrons from $F_{420}$ can be transferred to $NADP^+$ for use in biosynthesis through the $NADP^+$:$F_{420}$ oxidoreductase (Zeikus et al, 1977). In addition, the class of electron transporting proteins, the ferredoxins, have been isolated from certain members of the Order *Methanomicrobiales* (Moura et al, 1982; Terlesky and Ferry, 1988), and the *Methanococcales* (Hatchikian et al, 1989). An unusual polyferredoxin was isolated from a member of the Order *Methanobacteriales*, *Mb. thermoautotrophicum* strain ΔH (Hedderich et al, 1992; Steigerwald et al, 1992) but its role in electron transport has not been determined.

A recent discovery mentioned earlier (section 1.5.1.) was the discovery of an electron carrier that has been given the name methanophenazine (figure 14). Phenazines have been found in many bacteria, including *Pseudomonas* and *Nocardia* and are constituents of the pigments in these organisms while phenazines form components of antibiotics produced by *Streptomyces* spp. (Turner and Messenger, 1986). Experiments have shown that this molecule can serve as the electron carrier between $F_{420}H_2$

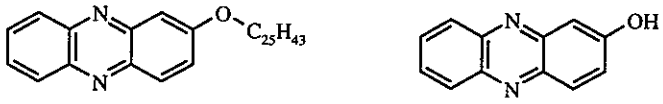



**Figure 14 -** Structure of methanophenazine and 2-hydroxyphenazine (Abken et al, 1998).

dehydrogenase or methyl viologen (MV) hydrogenase (mediated by b-type cytochromes) to the heterodisulfide reductase (Abken et al, 1998).

### 1.6.2. Redox reactions of $H_2$:$CO_2$ methanogenesis.

In the conversion of $CO_2$ to $CH_4$ there are 4 distinct 2 electron reduction steps. During growth on $H_2$:$CO_2$, the primary source of electrons is $H_2$ (Rouviere and Wolfe, 1988). The key protein in this reaction is a hydrogenase. This enzyme converts the $H_2$ into free protons ($H^+$) and electrons that are used in the reductive steps of methanogenesis.

The redox reactions of methanogenesis from $CO_2$ include the three reduction steps: $CO_2$ to formyl-MF, methenyl-$H_4$MPT to methylene-$H_4$MPT, and methylene-$H_4$MPT to methyl-$H_4$MPT (Figure 2). The involvement of hydrogenase has been proposed for the reduction and activation of $CO_2$ (Blaut, 1994; Deppenmeier et al, 1996). The next two reactions involving $H_4$MPT are reduction steps that convert the $C_1$-group from methenyl- to methylene- and methylene- to methyl- level. The methylene-$H_4$MPT dehydrogenase which catalyses the former reaction in *Mb. thermoautotrophicum* may be either $H_2$- or coenzyme $F_{420}$-dependent while the enzyme in *Methanosarcina* is strictly $F_{420}$-dependent.

In the terminal steps of methanogenesis the reduction of the methyl-CoM (methylreductase reaction) and the heterodisulfide reductase reaction are redox reactions where $H_2$ is required either directly as in the $H_2$-utilizing methylene-$H_4$MPT dehydrogenase and through the MV hydrogenase in the heterodisulfide reductase step, or indirectly as in the remaining steps linked through the coenzyme $F_{420}$.

To summarize, electrons are required in all of the steps in the conversion of the

substrate $CO_2$ to the methyl-level at CoM. These electrons are provided by hydrogen, often through the key cofactor, $F_{420}$.

### 1.6.3. Redox reactions of methanol methanogenesis.

In methanogenesis from methanol alone, reduction of methanol required the oxidation of 1 out of every 4 moles of methanol completely to $CO_2$ to produce sufficient electrons to reduce the other 3 moles of methanol to methane (Blaut, 1994). Thus, the electrons are produced via a reverse methanogenic pathway from $H_2$:$CO_2$ using the same enzymes but in the reverse direction to produce reduced coenzyme $F_{420}$ whereas, in $H_2$:$CO_2$ methanogenesis, the reactions consume reduced coenzyme $F_{420}$. This reduced coenzyme $F_{420}$ is used for the reduction of methanol to methane (Figure 12) in the absence of $H_2$.

Methanogenesis from methanol plus $H_2$ requires the latter substrate as the source of electrons for methane production and the hydrogenase is responsible for providing these electrons.

### 1.6.4. Redox reactions in acetate methanogenesis.

In the third major methanogenic pathway, acetate, the sole methanogenic substrate, is cleaved after the activation to acetyl-CoA. The carboxyl-portion is oxidized to $CO_2$ and the methyl-portion reduced to $CH_4$ (Blaut, 1994). The carboxyl group oxidation to $CO_2$ produces the reducing equivalents for the reduction of the methyl group (Ferry, 1992). This cleavage is accomplished through the action of the enzyme complex, carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS). CODH performs the cleavage of the methyl group from the acetyl-CoA and provides the electrons for the reduction of the methyl group to methane, while the ACS performs the activation of the acetate to acetyl-CoA. The two reactions occur at different sites on the same protein complex (Ragsdale and Kumar, 1996). The pathway as described in *Methanosarcina*, is fairly simple in structure (Figure 13), much more so than the pathways for $CO_2$ reduction (Figure 2) or methanol reduction (Figure 12) to methane.

Now that the key electron carriers and the enzymes involved in the electron transport pathways of the methanogens have been introduced, a closer look at several of the enzymes involved in electron transfer reactions will be made.

## 1.7. Hydrogenase, and other electron transfer enzymes in the Methanogens.

It is important to note that at several locations along the methanogenic pathway(s) there are reducing steps where electrons are required for the conversion of the various substrates into the ultimate end product, methane. Electrons are needed for reduction of $CO_2$, formyl-, methylene- and methyl- group, and the reduction of the heterodisulfide of CoM and CoB. Cofactor $F_{420}$ is required as the electron donor for the reactions involving $H_4$MPT and, depending upon the species, for the heterodisulfide reduction, though not directly, but using membrane reconstitution systems). Several enzymes that are used by the methanogens to provide electrons for the methanogenic pathway include the hydrogenase, formate dehydrogenase (FDH), carbon monoxide dehydrogenase (CODH), secondary alcohol dehydrogenases, and the $F_{420}H_2$ dehydrogenase.

### 1.7.1. Hydrogenase

The single most important enzyme activity involved in the process of producing the electrons for methanogenesis in the presence of hydrogen is (are) the hydrogenase (s)

#### 1.7.1.1. Historical Overview

In 1931, Stephenson and Stickland found that *E. coli* could use molecular hydrogen to reduce a variety of substrates (Stephenson and Stickland, 1931). They were the first to describe an enzyme activity that converted molecular hydrogen, releasing or donating electrons to some factor (eg. $NAD^+$, $NADP^+$), and releasing the protons according to the following reaction:

$$H_2 \rightleftharpoons 2H^+ + 2e^- \qquad (1)$$

This activity was discovered in the cell-extracts of *E. coli*, which is a facultative anaerobe.

This enzyme activity has been found, and the enzyme purified, in a large number of Bacteria, like *E. coli* (Adams and Hall, 1979), *Proteus mirabilis* (Schoenmaker et al, 1979), *Alcaligenes eutrophus* H16 (Schneider and Schlegel, 1976), *Azotobacter vinelandii* (Sun and Arp, 1991), *Thiobacillus ferrooxidans* (Fischer et al, 1996) and *Desulfovibrio gigas* (Hatchikian et al, 1978), in eukaryotic organisms like the anaerobic protozoa *Trichomonas vaginalis*, and *Trichomonas foetus* (associated with an organelle known as a hydrogenosome; Lindmark et al, 1975; Lindmark and Müller, 1973), and algae like *Scenedesmus obliquus* (Schnackenberg et al, 1993). In the Archaea, there are many examples of organisms that carry a hydrogenase enzyme, including *P. furiosus* (Bryant and Adams, 1989), *Pyrodictium brockii* (Pihl and Maier, 1991), and in particular, numerous species of the methanogens.

The enzyme activity may be followed by the reduction of various electron acceptors, depending on the source of the hydrogenase. Natural acceptors include the nicotinamides (either or both of $NAD^+$ and $NADP^+$), the flavins (FMN, FAD), cytochromes, and methanogenic cofactor $F_{420}$. The hydrogenases have also been assayed using artificial electron acceptors, including the viologen dyes (methyl and benzyl) and methylene blue. The reverse direction of the hydrogenase can be assayed by monitoring the oxidation of the same electron acceptors (pre-reduced electron donors) or by the manometric measurement of the production of $H_2$.

### 1.7.1.2. Physical properties and characteristics

The hydrogenase activity in general is $O_2$-sensitive, and early attempts, as well as more recent attempts (Van Dijk et al, 1979) at the purification of this enzyme were not very successful. Those attempts that did yield a pure preparation, did not either yield a very active sample or resulted in a very low recovery of enzyme.

Structurally, the hydrogenases vary in their native molecular weights and subunit sizes, and in their metal ion composition. Hydrogenases are iron-sulfur proteins which

are used typically for electron transferring reactions (Beinert, 1990). Iron-sulfur proteins are considered to be an ancient type of structure since the iron-sulfur centres of these proteins can be produced abiotically (Beinert, 1990). Hydrogenases can be divided into several groups based on their metal content.

One group of hydrogenases, termed the iron hydrogenases, are those with only the iron found in the iron-sulfur centres. Their structure and mechanism of activity has been reviewed recently (Adams, 1990). A second, probably more common form of the hydrogenase contains the metal atom, nickel, as the active-site metal. These [NiFe] hydrogenases are found in the methanogens, as well as the third type of hydrogenase, the [NiFeSe] hydrogenases. The selenium is found in the enzyme in the form of selenocysteine in which the sulfur atom of cysteine is replaced by a selenium atom. Selenium in enzymes is not uncommon and this type of modified amino acid is found in yeast enzymes (Stadtman, 1980). Genetic analysis in the methanogen, *Methanococcus voltae*, revealed the presence of four operons encoding different hydrogenases, two encoding hydrogenases that carry selenocysteine, and two that do not (Halboth and Klein, 1992).

### 1.7.1.3. Methanogenic hydrogenases

In $H_2$-utilizing methanogens, this enzyme activity provides the electrons for the stepwise reduction of the substrate (be it $CO_2$ or methanol) into methane. There may be several different classes of hydrogenase present in a single methanogen that have specific functions within the cell.

One class of hydrogenase provides the electrons to the methanogenic coenzyme $F_{420}$ which is directly involved as the carrier for the electrons in the methanogenic pathway from $CO_2$. This is the case in the majority of the $H_2$-utilizing methanogens. This coenzyme $F_{420}$-reactive or coenzyme $F_{420}$-reducing hydrogenase has been studied in detail in a number of methanogens and has been purified to apparent homogeneity in several species including *Methanobacterium thermoautotrophicum* strain ΔH (Fox et al, 1987) and strain Marburg (Graf and Thauer, 1981; Jacobson et al, 1982), *Methanobacterium*

*formicicum* (Baron and Ferry, 1989), *Methanococcus vannielii* (Yamazaki, 1982), *Methanococcus voltae* (Muth et al, 1987), *Methanosarcina barkeri* strain MS (Fauque et al, 1984), *Methanosarcina barkeri* strain Fusaro (Fiebig and Freidrich, 1989), and *Methanospirillum hungatei* (Sprott et al, 1987).

The coenzyme $F_{420}$-reactive hydrogenases are $O_2$ stable, nickel-containing enzymes that are generally very large in native form, and require FAD or FMN as a prosthetic group. FAD is an electron carrier that can transfer either one or two electrons at a time allowing the hydrogenase to transfer or accept electrons in either of these formats.

A second class of hydrogenase was isolated and found to not be reactive with cofactor $F_{420}$, but instead could only be assayed using an artificial electron acceptor, either methyl viologen (MV) or benzyl viologen (BV). The MV hydrogenase (viologen dye reducing hydrogenase; $F_{420}$-nonreactive; $F_{420}$-nonreducing hydrogenase) is smaller than its deazaflavin reactive counterpart and generally shows greater sequence similarity to the $NAD^+$ hydrogenases found in the Bacteria (Wu and Mandrand, 1993).

This hydrogenase has been purified from only a few methanogens including *Mb. thermoautotrophicum* strain Marburg (Coremans et al, 1992) and strain ΔH (Graf and Thauer, 1980), *Mb. formicicum* (Adams et al, 1986), *Methanobacterium* strain G2R (McKellar and Sprott, 1979), *Methanosarcina mazei* strain Gö1 (Deppenmeier et al, 1992), and *Ms. barkeri* strain MS (Kemner and Zeikus, 1994). An interesting discovery was made in *Mb. thermoautotrophicum* strain ΔH where a second variant of the MV hydrogenase, which differed slightly in physical (N-terminal sequence of one subunit) and kinetic properties (pH optimum) was found (Woo et al, 1993). This second hydrogenase introduced the possibility for the existence of several classes of the MV hydrogenase with different functions within the methanogens. This possibility has been taken advantage of in some recent models for methanogenesis (Deppenmeier et al, 1996).

Since the MV hydrogenase can only be assayed using artificial electron acceptors, the true natural electron acceptor for this enzyme is not known. The only leads are the discovery of an open reading frame (ORF) in the *mvh* operon encoding the MV

hydrogenase in *Mb. thermoautotrophicum* strain ΔH, with a sequence that appeared to code for a polyferredoxin (Reeve et al, 1989) and the discovery of methanophenazine (Abken et al, 1998). The purified polyferredoxin was not found to be reduced by the MV hydrogenase of *Mb. thermoautotrophicum* strain ΔH. However, insufficient amounts of polyferredoxin protein may have been used to perform this assay (Hedderich et al, 1992). The levels of the polyferredoxin appeared to decline to very low levels towards stationary phase but the activity of the hydrogenase did not change after this point. There is some argument for the polyferredoxin being used during exponential growth as a source of ferredoxin for the synthesis of the hydrogenase rather than functioning as the physiological electron acceptor for the MV hydrogenase.

Methanophenazine is a simple organic molecule that is highly hydrophobic (see section 1.6.1.) and has been proposed to be the natural electron acceptor for the MV hydrogenase, at least for the methanogen it was discovered in. This molecule was isolated from the membranes of *Methanosarcina mazei* strain Gö1 (Abken et al, 1998), but this molecule cannot be used directly in enzyme assays since it is not soluble in aqueous solutions, but a structurally similar compound, 2-hydroxyphenazine, was synthesized that apparently could accept electrons from MV hydrogenase and in the presence of cytochromes, the hydrogenase could be used to provide electrons for the reduction of the heterodisulphide reductase reaction. An attempt to isolate methanophenazine from other methanogenic species has failed (U. Deppenmeier, personal communication), and many other methanogens also do not have cytochromes. The search continues for the true physiological electron acceptor of this enzyme for many of the methanogens.

A third class of hydrogenase activity found only in the methanogenic bacteria is the previously mentioned $H_2$-forming $N^5$,$N^{10}$-methylenetetrahydromethanopterin dehydrogenase (section 1.5.1.). This hydrogenase is distinguished from all other hydrogenases in that it has no metal centres involved in catalysis (Zirngibl et al, 1990) and has been shown to use $H_2$ to reduce $F_{420}$ in a coupled enzyme system *in vivo* (Afting and Thauer, 1998).

### 1.7.2. Formate dehydrogenase

Formate utilization or production requires the enzyme, formate dehydrogenase (FDH). This enzyme has been found in a number of microorganisms in the Eukarya, the Bacteria, and the Archaea (Ferry, 1990). This enzyme catalyses the following reaction:

$$HCOO^- \rightleftharpoons CO_2 + H^+ + 2e^- \quad (2)$$

The purified enzymes vary in size and subunit composition, as well as in the presence or absence and type of bound cofactors and metal atoms. With the exception of *Pseudomonas oxalaticus* (Müller et al, 1978), FDHs found in the aerobic bacteria and yeasts do not contain metal centers or cofactors, while the FDHs of the anaerobic bacteria and archaea, contain either or both metal atoms and cofactors like FAD (Ferry, 1990). Typically, the FDHs found in the aerobic Bacteria are oxygen-stable whereas in the anaerobic Bacteria and Archaea, the FDHs are extremely oxygen-sensitive (Ferry, 1990).

The FDHs of several methanogenic Archaea have been purified. The enzymes from *Mb. formicicum* (Schauer and Ferry, 1982; Barber et al, 1983; Schauer and Ferry, 1986) and *Mc. vannielii* (Jones and Stadtman, 1981) have been studied extensively. Both of these enzymes are cofactor $F_{420}$ dependent, and are molybdenum-iron-sulfur proteins. The *Mb. formicicum* enzyme also contains FAD. The native enzymes are 105 kDa (*Mc. vannielii*) and 177 kDa (*Mb. formicicum*) and composed of only 2 subunits of 85 and 53 kDa in *Mb. formicicum* (Schauer and Ferry, 1986).

### 1.7.3. Carbon monoxide dehydrogenase

It has been long known that some methanogens can use carbon monoxide, CO, as a substrate for methanogenesis (Kluyver and Schnellen, 1947), eg., *Mb. thermoautotrophicum* strain ΔH (Daniels et al, 1977). The key enzyme in the utilization of CO, as well as in aceticlastic methanogenesis, is carbon monoxide dehydrogenase, or CODH. This enzyme reversibly converts CO to $CO_2$ releasing electrons for methanogenesis or alternatively, the enzyme cleaves acetate to CO oxidizing it into $CO_2$

and the methyl group. This enzyme complex is known to contain another enzyme activity, acetyl-CoA synthase (Ragsdale and Kumar, 1996).

The CODH catalyses the following reaction:

$$CO + H_2O \rightleftharpoons CO_2 + 2\,H^+ + 2\,e^- \quad (3)$$

In addition, the enzyme also catalyses the following acetyl-CoA synthase reaction in the methanogens:

$$CO + CH_3\text{-}H_4MPT + CoA\text{-}SH \rightleftharpoons CH_3CO_2\text{-}S\text{-}CoA + H_4MPT \quad (4)$$

An analogous reaction occurs in the acetyl-CoA synthesizing Wood/Ljungdahl pathway (Ljungdahl, 1986) of some Bacteria.

This enzyme activity has been found in a number of bacterial species including members of the genera *Clostridium* (Ragsdale et al, 1983) and *Acetobacterium* (Ragsdale et al, 1983). The enzyme is primarily used in acetyl-CoA synthesis. In methanogens, notably members of the genera, *Methanosarcina* and *Methanothrix* (Jetten et al, 1992), this enzyme is important in the aceticlastic methanogens,. Autotrophic methanogens, eg., *Methanobacterium thermoautotrophicum* strain ΔH use this enzyme in the primary carbon dioxide fixation step (Daniels et al, 1977).

The enzyme has been purified from several methanogens including *Ms. barkeri* (Krzycki and Zeikus, 1984), *Methanosarcina thermophila* (Terlesky et al, 1986), *Methanosarcina frisia* strain Gö1 (Eggen et al, 1996) and *Methanothrix soehngenii* (Jetten et al, 1989). The enzyme is a nickel/iron-sulfur enzyme, similar to some of the hydrogenases described earlier, and is very large (between 214-250 kDa) made up of typically 2 different subunits combining in a $\alpha_2\beta_2$ configuration (with exception of the CODH from *Ms. thermophila*, having 5 subunits in a $\alpha\beta\gamma\delta\varepsilon$ configuration). Only the enzyme from *Mtx. soehngenii* was reported to be $O_2$-stable (Jetten et al, 1989).

The electron acceptor for the CODH in these species has been identified as the

iron-sulfur protein, ferredoxin (Terlesky and Ferry, 1988), although methyl viologen can serve as an artificial electron acceptor. The methanogenic cofactor $F_{420}$ is reduced in the presence of CO, by extracts of *Mb. thermoautotrophicum* strain ΔH (Daniels et al, 1977) and by purified enzyme of *Mtx. soehngenii* (Jetten et al, 1989).

### 1.7.4. Secondary alcohol dehydrogenases

Alcohol dehydrogenases are found in numerous Bacteria and Archaea. Early in the studies on the methanogenic bacteria, ethanol was found to be a substrate of methanogenesis. However, this turned out to be incorrect as *Mb. omelianskii*, the methanogen that ethanol utilization was observed in, was a mixed culture (Bryant et al, 1967). However, ethanol can serve as an electron donor for methanogenesis in a limited number of species (Widdel, 1986).

Methanogenic bacteria use a limited number of substrates as electron donors, mainly $H_2$ or electrons derived from the oxidation of a methanogenic substrate that is disproportionated (eg. methanol). However, there have been recent reports of isolates that can use secondary alcohols as electron donors (Zellner and Winter, 1987). In particular, 2-propanol and 2-butanol have been used by some methanogens to reduce either $CO_2$ (Zellner et al, 1989) or methanol (Lalitha and Krishnan, 1991) to form methane. The enzyme involved in the release of the electrons from these alcohols is the secondary alcohol dehydrogenase.

Secondary alcohol dehydrogenases have been also found in members of the Bacteria and purified from the *Pseudomonas maltophilia* MB11L (Britt et al, 1992). The archaeal enzymes have been purified from *Methanogenium liminatans* (Bleicher and Winter, 1991), *Methanogenium thermophilum* (Widdel and Wolfe, 1989), and *Mb. palustre* (Bleicher and Winter, 1991). The cofactor specificity of these enzymes were either for cofactor $F_{420}$ (*Mg. liminatans* and *Mg. thermophilum*), or $NADP^+$ (*Mb. palustre*). Structurally, the enzymes are large, ranging from 150 to 175 kDa and composed of a single subunit ranging in size from 39 to 44 kDa (Bleicher and Winter, 1991).

### 1.7.5. $F_{420}H_2$ dehydrogenase

Methanogens that grow solely or can grow solely on methanol or acetate have no need for a hydrogenase to provide electrons for the reduction of their substrate to methane. These methanogens have a heterodisulfide reductase system that is dependent on reduced cofactor $F_{420}$, as the electron donor. An $F_{420}H_2$-dependent enzyme activity was found to direct the electrons from $F_{420}H_2$ to the heterodisulfide reductase system (Deppenmeier et al, 1990). These organisms rely on a specific enzyme, the $F_{420}H_2$ dehydrogenase to utilize the $F_{420}H_2$ in the cell for the terminal step of methanogenesis.

This enzyme has only been purified from the methanol-utilizing methanogens, *Methanolobus tindarius* (Haase et al, 1992) and *Methanosarcina mazei* strain Gö1 (Abken and Deppenmeier, 1997). A similar enzyme from the non-methanogenic bacterium, *Archaeoglobus fulgidus* has also been purified (Kunow et al, 1994) that transfers electrons from $F_{420}H_2$ to a quinone. It is suggested to be specifically involved in methanogenesis from methanol in the *Methanosarcinaceae* (Gottschalk et al, 1996).

Assay of this enzyme requires a rather complex system of electron acceptors. The enzyme activity is followed by the oxidation of reduced cofactor $F_{420}$, with methyl viologen as the electron acceptor. To prevent interference by the reduced MV dye, the electron acceptor, metronidazole (MTZ), is used to take the electrons from the reduced MV. The MTZ decomposes upon reduction.

Structurally, the enzyme is heteromultimeric with five different subunits (45, 40, 22, 18, and 17 kDa in *Ml. tindarius* and 40, 37, 22, 20, and 16 kDa in *Ms. mazei*) that combine into a rather large holoenzyme with a native molecular mass of approximately 115 kDa in *Ms. mazei* (Abken and Deppenmeier, 1997) and 120 kDa in *Ml. tindarius* (Haase et al, 1992). The enzyme from *Ml. tindarius* was reported to be devoid of any iron-sulfur centres whereas the enzyme from *Ms. mazei* does have these centres. Both were found to be $O_2$-stable enzymes, for a few days, at least, as the enzymes were both purified aerobically.

This enzyme is a major protein in both *Ml. tindarius* and *Ms. mazei* and is involved intimately in methanogenesis from methanol (Figure 13) according to a model

proposed by G. Gottschalk (Deppenmeier et al, 1996).

### 1.8. Enter *Methanosphaera stadtmanae*, a case study.

In 1983, an interesting spherical-shaped methanogen was discovered in the faecal matter from an unidentified human subject. This methanogen was found to have a rather strict substrate requirement, converting only methanol, in the presence of $H_2$, to methane. This methanogen was the first isolate of a new genus of methanogens, called *Methanosphaera*, and was given a species name of *stadtmanae*. Actually *stadtmaniae* was first used but the official name has since become *stadmanae*, in honour of Thressa Stadtman, a pioneer in the study of anaerobic microorganisms (Miller and Wolin, 1985).

Along with its sister isolate, *Msph. cuniculi*, isolated from a rabbit caecum (Biavati et al, 1988), it uses methanol plus $H_2$, and not methanol alone for methanogenesis. This is unlike *Methanosarcina* spp., which can use methanol $\pm$ $H_2$. Cytochromes were not detected in the membranes of this new genus which is contrary to the findings in *Methanosarcina* spp. (Miller and Wolin, 1985). B-type cytochromes are important in methanol methanogenesis in *Methanosarcina* spp. (Deppenmeier et al, 1996) and are proposed to be involved as the electron carrier along with methanophenazine between the $F_{420}H_2$ dehydrogenase and the heterodisulfide reductase reaction in methanogenesis from methanol (Figure 13).

Another point that states the peculiarity of this isolate is the finding that there is no detectable cofactor $F_{420}$-dependent hydrogenase in the cell extracts of this methanogen (Miller and Wolin, 1986). This is unusual in that all other known hydrogenotrophic methanogens carry a $F_{420}$-dependent hydrogenase, even if it is the only hydrogenase in that species (*Msp. hungatei*; Sprott et al, 1987), only *Mb.* strain G2R (McKellar and Sprott, 1979) has a single large MV-only hydrogenase. With the absence of a coenzyme $F_{420}$ reducing hydrogenase, a conventional mechanism for the reduction of this cofactor is absent.

# 2. Project Objectives

## 2.1. Objectives.

The primary objective of this project is to characterize the enzymatic elements involved in the electron transport pathway of *Methanosphaera stadtmanae*, a methanogen that grows only on methanol in the presence of $H_2$. This methanogen has no $F_{420}$-reducing hydrogenase and although it uses methanol as its primary (sole) methanogenic substrate, it does not carry cytochromes in its membranes. This is unlike other methanol-utilizers such as *Methanosarcina* strain Gö1 which has both b- and c-type cytochromes identified (Kamlage and Blaut, 1992).

The first part of the project will involve the physical and kinetic characterization of the MV hydrogenase of *Msph. stadtmanae* and a comparison of it to similar enzymes found in other methanogenic bacteria since it appears that the hydrogenase is the sole mechanism for providing electrons for both methanogenesis and for biosynthesis.

The second part of the project involves the study of a mechanism of $F_{420}$ reduction in *Msph. stadtmanae* since the primary source of electrons, $H_2$, is not directly linked to $F_{420}$ by the hydrogenase. Similar $F_{420}$-linked enzyme mechanisms will be studied in some of the other methanogens for comparison.

The various findings that will be discussed in this thesis have led to the construction of a model for the electron pathway in *Msph. stadtmanae* made with respect to the primary source of electrons for the cell ($H_2$) and the methanogenic cofactor $F_{420}$, which will conclude this thesis.

# 3. Materials and Methods

## 3.1. Universal Methods and Protocols -

**3.1.1. Organisms** - The organisms used for this thesis include the primary organism for the study, *Methanosphaera stadtmanae* (DSM 3091), along with *Methanosphaera cuniculi* (DSM 4103), *Methanobacterium bryantii* strain M.o.H. (DSM 863), *Methanobacterium thermoautotrophicum* strain Marburg (DSM 2133), *Methanobacterium* strain G2R (a gift from Dennis Sprott of the National Research Council in Ottawa), *Methanococcus thermolithotrophicus* (DSM 2095), *Methanococcus voltae* (DSM 1537), *Methanolobus tindarius* (DSM 2278), *Methanosaeta concilii* strain GP6 (DSM 3671), *Methanospirillum hungatei* strain GP1 (DSM 1101), and *Methanosarcina barkeri* strain Fusaro (DSM 804). Nonmethanogenic bacteria that were used in this thesis included the sulfate reducer *Desulfovibrio desulfuricans* (DSM 1924), and *Desulfovibrio desulfuricans* strain B203, a known mercury methylator (a gift from Cindy Gilmour).

**3.1.2. Growth Media** - The organisms above were cultured as per their medium formulations as follows (note that the formulations are included in tabular form at the end of the thesis in Appendix A and for practical considerations the amounts of the some of the chemicals, eg., the vitamins and mineral components, are added from stock mixture solutions containing these elements):

The culture medium used for *Mb.* strain G2R (Jarrell et al, 1982) contained (per liter): $Na_2CO_3$, 0.48 g; $(NH_4)_2SO_4$, 0.45 g; $K_2HPO_4$, 0.29 g; $KH_2PO_4$, 0.18 g; $MgSO_4 \cdot 7\ H_2O$, 0.12 g; $CaCl_2 \cdot 2\ H_2O$, 0.06 g; NaCl, 0.054 g; $FeSO_4 \cdot 7\ H_2O$, 0.021 g; Na Acetate, 0.42 g; resazurin, 0.5 mg; $Na_2S \cdot 9\ H_2O$, 0.45 g; nitriloacetic acid, 20.2 mg; $FeCl_3 \cdot 6\ H_2O$, 2.1 mg; $CoCl_2 \cdot 6\ H_2O$, 2.0 mg; $MnCl_2 \cdot 4\ H_2O$, 1.0 mg; $ZnCl_2$, 1.0 mg; $NiCl_2 \cdot 6\ H_2O$, 1.0 mg; $CuSO_4 \cdot 2\ H_2O$, 0.5 mg; $Na_2MoO_4 \cdot 2\ H_2O$, 0.5 mg; pyridoxine-HCl, 0.1 mg; riboflavin, 0.05 mg; thiamine, 0.05 mg; nicotinic acid, 0.05 mg; p-aminobenzoic acid, 0.05 mg; lipoic acid, 0.05 mg; biotin, 0.02 mg; folic acid, 0.02 mg; and cyanocobalamin, 0.01 mg.

The final pH of the medium was pH 6.5 (after being made anaerobic, see after this listing).

The medium used for *Mb. bryantii* strain M.o.H. (Jarrell et al, 1982) contained (per litre): NaCl, 0.30 g; $MgCl_2 \cdot 6\ H_2O$, 0.064 g; $NH_4Cl$, 1.00 g; Na acetate, 0.42 g; $KH_2PO_4$, 0.60 g; $K_2HPO_4$, 0.60 g; $CaCl_2 \cdot 2\ H_2O$, 0.032 g; $Na_2CO_3$, 0.80 g; resazurin, 0.5 mg; $Na_2S \cdot 9\ H_2O$, 0.45 g; $FeCl_3 \cdot 6\ H_2O$, 2.1 mg; $CoCl_2 \cdot 6\ H_2O$, 2.0 mg; $MnCl_2 \cdot 4\ H_2O$, 1.0 mg; $ZnCl_2$, 1.0 mg; $NiCl_2 \cdot 6\ H_2O$, 1.0 mg; $CuSO_4 \cdot 2\ H_2O$, 0.5 mg; $Na_2MoO_4 \cdot 2\ H_2O$, 0.5 mg; nitriloacetic acid, 20.2 mg; pyridoxine-HCl, 0.1 mg; riboflavin, 0.05 mg; thiamine, 0.05 mg; nicotinic acid, 0.05 mg; p-aminobenzoic acid, 0.05 mg; lipoic acid, 0.05 mg; biotin, 0.02 mg; folic acid, 0.02 mg; and cyanocobalamin, 0.01 mg. The final pH of the medium was pH 6.5 (after being made anaerobic, see after this listing).

The medium used for *Mb. thermoautotrophicum* strain Marburg (Fuchs et al, 1978) contained (per liter): $KH_2PO_4$, 0.42 g; $K_2HPO_4$, 0.23 g; $MgCl_2 \cdot 6\ H_2O$, 0.04 g; $CaCl_2 \cdot 2\ H_2O$, 0.03 g; NaCl, 0.595 g; $NH_4Cl$, 0.70 g; $Na_2CO_3$, 0.16 g; resazurin, 0.5 mg; $Na_2S \cdot 9\ H_2O$, 0.45 g; nitriloacetic acid, 20.2 mg; $FeCl_3 \cdot 6\ H_2O$, 2.1 mg; $CoCl_2 \cdot 6\ H_2O$, 2.0 mg; $MnCl_2 \cdot 4\ H_2O$, 1.0 mg; $ZnCl_2$, 1.0 mg; $NiCl_2 \cdot 6\ H_2O$, 1.0 mg; $CuSO_4 \cdot 2\ H_2O$, 0.5 mg; and $Na_2MoO_4 \cdot 2\ H_2O$, 0.5 mg. The final pH of the medium was pH 7.0 (after being made anaerobic, see after this listing).

The medium used for *Mc. thermolithotrophicus* when grown on $H_2$ plus $CO_2$ (Huber et al, 1982) as the methanogenic substrates contained (per liter): NaCl, 29.2 g; $MgCl_2 \cdot 6\ H_2O$, 5.10 g; $NH_4Cl$, 1.00 g; KCl, 0.34 g; $KH_2PO_4$, 0.28 g; $K_2HPO_4$, 0.28 g; $CaCl_2 \cdot 2\ H_2O$, 0.10 g; resazurin, 0.5 mg; $NaWO_4 \cdot 2\ H_2O$, 0.07 mg; $NaSeO_4$, 0.04 mg; $Na_2CO_3$, 0.20 g; $Na_2S \cdot 9\ H_2O$, 0.45 g; nitriloacetic acid, 20.2 mg; $FeCl_3 \cdot 6\ H_2O$, 2.1 mg; $CoCl_2 \cdot 6\ H_2O$, 2.0 mg; $MnCl_2 \cdot 4\ H_2O$, 1.0 mg; $ZnCl_2$, 1.0 mg; $NiCl_2 \cdot 6\ H_2O$, 1.0 mg; $CuSO_4 \cdot 2\ H_2O$, 0.5 mg; and $Na_2MoO_4 \cdot 2\ H_2O$, 0.5 mg. The final pH of the medium was pH 6.0 (after being made anaerobic, see after this listing).

The medium used for *Mc. thermolithotrophicus* when grown on formate (R. Sparling, Ph.D. Thesis) as the methanogenic substrate contained (per liter): KCl, 0.34 g; $NH_4Cl$, 1.00 g; $MgCl_2 \cdot 6\ H_2O$, 5.10 g; $CaCl_2 \cdot 2\ H_2O$, 0.10 g; $K_2HPO_4$, 0.25 g; $Na_2S \cdot 9\ H_2O$,

0.45 g; 2-(N-morpholino)-ethanesulfonic acid (MES), 9.75 g; piperazine-N,N'-bis-2-ethanesulfonic acid (PIPES), 15.12 g; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 11.90 g; resazurin, 0.5 mg; $NaWO_4 \cdot 2\ H_2O$, 0.07 mg; $NaSeO_4$, 0.04 mg; NaCl, 11.68 g; Na formate, 20.40 g; nitriloacetic acid, 20.2 mg; $FeCl_3 \cdot 6\ H_2O$, 2.1 mg; $CoCl_2 \cdot 6\ H_2O$, 2.0 mg; $MnCl_2 \cdot 4\ H_2O$, 1.0 mg; $ZnCl_2$, 1.0 mg; $NiCl_2 \cdot 6\ H_2O$, 1.0 mg; $CuSO_4 \cdot 2\ H_2O$, 0.5 mg; and $Na_2MoO_4 \cdot 2\ H_2O$, 0.5 mg. The final pH of the medium was pH 6.0 (after being made anaerobic, see after this listing).

The medium used for *Mc. voltae* (DSM Catalogue of Strains, 1993) contained (per liter): KCl, 0.34 g; $MgCl_2 \cdot 6\ H_2O$, 4.00 g; $MgSO_4 \cdot 7\ H_2O$, 3.45 g; $NH_4Cl$, 0.25 g; $CaCl_2 \cdot 2\ H_2O$, 0.14 g; $K_2HPO_4$, 0.14 g; yeast extract, 2.00 g; tryptone/trypticase, 2.00 g; NaCl, 18.00 g; $NaHCO_3$, 5.00 g; $Fe(NH_4)_2(SO_4)_2 \cdot 7\ H_2O$, 0.002 g; resazurin, 0.5 mg; $Na_2S \cdot 9\ H_2O$, 0.45 g; Na acetate, 1.00 g; nitriloacetic acid, 20.2 mg; $FeCl_3 \cdot 6\ H_2O$, 2.1 mg; $CoCl_2 \cdot 6\ H_2O$, 2.0 mg; $MnCl_2 \cdot 4\ H_2O$, 1.0 mg; $ZnCl_2$, 1.0 mg; $NiCl_2 \cdot 6\ H_2O$, 1.0 mg; $CuSO_4 \cdot 2\ H_2O$, 0.5 mg; $Na_2MoO_4 \cdot 2\ H_2O$, 0.5 mg; pyridoxine-HCl, 0.1 mg; riboflavin, 0.05 mg; thiamine, 0.05 mg; nicotinic acid, 0.05 mg; p-aminobenzoic acid, 0.05 mg; lipoic acid, 0.05 mg; biotin, 0.02 mg; folic acid, 0.02 mg; and cyanocobalamin, 0.01 mg. The final pH of the medium was pH 7.0 (after being made anaerobic, see after this listing).

The medium used for *Ml. tindarius* (König and Stetter, 1982) contained (per liter): KCl, 0.35 g; $MgCl_2 \cdot 6\ H_2O$, 4.00 g; $MgSO_4 \cdot 7\ H_2O$, 3.45 g; $NH_4Cl$, 0.25 g; $CaCl_2 \cdot 2\ H_2O$, 0.14 g; $K_2HPO_4$, 0.14 g; NaCl, 18.00 g; $NaHCO_3$, 1.00 g; $Fe(NH_4)_2(SO_4)_2 \cdot 7\ H_2O$, 0.002 g; resazurin, 0.5 mg; $Na_2S \cdot 9\ H_2O$, 0.45 g; methanol, 0.4%; nitriloacetic acid, 20.2 mg; $FeCl_3 \cdot 6\ H_2O$, 2.1 mg; $CoCl_2 \cdot 6\ H_2O$, 2.0 mg; $MnCl_2 \cdot 4\ H_2O$, 1.0 mg; $ZnCl_2$, 1.0 mg; $NiCl_2 \cdot 6\ H_2O$, 1.0 mg; $CuSO_4 \cdot 2\ H_2O$, 0.5 mg; $Na_2MoO_4 \cdot 2\ H_2O$, 0.5 mg; pyridoxine-HCl, 0.1 mg; riboflavin, 0.05 mg; thiamine, 0.05 mg; nicotinic acid, 0.05 mg; p-aminobenzoic acid, 0.05 mg; lipoic acid, 0.05 mg; biotin, 0.02 mg; folic acid, 0.02 mg; and cyanocobalamin, 0.01 mg. The final pH of the medium was pH 6.5 (after being made anaerobic, see after this listing).

The medium used for *Mst. concilii* strain GP6 (Patel, 1984) contained (per liter): $KH_2PO_4$, 0.30 g; NaCl, 0.60 g; $MgCl_2 \cdot 6\ H_2O$, 0.10 g; $CaCl_2 \cdot 2\ H_2O$, 0.08 g; $NH_4Cl$, 1.00 g;

$K_2CO_3 \cdot 1.5\ H_2O$, 4.00 g; resazurin, 0.5 mg; Na acetate, 6.80 g; $Na_2S \cdot 9\ H_2O$, 0.45 g; nitriloacetic acid, 20.2 mg; $FeCl_3 \cdot 6\ H_2O$, 2.1 mg; $CoCl_2 \cdot 6\ H_2O$, 2.0 mg; $MnCl_2 \cdot 4\ H_2O$, 1.0 mg; $ZnCl_2$, 1.0 mg; $NiCl_2 \cdot 6\ H_2O$, 1.0 mg; $CuSO_4 \cdot 2\ H_2O$, 0.5 mg; $Na_2MoO_4 \cdot 2\ H_2O$, 0.5 mg; pyridoxine-HCl, 0.1 mg; riboflavin, 0.05 mg; thiamine, 0.05 mg; nicotinic acid, 0.05 mg; p-aminobenzoic acid, 0.05 mg; lipoic acid, 0.05 mg; biotin, 0.02 mg; folic acid, 0.02 mg; and cyanocobalamin, 0.01 mg. The final pH of the medium was pH 7.0 (after being made anaerobic, see after this listing).

The basic medium for *Ms. barkeri* strain Fusaro (Kandler and Hippe, 1977) contained (per liter): $K_2HPO_4$, 0.35 g; $KH_2PO_4$, 0.23 g; $NH_4Cl$, 0.50 g; yeast extract, 2.00 g; $MgSO_4 \cdot 7\ H_2O$, 0.50 g; $CaCl_2 \cdot 2\ H_2O$, 0.25 g; NaCl, 2.25 g; $NaHCO_3$, 0.85 g; $Na_2CO_3$, 0.50 g; $Na_2S \cdot 9\ H_2O$, 0.45 g; resazurin, 0.5 mg; nitriloacetic acid, 20.2 mg; $FeCl_3 \cdot 6\ H_2O$, 2.1 mg; $CoCl_2 \cdot 6\ H_2O$, 2.0 mg; $MnCl_2 \cdot 4\ H_2O$, 1.0 mg; $ZnCl_2$, 1.0 mg; $NiCl_2 \cdot 6\ H_2O$, 1.0 mg; $CuSO_4 \cdot 2\ H_2O$, 0.5 mg; $Na_2MoO_4 \cdot 2\ H_2O$, 0.5 mg; pyridoxine-HCl, 0.1 mg; riboflavin, 0.05 mg; thiamine, 0.05 mg; nicotinic acid, 0.05 mg; p-aminobenzoic acid, 0.05 mg; lipoic acid, 0.05 mg; biotin, 0.02 mg; folic acid, 0.02 mg; and cyanocobalamin, 0.01 mg. The medium for growth on $CO_2$ as the methanogenic substrate used $H_2$:$CO_2$ as the gas phase. For growth on methanol, methanol was added to a final concentration of 0.4% and the gas phase was $N_2$:$CO_2$. For growth on acetate, Na acetate was added to the medium to a final concentration of 200 mM under an atmosphere of $N_2$:$CO_2$. The final pH of the medium was pH 6.5 (after being made anaerobic, see after this listing).

The medium used for *Msph. cuniculi* (Biavati et al, 1988) contained (per liter): tryptone/trypticase, 1.00 g; yeast extract, 1.00 g; $K_2HPO_4$, 0.30 g; $KH_2PO_4$, 0.30 g; $(NH_4)_2SO_4$, 0.30 g; NaCl, 0.61 g; $MgSO_4 \cdot 7\ H_2O$, 0.13 g; $CaCl_2 \cdot 2\ H_2O$, 0.08 g; Na acetate, 2.50 g; $NaSeO_4$, 0.04 mg; $NaHCO_3$, 3.00 g; resazurin, 0.5 mg; $Na_2S \cdot 9\ H_2O$, 0.45 g; methanol, 0.4%; nitriloacetic acid, 20.2 mg; $FeCl_3 \cdot 6\ H_2O$, 2.1 mg; $CoCl_2 \cdot 6\ H_2O$, 2.0 mg; $MnCl_2 \cdot 4\ H_2O$, 1.0 mg; $ZnCl_2$, 1.0 mg; $NiCl_2 \cdot 6\ H_2O$, 1.0 mg; $CuSO_4 \cdot 2\ H_2O$, 0.5 mg; $Na_2MoO_4 \cdot 2\ H_2O$, 0.5 mg; pyridoxine-HCl, 0.1 mg; riboflavin, 0.05 mg; thiamine, 0.05 mg; nicotinic acid, 0.05 mg; p-aminobenzoic acid, 0.05 mg; lipoic acid, 0.05 mg; biotin, 0.02 mg; folic acid, 0.02 mg; and cyanocobalamin, 0.01 mg. The final pH of the medium

was pH 6.9 (after being made anaerobic, see after this listing).

The medium used for *Msph. stadtmanae* (Miller and Wolin, 1985) contained (per liter): tryptone/trypticase, 2.00 g; yeast extract, 2.00 g; $K_2HPO_4$, 0.30 g; $KH_2PO_4$, 2.80 g; $(NH_4)_2SO_4$, 0.30 g; NaCl, 0.61 g; $MgSO_4 \cdot 7\ H_2O$, 0.13 g; $CaCl_2 \cdot 2\ H_2O$, 0.08 g; Na acetate, 0.50 g; $NaSeO_4$, 0.04 mg; $Na_2CO_3$, 1.70 g; $NH_4Cl$, 1.00 g; resazurin, 0.5 mg; $Na_2S \cdot 9\ H_2O$, 0.45 g; methanol, 0.4%; nitriloacetic acid, 20.2 mg; $FeCl_3 \cdot 6\ H_2O$, 2.1 mg; $CoCl_2 \cdot 6\ H_2O$, 2.0 mg; $MnCl_2 \cdot 4\ H_2O$, 1.0 mg; $ZnCl_2$, 1.0 mg; $NiCl_2 \cdot 6\ H_2O$, 1.0 mg; $CuSO_4 \cdot 2\ H_2O$, 0.5 mg; $Na_2MoO_4 \cdot 2\ H_2O$, 0.5 mg; pyridoxine-HCl, 0.1 mg; riboflavin, 0.05 mg; thiamine, 0.05 mg; nicotinic acid, 0.05 mg; p-aminobenzoic acid, 0.05 mg; lipoic acid, 0.05 mg; biotin, 0.02 mg; folic acid, 0.02 mg; and cyanocobalamin, 0.01 mg. The final pH of the medium was pH 6.9 (after being made anaerobic, see after this listing). For studies in the presence of 2-propanol, 2-propanol was added from a sterile stock solution to a final concentration of 0.6%.

The medium for *Msp. hungatei* strain GP1 (Patel et al, 1976) contained (per liter): NaCl, 0.30 g; $MgCl_2 \cdot 6\ H_2O$, 0.064 g; $NH_4Cl$, 0.40 g; Na acetate, 0.42 g; $K_2HPO_4$, 0.82 g; $CaCl_2 \cdot 2\ H_2O$, 0.064 g; resazurin, 0.5 mg; $Na_2CO_3$, 1.00 g; $Na_2S \cdot 9\ H_2O$, 0.45 g; nitriloacetic acid, 20.2 mg; $FeCl_3 \cdot 6\ H_2O$, 2.1 mg; $CoCl_2 \cdot 6\ H_2O$, 2.0 mg; $MnCl_2 \cdot 4\ H_2O$, 1.0 mg; $ZnCl_2$, 1.0 mg; $NiCl_2 \cdot 6\ H_2O$, 1.0 mg; $CuSO_4 \cdot 2\ H_2O$, 0.5 mg; $Na_2MoO_4 \cdot 2\ H_2O$, 0.5 mg; pyridoxine-HCl, 0.1 mg; riboflavin, 0.05 mg; thiamine, 0.05 mg; nicotinic acid, 0.05 mg; p-aminobenzoic acid, 0.05 mg; lipoic acid, 0.05 mg; biotin, 0.02 mg; folic acid, 0.02 mg; and cyanocobalamin, 0.01 mg. The final pH of the medium was pH 7.0 (after being made anaerobic, see below).

All of the media were made anaerobic by the use of the gassing manifold system, a modification of the Balch gassing manifold system (Balch & Wolfe, 1979). This system is connected to the source of gases to be used for the headspace ($H_2$:$CO_2$ for *Msph. stadtmanae*, *Msph. cuniculi*, *Mb. thermoautotrophicum*, *Mb.* strain G2R, *Ms. barkeri*, *Mc. voltae*, and *Msp. hungatei*; $N_2$:$CO_2$ for *Ml. tindarius*, *Mc. thermolithotrophicus* when grown on formate, *Ms. barkeri* when grown on methanol, and *Mst. concilii*) and a vacuum source, typically a water aspirator vacuum system. The

choice of either the gas pressurization or evacuation was controlled by a two-way stopcock. The media were pressurized to 0.2 atm over standard atmospheric pressure on the gassing manifold system.

Inoculation of media was typically performed by use of sterile 50 mL glass syringes, made anaerobic by washing the interior of the syringe with sterile, anaerobic 2 mM $Na_2S$ solution. Typical inoculum size was 10% of the volume of the medium, ie., 50 mL of inoculum into 500 mL of culture medium in the bottle, except for *Mst. concilii* which was inoculated at 20% volume.

**3.1.3. Growth Monitoring** - For growth curves and study of the respective enzyme activities culture density was measured using stoppered Balch-Wolfe culture tubes (Bellco Glass Inc., New Jersey) and growth was monitored on an LKB Novaspec II spectrophotometer (Pharmacia, Sweden) fitted with an adaptor to hold the Balch tubes. The growth density was measured at $\lambda = 660$ nm.

**3.1.4. Harvest of Cultures** - For the study and purification of the methyl viologen-reducing hydrogenase of *Msph. stadtmanae*, and for the enzymatic study of other hydrogenotrophic methanogens aerobically, the cells were first prepared by replacing the $H_2$:$CO_2$ gas phase used by the organism for growth, with $N_2$ in a method similar to that used to prepare the media, ie., using the gassing manifold system to alternately evacuate the headspace and replacing it with $N_2$, after this treatment, the cells were harvested aerobically via centrifugation. For strictly anaerobic studies (early hydrogenase and the $F_{420}H_2$ dehydrogenase studies), the cultures and extracts were handled using an Anaerobic Chamber (Coy Instruments, Michigan) which contained an atmosphere of $N_2$:$H_2$ (95:5), and used palladium catalyst in order to remove any trace amounts of $O_2$ that may have entered through the airlock. The centrifuge bottles (Nalgene) used were fitted with rubber o-rings to prevent or reduce $O_2$ entry, thus allowing the anaerobic centrifugation of the cultures. Typically, the cells were harvested at 22,000 × g, on a refrigerated Sorvall RC-5 centrifuge (Dupont Instruments). Cells not used immediately (either aerobic or

anaerobic) were stored at -60°C until needed.

**3.1.5. Cell Disruption** - The primary method of cell disruption, regardless of culture, was via the French Pressure Cell (SLM/Aminco, Rochester). Dependent on the organism, the cells were either pressed at 20,000 or 38,000 psi. The only cultures that required pressing at the higher pressure were both species of *Methanosphaera*. For anaerobic French press, the cell was first pre-treated with an anaerobic 2mM $Na_2S$ solution in order to scrub out the $O_2$ from the interior of the cell, and the inlet and outlet connections were fitted with tubing and needles to prevent exposure of the cell suspension(s) to atmospheric $O_2$. For organisms that were less sturdy, specifically the marine organism, *Methanococcus thermolithotrophicus*, the cells were lysed simply by the resuspension of the cell pellet with a low-salt buffer (eg., Tris-Cl).

**3.1.6. High-Speed Centrifugation** - Preparation of cell-free extracts following French press was performed via ultracentrifugation, in order to remove cell debris and membrane fragments, at a speed of 120,000 × g for 1.0 hr at 4°C in a 60Ti (Beckman Industries) rotor on either a L3-50 or L8-80 ultracentrifuge (Beckman Industries) using metal capped, clear polycarbonate ultracentrifuge tubes with a 38.5 mL nominal capacity (Nalgene/Beckman). This high-speed supernatant was then processed further.

**3.1.7. Enzyme Assays** - Anaerobic enzyme assays were performed typically by using stoppered (size 00) 12mm x 75mm Pyrex culture tubes (Corning) as described previously (Daniels & Wessels, 1984), with extracts or other solutions added with gas-tight microsyringes (Hamilton Co., Reno) that were scrubbed of $O_2$ by an anaerobic 2mM $Na_2S$ solution. Generally, the assay solution volume was 2.0 mL.

The enzyme activities were followed by change in absorbance spectrophotometrically on the LKB Novaspec II spectrophotometer and the reaction rates were recorded on a Fisherbrand Recordall series 5000 chart recorder (Fisher Scientific Ltd.), which recorded the changes in absorbance continuously.

**3.1.8. Kinetic Analysis of Enzyme Activities** - For the estimation of the kinetic parameters for enzyme activity, $K_m$ and $V_{max}$, the data acquired were plotted using the classical plotting methods of Henri-Michaelis-Menten, plotting velocity (v) versus substrate concentration ([S]) (Michaelis and Menten, 1913) and Lineweaver-Burk, plotting the respective inverts of the same parameters of Michaelis and Menten, (1/v versus 1/[S]) (Lineweaver and Burk, 1933). Alternative methods of replotting kinetic data included the methods of Woolf-Augustinsson-Hofstee (also referred to as the method of Eadie-Hofstee), which plots v versus v/[S] (Hofstee, 1959) and Eadie-Scatchard, which plots v/[S] versus v (Scatchard, 1949), which are useful in cases where there are significant errors in the enzyme velocity determinations (Segel, 1975) and are not sensitive to the low substrate concentration values that the Lineweaver-Burk plot is subject to. Another alternative method is the plotting method of Hanes-Woolf, plotting [S]/v versus [S] (Hanes, 1932), which is useful when the substrate values increment at an even spacing (Segel, 1975).

Data were plotted using either SigmaPlot for Windows 3.1 (works on Windows '95 as well), version 1.02 (Jandel Scientific), used to plot the linear (mathematically smoothed) kinetic plots or CurveExpert for Windows '95, version 1.3 (D. Hyams, Starkville, MS), used to plot the Michaelis-Menten kinetic plots. Both programs also provided correlation coefficients ($r^2$) for the respective graph types.

**3.1.9. Protein Determination** - Protein concentrations were determined using the micro-method of Coomassie Brilliant Blue G dye (Sigma Chemical Co., St. Louis, MO) binding of Bradford (Bradford, 1976), using Bovine Serum Albumin (Sigma Chemical Co., St. Louis, MO) as the protein standard. Protein elution profiles off column chromatography or from sucrose density gradients were measured on a Milton-Roy 3000 spectrophotometer at 280 nm using either quartz or plastic (methacrylate) cuvettes (Fisher Scientific).

### 3.2. MV Hydrogenase Specific Methods -

**3.2.1. Enzyme Assays -** Assay tubes were constructed as described above (section 3.1.7). Hydrogenase assay tubes contained 2.0 mL of 50 mM Tris-Cl, pH 8.0, $\pm MgCl_2$, under $H_2$ as the headspace gas, with either methyl viologen ($\lambda$ = 560 nm, c=2.0 mM, $\varepsilon_{560}$ = 8.0 $mM^{-1} \bullet cm^{-1}$) or coenzyme $F_{420}$ ($\lambda$ = 401 nm, c=7.5-12 $\mu$M, $\varepsilon_{401}$ = 25.8 $mM^{-1} \bullet cm^{-1}$) as the electron acceptor. Also tested as possible electron acceptors were flavin adenine dinucleotide (FAD) ($\lambda$ = 450 nm, c=14 $\mu$M, $\varepsilon_{450}$ = 11.3 $mM^{-1} \bullet cm^{-1}$), flavin mononucleotide (FMN) ($\lambda$ = 450 nm, c=14 $\mu$M, $\varepsilon_{450}$ = 12.2 $mM^{-1} \bullet cm^{-1}$), nicotinamide adenine dinucleotide ($NAD^+$) ($\lambda$ = 340 nm, c=0.285 mM, $\varepsilon_{340}$ = 6.22 $mM^{-1} \bullet cm^{-1}$), nicotinamide adenine dinucleotide phosphate ($NADP^+$) ($\lambda$ = 340 nm, c=0.285 mM, $\varepsilon_{340}$ = 6.2 $mM^{-1} \bullet cm^{-1}$), phenazine ($\lambda$ = 365 nm, c=1.0 mM), clostridial ferredoxin (c=25 $\mu$g/mL), horse heart cytochrome $C_3$ (c=25, 50 $\mu$g/mL) (Sigma Chemicals, St.Louis), phenazine methosulfate, 2,6-dichlorophenol indophenol and methylene blue (Fisher Scientific). Reactions were followed, at their appropriate wavelength, for the specific electron acceptor following the addition of the cell extract.

Inhibitors of hydrogenase activity were tested with methyl viologen as the electron acceptor and using 50 mM HEPES, pH 7.0 as the assay buffer. Inhibitors that were tested included $CuCl_2$, $CdCl_2$, $CoCl_2$, $HgCl_2$, $NiCl_2$, $ZnCl_2$ (all tested at 1mM final concentration), NaCN (1 mM), $NaN_3$ (0.02% or 3 mM), 2-bromoethanesulfonic acid (5 mM), p-hydroxymercuribenzoic acid (25 $\mu$M), and 2,6-dichlorophenol indophenol. Acetylene gas was also tested for its ability to inhibit the hydrogenase, as it was shown that growth and methanogenesis was inhibited in whole cells by the addition of acetylene into the gas phase of methanogenic cultures (Sprott et al, 1982).

Stabilization of the hydrogenase was performed by the chemical additions (to a final concentration of 20%) of either glycerol, ethylene glycol, acetone, ethanol, methanol, or sucrose. Reducing agents were added to a final concentration of 20 mM.

**3.2.2. Hydrogenase Reactivation** - Aerobically handled hydrogenase must be reactivated in order to achieve maximum activity, and since the activity is $O_2$-inhibited,

this requires the removal of $O_2$ from the hydrogenase sample. This is achieved by placing the sample inside a Pyrex culture tube (assay tube), and cycling evacuation of the headspace of the sample and repressurizing with $N_2$ for 5 cycles. Immediately after this degas procedure the sample was assayed for hydrogenase activity. This process eliminates most, if not all, of the lag period in activity that would be observed if there were no reactivation procedure.

**3.2.3. Column Chromatography** - Several methods of column chromatography were used in order to purify the MV-hydrogenase of *Msph. stadtmanae*. Resins used for low pressure liquid column chromatography were for ion-exchange: DEAE Cellufine (Amicon) and Express-Ion Q exchanger (Whatman, through Sigma Chemicals, St. Louis). For high pressure column chromatography, the BioLogic system (BioRad, Mississauga, ON) was used, with the ion exchanger Mono Q.

Resins employed for size-exclusion chromatography, for purification or for molecular weight determination, were: Sepharose 6B (fractionation range of 10,000-4,000,000), Sephacryl S-200HR (fractionation range of 5,000-250,000) (Sigma Chemicals, St.Louis), and Sephadex G-200 (fractionation range of 5,000-600,000) (Pharmacia Biotech, Sweden).

Hydrophobic interaction chromatography studies were performed on Phenyl-Sepharose CL-4B (Sigma Chemicals, St.Louis) and t-Butyl resin (BioRad, Mississauga, ON) on the BioLogic system.

Chromatography by adsorption onto inorganic hydroxylapatite was performed using prepared material specifically for column use, Hydroxylapatite HT (BioRad, Mississauga, ON), which allowed for reasonable flow rates compared to the batch material available (Sigma Chemical Co., St. Louis, MO).

Chromatofocusing, a form of ion exchange chromatography that uses ampholytes to elute proteins based on pH, was performed on Polybuffer Exchanger 94 (Sigma Chemical Co., St.Louis, MO). For low pH elution, 50 mM Glycine buffer, pH 2.5 was used.

**3.2.4. Protein Concentration Methods** - The methods used to concentrate protein solutions for column chromatography included ultrafiltration with either a YM1, YM10, or YM30 membrane (Amicon Inc., Beverly, MA) or the use of Aquacide II (Calbiochem-Novabiochem, San Diego, CA). With Aquacide, the sample to be concentrated is held in dialysis tubing (MWCO = 6,000-8,000) and the Aquacide is packed around the filled dialysis tubing. This draws the water out of the dialysis bag, thus concentrating the sample within the bag.

**3.2.5. Electrophoresis** - All chemicals were purchased from Sigma (Sigma Chemical Co., St. Louis, MO) and were of electrophoresis grade. Electrophoresis on slab acrylamide gels was used to determine the purity of the preparations of hydrogenase and to determine the molecular weight of its component subunits as well as its native form. The same electrophoresis unit, the Hoefer 600 series, (Hoefer Instruments) was used for both SDS- denaturing and native, non-denaturing electrophoresis.

SDS-PAGE was performed by the method of Laemmli (Laemmli, 1970), typically using an acrylamide concentration of 10%. High molecular weight markers were available to calibrate the SDS-PAGE gels: bovine erythrocyte carbonic anhydrase (29,000); egg albumin (45,000); bovine albumin (66,000); rabbit muscle phosphorylase B (97,400); *Escherichia coli* β-galactosidase (116,000); and rabbit muscle myosin (205,000) (Sigma Chemicals, St. Louis, MO) as well as low molecular weight markers: α-lactalbumin (14,400); trypsin inhibitor (20,100); carbonic anhydrase (30,000); ovalbumin (43,000); albumin (67,000); and phosphorylase B (94,000) (Pharmacia Biotech, Sweden).

Native electrophoresis was typically performed on an acrylamide gradient of 3.5-25.2%, run overnight (at least 16hrs) at a constant 150 V. High molecular weight native markers were used to calibrate the gradient gel: bovine serum albumin (67,000); beef heart lactate dehydrogenase (140,000); beef liver catalase (232,000); horse spleen ferritin (440,000); and hog thyroid thyroglobulin (669,000) (Pharmacia Biotech, Sweden).

Staining of either the SDS-PAGE or Native PAGE gels was performed typically with either Coomassie Brilliant Blue R stain, or with the Silver Staining kit of Sigma to

visualize low concentration protein bands. Also used was staining with Sypro Orange (BioRad, Mississauga, ON), which allowed documentation of the PAGE gel by the GelDoc system of BioRad.

**3.2.6. Activity Staining** - The hydrogenase could be activity stained after native gel electrophoresis by incubation of the PAGE gel in hydrogenase assay buffer (2 mM MV in 50 mM Tris-Cl, pH 8.0 or 10 mM MV in 50 mM Tris-Cl, pH 8.5) inside the anaerobic chamber, under an atmosphere of approximately 5 ± 2% $H_2$. Bands of dark blue (reduced MV) were visualized after a few minutes (depending on the concentration of the hydrogenase in the sample). The bands of reduced MV could be fixed in the gel by the addition of 2,3,5-triphenyltetrazolium chloride from a 1.5% stock solution (Sigma Chemicals, St. Louis) which would react with the reduced MV to produce red bands.

**3.2.7. Chemical Determinations** - Several chemical methods were employed in the characterization of the physical properties of the MV hydrogenase of *Msph. stadtmanae*. Acid labile sulfide was determined using the micro-method of Beinert (1983) using $Na_2S$ (Sigma Chemical Co., St. Louis, MO) as the standard. Iron determinations were made using the Ferrozine method of iron determination from biological samples (Fish, 1988), using ferric ammonium citrate (Sigma Chemical Co., St. Louis, MO) as standard.

### 3.3. $F_{420}$ and $F_{420}H_2$ Dehydrogenase Specific Methods -

**3.3.1. $F_{420}$ Purification** - The methanogenic cofactor, $F_{420}$, was harvested after a 6-8 hour culture incubation from the thermophilic methanogen, *Methanococcus thermolithotrophicus*, grown on sodium formate as the primary growth and methanogenic substrate. The purification protocol involved the anaerobic harvest of the cells of *Mc. thermolithotrophicus*, after which the cells were resuspended aerobically with 40% methanol, pre-heated to 80°C. This causes the immediate lysis of the cells, and this suspension is heated at 80°C for a further 30 minutes minimum, with periodic vortexing to mix the suspension and denature the proteins. Following centrifugation at 22,000 × g

for 30 minutes, the supernatant is applied to a column of DEAE Sephadex A-25, pre-equilibrated in 50mM Tris-Cl, pH 8.0. After washing with the equilibration buffer, a reddish cofactor is eluted slowly off the column with a step gradient of 0.3M $NH_4HCO_3$. This reddish compound is completely separated from the cofactor $F_{420}$ using this step, while a step to 0.6M $NH_4HCO_3$ removes the red compound from the column, and begins to elute the $F_{420}$. A further step to 1.0M elutes the cofactor completely from the column. The cofactor $F_{420}$ can be separated from the $NH_4HCO_3$ and concentrated by evaporation on a Büchi Rotavapor-R rotary evaporator (Fisher Scientific Ltd.) at 80°C.

**3.3.2. $F_{420}$ Reduction** - The reduction of the cofactor $F_{420}$ was performed chemically using sodium borohydride, under $N_2$. The excess borohydride was destroyed by the addition of concentrated HCl to a pH of approximately 2.0 (solution just becomes cloudy), then the pH was adjusted to neutrality, just as the cloudiness disappears (pH 6.0-8.0) with a concentrated KOH solution (10.0M).

**3.3.3. Enzyme Assays -** Assay tubes for carbon monoxide dehydrogenase (CODH) were composed of 50 mM Tris-Cl, pH 8.0 using carbon monoxide (Linde Welders Supply, Winnipeg) as the headspace gas. Either of the electron acceptors, methyl viologen or cofactor $F_{420}$ were used for the assay.

Assay tubes for the $F_{420}H_2$ dehydrogenase contained a similar ion composition to the assay described previously (Haase et al, 1989); namely, 40 mM potassium phosphate, 20% sucrose, pH 7.0, under a headspace of $N_2$, though for most of the later experiments, 200 mM potassium phosphate was used. Reduced coenzyme $F_{420}$ was used as the electron donor and methyl viologen (0.25 mM) with metronidazole (0.5 mM) as the electron acceptors. The reaction was followed with the oxidation of $F_{420}$ at 401 nm ($\varepsilon_{401}$ = 29.5 $mM^{-1} \cdot cm^{-1}$) after the addition of the MV. An alternative electron acceptor was also employed, phenazine (Abken et al, 1998) which could be used to replace the combination of metronidazole and methyl viologen. For this assay, the oxidation of $F_{420}H_2$ was followed at 420 nm ($\varepsilon_{420}$ = 45.5 $mM^{-1} \cdot cm^{-1}$) after the addition of the phenazine, instead

of at 401 nm because of interference caused by phenazine at the shorter wavelength.

Assay tubes for following reverse $F_{420}$-reducing hydrogenase (or $F_{420}H_2$-oxidizing non-dehydrogenase) activity used the same composition buffer as the $F_{420}H_2$ dehydrogenase assay (see above), but no electron acceptors (like metronidazole plus methyl viologen) were used, similar to the system employed previously (Livingston et al, 1987).

Assay tubes for the $NADP^+$:$F_{420}$ oxidoreductase were made up in 50 mM potassium phosphate, pH 8.5, using reduced coenzyme $F_{420}$ as the electron donor, and $NADP^+$ (0.3 mM) as the electron acceptor.

**3.3.4. Sucrose Density Gradient Centrifugation** - Sucrose linear gradients from 0-20% were made up in 11.8 mL thin-walled centrifuge tubes (Nalgene). Centrifugation was performed at 195,000 × g in a SW41 swinging bucket rotor at 4 °C for 4 hours on either a L3-50 or L8-80 ultracentrifuge (Beckman Industries). The gradient was eluted through the bottom of the tube by piercing with a 20 gauge needle, and the fractions of approximately 1.0-1.2mL were collected by a BioRad Model 2110 fraction collector (BioRad, Mississauga, ON).

# 4. Hydrogenase of *Methanosphaera stadtmanae*

## 4.1. Introduction

The enzyme activity, known as the hydrogenase, was first discovered in *E. coli* in the early 1930's (Stephenson and Stickland, 1931). The hydrogenase is an important enzyme in the metabolism of many microorganisms. This enzyme is involved in either the consumption (or uptake), or the production (or evolution) of the most simple of molecules, hydrogen. In the mode of $H_2$ uptake, the purpose of the $H_2$ is to provide reducing equivalents for biochemical reactions within the cell. In the mode of $H_2$ evolution, the purpose of $H_2$ production is for the removal of excess reductant from the cell. The hydrogenase (in either the $H_2$-evolving or $H_2$-uptake forms) may be found in a wide range of microorganisms encompassing the three Domains of Life, the Archaea, the Bacteria, and the Eukarya (Adams et al., 1980). The hydrogenase reaction is described by the following:

$$H_2 \rightleftharpoons 2H^+ + 2e^- \qquad (1)$$

In the hydrogenotrophic methanogens, this is an important reaction since the electrons produced in this reaction are used for the energy producing methanogenic pathway by reducing stepwise the substrate (eg. $CO_2$). In the mentioned example of $H_2$:$CO_2$ methanogenesis, there is a requirement of 4 moles of hydrogen for every mole of $CO_2$ consumed.

Although the hydrogenase is an important element in the biochemistry of the methanogens that use the $H_2$:$CO_2$ pathway, hydrogenase activity has been detected in methanogens that grow on the other methanogenic substrates for which there is no absolute requirement for $H_2$, e.g. the acetotrophic *Methanosaeta concilii* (Patel, 1984) and the methanol-utilizer, *Methanolobus tindarius* (König and Stetter, 1982).

In this chapter, the purification and characterization of the methyl viologen-only (MV) reducing hydrogenase of the methanol-utilizing methanogen, *Methanosphaera*

*stadtmanae* will be discussed with respect to both kinetic and physical properties. These properties will be compared to known hydrogenases of this type, of which several have been purified from other members of the methanogens (Adams et al, 1986; Deppenmeier et al, 1992; Kemner and Zeikus, 1994; McKellar and Sprott, 1979). This methanogen is interesting in that it grows obligately on $H_2$ plus methanol, and cannot grow on methanol alone unlike *Ml. tindarius* or *Methanosarcina* spp. (Miller and Wolin, 1985). In addition, this methanogen was reported to not carry cytochromes that are involved in the electron transfer reactions in other methanol-utilizing methanogens (Miller and Wolin, 1985).

## 4.2. Results and Discussion

### 4.2.1. Characteristics of the MV-Hydrogenase in Crude Extract.

#### 4.2.1.1. Reactivation of MV hydrogenase activity.

The nature of the activity of the MV hydrogenase of *Msph. stadtmanae* is an anaerobic type. The assay of the enzyme is performed under an atmosphere of hydrogen with the complete absence of oxygen. Studies have been made very early in the history of the hydrogenase enzyme from *E. coli* with respect to its interaction with molecular $O_2$ (Fisher et al, 1954). For several forms of the enzyme, it has been reported that the presence of $O_2$ causes the complete inactivation of the enzyme, and in some cases, this process is irreversible. In the examples in which this aerobic inactivation is reversible, the methods of the "reductive activation" of the enzyme vary, but generally involve the removal of the $O_2$, either physically (via evacuation and replacement with an $O_2$-free gas) or chemically (via the addition of some reducing agent like sodium dithionite) or the combination of both.

A comparison of using headspace gas replacement with either $H_2$ or $N_2$, in the presence or the absence of a reducing agent was tested for the effectiveness at recovering enzyme activity. The results are shown in Table 4.1.

**Table 4.1 - Reactivation of MV Hydrogenase activity from *Msph. stadtmanae*.**

| Method | Specific Activity (μmoles MV/min/mg protein) | Lag Period (before activity maximum is reached) |
|---|---|---|
| **none** | 4.6 | ~ 2 minutes |
| **degas with $H_2$** | 6.1 | none |
| **degas with $H_2$ + dithionite** | 4.8 | none |
| **degas with $N_2$** | 11.2 | none |
| **degas with $N_2$ + dithionite** | 4.5 | none |

It was found that the hydrogenase activity from *Msph. stadtmanae* was best activated by the degassing and repressurization with $N_2$ instead of $H_2$, which is unusual compared to that found in other methanogenic bacteria (Adams et al, 1986; Deppenmeier et al, 1992; Fiebig and Freidrich, 1989; Fox et al, 1987; Jin et al, 1983; McKellar and Sprott, 1979; Muth et al, 1987; Shah and Clark, 1990; and Yamazaki, 1982). This is at first surprising since $H_2$ is the substrate of the activity, however this is not unusual since hydrogenases from other bacteria have been found to be reactivated by the removal of $O_2$ and its replacement by gases other than $H_2$ including $N_2$, argon, and $CO_2$ (Baron and Ferry, 1989; Niviere et al, 1986). The addition of a reducing agent (sodium dithionite) was found to reduce the level of the hydrogenase activity recovered.

### 4.2.1.2. Electron Acceptor Specificity

The hydrogenase of *Msph. stadtmanae* was tested in crude form for its specificity for known electron acceptors. Included in the survey were methyl viologen, methylene blue, phenazine methosulfate, dichlorophenol indophenol, cofactor $F_{420}$, the flavins: flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), the nicotinamide nucleotides: nicotinamide adenine dinucleotide ($NAD^+$) and nicotinamide adenine dinucleotide phosphate ($NADP^+$), ferredoxin (from *Clostridium pasteurianum*), and cytochrome $c_3$ (from horse heart). The results of this survey are shown in Table 4.2.

**Table 4.2 - Electron Acceptor Specificity of Hydrogenase activity in Crude Extract from *Msph. stadtmanae***

| Electron Acceptor | Concentration | Specific Activity (µmole/min/mg protein) | E°' of Electron Acceptor |
|---|---|---|---|
| **Methyl Viologen** | 2.0 mM | 8.27 | -440mV |
| **$F_{420}$** | 10 µM | <0.01 | -340mV |
| **$F_{420}$ + FMN** | 10 µM $F_{420}$; 14 µM FMN | <0.01 | - |
| **$F_{420}$ + FAD** | 10 µM $F_{420}$; 14 µM FAD | <0.01 | - |
| **Cytochrome $c_3$** | 50 µg/mL | <0.01 | 250mV |
| **Ferredoxin** | 25 µg/mL | <0.01 | - |
| **Methylene Blue** | 0.2 mM | <0.01 | 11mV |
| **Dichlorophenol Indophenol** | 0.5 mM | <0.01 | 210mV |
| **Phenazine Methosulfate** | 0.25 mM | <0.01 | 80mV |
| **FMN** | 14 µM | 0.05 | -220mV |
| **FAD** | 14 µM | 0.09 | -219mV |
| **$NAD^+$** | 285 µM | <0.01 | -320mV |
| **$NADP^+$** | 285 µM | <0.01 | -324mV |

The most effective electron acceptor for the hydrogenase of *Msph. stadtmanae* was found to be the viologen dye, methyl viologen. Other electron acceptors that were reduced by this hydrogenase included the flavins (FAD, FMN), as both of these could be reduced at a low rate by the hydrogenase activity from *Msph. stadtmanae*. This property has been reported for the MV-hydrogenase of another methanogen as well (McKellar and Sprott, 1979). The hydrogenase activity from *Msph. stadtmanae* does not reduce either form of the nicotinamides, cytochrome $c_3$, or purified ferredoxin from *Clostridium*. None of the other artificial electron acceptors were reduced by the hydrogenase from *Msph. stadtmanae*.

As determined by previous investigators, the $F_{420}$-reducing hydrogenase from several methanogens required the addition of either FMN or FAD to the reaction mixture

(after certain steps in column chromatography where the associated flavin would be separated from the enzyme) in order to recover activity of the enzyme (Baron and Ferry, 1989; Muth et al, 1987). This was due to the loose association of the flavins with the enzyme which could be lost during purification. The flavins can serve as one electron to two electron switches for electron transfer to the methanogenic cofactor $F_{420}$. To test this possibility that the enzyme activity from *Msph. stadtmanae* may be able to reduce $F_{420}$ with the aid of the one-to-two electron switch, hydrogenase from *Msph. stadtmanae* was assayed with $F_{420}$ plus one of either FAD or FMN as electron acceptors, and the results are also shown in Table 2.

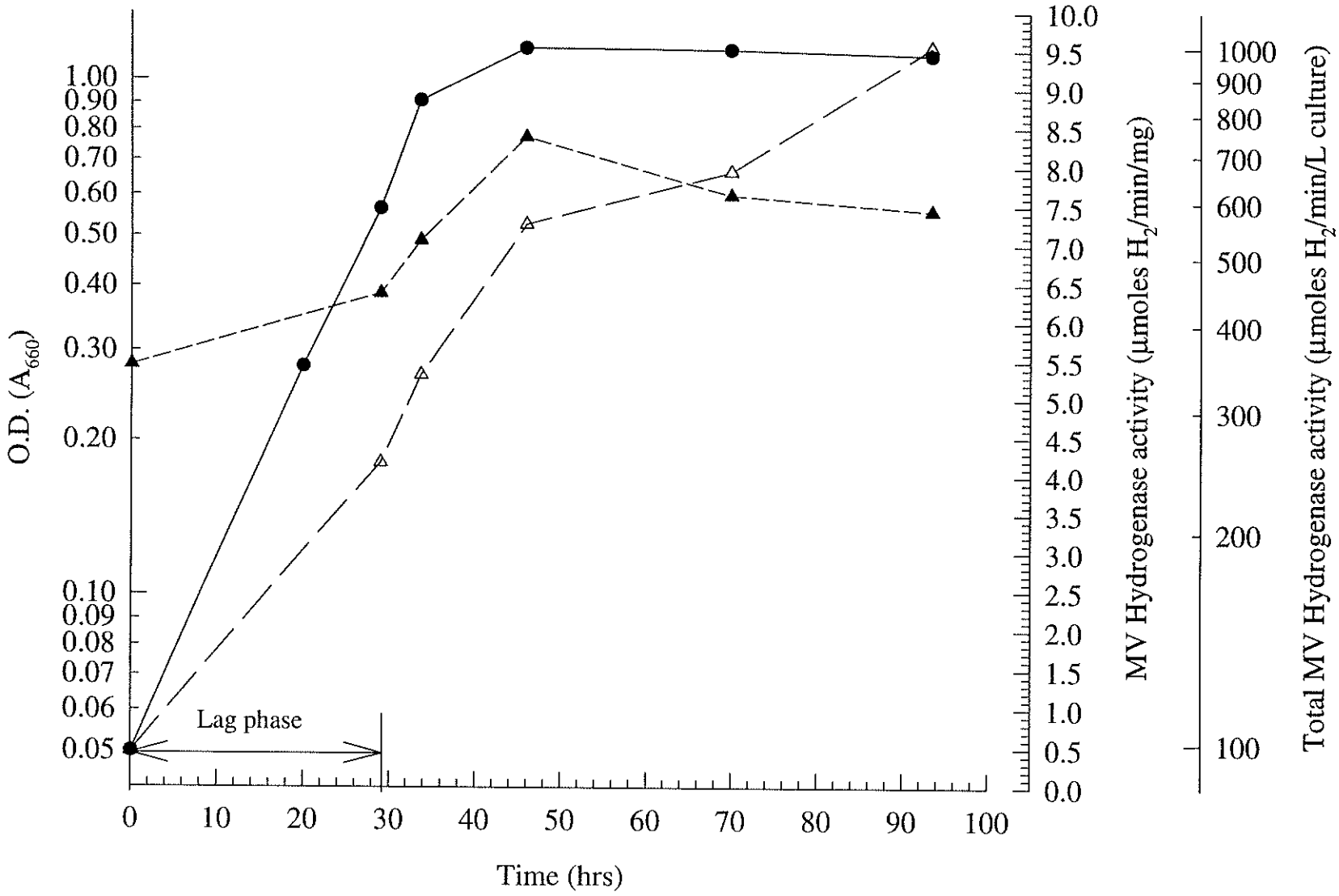
The results show that the MV hydrogenase does not react with cofactor $F_{420}$ directly, alone or in the presence of an electron conduit like the flavins. The natural electron acceptor for this form of the methanogenic hydrogenases still defies identification, though some have suggested that the gene product of the operon which encodes the MV hydrogenase in the *Mb. thermoautotrophicum* strains, which forms a polyferredoxin, may be the natural electron acceptor for this enzyme but this was not conclusively proven with pure polyferredoxin from *Mb. thermoautotrophicum* strain ΔH (Steigerwald et al., 1992) and strain Marburg (Hedderich et al., 1992).

#### 4.2.1.3. Growth phase dependence on MV Hydrogenase activity.

The phase in the growth cycle of the organism where the enzyme activity is the highest is important to determine the best time to harvest the cells for maximum recovery of enzyme activity. This survey of MV hydrogenase activity level with respect to the growth phase is illustrated in figure 1.

This survey shows that the best time to achieve maximum activity is during late-log phase, when the peak of enzyme activity occurs since the enzyme activity appears to be maintained at a relatively constant level throughout exponential phase of the growth cycle.

**Figure 1 -** Growth phase dependence on the activity of the MV hydrogenase from *Msph. stadtmanae*. Assays were performed in triplicate. (●) - Optical density; (▲) - MV hydrogenase specific activity; (△) - MV hydrogenase total activity.


O.D. ($A_{660}$)
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.09
0.08
0.07
0.06
0.05
Lag phase
0
10
20
30
40
50
60
70
80
90
100
Time (hrs)
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MV Hydrogenase activity (µmoles $H_2$/min/mg)
1000
900
800
700
600
500
400
300
200
100
Total MV Hydrogenase activity (µmoles $H_2$/min/L culture)

#### 4.2.1.4. pH dependence of the MV Hydrogenase activity.

A characteristic that is important in the study of any enzyme activity is its pH dependence. The MV hydrogenase of *Msph. stadtmanae* was assayed in crude extract over a pH range of 5.5 to 9.0, to determine the pH of maximum activity.
The pH profile is shown in Figure 2. Based on this survey, the optimum pH was determined to be about pH 8.5 in crude extract. An interesting side note, methyl viologen was found to act like a pH indicator with a transition that occurs at high pH; this was observed when attempting to assay the hydrogenase at the high pHs (8.5 and up). Because the viologen dye appeared to be reduced without the addition of enzyme extract, the activity levels at the higher pH values may not be accurate. This property will also be important in the following chapter on the $F_{420}H_2$ dehydrogenase activity from *Msph. stadtmanae*.

#### 4.2.1.5. Temperature dependence of the MV Hydrogenase activity.

The MV hydrogenase activity in crude extract from *Msph. stadtmanae* was tested for its temperature maximum. The temperature profile of the hydrogenase is shown in Figure 3. The temperature at which maximum activity occurs is at 47°C, after which the activity declines sharply. This value is not unusual for a mesophilic organism, similar to the $F_{420}$ hydrogenase from *Methanococcus voltae* (37°C optimal, Muth et al., 1987), but differs from the reports of the purified MV hydrogenase from *Methanosarcina mazei* strain Gö1 (80°C optimal, Deppenmeier et al, 1992) and the $F_{420}$ hydrogenase *Ms. barkeri* strain Fusaro (55°C optimal, Michel et al., 1995). It, however, will be shown later that there is a difference in the temperature optimum of the hydrogenase between the activity in crude versus purified systems.

#### 4.2.1.6. Temperature Stability.

The stability of the enzyme to applied heat is important to determine since the stability could be taken advantage of in the purification of the enzyme. A preliminary thermal stability at high temperature study was performed at 80°C (figure 4). This

**Figure 2 -** pH dependence on the activity of the MV hydrogenase in crude extract from *Msph. stadtmanae*. Assays were performed in triplicate.

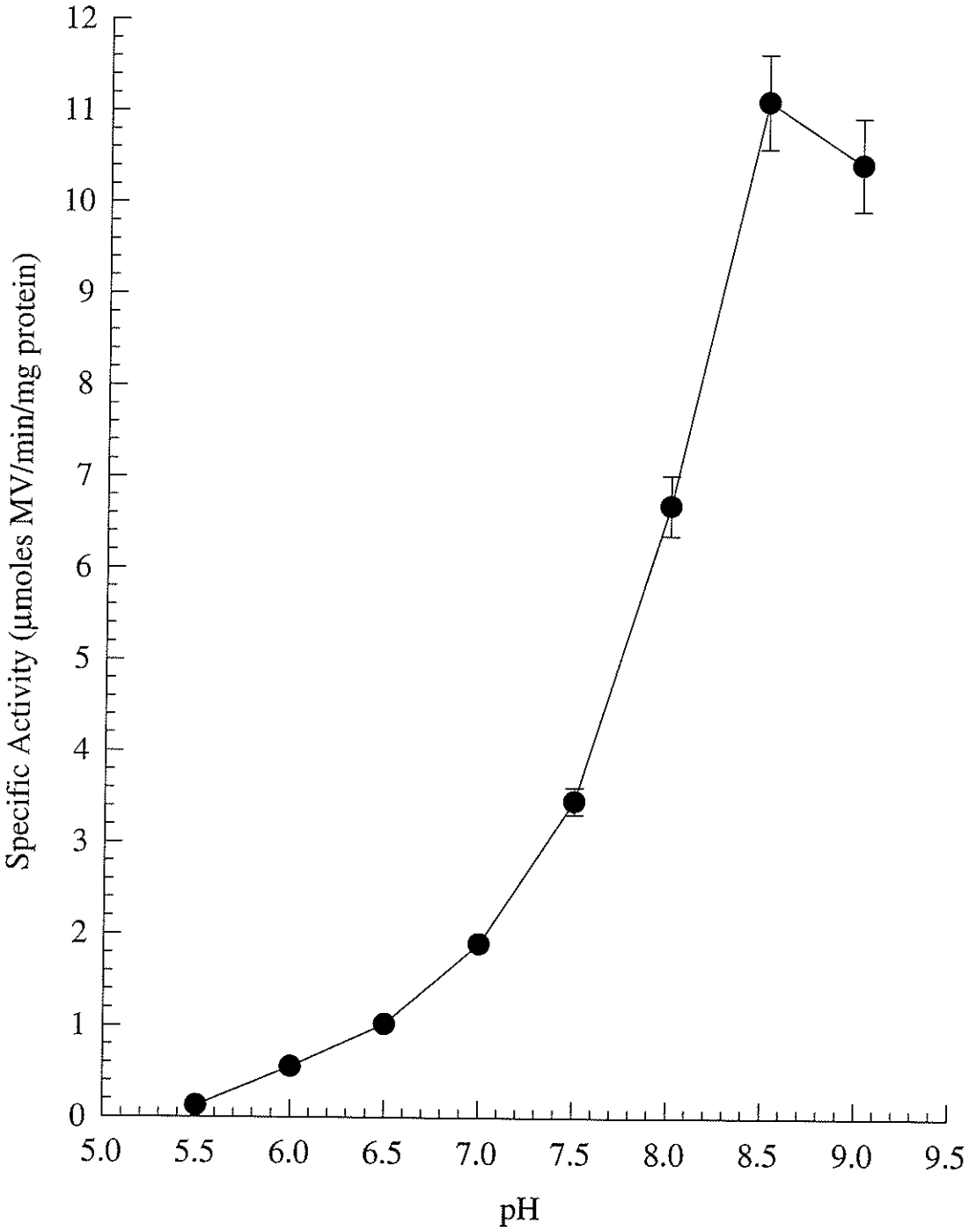

Specific Activity (µmoles MV/min/mg protein)
0
1
2
3
4
5
6
7
8
9
10
11
12
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
pH

**Figure 3 -** Temperature dependence on the activity of the MV hydrogenase in crude extract from *Msph. stadtmanae*. Assays were performed in triplicate.

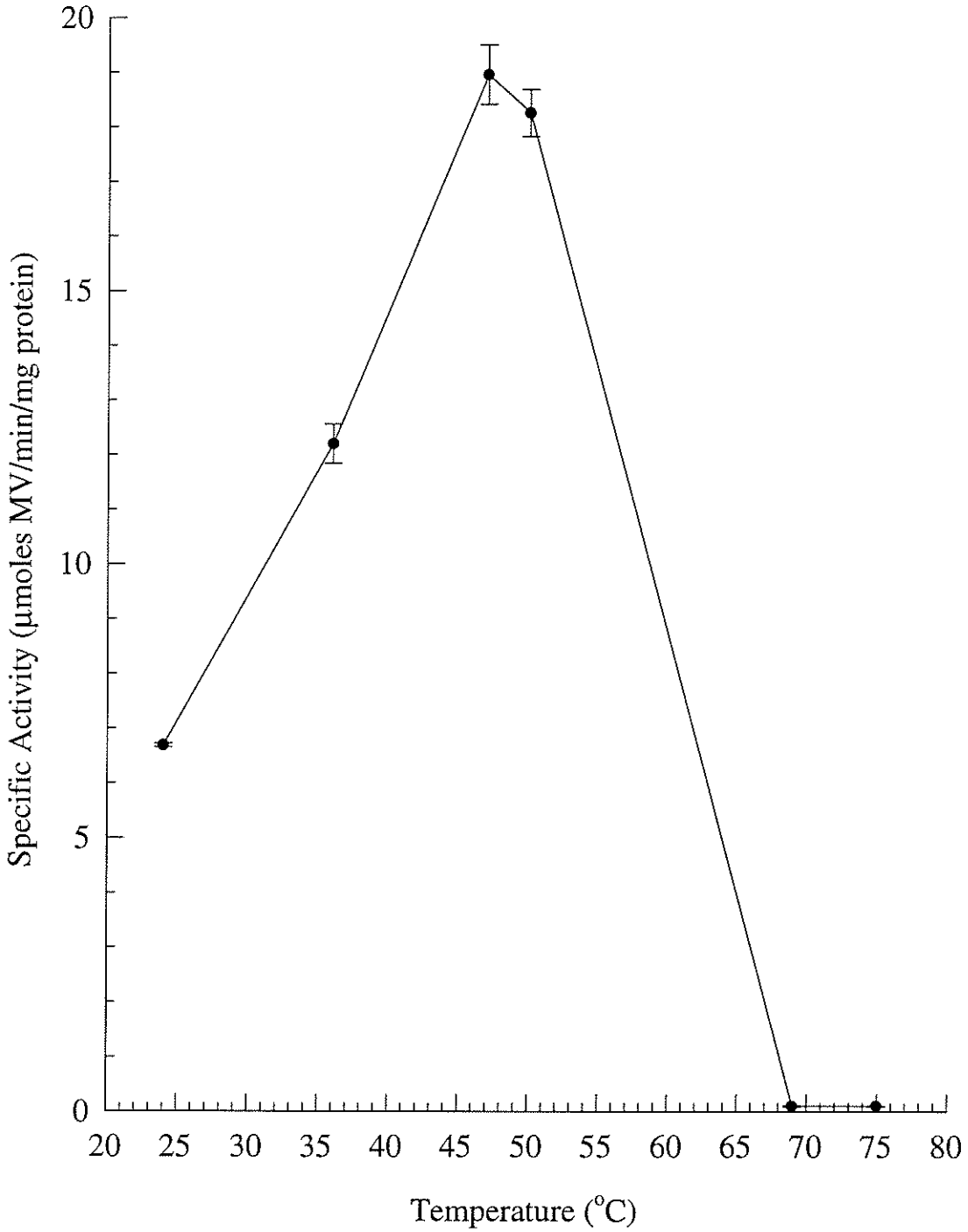
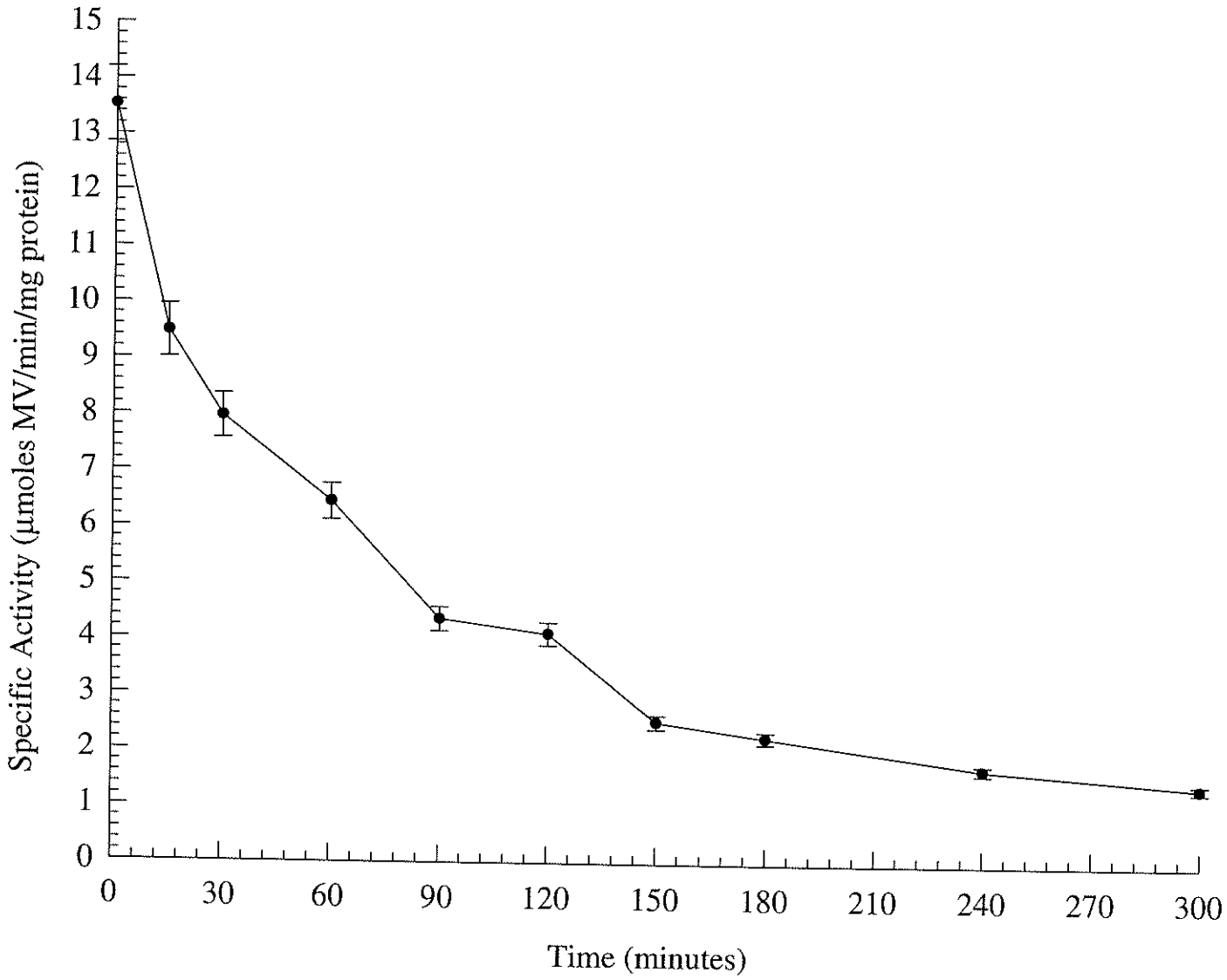

20
15
10
5
0
Specific Activity (µmoles MV/min/mg protein)
20
25
30
35
40
45
50
55
60
65
70
75
80
Temperature (°C)

**Figure 4 -** Temperature stability (at 80°C) of the activity of the MV hydrogenase in crude extract from *Msph. stadtmanae*. Assays were performed in triplicate.


Specific Activity (µmoles MV/min/mg protein)
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0
30
60
90
120
150
180
210
240
270
300
Time (minutes)

showed that the hydrogenase activity has a half-life of approximately 1 hour at this temperature. This stability may prove to be useful for the purification of the hydrogenase activity. It is interesting that the enzyme activity is stable at 80°C, but the enzyme shows no activity at this temperature (see section 4.2.1.5.).

#### 4.2.1.7. General Stability (+/- Effectors)

The general stability of the hydrogenase is necessary to know since the enzyme must be able to survive an extended period in cell extract form for the purposes of purification. A survey of the hydrogenase activity, in unamended extract or with the addition of other chemical components that may protect the enzyme was performed. The results are shown on Figure 5. There was an increase in hydrogenase activity in the aerobically treated extract over the first couple of days but then a decline to near the original levels was detected in the unamended extract. Of particular note, both of the reducing agents, 2-mercaptoethanol and more pronounced in dithiothreitol (DTT), the activity of the hydrogenase was found to be decreased initially, but the pattern of an increase in activity does occur here in days 2 and 4 before the activity declines to below the original extract. In general, the activity appears to be stable over a long period in cell extract and stored at 4°C, and the presence of reducing agents appear to not be useful in retaining full activity.

#### 4.2.1.8. Inhibitors of MV Hydrogenase Activity.

In the literature, many different types of molecules have been found to inhibit the activity of the various types of hydrogenases that currently exist. These include salts of metals like mercury ($Hg^{2+}$), a metal chelator like ethylene diamine tetraacetic acid (EDTA), anions like cyanide ($CN^-$) and azide ($N_3^-$), and gases like acetylene, carbon monoxide, and of course, oxygen.

Several different inhibitors have been tested as to their effect on the activity of the MV hydrogenase of *Msph. stadtmanae*, including several heavy metal ions, EDTA, and the toxic anions, $CN^-$ and $N_3^-$. The results of this survey are shown on Table 4.3.

**Figure 5 -** General stability of the MV hydrogenase activity in crude extract from *Msph. stadtmanae*. Extract was stored at 4°C. Assays were performed in triplicate. Legend: (•) - no additions; (■) - plus glycerol; (▲) - plus ethylene glycol; (▼) - plus sucrose; (♦) - plus ethanol; (○) - plus methanol; (□) - plus acetone; (△) - plus dithiothreitol; (▽) - plus 2-mercaptoethanol.

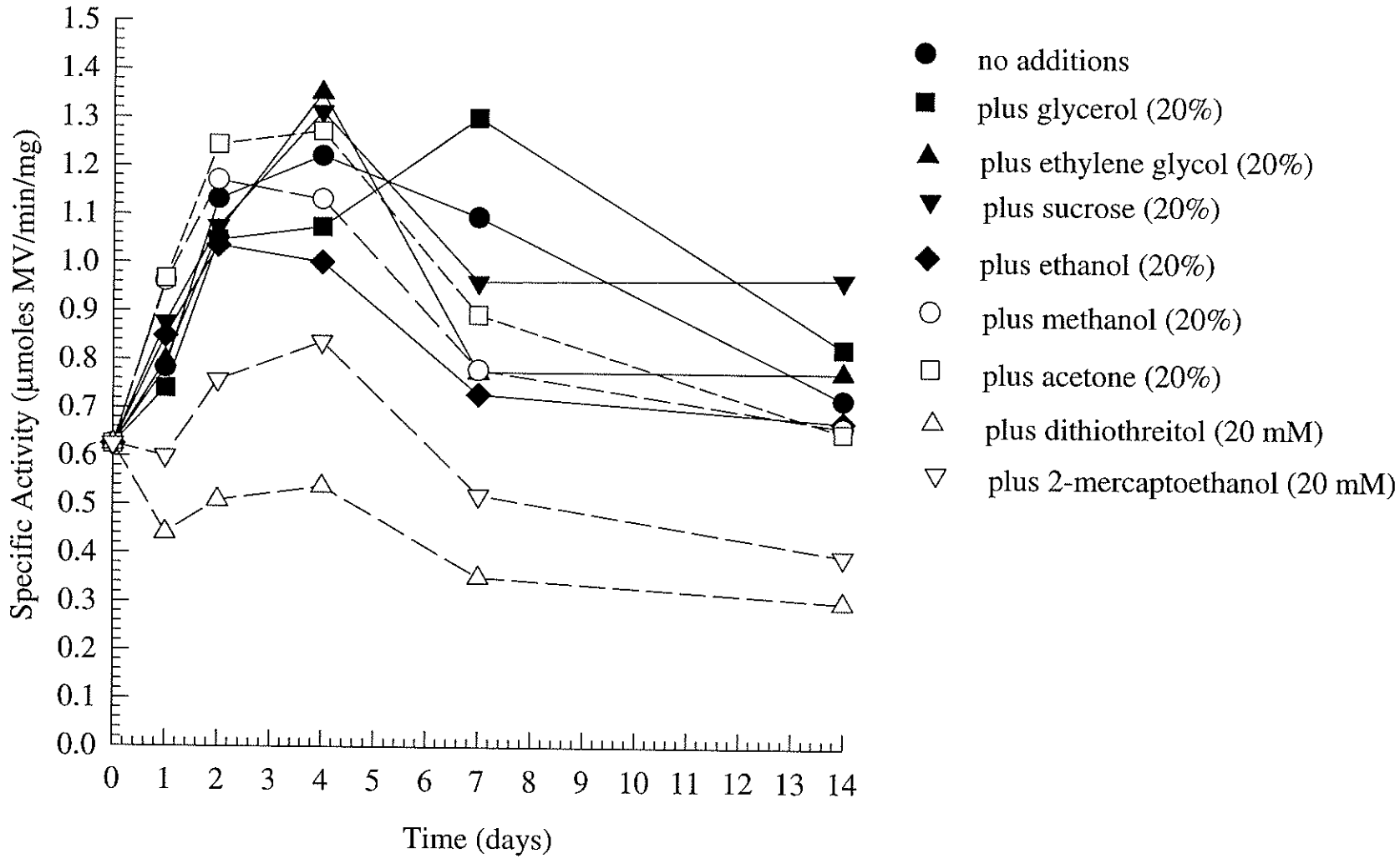

Specific Activity (µmoles MV/min/mg)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (days)
no additions
plus glycerol (20%)
plus ethylene glycol (20%)
plus sucrose (20%)
plus ethanol (20%)
plus methanol (20%)
plus acetone (20%)
plus dithiothreitol (20 mM)
plus 2-mercaptoethanol (20 mM)

**Table 4.3 - Effect of enzyme inhibitors on MV Hydrogenase activity**

| Inhibitor Added | Concentration | % Relative Activity |
|---|---|---|
| **None** | - | 100.0 |
| **2-BES** | 4 mM | 114.3 |
| **$Cd^{2+}$** | 1 mM | 57.1 |
| **$CN^-$** | 1 mM | 130.9 |
| **$Cu^{2+}$** | 1 mM | 52.4 |
| **EDTA** | 10 mM | 138.1 |
| **$Hg^{2+}$** | 1 mM | 21.4 |
| **$N_3^-$** | 3 mM | 100.0 |
| **p-HMB** | 20 µM | 113.1 |
| **$Zn^{2+}$** | 1 mM | 70.2 |

- Note: all assays performed at pH 7.0, using 2 mM MV as electron acceptor and using crude enzyme extract. 100% is equivalent to 3.2 µmoles MV/min/mg. All assays were performed in triplicate.

Some interesting characteristics are discovered pertaining to the hydrogenase of *Msph. stadtmanae*, for example, a significant stimulation of activity occurs in the presence of $CN^-$ and EDTA. Slight stimulation is observed by the addition of 2-bromoethanesulfonic acid (2-BES), a methanogenic inhibitor which mimics the methanogenic cofactor, CoM. Stimulation is also observed with the addition of p-hydroxymercuribenzoic acid (p-HMB), a potent inhibitor of dehydrogenase and transferase enzymes (Zollner, 1989) and the MV hydrogenase from *Methanobacterium* strain G2R (McKellar and Sprott, 1979). Inhibition occurs by the addition of the heavy metals, $Hg^{2+}$, $Cu^{2+}$, $Cd^{2+}$ and $Zn^{2+}$.

### 4.2.1.9. Kinetic Characteristics of the MV hydrogenase.

The substrates of the hydrogenase are hydrogen and the electron acceptor, in this case, methyl viologen, an artificial electron acceptor, as the physiological acceptor is not known. The kinetic values, $K_m$ and $V_{max}$ have been determined in the crude extract activity of the MV hydrogenase of *Msph. stadtmanae*. The kinetic plots for the MV

hydrogenase with respect to methyl viologen are shown in Figures 6 to 8.

The first plot is the classical Michaelis-Menten plot (Figure 6). On Figure 7, both the Lineweaver-Burk (double reciprocal, 1/v vs 1/[S]) plot (Figure 7a) and the Woolf-Augustinsson-Hofstee plots (Figure 7b) are illustrated. There are two other replots of kinetic data available, the Hanes-Woolf plot (Figure 8a) and the Eadie-Scatchard plot (Figure 8b). All of these various replots of the kinetic data provide a similar $K_m$ for MV for the hydrogenase activity (ranging from 1.7-2.4 mM MV), while the Michaelis-Menten plot (3.4 mM MV) provides a value about double that given for the replots. Since the best method for assessing the $K_m$ is the Michaelis-Menten, that value will be used from this point on in crude extract. The variations in the values between the methods is mainly due to imperfections in the data (H. Duckworth, personal communication).

## 4.3. Development of a Protocol for the Purification of the MV Hydrogenase.

There are many methods available to purify proteins and enzymes. The methods can be grouped into the following:

(1) Preparative - these methods include salting out procedures (eg. ammonium sulphate precipitation), alcohol precipitation, centrifugation (low- and high-speed forms), and concentration methods (ultrafiltration, Aquacide®).

(2) Chromatographic - use of the various adsorption, filtration methods in the form of column chromatography including ion exchange, size exclusion (gel filtration), hydrophobic interaction, affinity, and adsorption onto inorganic matrices (hydroxylapatite).

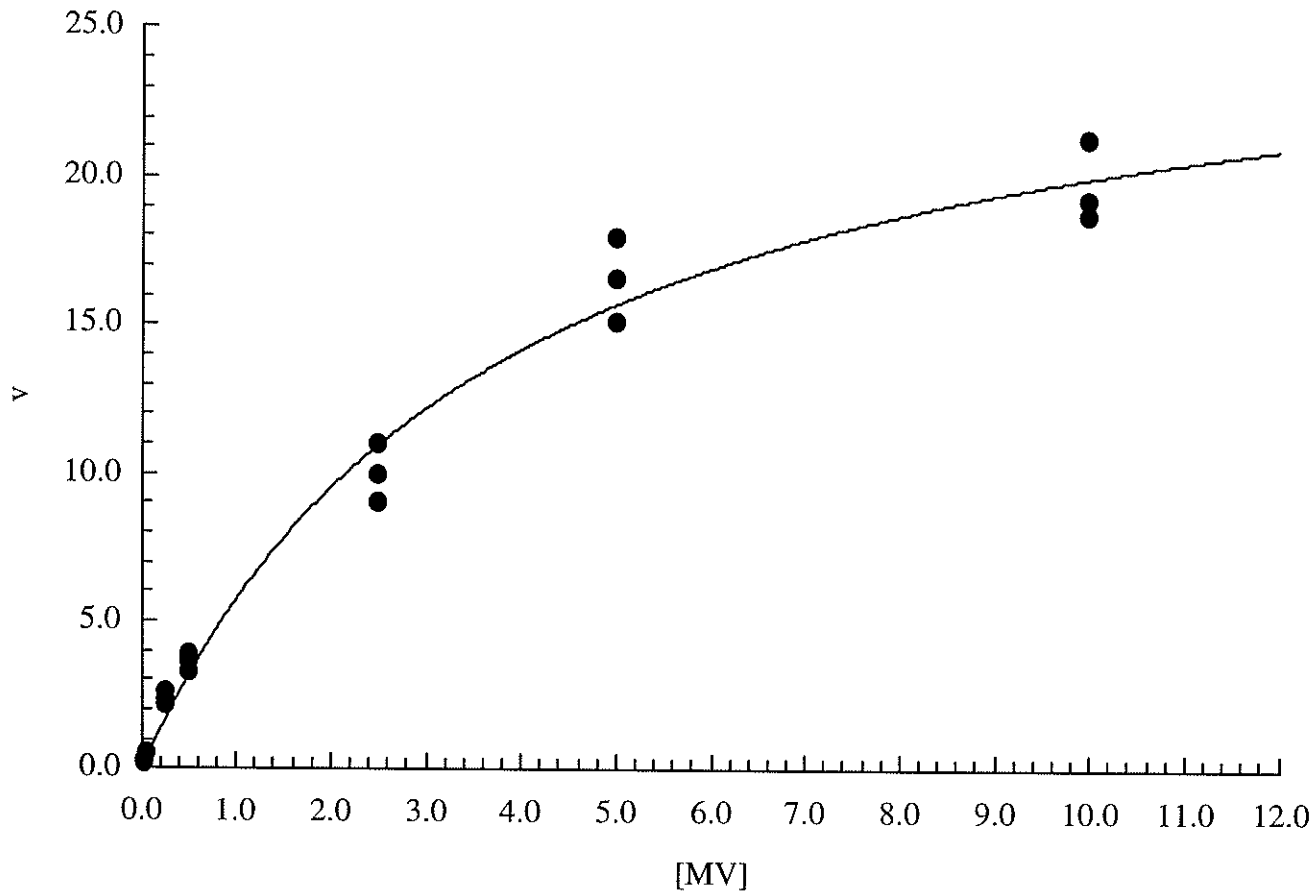
(3) Electrophoretic - typically used to gauge the purity of the preparation (either SDS-denaturing or native), can be used as a preparative method (native along with electroelution).

### 3.3.1. Preparative Methods

#### 3.3.1.1. Centrifugation and Sucrose density gradients

Starting with simple fractionation protocols involving ultracentrifugation, the MV

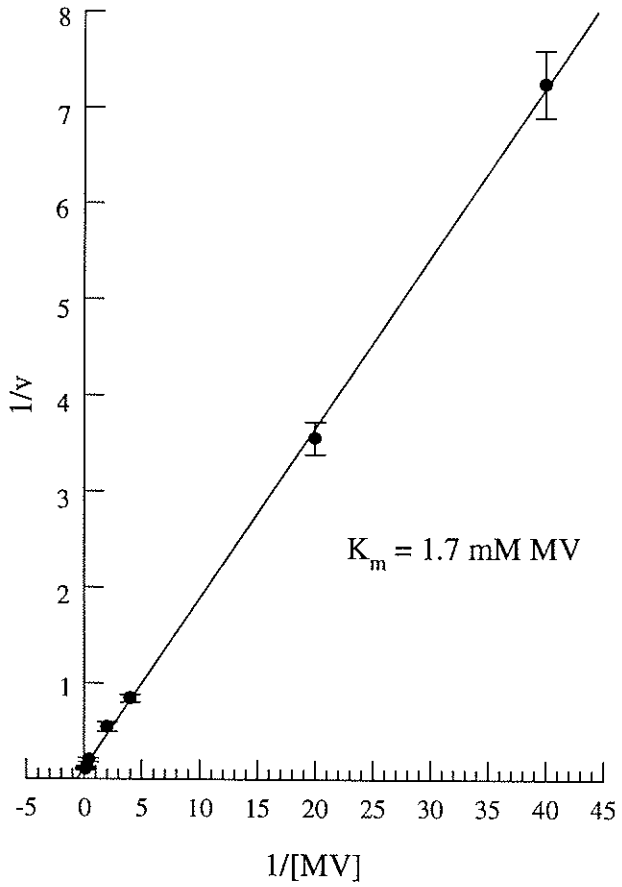
**Figure 6 -** Michaelis-Menten kinetic plot for the MV hydrogenase from *Msph. stadtmanae*. Assays were performed in triplicate. $K_m$ = 3.4 mM, $r^2$ = 0.996.




$K_m$ = 3.4 mM MV
$V_{max}$ = 26.2 µmoles MV/min/mg

**Figure 7 -** Replots of kinetic data for the MV hydrogenase from *Msph. stadtmanae*. (a) Lineweaver-Burk plot, $K_m$ = 1.67 mM, $r^2$ = 0.999. (b) Woolf-Augustinsson-Hofstee plot, $K_m$ = 1.94 mM, $r^2$ = 0.919. Assays were performed in triplicate.

a) Lineweaver-Burk Kinetic Plot




b) Woolf-Augustinsson-Hofstee Kinetic Plot

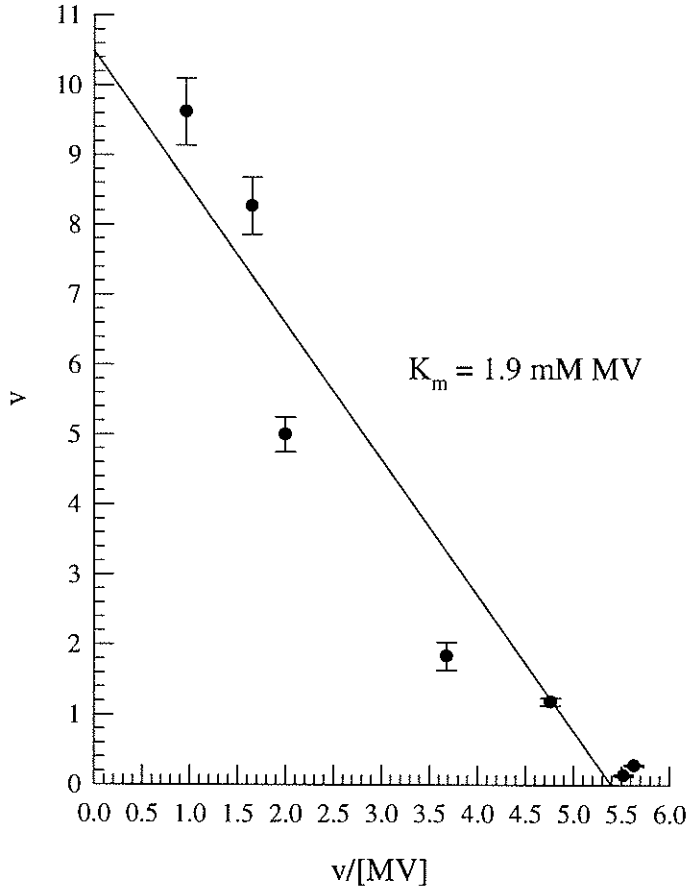
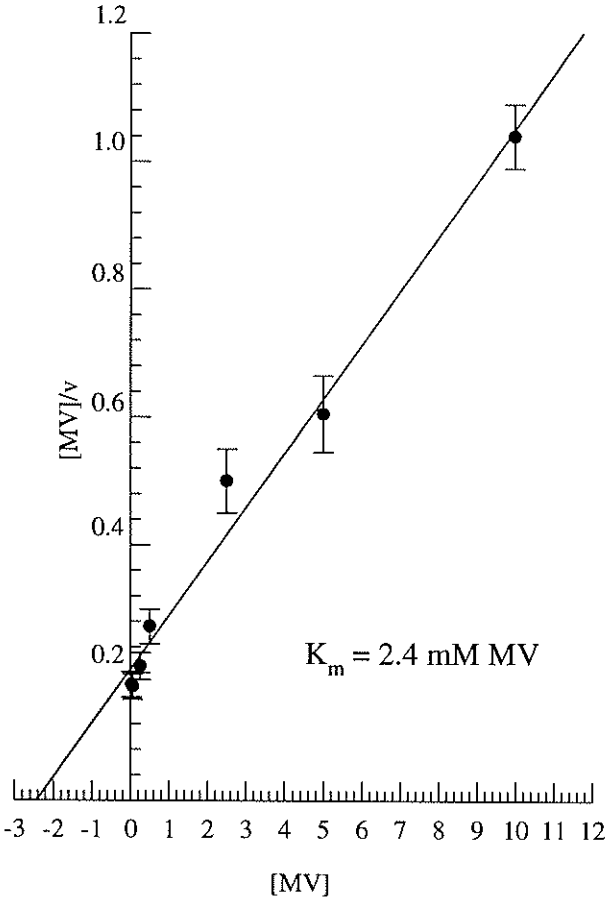
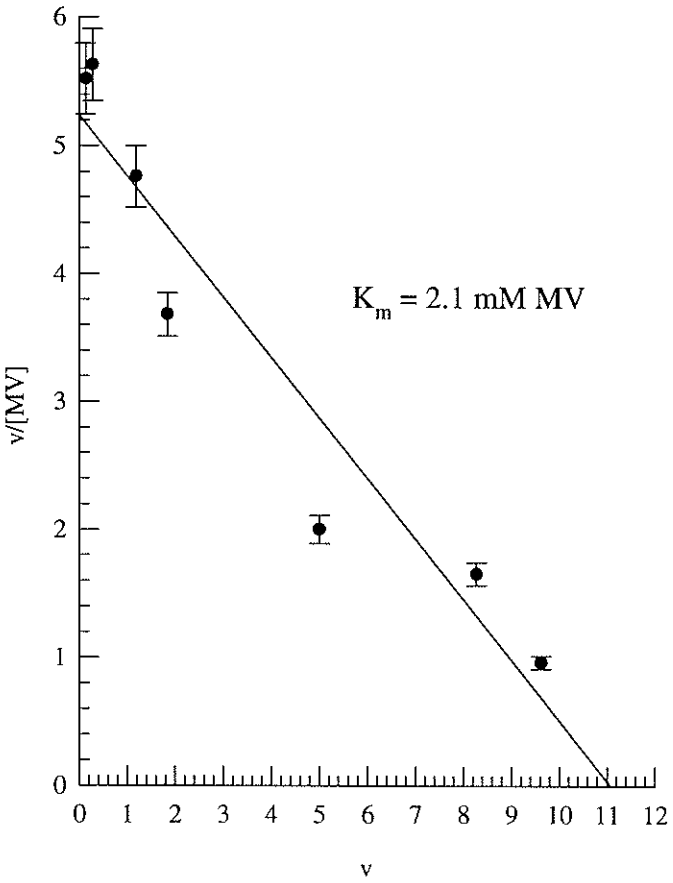


**Figure 8 -** Replots of kinetic data for the MV hydrogenase from *Msph. stadtmanae*. (a) Hanes-Woolf plot, $K_m$ = 2.38 mM, $r^2$ = 0.983. (b) Eadie-Scatchard plot, $K_m$ = 2.12 mM, $r^2$ = 0.919. Assays were performed in triplicate.

a) Hanes-Woolf Kinetic Plot




b) Eadie-Scatchard Kinetic Plot



hydrogenase activity appears to be a soluble activity, as judged by the consistent high recovery in the supernatant following ultracentrifugation of the crude extract (70 to 95%).

A linear sucrose density gradient (0-20% sucrose) was performed on cell-free extract/ high-speed supernatant (figure 9). The activity profile shows that the hydrogenase is of low density as the activity had eluted from the gradient at the upper (low sucrose concentration) end of the gradient. The location of the hydrogenase in this profile is similar to that observed for the MV hydrogenase activities detected from most of the other methanogens tested, and will be seen in Chapter 6. The gradients, however, were not calibrated with known protein molecular weight markers.

#### 4.3.1.2. Ammonium sulphate precipitation

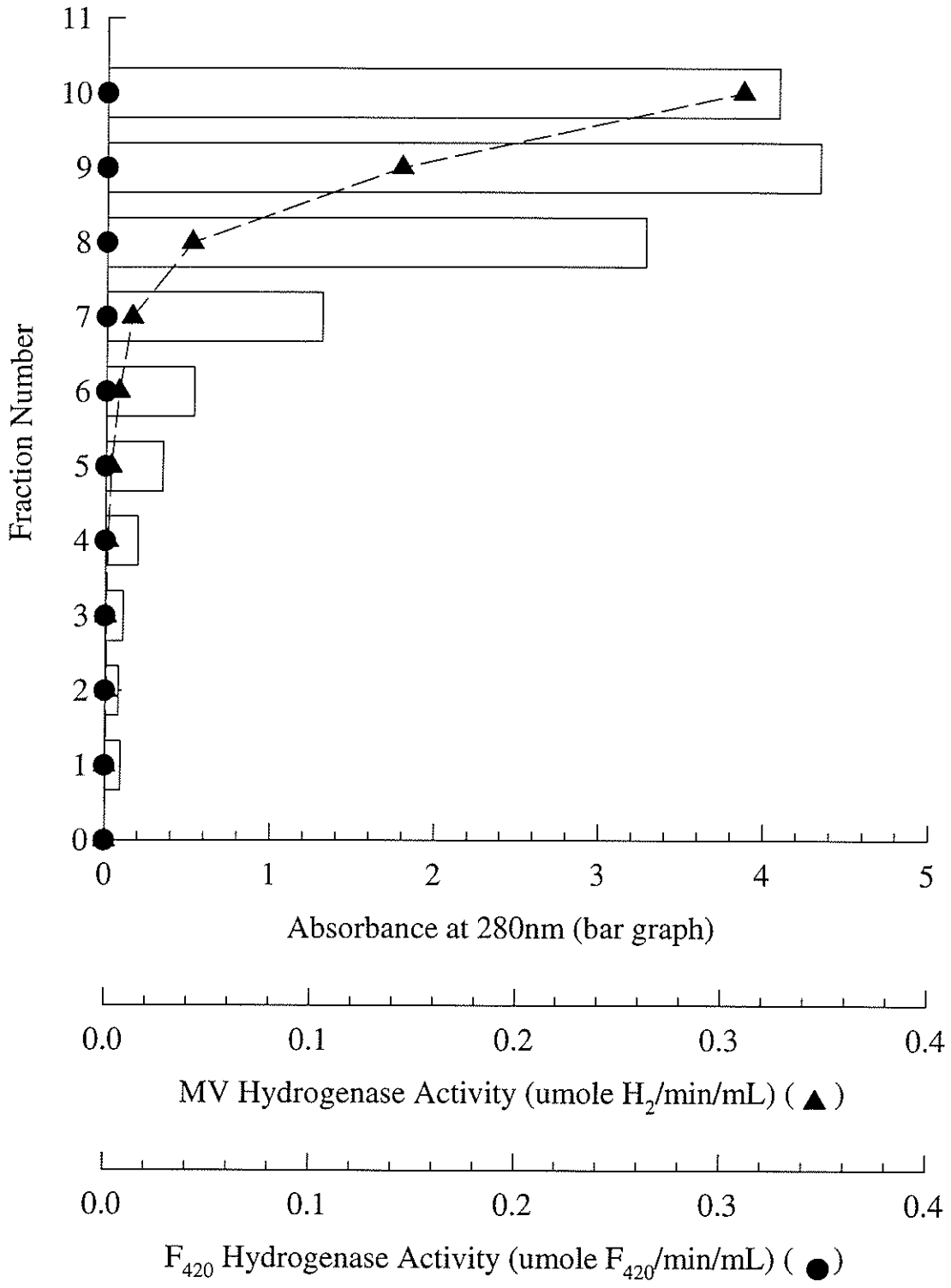
The method of salting-out proteins has been found to be very useful in the goal of purifying proteins. The most common salt used is ammonium sulphate, $(NH_4)_2SO_4$. The fractionation distribution of a particular protein along a profile from 0-100%$(NH_4)_2SO_4$ can be useful as a step in the purification of a protein, as well, it provides evidence as to its hydrophobic character, since hydrophobic proteins will precipitate in the presence of $(NH_4)_2SO_4$ at a lower concentration than a less or non-hydrophobic protein.

A simple ammonium sulphate fractionation profile for the MV hydrogenase from crude extract is shown on Table 4.4. Most of the hydrogenase was precipitated between 35 and 65%, and some remained in solution at saturation. This suggested that there is some hydrophobic character or as association with material that is hydrophobic in nature.

**Table 4.4 - $(NH_4)_2SO_4$ Fractionation of MV Hydrogenase Activity.**

| $(NH_4)_2SO_4$ concentration | Activity (µmoles MV/min) | Specific Activity (µmoles MV/min/mg) | Recovery (%) |
|---|---|---|---|
| **0-30% pellet** | 243.8 | 4.90 | 23.5 |
| **30-65% pellet** | 754.5 | 3.69 | 72.7 |
| **65% supernatant** | 39.7 | 0.05 | 3.8 |

**Figure 9 -** Linear sucrose density gradient profile for the MV hydrogenase in crude extract from *Msph. stadtmanae*. Assays were performed in triplicate.


Fraction Number
11
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
Absorbance at 280nm (bar graph)
0.0
0.1
0.2
0.3
0.4
MV Hydrogenase Activity (umole $H_2$/min/mL) ( ▲ )
0.0
0.1
0.2
0.3
0.4
$F_{420}$ Hydrogenase Activity (umole $F_{420}$/min/mL) ( ● )

### 4.3.1.3. Protein Concentration Methods.

Most chromatographic methods result in the dilution of the protein of interest, in particular, those methods that employ linear gradients in order to develop the column. There are several methods that are available to attempt to concentrate the protein/enzyme for use in the next step in the protocol. Most commonly used are the diafiltration or ultrafiltration membranes that use gas pressure to force liquid through a membrane that can keep molecules of a specific size (assuming all are globular proteins) from passing through. Membranes available have molecular weight cut-off (MWCO) limits of 30,000 MW, 10,000 MW, and 1,000 MW.

Early work involving these membranes did not involve checking whether the hydrogenase went through the membrane, so the recoveries of activity in subsequent steps were affected, mainly by the passage of the activity through these filters. Obviously one should not assume that the hydrogenase, even if a size estimate was determined via other means (ie., size exclusion chromatography), will be retained on such a membrane.

An example of the flow of partially purified hydrogenase activity through even a membrane with a MWCO of 10,000 and with a MWCO of 30,000 (in the presence of 90% saturation of ammonium sulphate) is shown in Table 4.5.

**Table 4.5 - Retentivity of MV Hydrogenase activity on ultrafiltration membrane.**

| Fraction | Type | Activity (U) | Recovery (%) |
|---|---|---|---|
| **Retentate (Concentrate)** | YM10 | 186.8 | 52.5 |
| **Filtrate** | YM10 | 169.0 | 47.5 |
| **Retentate (+ Amm. $SO_4$)** | YM30 | 142.7 | 72.8 |
| **Filtrate (+ Amm. $SO_4$)** | YM30 | 53.2 | 27.2 |

This shows that the physical properties of the hydrogenase appear to not allow the use of this method for the concentration of the hydrogenase activity, however, it introduces the possibility of using this method for the filtration of the activity in order to remove other proteins from the mixture that would be held back from passing through the

membrane (ie., a possible purification step).

### 4.3.2. Chromatographic Properties of the MV Hydrogenase.

Column chromatography is an effective way to purify proteins from the milieu of biomolecules from the extracts of microorganisms or the cellular components from the organs of higher organisms. There are a variety of chromatographic materials from which to choose to attempt the purification of the chosen enzyme, including the commonly used ion-exchange (separation based on charge), hydrophobic interaction (separation based on hydrophobicity), gel permeation/gel filtration/size-exclusion (separation based on size) and numerous affinity resins that are available.

#### 4.3.2.1. Ion-exchange chromatography.

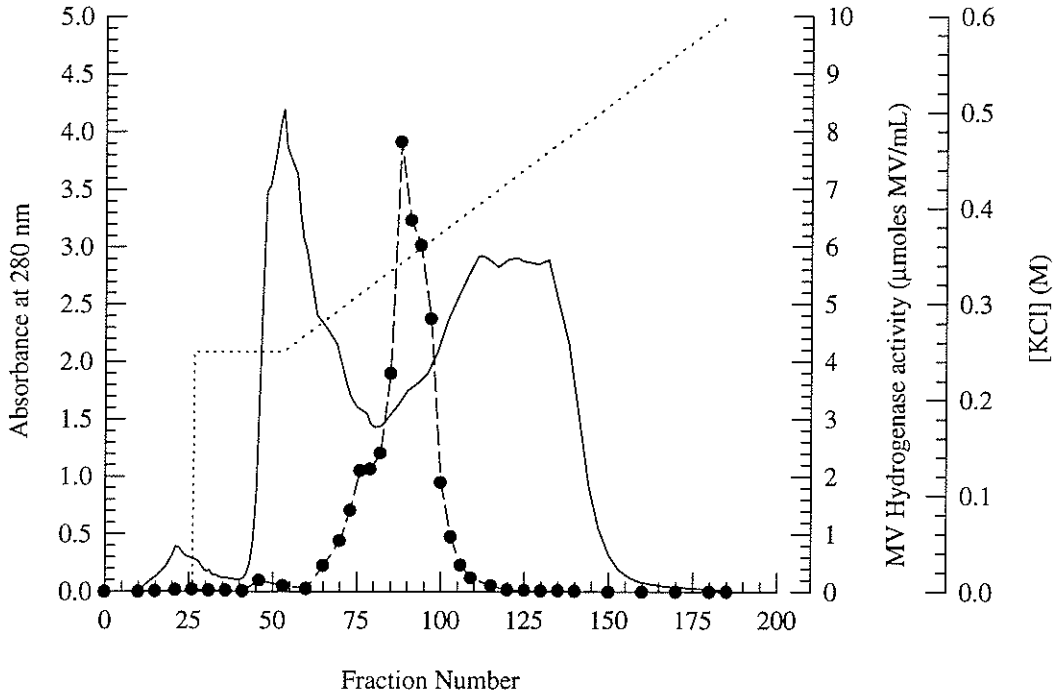
Ion-exchange chromatography is a method that separates molecules based on their differential mobilities on a fixed charged (either anionic or cationic) resin and the mobile phase containing a fixed or continuously changing concentration of ions (salts). At all pHs, a protein will be charged, either positively (cation) or negatively (anion), depending on their pI, the point at which the protein has a net zero charge.

The first thing to determine in the analysis of a protein chromatographically on ion-exchange is the nature of the charge at which the protein is most stable (likely near the internal physiological pH of the source organism, around pH 7.0-8.0).

Diethylaminoethyl (DEAE)-based resins are weak anionic exchangers. This method was found to be the most useful and reliable method in the purification attempt. A typical elution profile at pH 8.0 from ion-exchange is shown in figure 10a. Another elution profile performed at pH 5.5 is shown in figure 10b. As can be seen from the differences in the two elution profiles, a significantly cleaner profile is obtained at pH 5.5, with less protein (or 280 nm-absorbing material) binding to the column used. There were consistently high recoveries and high purification factors achieved using this type of resin (see Table 4.6). Also of note, it required large amounts of wash buffer in order to reach a low absorbance for the eluate and some hydrogenase activity was found to slowly

**Figure 10 -** Ion exchange elution profiles for the MV hydrogenase from *Msph. stadtmanae* from DEAE Cellufine. Column dimensions: 2.5 × 15.0 cm. (a) 50 mM Tris-Cl, pH 8.0. (b) 50 mM Piperazine, pH 5.5. Flow rate: 0.75 mL/min. (●) - MV hydrogenase activity.

a) 50 mM Tris-Cl, pH 8.0




b) 50 mM Piperazine-Cl, pH 5.5

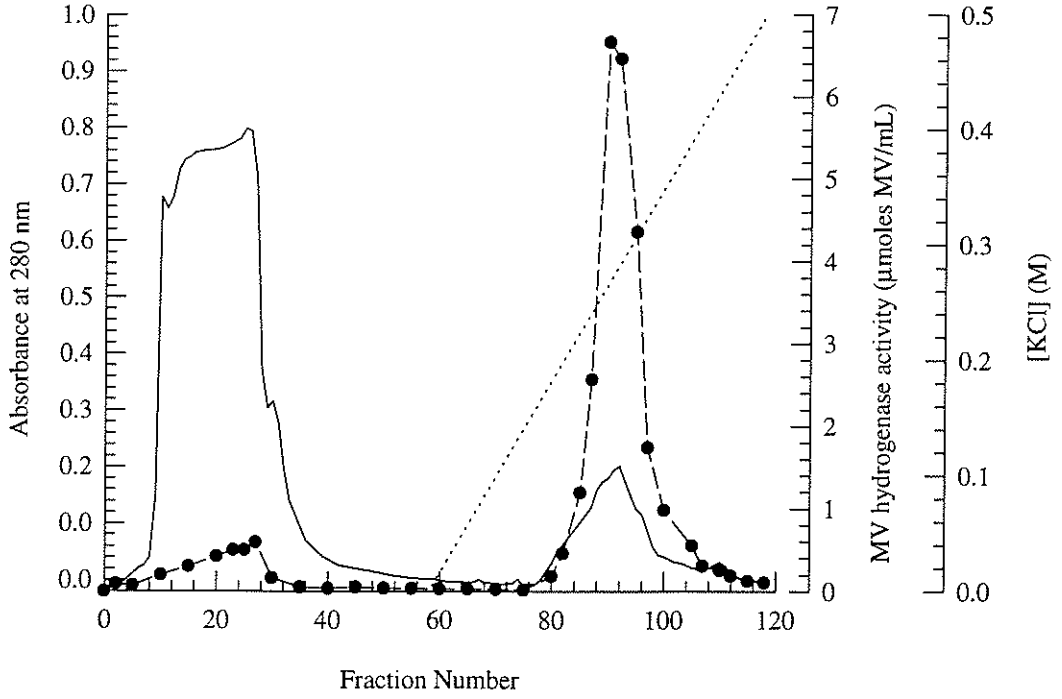


**Table 4.6 - Recoveries and Specific activities of MV Hydrogenase off IE (figure 10).**

| Resin/Stage | pH | Specific Activity (μmoles MV/min/mg) | Purifcation (-fold) | Recovery (%) |
|---|---|---|---|---|
| **Crude Extract** | 8.0 | 6.56 | - | 100 |
| **DEAE Sephacel** | 8.0 | 75.88 | 12.6 | 81 |
| **Crude Extract** | 5.5 | 2.32 | - | 100 |
| **DEAE Cellufine** | 5.5 | 54.43 | 64.1 | 56 |

elute during the extended wash phase of the column at this pH, prior to the application of the gradient.

### 4.3.2.2. Hydrophobic interaction chromatography.

The method of HIC has been employed in the purification of several hydrogenases previously (Deppenmeier et al, 1992; Jacobson et al, 1982). Phenyl-Sepharose CL-4B was used to attempt to resolve the hydrogenase from the other proteins in the cell extract. A typical elution profile is shown on Figure 11.

Generally, there is some hydrogenase that consistently washes through during the loading phase, while there appears to be two peaks of hydrogenase activity that elute from the column, one when the salt (which encourages hydrophobic interaction) is removed (via linear or step gradient), and another which elutes when the column is developed with the addition of a detergent (eg. CHAPS). However, the activities and recoveries of the hydrogenase during most of these trials were very low (Table 4.7). It is not proven, however, that these two activity peaks are indicative of (at least) two different hydrogenase species in *Msph. stadtmanae* (more on this later). Note that the buffer changes are using step gradients, so many other proteins are eluted at the same time.

**Figure 11 -** Hydrophobic interaction elution profile for the MV hydrogenase from *Msph. stadtmanae* from Phenyl-Sepharose CL-4B. The material applied was a crude extract sample. Column dimensions: 2.5 × 15.0 cm. Flow rate: 0.80 mL/min. Buffer: 50 mM Tris-Cl, pH 8.0. (●) - MV hydrogenase activity.

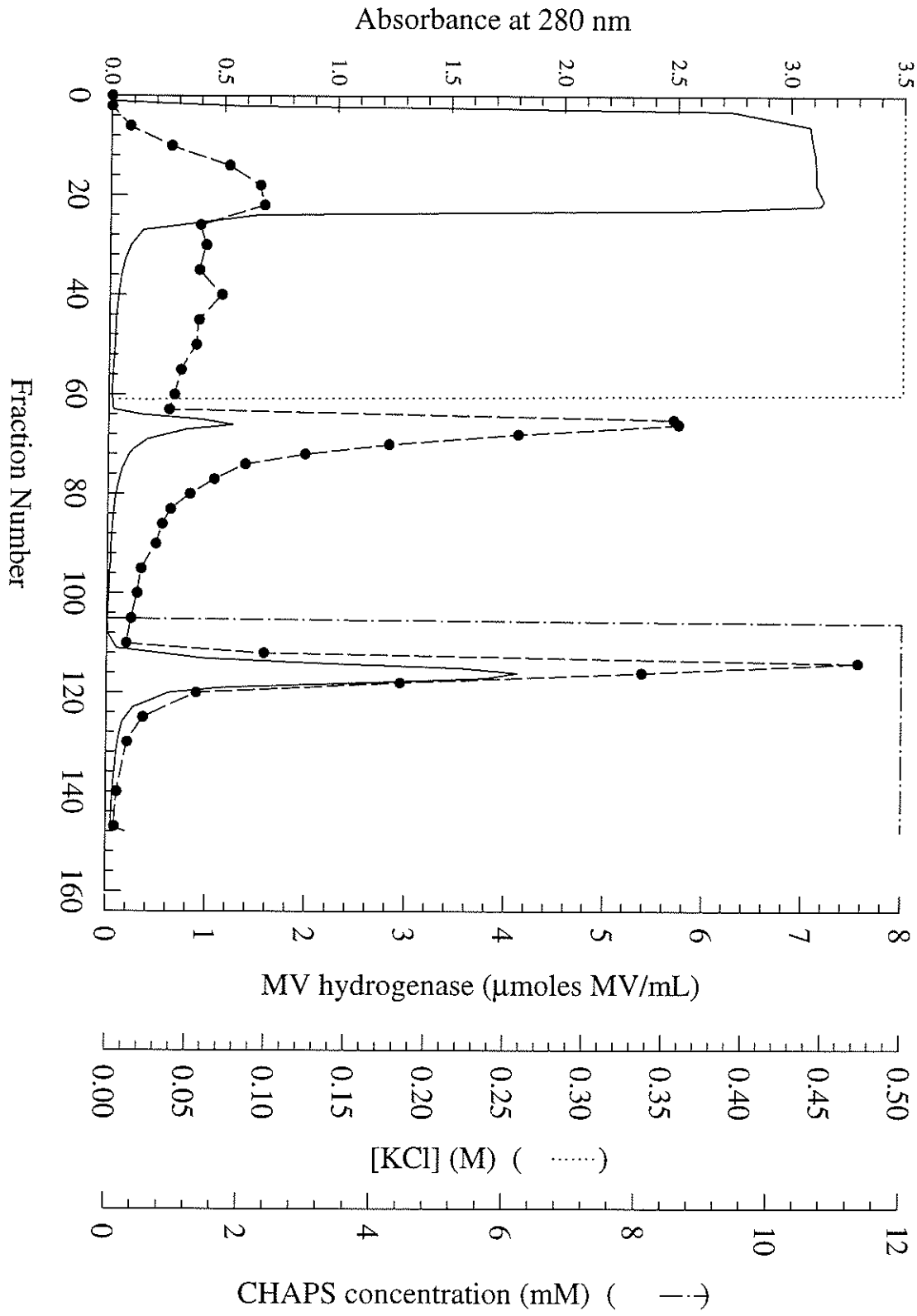

Absorbance at 280 nm
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Fraction Number
0
20
40
60
80
100
120
140
160
MV hydrogenase (µmoles MV/mL)
0
1
2
3
4
5
6
7
8
[KCl] (M) ( ····· )
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
CHAPS concentration (mM) ( —·— )
0
2
4
6
8
10
12

**Table 4.7 - Recoveries and Specific activities of MV Hydrogenase off HI.**

| Stage | pH | Specific Activity (μmoles MV/min/mg) | Purifcation (-fold) | Recovery (%) |
|---|---|---|---|---|
| **Crude Extract** | 8.0 | 7.42 | - | 100 |
| **Phenyl-Sepharose CL-4B (first peak)** | 8.0 | 15.59 | 2.1 | 13.2 |
| **Phenyl-Sepharose CL-4B (second peak)** | 8.0 | 2.33 | 0.3 | 8.4 |

### 4.3.2.3. Hydroxylapatite adsorption chromatography.

This method of binding to an inorganic packing material has been used on several occasions in the purification of hydrogenases from other bacteria, including methanogens (Deppenmeier et al, 1992). Binding of acidic proteins is promoted in the presence of salt, generally in the form of NaCl, and binding is promoted in general at a pH near neutrality. The testing of several pHs, the elution profile did not differ for the hydrogenase or the other proteins in the crude extract (Figure 12a, 12b and 12c).

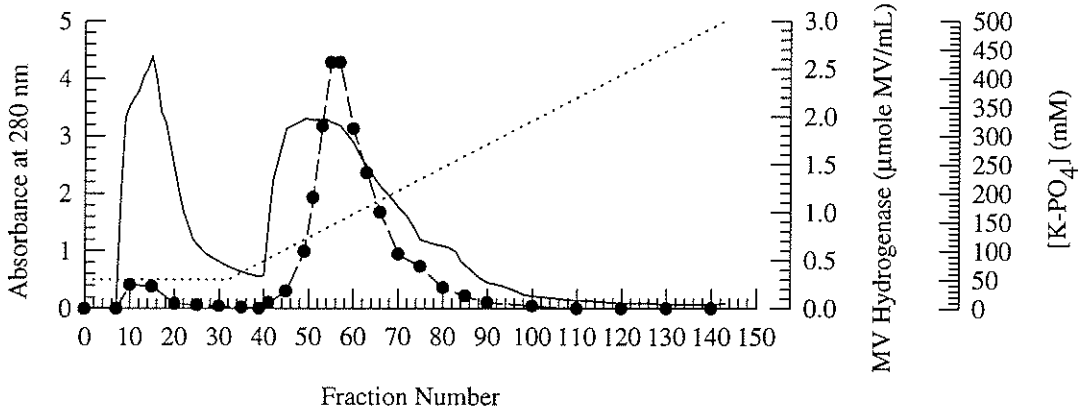
In general, the method of hydroxylapatite binding yields good recovery and decent purification. The alteration of pH does lead to a improved recovery at pHs 7.0 and 7.5 and a slight enhancement of purification as the pH is increased (Table 4.8). The problem, however, is the resolution that the method provides. Based on all of the chromatograms, most of the protein that does bind to hydroxylapatite also elutes at or near where the hydrogenase activity elutes.

### 4.3.2.4. Chromatofocusing.

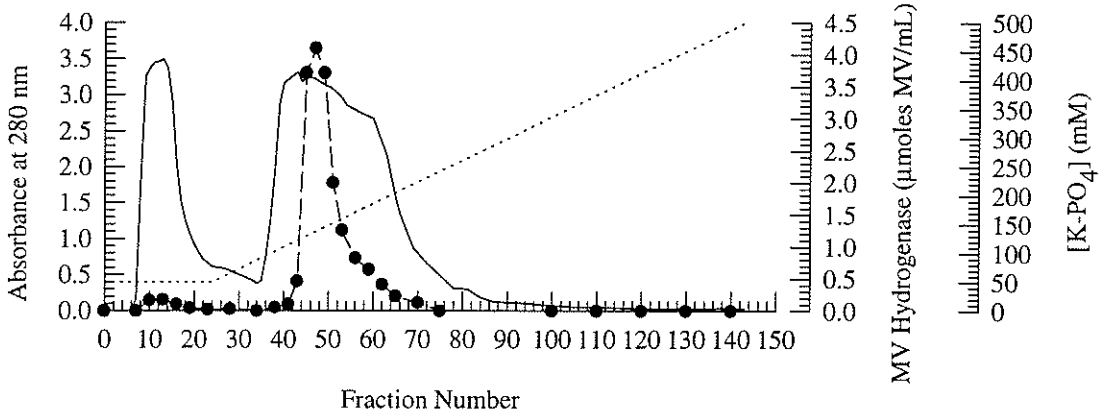
The method of chromatofocusing uses the specific isoelectric point of the protein of interest in order to achieve separation of the enzyme from the other proteins in mixture. Theoretically, because each protein has a different amino acid sequence, each should have a distinct isoelectric point (pI), therefore there should be no protein with the identical pI to the one being purified.

**Figure 12 -** Hydroxylapatite adsorption elution profile at various pH values for the MV hydrogenase from *Msph. stadtmanae* from Hydroxylapatite HT. (a) pH 6.5; (b) pH 7.0; (c) pH 7.5. Column dimensions: 2.5 × 6.0 cm. Flow rate: 0.90 mL/min. Buffer: 50 mM K-$PO_4$, 0.2 M NaCl. (●) - MV hydrogenase activity.

a) pH 6.5




b) pH 7.0




c) pH 7.5

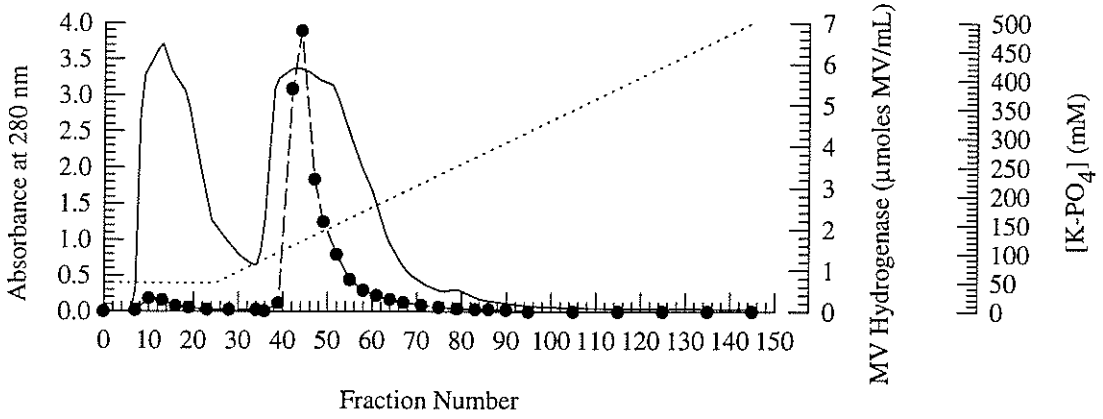


**Table 4.8 - Recoveries and Specific activities of MV Hydrogenase off HA.**

| Stage | pH | Specific Activity (µmoles MV/min/mg) | Purifcation (-fold) | Recovery (%) |
|---|---|---|---|---|
| **Crude Extract** | 6.5 | 6.35 | - | 100 |
| **Hydroxylapatite Pool** | 6.5 | 5.65 | 0.9 | 43.5 |
| **Crude Extract** | 7.0 | 4.75 | - | 100 |
| **Hydroxylapatite Pool** | 7.0 | 7.29 | 1.5 | 67.1 |
| **Crude Extract** | 7.5 | 5.00 | - | 100 |
| **Hydroxylapatite Pool** | 7.5 | 8.77 | 1.8 | 93.4 |

Typically, one performs this procedure with material that has already been clarified to a certain degree prior to its' use. For the following examples, ion exchange and hydroxylapatite chromatography has already been performed.

The results for the elution of the MV hydrogenase activity from Polybuffer Exchanger 94 (a chromatofocusing resin from Pharmacia) revealed an extremely low pH was required to elute the enzyme from the resin (Figure 13) and indeed the application of a pH 2.5 glycine buffer was required in order to remove the enzyme from the column. Note that for the trials involving this method the material that was applied was already partially purified via ion exchange and hydroxylapatite chromatography (see more on this form of chromatography later). The results from chromatofocusing suggested that the protein that represents the hydrogenase activity was likely very acidic, with a pI of less than 4.0. This is supported by the
chromatography of the hydrogenase on DEAE Cellufine at pH 5.5, in which hydrogenase activity was found to still bind and require almost the same concentration of salt (KCl) to elute (see figure 10b). The recovery of the activity from this column was very low and the specific activity had actually decreased from the starting material (see Table 4.9), so this method is not a likely step to be employed in the purification of the MV hydrogenase from *Msph. stadtmanae*. In addition, the step down to pH 2.5 eluted other proteins that were still bound to the column as well.

**Figure 13 -** Chromatofocusing elution profile for the MV hydrogenase from *Msph. stadtmanae* from Polybuffer Exchanger 94. Column dimensions: 1.5 × 6.0 cm. Flow rate: 0.9 mL/min. Buffer: Polybuffer pH range 7 to 4, followed by 25 mM Glycine, pH 2.5. (●) - MV hydrogenase activity.

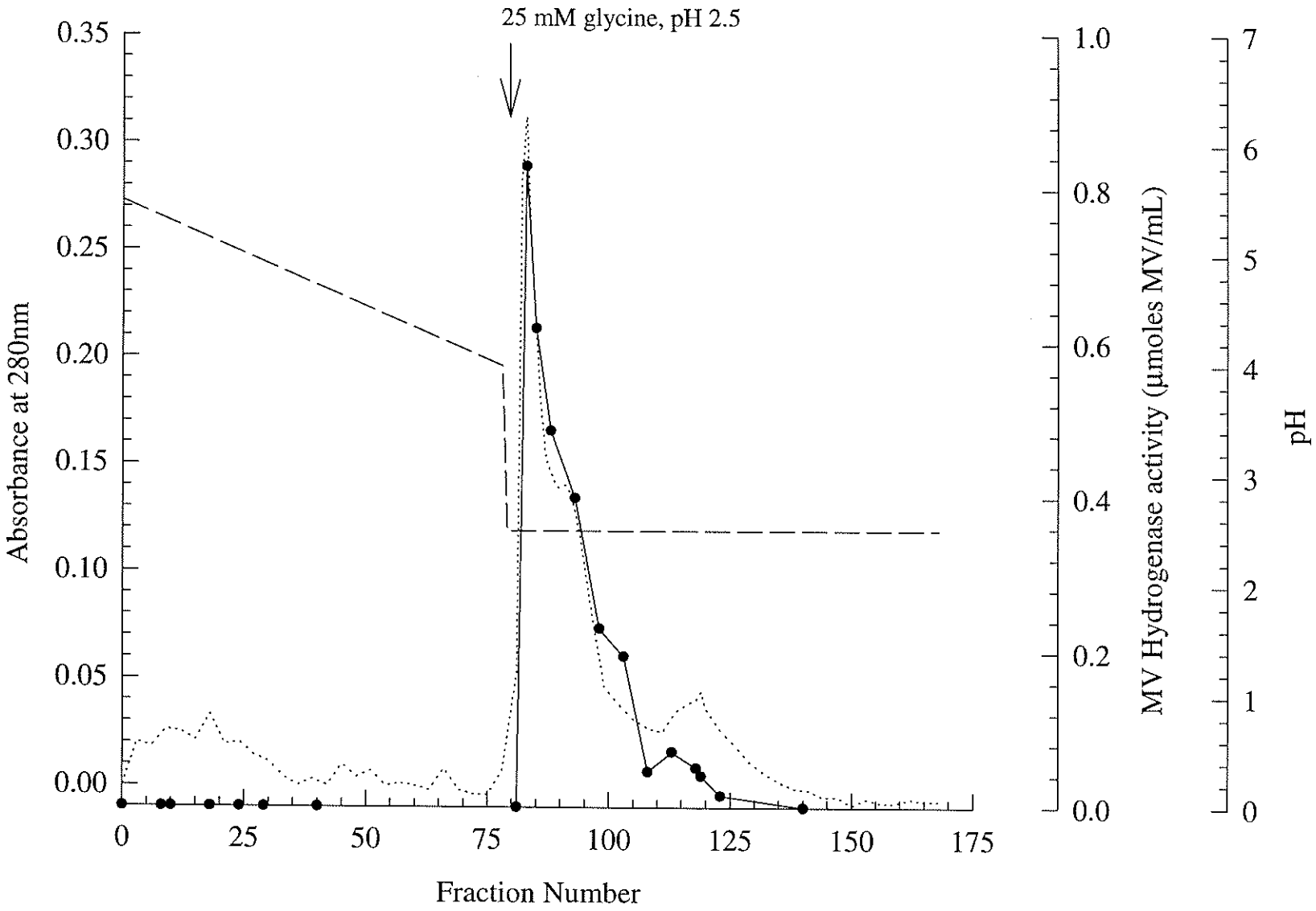

25 mM glycine, pH 2.5
Absorbance at 280nm
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
25
50
75
100
125
150
175
Fraction Number
1.0
0.8
0.6
0.4
0.2
0.0
MV Hydrogenase activity (µmoles MV/mL)
7
6
5
4
3
2
1
0
pH

**Table 4.9 - Summary of Purification Attempt Involving Chromatofocusing**

| Stage | Specific Activity (μmoles MV/min/mg) | Purification (-fold) | Recovery (%) |
|---|---|---|---|
| **Crude Extract** | 5.8 | - | 100 |
| **Ion Exchange, pH 8.0** | 145.5 | 25.1 | 168 |
| **Hydroxylapatite HT** | 59.9 | 10.3 | 84 |
| **Polybuffer Exchanger** | 24.5 | 4.2 | 1.6 |

### 4.3.2.5. Metal chelate affinity chromatography (MCAC).

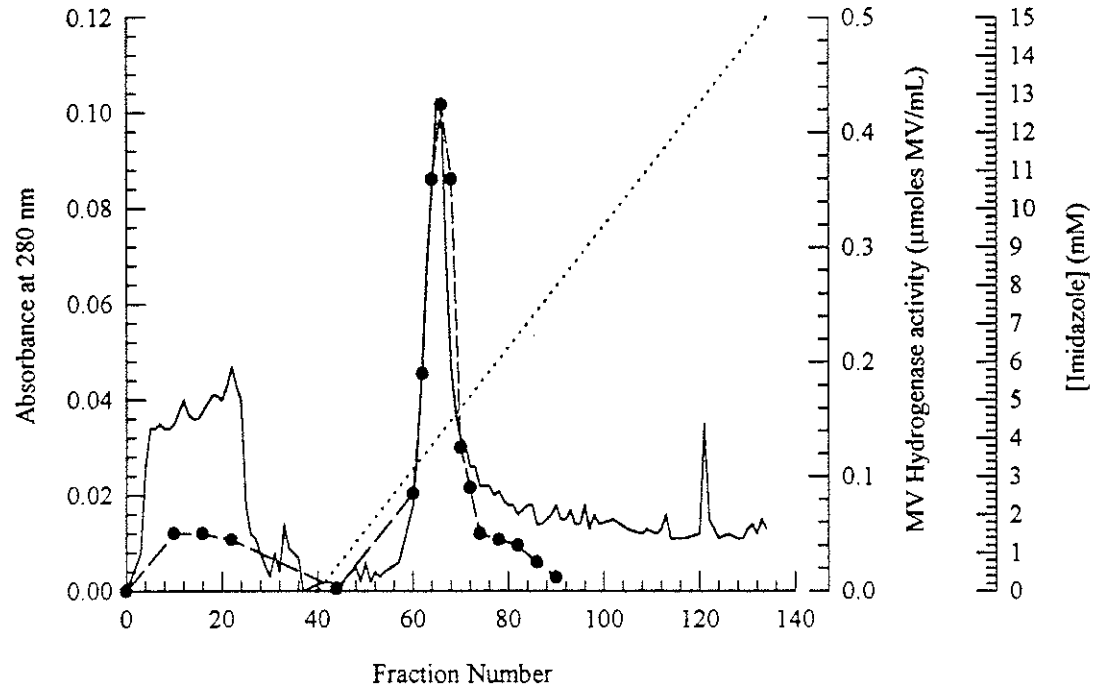
The use of this exotic form of affinity chromatography has been rather limited since its discovery (Porath and Olin, 1983). This type of affinity matrix takes advantage of the affinity of the protein of interest for binding to metal ions that are ionically bound to the stationary phase. The choice of metal ion is left up to the experimenter and the particular protein (which ever works best). This form of chromatography has been used successfully on one reported occasion for the purification of a hydrogenase, the $F_{420}$ hydrogenase from *Msp. hungatei* (Choquet and Sprott, 1990). The mechanism of binding has been proposed to be a combination of both ionic and hydrophobic interactions (Porath, 1992).

For the experimental trials on the MV hydrogenase activity from *Msph. stadtmanae*, the two metals primarily used were nickel ($Ni^{2+}$) and zinc ($Zn^{2+}$). Other metals were tried, including cobalt ($Co^{2+}$) and copper ($Cu^{2+}$), but the MV hydrogenase had little or no affinity for either of them.

The elution profiles from a $Ni^{2+}$ charged column (Figure 14a) and a $Zn^{2+}$ charged column (Figure 14b) are shown. Of the two, $Zn^{2+}$ displays the lowest binding ability for the hydrogenase activity, with most passing through the wash (or this could be another indication that there may be two or more groups of hydrogenase activity in *Msph. stadtmanae*). The level of purification is not very impressive (Table 4.10) for either of these columns, and in fact, the actual specific activity was found to decrease in cases where this was a second or third column running in a series.

**Figure 14 -** Metal chelate affinity elution profile for the MV hydrogenase from *Msph. stadtmanae* from: (a) $Ni^{2+}$, and (b) $Zn^{2+}$-charged MCAC columns. Column dimensions: 1.5 × 10.0 cm. Flow rate: 0.90 mL/min. Buffer: 50 mM Tris-Cl, 0.2 M NaCl. (●) - MV hydrogenase activity.

a) $Ni^{2+}$-charged




b) $Zn^{2+}$-charged

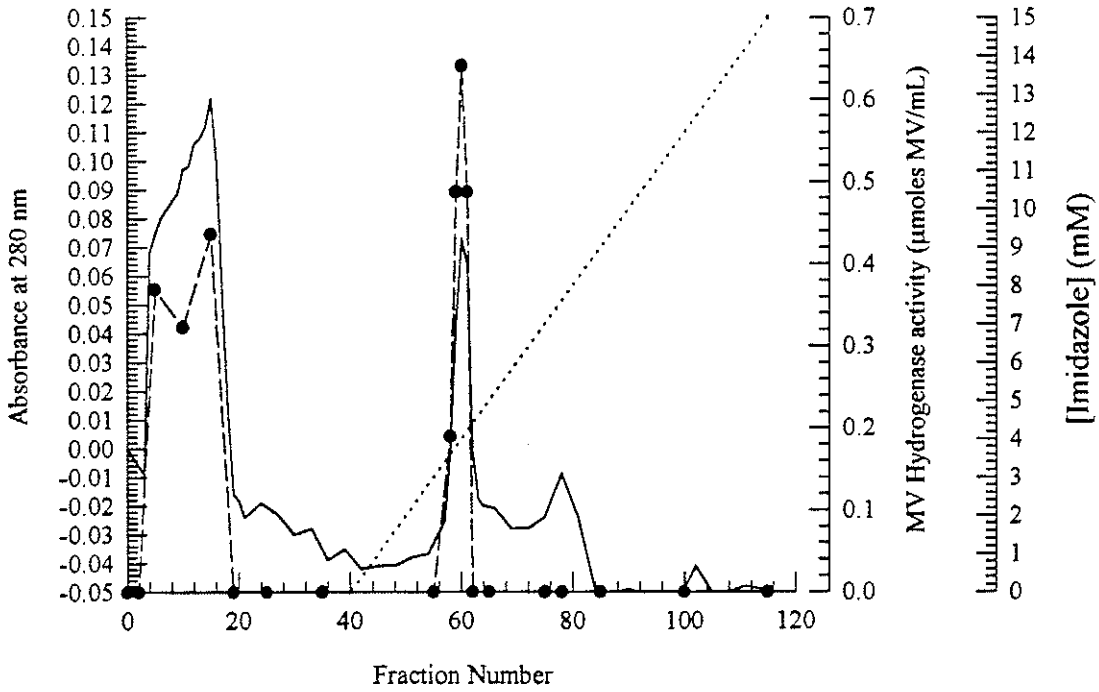


**Table 4.10 - Specific activities and recoveries of MV hydrogenase from MCAC.**

| Stage | Metal Chelate | Specific Activity (μmoles MV/min/mg) | Purification (-fold) | Recovery (%) |
|---|---|---|---|---|
| **Crude Extract** | - | 3.10 | - | 100 |
| **MCAC** | $Ni^{2+}$ | 5.09 | 1.6 | 14.3 |
| **Crude Extract** | - | 3.11 | - | 100 |
| **MCAC** | $Zn^{2+}$ | 3.38 | 1.5 | 8.6 |

### 4.3.2.6. Size-exclusion chromatography

This form of chromatography is useful, mainly for the estimation of the native molecular weight of the protein of interest. This can, however, be useful as a step in the purification of a protein, depending on the molecular weights of the other proteins in the mixture.

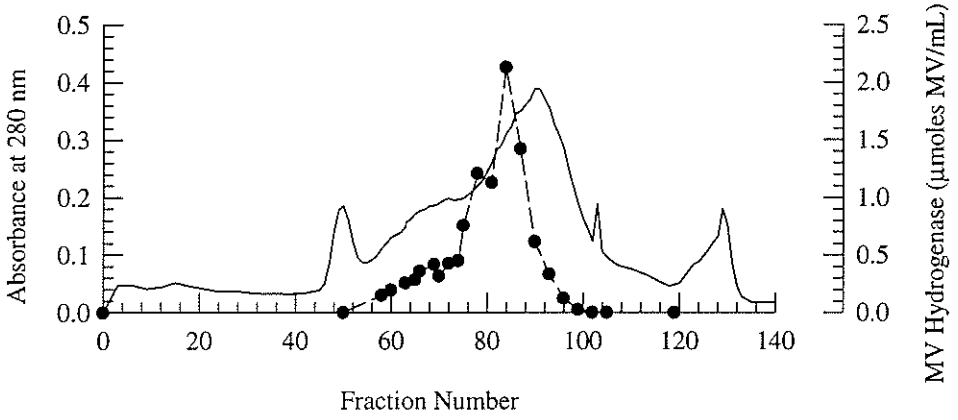
There were several resins available for this type of chromatography with different size-exclusion ranges. A large range agarose-based resin, Sepharose 6B, with a fractionation range of 10,000-4,000,000 MW. A chromatogram for the MV hydrogenase from *Msph. stadtmanae* is shown in figure 15a. The estimation for the molecular size of the hydrogenase is approximately 177,000 MW using this resin, though a split peak for hydrogenase activity is displayed.

A second type of resin with a narrower range, Sephacryl S-200HR, an acrylamide-based resin with a fractionation range of 10,000-200,000 MW was employed to see if a greater resolution of the MV hydrogenase could be obtained. A chromatogram for the MV hydrogenase activity is shown in figure 15b. This shows that the activity elutes at or near the void volume for this column, so another resin with a greater range was tested.

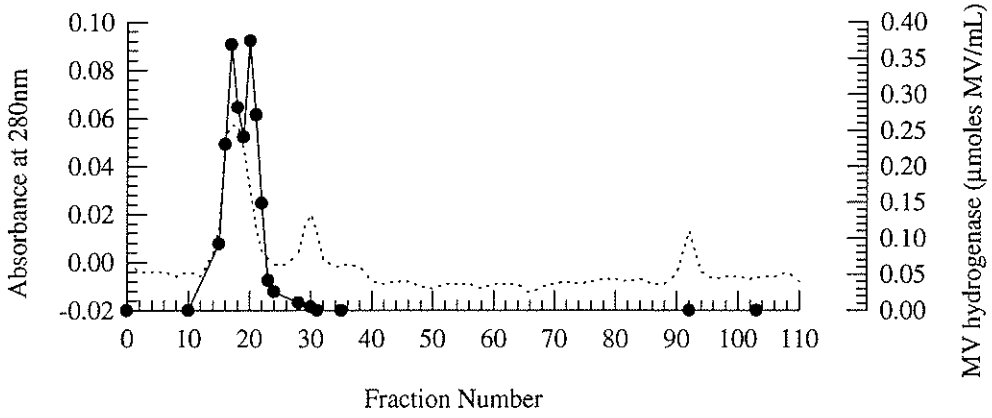
The third resin, Sephadex G-200, with a fractionation range of 5,000-600,000 MW, was tested and a chromatogram is shown in figure 15c. This resin was not calibrated so a size based on this column is not available, but the activity profile indicated that there were at least two peaks, one that is reasonably large and a second smaller peak.

**Figure 15** - Size-exclusion elution profile from various resin types for the MV hydrogenase from *Msph. stadtmanae*. (a) Sepharose 6B, column dimensions: 2.5 × 90.0 cm. Flow rate: 0.40 mL/min. (b) Sephacryl S-200 HR, column dimensions: 1.6 × 90.0 cm. (c) Sephadex G-200, 2.5 × 90.0 cm. Buffer: 50 mM Tris-Cl, 0.2 M NaCl, pH 8.0. (●) - MV hydrogenase activity.

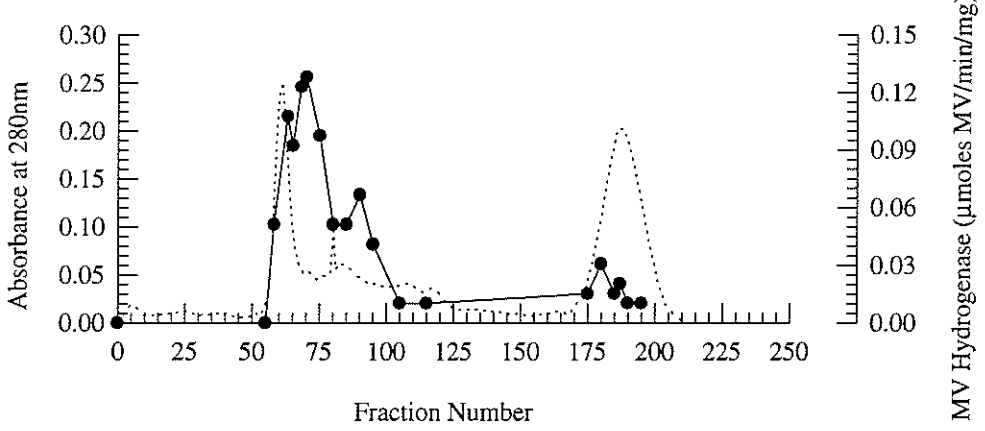
a) Sepharose 6B




b) Sephacryl S-200HR




c) Sephadex G-200



### 4.3.3. Electrophoretic methods - Native gradient PAGE.

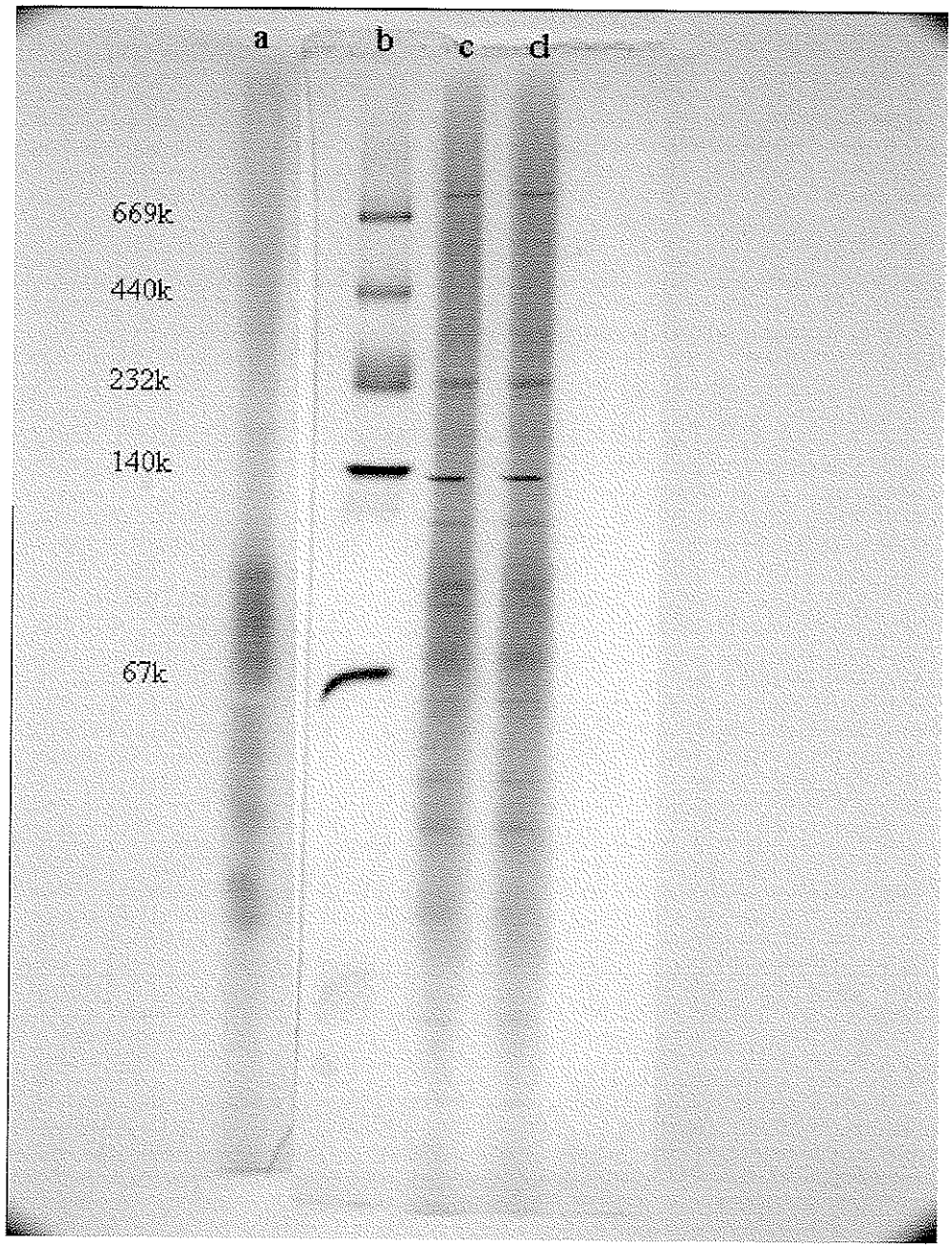
Native polyacrylamide gel electrophoresis is a useful method that can be used to determine the purity and the molecular size of a particular protein. There are two ways of performing PAGE under non-denaturing conditions: (1) Using a series of single percentage acrylamide gels, or (2) using a gradient gel. The advantage of the gel series is that one will not elute any protein off the gel if it is smaller than the exclusion limit of the gradient gel, but requires one to run 4 or 5 different gels in order to estimate the size of the unknown protein, plus there is a requirement for a fairly highly concentrated sample of the protein being analyzed. The advantage of the gradient gel is that one can perform just a single experiment and the bands of protein obtained are very narrow, ie., of high resolution.

Native PAGE is also useful since the protein is not denatured. Therefore, one can assay for the activity of the enzyme/protein in the gel itself and determine precisely where the enzyme is on the gel, if a method of activity staining is available. For the hydrogenase, fortunately, there is a method available. All that is required is the addition of a chemical, 2,3,5-triphenyltetrazolium chloride (TPTC) to the standard assay solution. The hydrogenase will reduce the methyl viologen in the assay buffer in the presence of $H_2$, and the MV will reduce the TPTC producing an insoluble red precipitate (Fiebig and Freidrich, 1989).

Native gradient PAGE was performed on the cell extract of *Msph. stadtmanae* and along with the activity staining method just described, several regions within the sample lane displayed MV reducing activity (Figure 16). This lends evidence for the presence of more than one hydrogenase entity in the extracts of *Msph. stadtmanae*, but does not state that these differentially migrating forms of the MV-reducing regions are different hydrogenase activities. They could just be different aggregate forms of the hydrogenase activity found in the cell. Only the purification of two or more different hydrogenases will support the presence of multiple hydrogenase activities that are structurally distinct.

Another observation with respect to the activity and protein staining, the areas that do stain for hydrogenase activity do not stain very strongly for protein.

**Figure 16 -** Native gradient PAGE of crude extract from *Msph. stadtmanae* activity stained for MV hydrogenase. Activity staining performed in an anaerobic chamber under approximately 4% $H_2$. Lanes: (a) activity stain only; (b) MW markers; (c) and (d) activity and protein stained extract (both same). Molecular weight markers were: bovine serum albumin, 67,000; lactate dehydrogenase, 140,000; catalase, 232,000; ferritin, 440,000; and thyroglobulin, 669,000.


a
b
c
d
669k
440k
232k
140k
67k

### 4.3.4. Evidence for multiple hydrogenase activities in *Msph. stadtmanae*.

With the variety of chromatographic methods employed in the attempt to purify the MV hydrogenase from *Msph. stadtmanae*, some interesting observations were made.

For example, ion exchange elution profiles consistently showed an activity peak that had a shoulder (at pH 8.0), and with a drastic change in the pH (due to the finding that the hydrogenase appeared to have a very low pI) to pH 5.5, a second peak of activity appeared to slowly elute from the column prior to the application of the salt gradient.

The hydrophobic interaction resin also showed the presence of two peaks of activity, one that eluted with the removal of the salt (required for binding), and a second one that eluted with the addition of detergent (either CHAPS or Triton X-100). Also of note is the presence of hydrogenase activity that does not bind and comes through in the load and wash.

## 4.4. Purification of the MV-Hydrogenase from *Msph. stadtmanae*.

With the amount of information obtained by the preliminary chromatographic and preparative methods for the purification of the MV hydrogenase activities from *Msph. stadtmanae*, it was just a matter of using the right combination of methods in order to effect the purification of the MV hydrogenase activity.

The purification protocol that will be described here was found to be effective, though a rather long process that would take about one week to perform. This protocol was found to be able to purify not just one, but apparently at least two differentially migrating hydrogenase activities from *Msph. stadtmanae*.

**Purification Protocol:**

**Step 1. Cell extract generation.**

Starting with 4.0 L of late-log phase culture of *Msph. stadtmanae*, the cells were harvested aerobically, with a pre-treatment of gas headspace exchange of the $H_2$:$CO_2$ with $N_2$. The cells were centrifuged at 22,000 × g at 4 °C in a Sorvall RC-5 refrigerated centrifuge. The cells were washed with 50 mM Tris-Cl, pH 8.1 and the wet weight of

cells was determined. The cells were again resuspended with 50 mM Tris-Cl, pH 8.1 (10 mL of buffer per g wet weight of cell paste), the serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 5 mM. The cell suspension was passed through a French pressure cell at 38,000 psi, three times. The crude cell extract was then ultracentrifuged on a Beckman L8-80 ultracentrifuge for 1 hour at 120,000 × g at 4°C.

**Step 2. pH precipitation.**

The high speed supernatant (HSS), containing the majority of the hydrogenase activity (in this trial, the activity recovered was higher than was detected in the crude extract, but typically ranges between 70-95% recovery), was then dialyzed at 4°C against 25 mM citrate-phosphate buffer, pH 4.5 in the presence of the acid protease inhibitor, pepstatin A at 1 $\mu$M final concentration. One exchange of buffer was made at 4 hours and left overnight. The following day, the dialyzate (which has become cloudy) was ultracentrifuged at 120,000 × g for 1 hour at 4°C.

**Step 3. pH adjustment.**

The supernatant containing the hydrogenase activity was then again dialyzed against the starting buffer, 50 mM Tris-Cl, pH 8.1, plus PMSF, with one buffer exchange after 4 hours and left overnight at 4°C.

**Step 4. Ammonium sulphate precipitation.**

The next step involved adding ammonium sulphate (enzyme grade) to 90% saturation. This was far higher than previously stated in section 3.3.1.2., but it was found that the majority of the hydrogenase activity would not precipitate, even at saturating conditions at this point (Table 4.11). This is unusual compared to the results on table 4 which show that the hydrogenase activity would precipitate between 35-65%. These results suggest that the pH precipitation probably removed material that the hydrogenase activity was associated with that caused it the precipitate at that range of ammonium

**Table 4.11 - Ammonium sulphate precipitation of MV hydrogenase activity.**

| $(NH_4)_2SO_4$ Percentage | Activity (μmoles MV/min) | Specific Activity (μmoles MV/min/mg) | Fractional Distribution (%) |
|---|---|---|---|
| **90% pellet** | 34.9 | 2.9 | 32.5 |
| **90% supernatant** | 72.6 | 227.0 | 67.5 |

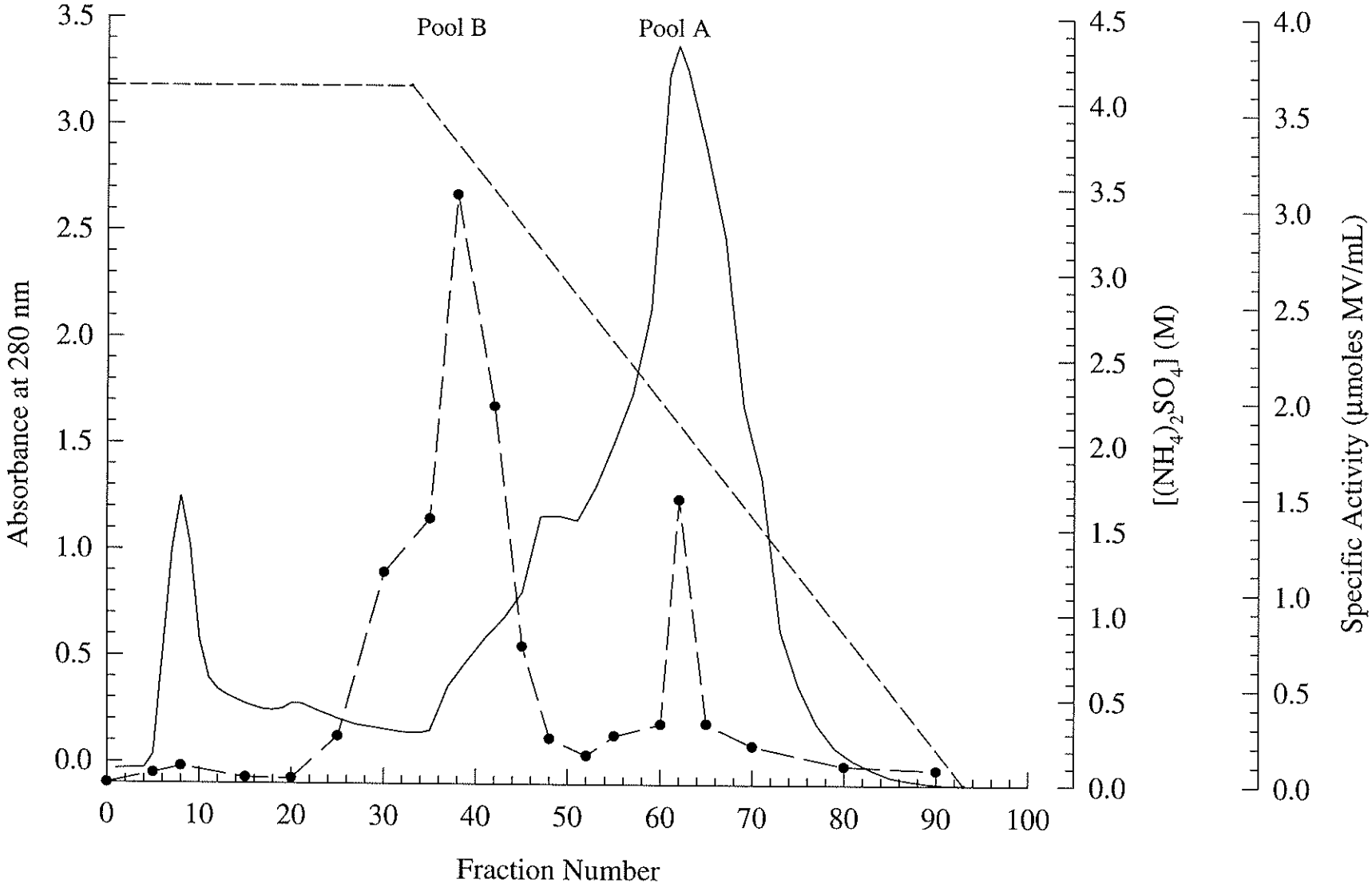
sulphate concentration.

After centrifugation of the ammonium sulphate treated extract at 22,000 × g, for 30 minutes, the supernatant was concentrated on a YM30 Amicon ultrafiltration membrane and brought to a more manageable volume (since the addition of ammonium sulphate significantly increases the volume). Note that at this high concentration of ammonium sulphate, the viscosity of the supernatant was very high so concentration of the sample via this method was very slow. Most of the activity was retained, although some did pass through. It was found that if the extract is still relatively concentrated, less hydrogenase would pass through.

**Step 5. Hydrophobic interaction chromatography.**

After concentration, more ammonium sulphate was added to 100% saturation. No further material precipitated in this step. This saturated solution was then applied to the first of the columns, Phenyl-Sepharose CL-4B (2.5 × 3.0 cm), pre-equilibrated with saturated ammonium sulphate in 50 mM Tris-Cl, pH 8.1. The column was washed with the saturated ammonium sulphate buffer for about 10 column volumes, then the column was developed with a gradient from saturated ammonium sulphate (approximately 4.0 M at 4 °C) to 0.0 M ammonium sulphate in 50 mM Tris-Cl, pH 8.1. The elution profile is shown in figure 17. The profile indicates that there are two hydrogenase activities (which appear to be fairly resolved, but are not, see later), one which elutes very early in the gradient (approximately 3.9 M), while the other eluted at about 2.2 M.

**Figure 17 -** Hydrophobic interaction elution profile for the MV hydrogenase from *Msph. stadtmanae* from Phenyl-Sepharose CL-4B. Column dimensions: 2.5 × 5.0 cm. Flow rate: 0.90 mL/min. Buffer: 50 mM Tris-Cl, saturated $(NH_4)_2SO_4$, pH 8.1. (●) - MV hydrogenase activity.


Pool B
Pool A
Absorbance at 280 nm
[(NH4)2SO4] (M)
Specific Activity (µmoles MV/mL)
Fraction Number
0
10
20
30
40
50
60
70
80
90
100
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5

**Step 6. Dialysis.**

The two pools of hydrogenase activity were then dialyzed separately at 4°C against 50 mM Tris-Cl, pH 8.1 (as there is still a significant amount of ammonium sulphate present in the pools) with one exchange of buffer at 4 hours and then left overnight.

**Step 7. Ion exchange chromatography.**

The two pools were then processed separately on a DEAE Cellufine (Amicon) column (1.0 × 5.0 cm) pre-equilibrated in 50 mM Tris-Cl, pH 8.1. After approximately 10 column volumes of wash buffer (50 mM Tris-Cl, pH 8.1), a gradient of 0 to 500 mM KCl was used to develop the column. The two separate elution profiles demonstrated that the two hydrogenase activities were not completely separated by the Phenyl-Sepharose column (Figure 18a and Figure 18b) and in fact were still mixed together in a certain degree, but the two pools appeared to have different ratios of the two activities. The activities could not be resolved on DEAE Cellufine under these conditions, even though the elution conditions for the two peaks were not close together (early hydrogenase peak eluted at 30 mM KCl while the second peak required 180 mM KCl) because of the broadness of the peaks.

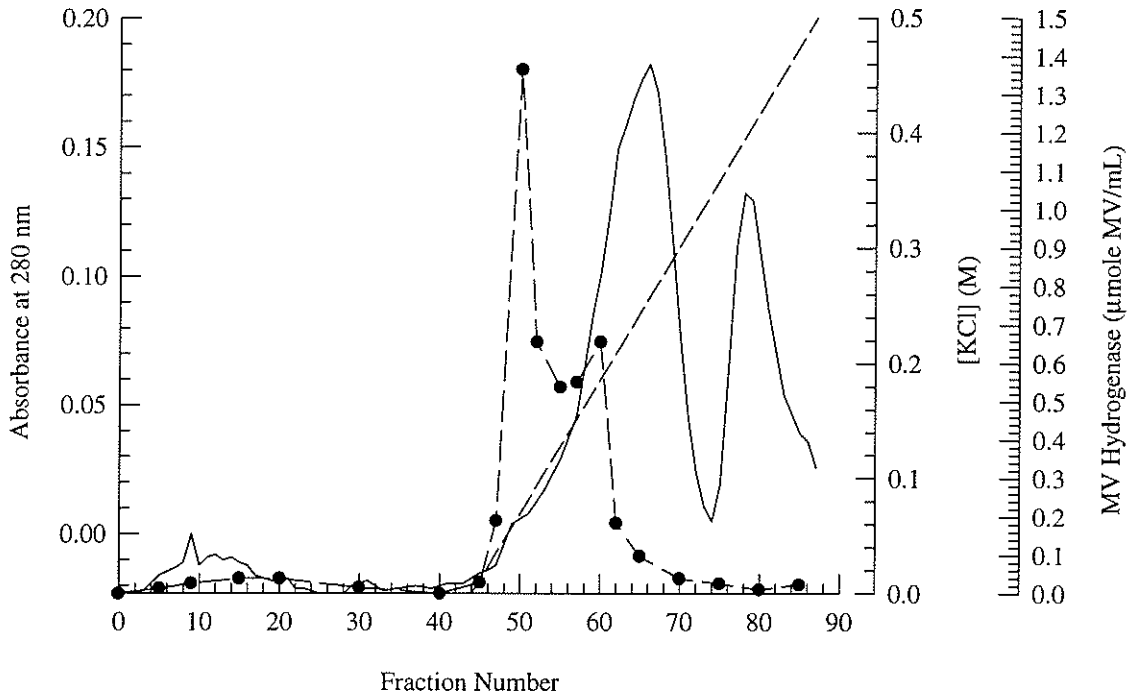
**Step 8. Hydroxylapatite adsorption chromatography.**

To these pools, solid HEPES buffer salt was added to a final concentration of 50 mM, the final pH was near 7.0 without adjustment. Note that there is no K-$PO_4$ added, this is important as will be revealed later.

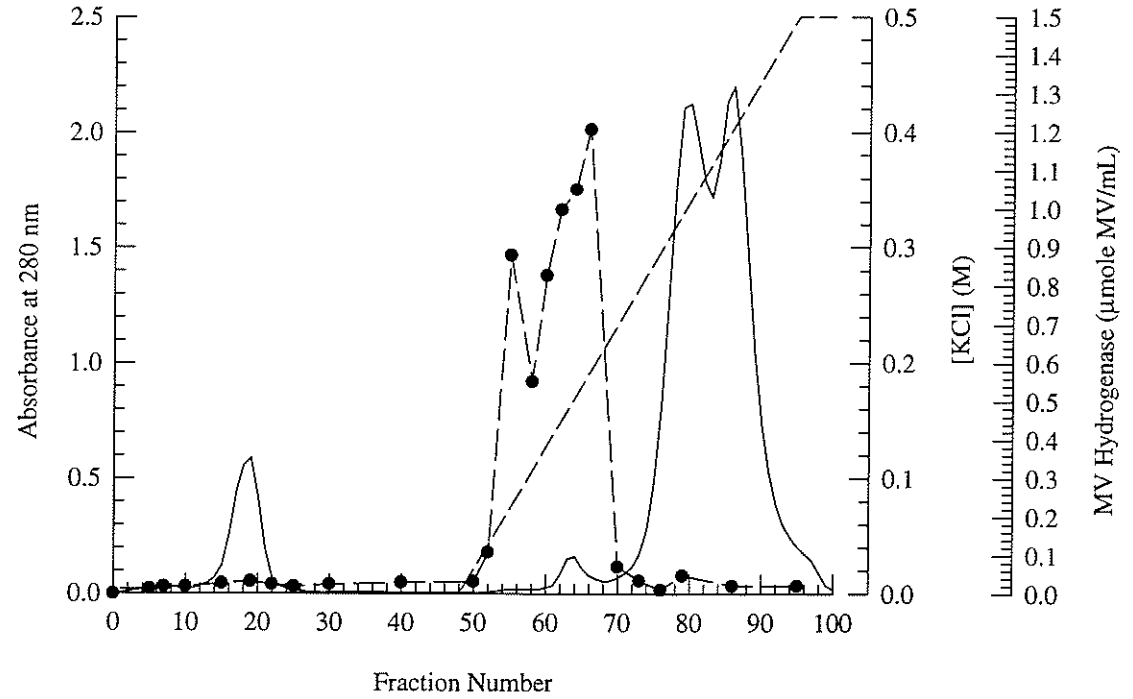
The ion-exchange pools were processed on a Hydroxylapatite HT (BioRad) column (1.0 × 5.0 cm), pre-equilibrated in 1 mM K-$PO_4$, 0.2 M NaCl, pH 7.0. The column was washed with 1 mM K-$PO_4$, 0.2 M NaCl, pH 7.0 for approximately 10 column volumes. It was at this point that one of the hydrogenase activities began to elute slowly from the column as a reddish band. After the band had eluted, the gradient from 1 to 150 mM K-$PO_4$ was started to elute the second hydrogenase activity, which eluted rather early

**Figure 18 -** Ion exchange elution profiles for the MV hydrogenase from *Msph. stadtmanae* from DEAE Cellufine. (a) HI pool 1 peak. (b) HI pool 2. Column dimensions: 1.0 × 5.0 cm. Flow rate: 1.0 mL/min. Buffer: 50 mM Tris-Cl, pH 8.1. (●) - MV hydrogenase activity.

a)


Absorbance at 280 nm
0.20
0.15
0.10
0.05
0.00
0.5
0.4
0.3
0.2
0.1
0.0
[KCl] (M)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
MV Hydrogenase (µmole MV/mL)
0
10
20
30
40
50
60
70
80
90
Fraction Number

b)


Absorbance at 280 nm
2.5
2.0
1.5
1.0
0.5
0.0
0.5
0.4
0.3
0.2
0.1
0.0
[KCl] (M)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
MV Hydrogenase (µmole MV/mL)
0
10
20
30
40
50
60
70
80
90
100
Fraction Number

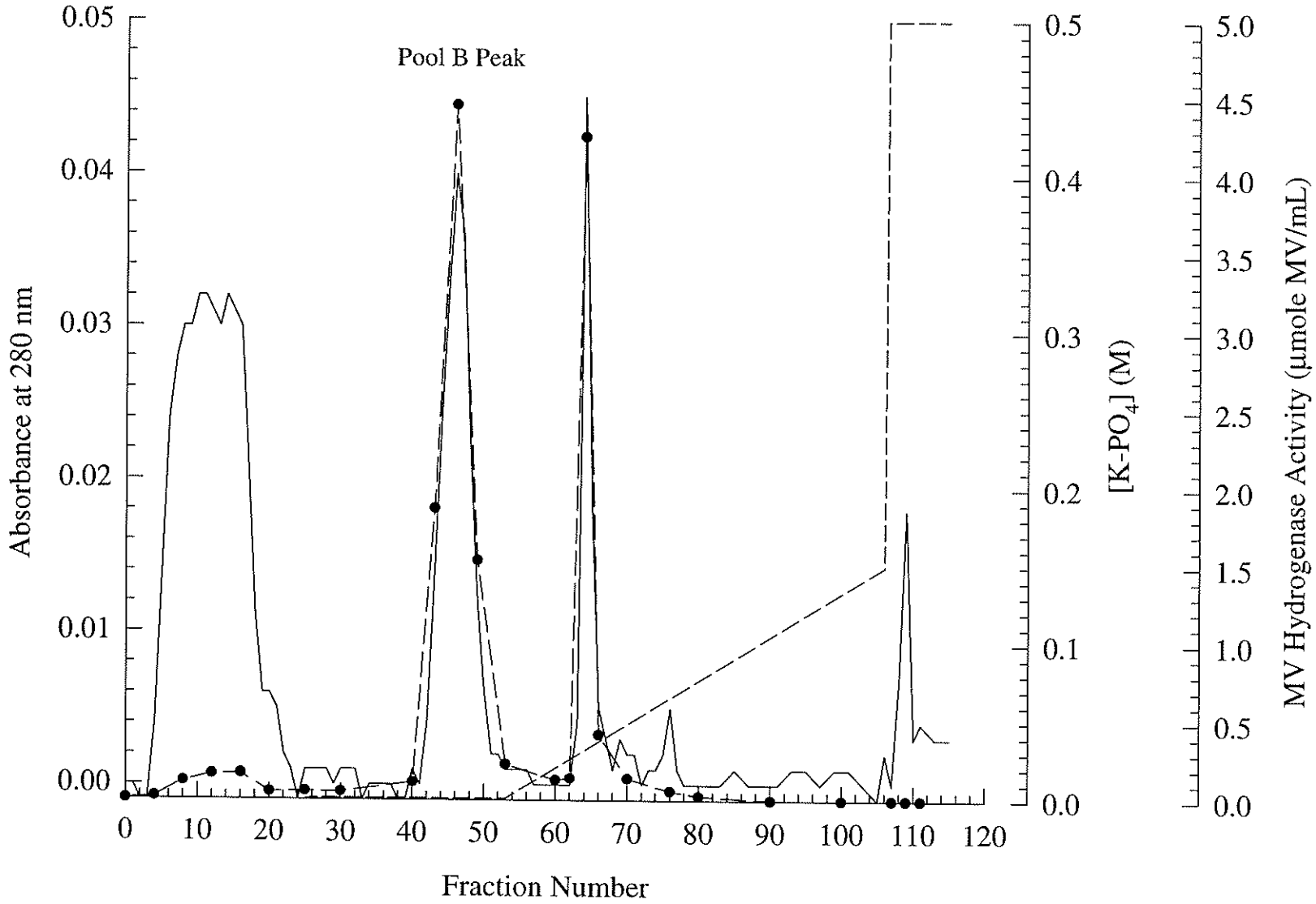
in the gradient (figure 19). Thus there are two hydrogenase activities that could be separated using hydroxylapatite chromatography. The hydrogenases eluted at 1 mM and at 30 mM K-$PO_4$ concentrations. Judging by the elution profile, the two peaks are distinct from each other (along with the elution properties of the activities on both DEAE- and on hydrophobic interaction) and the hydrogenase activity peaks were found to coincide precisely with the protein peaks.

The summary of the purification protocol including the recovery and level of purification achieved is shown on Table 4.12. The final purification factor for the MV hydrogenase was determined to be for the best preparation 185.9-fold with a final specific activity of 650.8 µmoles MV/min/mg protein or 325.4 µmoles $H_2$/min/mg protein. Of note, the specific activity off the ion-exchange was actually higher than that detected in the final value off hydroxylapatite. An elevated value of specific activity has been observed in the MV hydrogenase previously in pools off IE, and has also been previously

**Table 4.12 - Summary of purification of MV hydrogenase**

| Stage | Protein (mg) | Activity (U) | Specific Activity (U/mg) | Purification (-fold) | Yield (%) |
|---|---|---|---|---|---|
| **Crude extract** | 159.7 | 565.7 | 3.5 | - | 100 |
| **High-speed supernatant** | 184.7 | 1246.2 | 6.7 | 1.9 | 220 |
| **pH precipitation supernatant** | 7.3 | 651.9 | 89.3 | 25.5 | 115 |
| **Ammonium sulphate supernatant** | 0.32 | 72.6 | 227.0 | 64.9 | 12.8 |
| **Phenyl-sepharose pool A** | 0.26 | 66.0 | 256.7 | 73.3 | 11.7 |
| **Ion-exchange pool A** | 0.03 | 15.8 | 563.8 | 161.1 | 2.8 |
| **Hydroxylapatite pool A** | 0.04 | 10.8 | 251.9 | 71.9 | 1.9 |
| **Phenyl-sepharose pool B** | 0.37 | 48.2 | 130.3 | 37.5 | 8.5 |
| **Ion-exchange pool B** | 0.05 | 64.5 | 1217.2 | 347.8 | 11.4 |
| **Hydroxylapatite pool B** | 0.05 | 10.8 | 650.8 | 185.9 | 6.2 |

**Figure 19 -** Hydroxylapatite HT elution profile for the MV hydrogenases from *Msph. stadtmanae*. Column dimensions: 1.0 × 5.0 cm. Flow rate: 1.2 mL/min. Buffer: 1 mM K-$PO_4$, 0.2 M NaCl, pH 7.0. (●) - MV hydrogenase activity.


Pool B Peak
Absorbance at 280 nm
0.05
0.04
0.03
0.02
0.01
0.00
0
10
20
30
40
50
60
70
80
90
100
110
120
Fraction Number
0.5
0.4
0.3
0.2
0.1
0.0
[K-PO4] (M)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MV Hydrogenase Activity (µmole MV/mL)

observed in other laboratories (L. Daniels, personal communications). This has been attributed to salt effects as elution requires increased levels of salts to displace protein or other biomolecules off the IE resin.

### 4.5. Properties of the purified MV hydrogenase.

#### 4.5.1. Physical characteristics.

Based on SDS-PAGE of the two pools of activity, these hydrogenases are probably the same protein, both have 2 subunits (figure 20), assigned as α (estimated MW = 68,750) and β (estimated MW = 63,500). They are very similar in size based on the migration distances on the gel. The hydrogenase (both pools) appear to have a native molecular weight of approximately 49,500 as estimated using native gradient PAGE (figure 21). This native size does not correspond to the total of the MW provided by the subunit masses estimated by SDS-PAGE. This may partially be due to the Stokes radius of the native protein.

#### 4.5.2. pH profiles.

The pH profiles for both MVH I and MVH II were determined using a pH range of 5.0 to 9.5 (broader than that used in the pH profile determined in crude). The profiles for both enzyme preparations are shown on figure 22a and figure 22b. The activity for both hydrogenases were found to rise with the pH tested. Both had a maximum of pH optimum of >9.5 (the upper limit of the pH survey). This profile is similar to the profile obtained for the MV reducing activity found for the $F_{420}$ hydrogenase from *Mb. thermoautotrophicum* strain ΔH (Fox et al, 1987), however, it differs from the pH optimum found in crude extract (section 4.2.1.4.). The pH profiles for the two MV hydrogenase pools suggest that they are the same protein. This is unlike that determined for the two MV hydrogenases separated from *Mb. thermoautotrophicum* ΔH previously (Woo et al, 1993) in which the two MV hydrogenases had similar subunit composition, but differed in pH profile and the N-terminal amino acid sequence of one subunit.

**Figure 20 -** SDS-PAGE of the purified MV hydrogenases from *Msph. stadtmanae*. 10% acrylamide gel with the following MW standards: phosphorylase B, 94,000; albumin, 67,000; ovalbumin, 43, 000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100, and α-lactalbumin, 14,400.

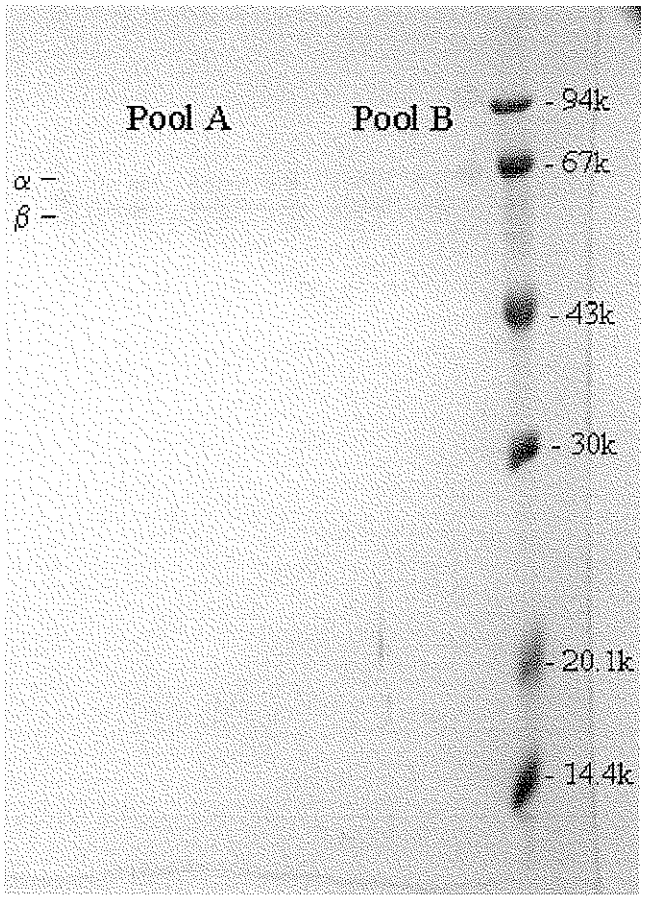

Pool A
Pool B
α
β
- 94k
- 67k
- 43k
- 30k
- 20.1k
- 14.4k

**Figure 21 -** Native gradient PAGE of the MV hydrogenase (Pool B) in duplicate from *Msph. stadtmanae*. Gradients were 3.5-25.2% acrylamide.. Gel run at constant 150 V for 18 hours at 4°C.

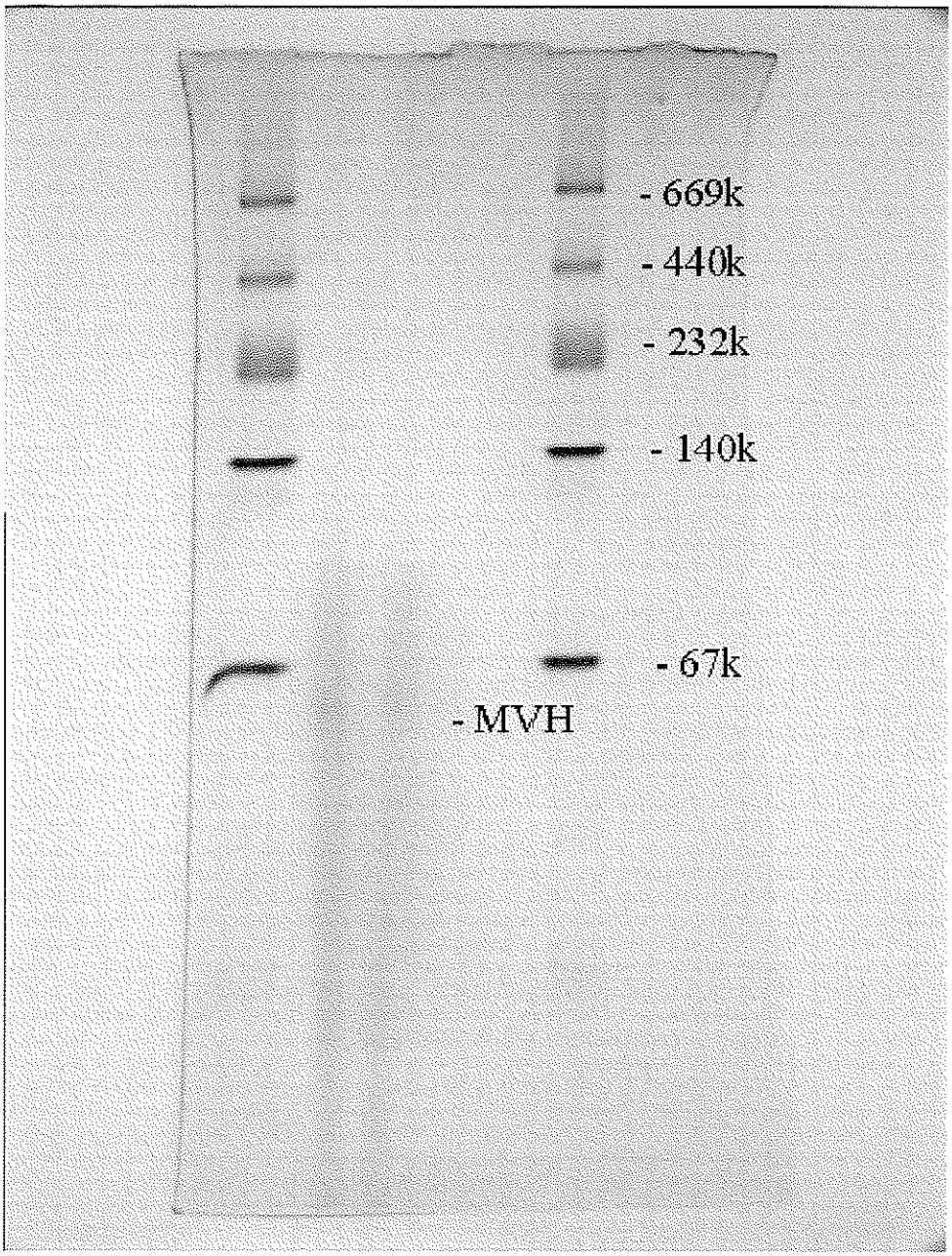
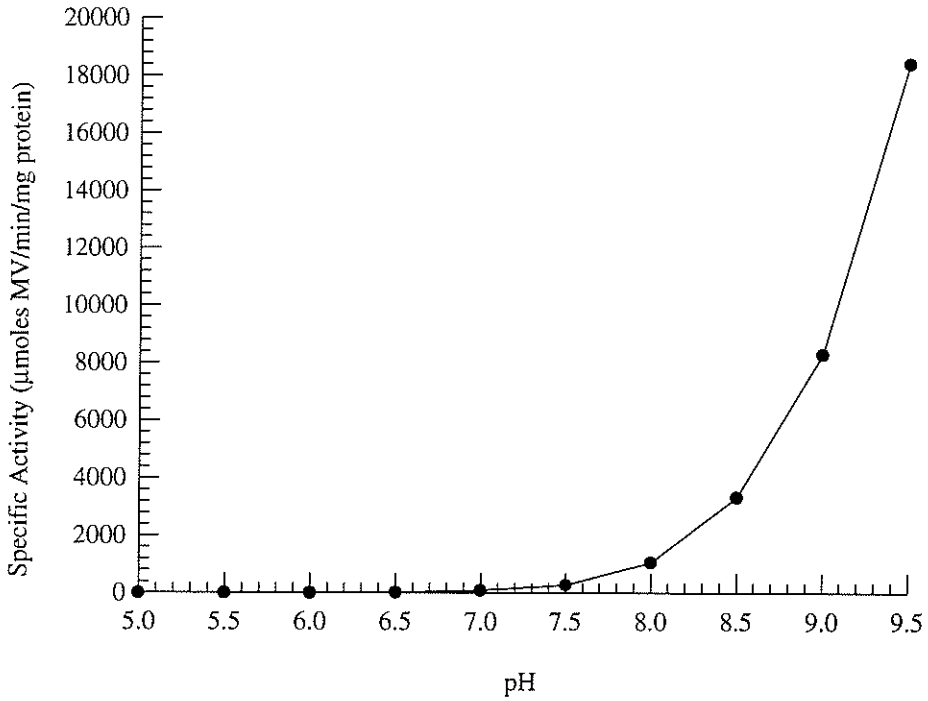
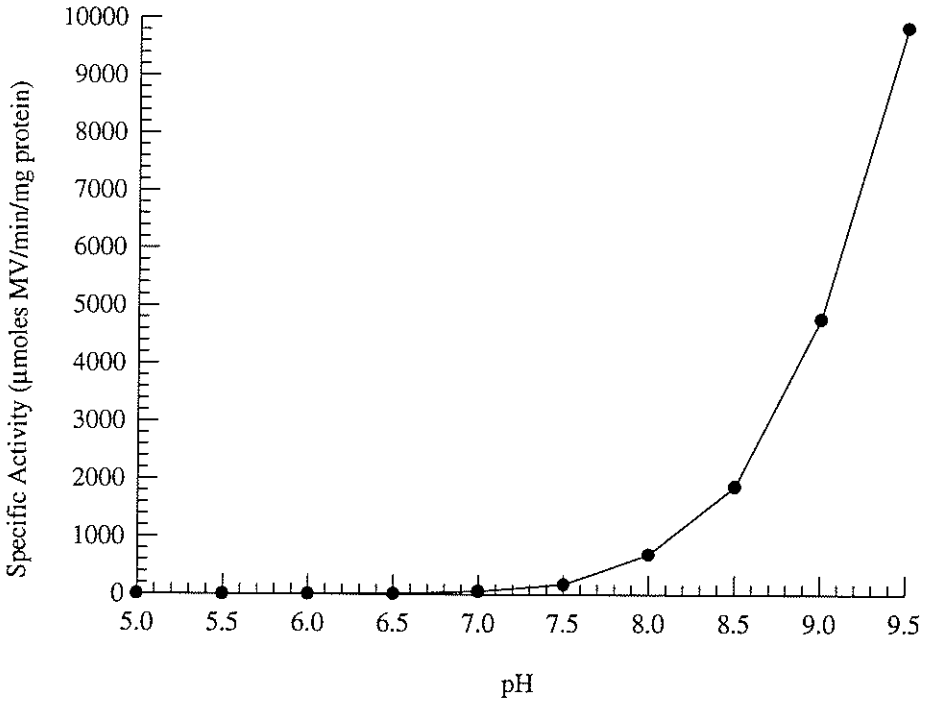

- 669k
- 440k
- 232k
- 140k
- 67k
- MVH

**Figure 22 -** pH profiles for the MV hydrogenases, (a) Pool B and (b) Pool A from *Msph. stadtmanae*. In all cases, 2 mM MV was used as the electron acceptor.

a) Pool B




b) Pool A



### 4.5.3. Kinetic characteristics.

#### 4.5.3.1. Apparent $K_m$ and $V_{max}$.

The kinetic parameters for both preparations of the MV hydrogenase were performed with the artificial electron acceptor, MV. The various types of kinetic plots and replots are shown on figures 23a, 24a and 25a for MVH Pool B and figures 23b, 24b and 25b for MVH Pool A. The Michaelis-Menten plots show no difference in the apparent $K_m$ for the two activities, and both the Lineweaver-Burk and the Woolf-Augustinsson-Hofstee plots indicate the $K_m$'s for both are similar, if not identical. It is interesting that like the work with crude extract, the values for the apparent $K_m$ between Michaelis-Menten and the replots for both hydrogenases do not agree, in addition to being 2 times higher in purified form. Further analyses were performed on only Pool B.

#### 4.5.3.2. Turnover Number ($k_{cat}$) and Catalytic Efficiency.

The turnover number of an enzyme, the $k_{cat}$, is used to determine the rate of reaction per enzyme unit, represented as moles of substrate converted per mole of enzyme per second. The fastest known enzymes are catalase ($k_{cat} = 4.0 \times 10^7$) and carbonic anhydrase ($k_{cat} = 1.0 \times 10^6$) (Zubay, 1993). Based on the native molecular mass of the MV hydrogenase, the turnover number for the MV hydrogenase (Pool B) from *Msph. stadtmanae* was calculated to be $k_{cat} = 2.4 \times 10^8\ s^{-1}$.

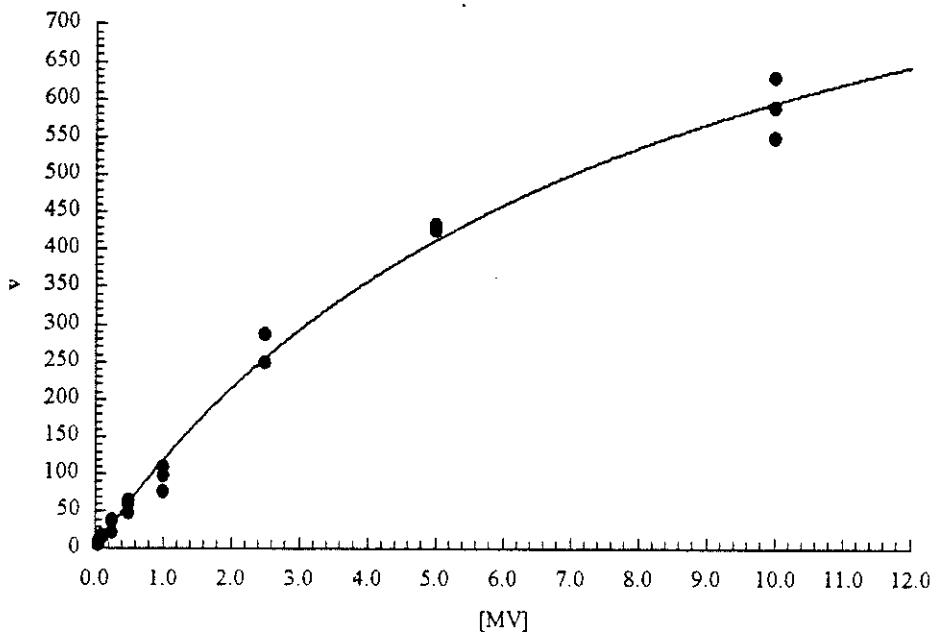
A more useful parameter is the catalytic efficiency constant, $k_{cat}/K_m$. For the MV hydrogenase, $k_{cat}/K_m = 3.0 \times 10^{10}\ M^{-1} \cdot s^{-1}$. A comparison to reported kinetic values with respect to MV are shown on Table 4.13. Note that some of the values on the table are those reported for the MV-reducing activity of the $F_{420}$-reducing hydrogenases from several methanogens.

### 4.5.4. Metals composition and acid-labile sulphide.

The hydrogenase, regardless of source, has always been known as a metallo-enzyme (with the exception of the $H_2$-forming Methylene-$H_4$MPT dehydrogenase; Zirngibl et al, 1989). Specifically they are iron-sulphur proteins (Beinert, 1990) with the

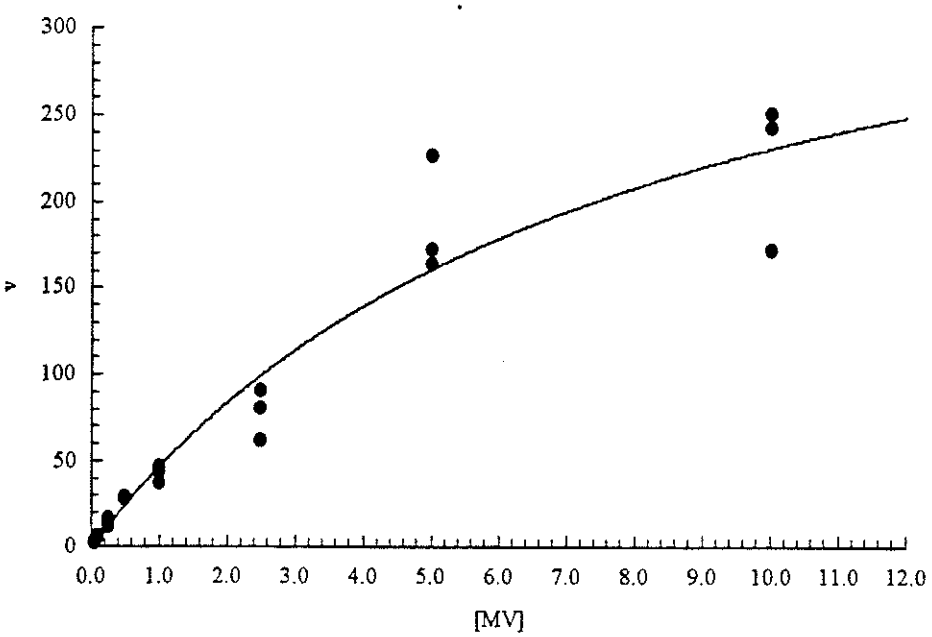
**Figure 23 -** Michaelis-Menten kinetic plots for the purified hydrogenases, (a) Pool B, $r^2 = 0.996$, $K_m = 7.9$ and (b) Pool A, $r^2 = 0.966$, $K_m = 7.8$, from *Msph. stadtmanae*. Assays performed at pH 8.0 in 50 mM Tris-Cl. Each point performed in triplicate.

a) Pool A Preparation




$K_m$ = 7.9 mM MV

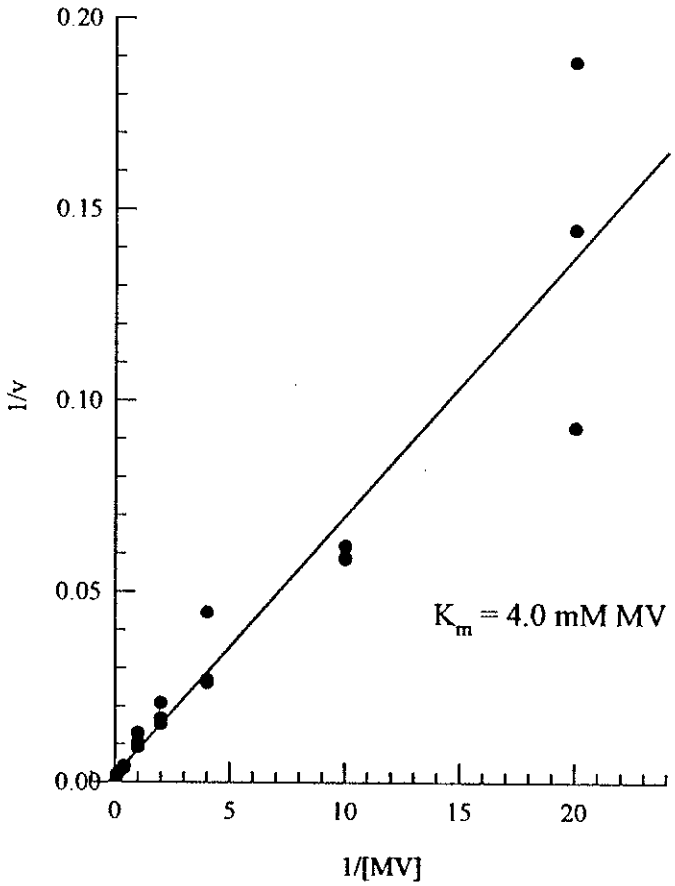
b) Pool B Preparation




$K_m$ = 7.8 mM MV

**Figure 24 -** Lineweaver-Burk kinetic replots for MVH Pools A and B. (a) Pool B, $r^2 = 0.897$, $K_m = 4.0$ and (b) Pool A, $r^2 = 0.993$, $K_m = 4.0$ All assays performed in triplicate and in 50 mM Tris-Cl, pH 8.0.

a) Pool B Preparation




b) Pool A Preparation

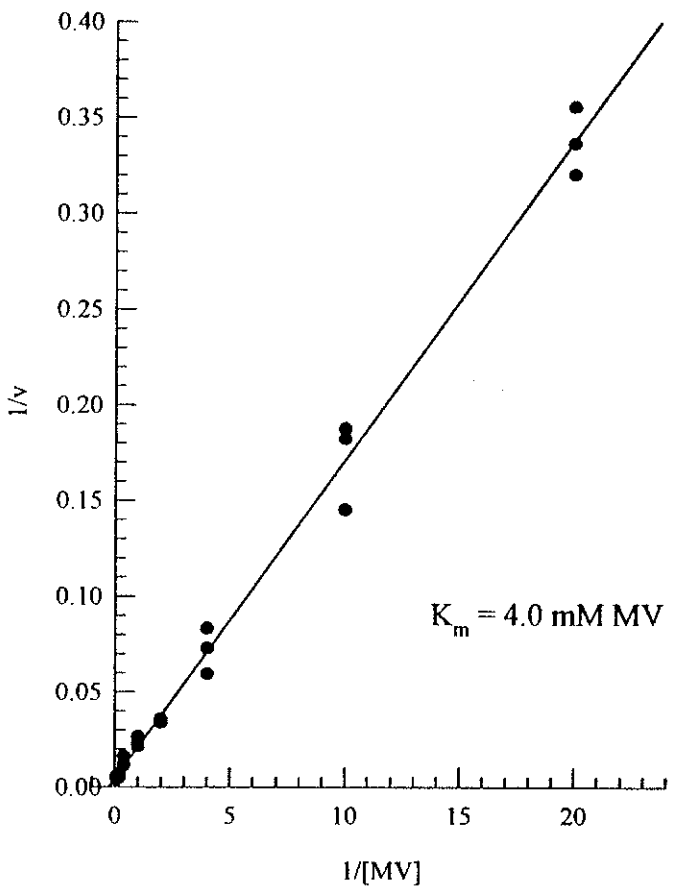
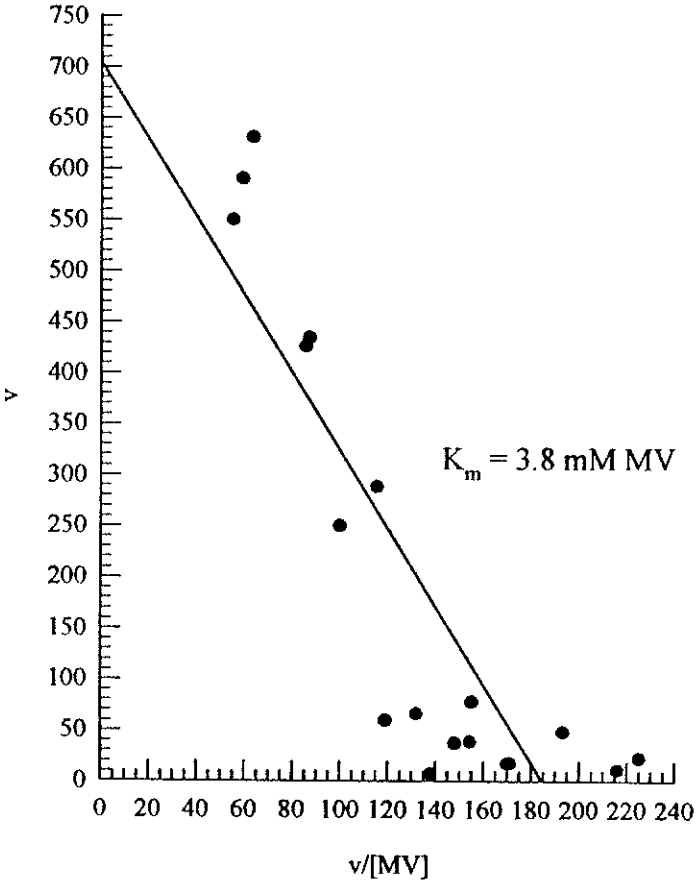


**Figure 25 -** Woolf-Augustinsson-Hofstee kinetic replots for MVH Pools A and B. (a) Pool B, $r^2 = 0.755$, $K_m = 3.8$ and (b) Pool A, $r^2 = 0.659$, $K_m = 4.2$. All assays performed in triplicate and in 50 mM Tris-Cl, pH 8.0.

a) Pool B Preparation




b) Pool A Preparation

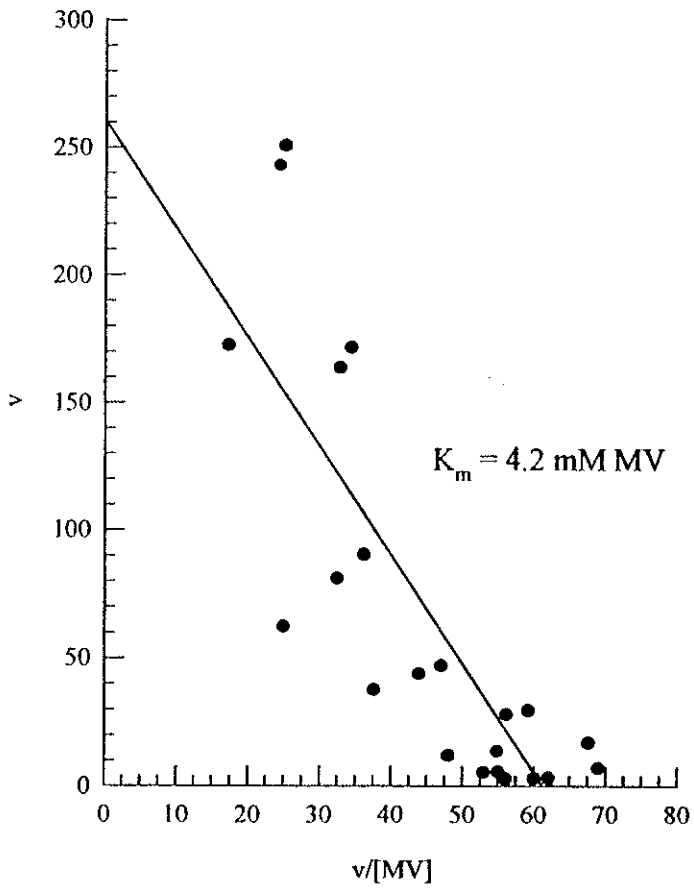


**Table 4.13 - Comparison of Kinetic Parameters with Known Methanogenic Hydrogenases.**

| Organism | MV $K_m$ | $k_{cat}/K_m$ | Reference |
|---|---|---|---|
| *Mb.* strain G2R (MV) | 0.3 mM | not reported | McKellar and Sprott, 1979 |
| *Mb. thermoautotrophicum* strain ΔH ($F_{420}$) | 0.4 mM | $9.5 \times 10^6$ | Jacobson et al, 1981 |
| *Mb. thermoautotropicum* strain ΔH ($F_{420}$) | 1.5 mM | $1.9 \times 10^7$ | Livingston et al, 1987 |
| *Mc. voltae* ($F_{420}$) | 1.2 mM | $8.8 \times 10^5$ | Muth et al, 1987 |
| *Ms. barkeri* strain Fusaro ($F_{420}$) | 3.3 mM | $2.8 \times 10^6$ | Fiebig and Freidrich, 1989 |
| *Ms. barkeri* strain Fusaro ($F_{420}$) | 3.4 mM | $6.9 \times 10^7$ | Michel et al, 1995 |
| *Ms. mazei* strain Gö1 (MV) | 8.0 mM | $5.8 \times 10^4$ | Deppenmeier et al, 1994 |
| *Msph. stadtmanae* (MV) | 7.9 mM | $3.0 \times 10^{10}$ | This Thesis |

- Note: electron carrier in brackets indicate type of hydrogenase, either MV- or $F_{420}$-reducing.

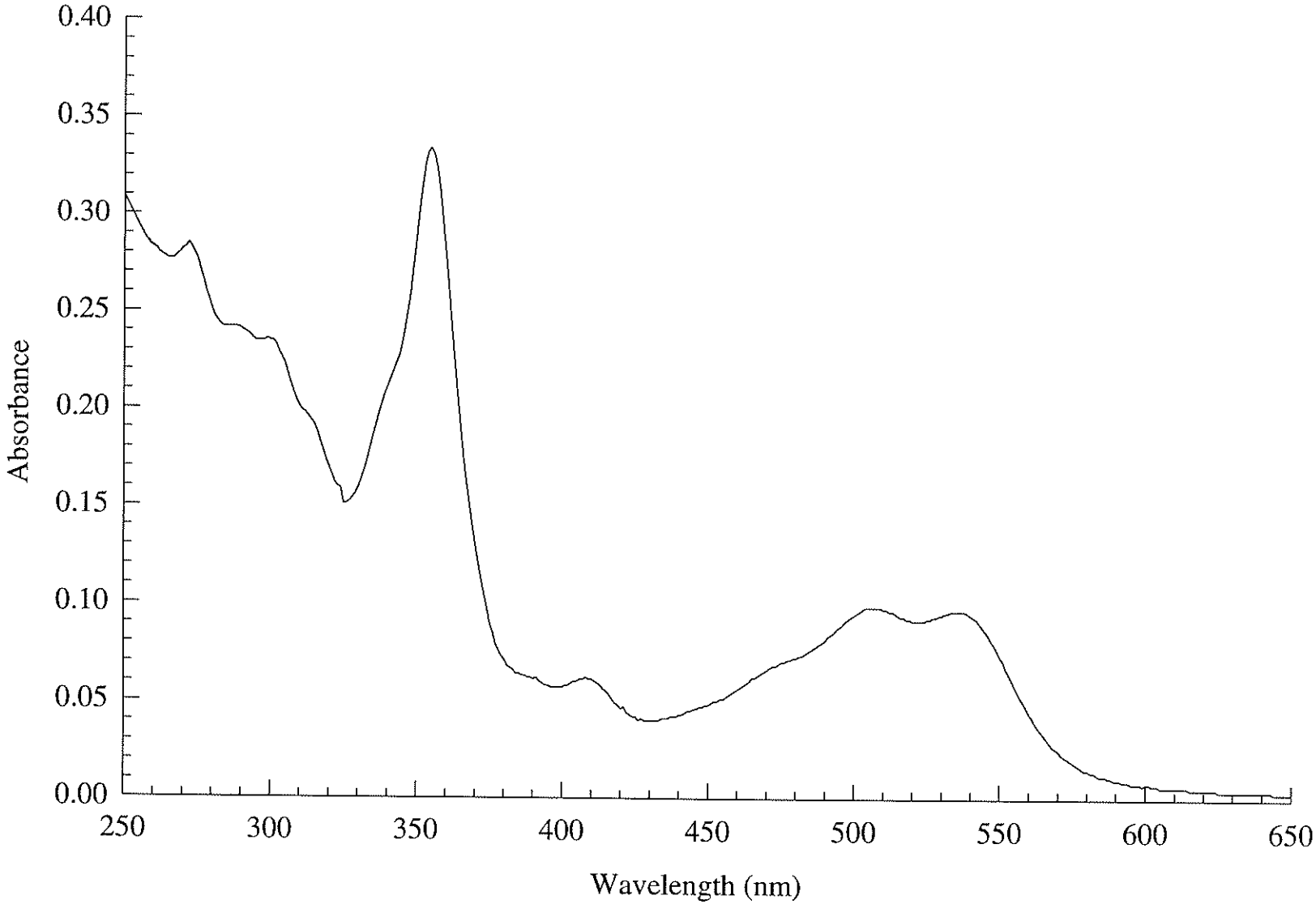
iron-sulphur being used for electron transfer. The hydrogenases from the methanogenic bacteria typically contain nickel, in addition to the iron-sulfur moieties (Wu and Mandrand, 1993), so an attempt was made to determine if the hydrogenase from *Msph. stadtmanae* also carried these metals as well. The levels of iron detected in the hydrogenase sample, per mole of hydrogenase was determined to be 11.2 ± 0.7 moles, while the amount of acid-labile sulphide was determined to be 18.5 ± 3.1 moles per mole hydrogenase.

### 4.5.5. Spectral properties.

The purified hydrogenases from other methanogenic bacteria, as well as eubacteria display distinctive spectral properties, with absorption peaks at specific locations in the absorption spectrum of the protein sample. The spectrum for MV hydrogenase is shown in figure 26.

The spectrum for MV hydrogenase is unusual with respect to known

**Figure 26 -** Absorption spectrum for MV hydrogenase from *Msph. stadtmanae*. Spectrum determined on a Pharmacia Ultrospec 4000 UV/Vis spectrophotometer.


0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Absorbance
250
300
350
400
450
500
550
600
650
Wavelength (nm)

hydrogenases. A complex spectrum with six major peaks, two peaks, one at 350 nm and one at 525 nm are not typical for known hydrogenases and suggest a similarity to cobamides (Weissbach et al, 1966). Visually, the hydrogenase preparation is red-pink in colour, which agrees with the cobamide possibility.

### 4.5.6. Comparison to known MV hydrogenases.

Inevitably, the properties of any enzyme purified must be compared to similar proteins purified from related organisms. The physical properties and composition of the two hydrogenases purified from *Msph. stadtmanae* can now be compared to the purified MV hydrogenases from other methanogenic bacteria. The physical properties, like native molecular weight and the subunit size and composition, are compared to published MV hydrogenases from other methanogens (Table 4.14). The subunit sizes are in the same range as those reported for many of the MV hydrogenases purified so far. The native size is another story, there are no reported hydrogenases, archaebacterial or eubacterial, that are that size in native form, the closest is the hydrogenase that contains no metal centers in the methanogens (methylene-$H_4$MPT dehydrogenase) with a molecular mass of approximately 43 kDa (Zirngibl et al., 1990).

The metals composition comparison with reported MV hydrogenases is shown in Table 4.15. Some of the MV hydrogenases listed did not report the metals composition of their preparation (e.g., *Mb. thermoautotrophicum* strain ΔH and *Mc. jannaschii*). The sulphide that was detected is higher than that found in the preparations obtained from both *Mb. formicicum* and *Ms. barkeri* strain MS, but the iron levels for the hydrogenase from *Msph. stadtmanae* are similar.

## 4.6. Summary

The MV hydrogenase activity from *Msph. stadtmanae* has been purified. Some of the data acquired suggested that there may be several forms of the hydrogenase in the extracts of *Msph. stadtmanae*. This finding is not unusual since this was reported in other methanogens (*Mc. voltae*, Halboth and Klein, 1990; *Mb. thermoautotrophicum* strain ΔH,

**Table 4.14 - Physical Properties of MV Hydrogenases from the Methanogenic Archaea**

| Organism | Native Molecular Weight (kDa) | Subunit Structure | Reference |
|---|---|---|---|
| *Methanobacterium* strain G2R | 900 | 80; 50.7; 38.5 | McKellar and Sprott, 1979 |
| *Methanobacterium formicicum* strain MF | 70 | 42.6; 34; 23.5 | Jin et al, 1983 |
| *Methanobacterium thermoautotrophicum* strain ΔH (enzyme I) | not reported | 52; 40 | Kojima et al, 1983 |
| *Methanobacterium thermoautotrophicum* strain ΔH (enzyme II) | 145 | 50; 44; 36; 15 | Woo et al, 1993 |
| *Methanobacterium thermoautotrophicum* strain Marburg | 230 | 51; 41; 17 | Braks et al, 1994 |
| *Methanococcus jannaschii* | 475 | 43; 31 | Shah and Clark, 1990 |
| *Methanosarcina barkeri* strain MS | 98 | 57; 35 | Kemner and Zeikus, 1994 |
| *Methanosarcina mazei* strain Gö1 | 79 | 60; 40 | Deppenmeier et al, 1992 |
| *Methanosphaera stadtmanae* | 49.5 | 68.8; 63.5 | This Thesis |

**Table 4.15 - Metals Composition of the MV Hydrogenases of the Methanogenic Archaea**

| Organism | Iron (moles/mole enzyme) | Acid Labile Sulfide (moles/mole enzyme) | Other Metals (moles/mole enzyme) | Reference |
|---|---|---|---|---|
| ***Methanobacterium formicicum*** **strain MF** | 10 | 8 | 1.0 Ni; 1.0 Zn; 2.0 Cu | Adams et al, 1986 |
| ***Methanobacterium thermoautotrophicum*** **strain ΔH** | n.d. | n.d. | n.d. | Woo et al, 1993 |
| ***Methanococcus jannaschii*** | n.d. | n.d. | n.d. | Shah and Clark, 1990 |
| ***Methanosarcina mazei*** **strain Gö1** | 15.1 | 13.3 | 0.8 Ni | Deppenmeier et al, 1992 |
| ***Methanosarcina barkeri*** **strain MS** | 8 ± 2 | 8 ± 2 | 0.5 Ni | Kemner and Zeikus, 1994 |
| ***Methanosphaera stadtmanae*** | 11.2 ± 0.7 | 18.5 ± 3.1 | n.d. | This Thesis |

Woo et al, 1993) and has been reported from other bacteria (Ackrell et al., 1966; Adams et al., 1981; Prickril et al., 1987).

The hydrogenase from *Msph. stadtmanae* appears to be a heterodimer of two subunits (68.8 + 63.5 kDa) based on SDS PAGE, but the native PAGE results give a mass of approximately 50 kDa. The reason for this migration of the hydrogenase activity to the low molecular mass (higher acrylamide concentration) region of the gradient gel is not known, but partly may be attributed to the Stokes radius of the native protein. Another reason may be the overall shape of the molecule as well, that it may not be the assumed globular form. There were no confirmatory analyses performed (gel filtration, sucrose gradient) on the pure sample, so it is not known whether this value can be accepted on it's own. A sucrose gradient analysis was performed on the crude extract and did show that the hydrogenase activity was a low density activity, however, this gradient was not calibrated with molecular weight markers (as the method was taken from a purification protocol), and other factors become important in density determinations (ie. lipids).

The two hydrogenase preparations have different specific activities, but appear to have almost identical apparent $K_m$s of 7.9 and 7.8 (Michaelis-Menten values) for MVH samples I and II respectively. Both preparations of the MV hydrogenase also displayed a similar pH profile, with a pH optimum for activity at pH 9.5 (the highest pH tested), which further supports that the two preparations are probably derived from the same enzyme. SDS PAGE of both samples showed that they contain the same size peptides as well.

The kinetic parameters for the MV hydrogenase from *Msph. stadtmanae* have been determined. The $K_m$ for the artificial electron acceptor, methyl viologen, as reported above, was calculated to be 7.9 mM, similar to that reported for the MV hydrogenase from *Ms. mazei* strain Gö1 (Deppenmeier et al, 1994). The catalytic efficiency parameter, $k_{cat}/K_m$, for the MV hydrogenase from *Msph. stadtmanae* was found to be very high ($3.0 \times 10^{10}$), higher than those previously reported for hydrogenases from other methanogenic bacteria (e.g., *Ms. barkeri* strain Fusaro, the $F_{420}$-reducing form using MV as electron acceptor, a $k_{cat}/K_m = 6.9 \times 10^7$). The values for the kinetic values, ie., the specific

activities and the $k_{cat}$ and $k_{cat}/K_m$, may be high due to possible error in the protein determination method. For the purification, the protein detected was extremely low so the values for these parameters were correspondingly high. An uncertainty in both the protein detected and the native molecular weight (and hence the enzyme concentration) will alter the value of $k_{cat}$ (since $k_{cat} = V_{max}/E_t$). This should be considered when looking at these values.

The hydrogenase from *Msph. stadtmanae* was also found to contain the expected prosthetic groups for a hydrogenase, ie., iron and acid-labile sulphide. The amounts detected suggest four units of one type of iron-sulphur cluster, the 3Fe-4S group. It is possible that this group may actually be a 4Fe-4S group as analytical methods have been known to lose an Fe from such a group (Beinert, 1990).

An unusual property for a hydrogenase of its type is the hydrophobic nature of the protein, or the lack of hydrophobic nature. After the pH precipitation step, the addition of ammonium sulphate to saturating conditions did not cause the hydrogenase activity to precipitate, suggesting that there are either very few or no hydrophobic regions on the surface of the enzyme. In addition, ammonium sulphate was required to be at saturating levels for the binding of the activity, and early in the gradient, one portion of the activity eluted off the column (approximately 3.9 M) while a second group eluted off at a lower concentration (1.7 M). This does not appear to indicate a hydrophobic protein. The initial ammonium sulphate precipitation study could indicate that the hydrogenase is normally associated with other proteins that do have a hydrophobic character, ie., a possible membrane associated complex.

The spectral properties are unique. The close similarity to a cobamide spectrum is curious, it has been reported in the literature that cobamides could accept electrons from ferredoxin (Weissbach et al, 1966), so the precedence is there for involvement in electron transfer.

# 5. $F_{420}$ Reduction in *Methanosphaera stadtmanae*

## 5.1. Introduction

The major methanogenic cofactor, $F_{420}$, is involved in the electron transport systems of the methanogenic bacteria. The reduced form of $F_{420}$ ($F_{420}H_2$) is also the source of the electrons or reducing equivalents required for the biosynthetic pathways of the methanogens (Zeikus et al., 1977). The major cofactor for the biosynthetic pathways are the nicotinamide nucleotides (ie. $NAD^+$ and $NADP^+$), the enzyme link to these cofactors is the $NADP^+$:$F_{420}$ oxidoreductase (Zeikus et al., 1977). A link between $NAD^+$ and $NADP^+$ was discovered recently involving a coupled enzyme system, an $NAD^+$-dependent malate dehydrogenase and an $NAD^+$/$NADP^+$-reactive form, that could perform the NADPH:$NAD^+$ transhydrogenation reaction (Thompson et al., 1998). Therefore a mechanism for the reduction of cofactor $F_{420}$ is required for all of the methanogens.

In the hydrogenotrophic methanogens, the enzyme typically linked to the reduction of the cofactor $F_{420}$ is the hydrogenase. However, there are three examples of this type of methanogen previously reported to not carry a detectable $F_{420}$-linked hydrogenase activity, these include *Methanobacterium* strain G2R (McKellar and Sprott, 1979), *Methanosphaera cuniculi* (Biavati et al, 1988) and *Msph. stadtmanae* (Deppenmeier et al., 1989; Wong et al, 1994).

In the acetotrophic, or the acetate-utilizing methanogens, the enzyme carbon monoxide dehydrogenase (CODH) is a source of the electrons for methanogenesis, although not all CODHs in these methanogens have been found to be $F_{420}$-linked (only the CODH purified from *Methanothrix soehngenii* was determined to be cofactor $F_{420}$-reducing, Jetten et al., 1989). The process of methanogenesis from acetate is limited only to members of the two genera, *Methanosarcina* and *Methanothrix/Methanosaeta* (Ferry, 1993).

In the methanol-only methanogens, the enzymes of the methanogenic pathway involving $H_4$MPT become the source of the electrons for the reduction of $F_{420}$, as the

reactions involving this cofactor are used in the reverse order as they are used in the $H_2$:$CO_2$ pathway. In the methanol-utilizers, another enzyme is also involved with cofactor $F_{420}$ in the redox steps at the terminal end of the methanogenic pathway at the heterodisulfide reduction step, the $F_{420}H_2$ dehydrogenase (Deppenmeier et al., 1991). In these methanogens, the enzyme is used for the purpose of the oxidation of reduced cofactor $F_{420}$ ($F_{420}H_2$) in the terminal reaction involving the recycling of the heterodisulfide back into the component thiols of CoM and CoB.

In the organism of study, *Msph. stadtmanae*, a methanogen that strictly grows on methanol plus $H_2$, the only hydrogenase found in the cell-extracts is a methyl-viologen reducing hydrogenase activity (Deppenmeier et al ,1989), described in the previous chapter, which is unable to reduce the prime methanogenic cofactor $F_{420}$. Therefore, a mechanism for the reduction of $F_{420}$ is not known in *Msph. stadtmanae*, and the mechanism is not directly linked to the primary source of electrons, hydrogen. Since the primary source of electrons for the cell is not directly linked to the reduction of the cofactor $F_{420}$, and the production of $F_{420}H_2$ is required for the biosynthetic pathways, an alternative mechanism for the reduction of $F_{420}$ must exist.

One route may be through the enzyme, CODH, an enzyme that is involved in the conversion of acetate into methane in the acetotrophic methanogens, this enzyme activity has been detected in the related hydrogenotrophic *Methanobacterium thermoautotrophicum* strain ΔH, and was found also to be cofactor $F_{420}$-linked (Daniels et al., 1977). Another possible route is through the enzyme known as the $F_{420}H_2$ dehydrogenase, discovered in and purified from the methanol-utilizer, *Methanolobus tindarius* (Haase et al., 1992) and recently purified from the nutritionally diverse *Methanosarcina mazei* strain Gö1 (Abken and Deppenmeier, 1997). It is implicated as having a major role in methanogenesis from methanol alone (Deppenmeier et al., 1994).

The objective of this chapter is to study and attempt to determine the mechanism of cofactor $F_{420}$ reduction by the hydrogenotrophic *Msph. stadtmanae* in the absence of an $F_{420}$-reducing hydrogenase, and to characterize this activity. Unless otherwise stated, all assays were performed in triplicate and with <10% deviation from the mean.

## 5.1. Results and Discussion.

### 5.1.1. Levels of the Cofactor $F_{420}$ in *Methanosphaera stadtmanae*

A specific method for the enumeration of methanogens in culture, mixed or otherwise, is observing their fluorescence, due to the presence of cofactor $F_{420}$ in the cell, under a fluorescence microscope. Cultures of pure *Msph. stadtmanae* have been shown to fluoresce to a lesser degree than most other methanogenic bacteria. Analysis of the relative levels of this cofactor has been performed between *Msph. stadtmanae*, and two other methanogens, *Mb. thermoautotrophicum* strain Marburg, and *Mc. thermolithotrophicus*. The results of this comparison are shown in Table 5.1, along with

**Table 5.1 - Cofactor $F_{420}$ Levels in Various Methanogenic and Non-methanogenic Bacteria.**

| Organism | Cofactor $F_{420}$ Levels (nmole/mg protein) | Cofactor $F_{420}$ Levels (nmole/g dry weight) | Reference |
|---|---|---|---|
| *Methanosphaera stadtmanae* | 0.16 ± 0.02 | 80 ± 8 | This Thesis |
| *Methanobacterium thermoautotrophicum* strain Marburg | 5.90 ± 0.6 | 2,950 ± 300 | This Thesis |
| *Methanococcus thermolithotrophicus* | 27.10 ± 3.0 | 13,550 ± 1000 | This Thesis |
| *Methanobacterium thermoautotrophicum* strain ΔH | - | 2,500 | Daniels et al., 1985 |
| *Methanospirillum hungatei* | - | 3,700 | Eirich et al., 1979 |
| *Ferroglobus placidus* | 0.40 | 200 | Vorholt et al., 1997 |
| *Halobacterium halobium* | - | 58 | Lin and White, 1986 |
| *Mycobacterium avium* | - | 50 | Lin and White, 1986 |
| *Nocardia aurantia* | - | 850 | Daniels et al., 1985 |
| *Streptomyces griseus* | - | 2050 | Daniels et al., 1985 |

Note: Conversion of cofactor $F_{420}$ levels from nmole/mg protein to nmole/g dry weight made assuming 50% of dry weight attributed to protein. "-" denotes that value was not reported.

other published values for other methanogenic species.

The results of this survey indicate that the levels per mg protein in *Msph. stadtmanae* are indeed lower than those found in the related methanogen, *Mb. thermoautotrophicum* strain Marburg and significantly lower than the levels found in *Mc. thermolithotrophicus*. This would indicate that the cofactor is not used directly in the process of methanogenesis, as is the case in the other two species, but is probably used specifically in the process of cell biosynthesis. This, however, does not tell us the source and the enzymatic mechanism of the reduction of the cofactor, since the primary source of electrons for the cell is hydrogen, and the hydrogenase activities found in this organism are not cofactor $F_{420}$-linked.

In *Msph. stadtmanae*, the pathway of methanogenesis omits the major portion of the methanogenic pathway from $H_2$:$CO_2$ in its synthesis of methane from methanol plus $H_2$. At the same time it bypasses many of the enzymes that require reduced cofactor $F_{420}$ as an electron donor. Many of these enzymes and coenzymes have been found in low levels in the extracts of *Msph. stadtmanae* (Schwörer and Thauer, 1991) and their presence may be linked to cell biosynthesis (Choquet et al., 1994; Lin and Sparling, 1995). Of the coenzymes, methanofuran was found to be absent (van de Wijngaard et al., 1991), thus the methanogenic pathway from $CO_2$ is incomplete in *Msph. stadtmanae* and cannot produce methane from this substrate.

### 5.1.2. $F_{420}$ Reduction and CODH Activity in *Methanosphaera stadtmanae*

The carbon monoxide dehydrogenase is an essential element of the methanogenic pathway of the acetotrophic methanogens (Ferry, 1993) and is involved in the primary $CO_2$ fixation step into organic carbon in the fully autotrophic methanogens (Daniels et al., 1977). This activity has been detected in a number of methanogenic bacteria, including those which do not use acetate as a substrate for methanogenesis (eg. *Mb. thermoautotrophicum* strain ΔH), but instead use it for carbon fixation. Although it is an important enzyme in providing a source of electrons from CO or from acetate, this enzyme is usually not linked to the cofactor $F_{420}$ in these methanogens. Assay is typically

through the class of artificial dyes, the viologens, which includes benzyl and methyl viologen.

In the following study, several methanogens, including the species of interest, *Msph. stadtmanae*, were tested to determine whether, (a) the organism contains a CODH activity, and (b) the activity is coenzyme $F_{420}$-linked. For comparison, strains of the eubacterial sulfate-reducer, *Desulfovibrio desulfuricans* are included, but were only tested with methyl viologen as electron acceptor. The reasoning behind testing for this enzyme activity is the published finding of the CODH activity in the related methanogen, *Mb. thermoautotrophicum* strain ΔH, (both *Msph. stadtmanae* and *Mb. thermoautotrophicum* are members of the Order *Methanobacteriales*), and the association of this CODH activity with $F_{420}$ reduction (Daniels et al., 1977). In addition, acetate is required in the growth medium of *Msph. stadtmanae*, although it is not used as a methanogenic substrate (Miller and Wolin, 1984).

Table 5.2 summarizes the crude extract CODH activities found in the methanogenic species that cover all three methanogenic Orders, *Msph. stadtmanae*, *Mb. thermoautotrophicum* strain Marburg, *Methanococcus thermolithotrophicus*, and *Methanosaeta concilli*, with either MV or $F_{420}$ being tested as electron acceptor, along with the activities found in the bacterial strains of *Desulfovibrio desulfuricans*. Also in this table are reported values for CODH activities from other methanogenic bacteria.

These results show that *Msph. stadtmanae* does not carry a CODH in its cell extracts. Acetate is required as a fixed carbon source, as is the case in several other methanogens (*Mc. voltae* and *Msp. hungatei*). An interesting comparison can be made between the two strains of *Mb. thermoautotrophicum*, the published finding in strain ΔH is that the CODH is $F_{420}$-linked (Daniels et al., 1977), meanwhile, the CODH activity found in strain Marburg is not (see Table 5.2). This is not surprising since although the two strains are said to be of the same species, there is a significant difference at the nucleic acid sequence level with only about a 40% DNA:DNA homology (Nölling et al., 1993) between the two that could certainly account for the differences in enzyme characteristics. The differences are significant enough that a proposal for the

**Table 5.2 - CODH Activities from Selected Methanogenic Bacteria and Anaerobic Eubacteria**

| Organism | Specific Activity (μmole MV/min/mg) | Specific Activity (μmole $F_{420}$/min/mg) |
|---|---|---|
| *Methanosphaera stadtmanae* | 0.00[a] | 0.00[a] |
| *Methanosaeta concilii* | 2.28 ± 0.23[a] | 0.02 ± 0.002[a] |
| *Methanobacterium thermoautotrophicum* **strain Marburg** | 0.22 ± 0.02[a] | 0.00[a] |
| *Methanococcus thermolithotrophicus* | 0.54 ± 0.05[a] | not determined |
| *Methanobacterium thermoautotrophicum* **strain ΔH** | 0.40[b] | 0.79 μmole/mL[b] |
| *Methanococcus vannielii* | 0.33*[c] | 0.00[c] |
| *Methanosarcina barkeri* | 5.23[d] | 0.00[d] |
| *Methanosarcina frisia* **strain Göl** | 3.3[e] | 0.00[e] |
| *Methanosarcina thermophila* | 10.5[f] | 0.00[f] |
| *Methanothrix soehngenii* | 9.0[g] | 0.14[g] |
| *Desulfovibrio desulfuricans* **strain B203** | 0.00[a] | - |
| *Desulfovibrio desulfuricans* **(DSM 1924)** | 0.11[a] | - |

* - denotes that 2,3,5-triphenyltetrazolium chloride was used as electron acceptor instead of methyl viologen.

[a] - source: This Thesis; [b] - source: Daniels et al., 1977; [c] - source: DeMoll et al., 1987; [d] - source: Grahame and Stadtman, 1987; [e] - source: Eggen et al., 1996; [f] -source: Terlesky et al., 1996; [g] - source: Jetten et al., 1989.

reorganization of the Order *Methanobacteriales* be made with these two species placed in different families (D. Grahame, Methane Genes, Genomes, and Genesis Symposium S236, ASM General Meeting 1998).

The published data on the CODHs of the methanogenic bacteria indicate that all of the CODHs from these methanogens are $F_{420}$-nonreactive (DeMoll et al., 1987; Grahame and Stadtman, 1987; Terlesky et al., 1989) with the exception of the $F_{420}$-linked CODH purified from *Mtx. soehngenii* (Jetten et al., 1989). The results from this species are in doubt due to the fact that this species had been found to be an impure culture (DSM

Catalogue of Strains 1996), but it is unlikely that the contaminant would carry a CODH that is coenzyme $F_{420}$-dependent. The CODH activity from the acetotroph, *Mst. concilii* was found to be $F_{420}$-linked, although the activity was found to be low using this electron acceptor in comparison to the activity detected with MV as the electron acceptor. The percentage of the observed activity of the $F_{420}$ activity compared to the MV (expressed in $H_2$ equivalents) activity (the ratio of the $F_{420}$ to the MV activity) in *Mst. concilii* is 1.6%, similar to the reported value for the CODH found in *Mtx. soehngenii*, with a relative activity of 1.5%.

### 5.1.3. $F_{420}H_2$ Dehydrogenase Activity in *Methanosphaera stadtmanae*

An alternative mechanism for the reduction of cofactor $F_{420}$ is through the enzyme, $F_{420}H_2$ dehydrogenase. This enzyme was discovered and purified from the methanol-utilizing methanogen, *Methanolobus tindarius* (Haase et al., 1992) and also from *Ms. mazei* strain Gö1 (Abken and Deppenmeier, 1997). The enzyme activity is measured in the direction of the oxidation of reduced cofactor $F_{420}$ ($F_{420}H_2$), and the proposed physiological role for this enzyme in both *Ml. tindarius* and *Ms. mazei* growing on methanol is providing the electrons from $F_{420}H_2$ (reduced via the reverse methanogenic pathway in the methanol methanogenesis pathway) to the heterodisulfide reduction reaction performed by the heterodisulfide reductase in those methanogens in which this step is cofactor $F_{420}$-linked. Since *Msph. stadtmanae* is deficient in a means of reducing $F_{420}$, this enzyme may be a possible enzymatic mechanism for the reduction of this cofactor (opposite to that of the direction used in both *Ml. tindarius* and *Ms. mazei*).

Using the method of assay of this enzyme initially described by Deppenmeier et al (Deppenmeier et al., 1990) and used for the purification of the enzyme from *Ml. tindarius* (Haase et al., 1992), low, but measurable levels of $F_{420}H_2$ dehydrogenase activity were detected in the crude and cell-free extracts of *Msph. stadtmanae* (Wong et al., 1994). The assay system described requires 40 mM potassium phosphate buffer, pH 7.0, 20% sucrose, $F_{420}H_2$, 0.5 mM methyl viologen, and 0.5 mM metronidazole (MTZ). The essential reaction is described as follows:

$$F_{420}H_2 + MV_{(oxidized)} \rightleftharpoons F_{420} + MV_{(reduced)}$$

The problem with this system is that the absorption maxima for both of these electron carriers are very close to each other and can interfere with the measurement of the progression of the reaction, both would show an increase in absorbance in this system as oxidation of cofactor $F_{420}$ results in an increase in absorbance at 401 nm and reduction of MV results in an increase in absorbance at 560 nm. To solve this inherent problem, Deppenmeier's group added MTZ to the reaction mixture, which provides a sink for the electrons once the MV is reduced:

$$MV_{(reduced)} + MTZ_{(oxidized)} \rightarrow MV_{(oxidized)} + MTZ_{(reduced)}$$

Once the MTZ is reduced, it decomposes and has no further influence in the reaction system. To confirm this, tests were performed on the enzyme assay system without the addition of the enzyme extract in order to determine whether a chemical reaction could occur between these electron carriers directly. The results shown (Table 5.3) indicate that reduced cofactor $F_{420}$ cannot serve to reduce the artificial dye, methyl viologen, with or without metronidazole without the presence of an enzymatic factor, like the $F_{420}H_2$ dehydrogenase.

### 5.1.3.1. Levels of $F_{420}H_2$ Dehydrogenase Activity in *Msph. stadtmanae*

The preliminary study into the presence of the $F_{420}H_2$ dehydrogenase activity of *Msph. stadtmanae* was to determine the levels of the enzyme activity in the extracts of the cell, and to determine whether this could be a side reaction of an already described enzyme activity in the cell, the $NADP^+$:$F_{420}$ oxidoreductase, which is being studied elsewhere in this research laboratory.

The $F_{420}H_2$ dehydrogenase activity levels were determined in the anaerobic extracts of *Msph. stadtmanae* and were found to be very low (Table 5.4) in comparison to the published findings of the first described activity in *Ml. tindarius*.

**Table 5.3 - Chemical Oxidation of Assay Components Survey**

| Assay Components | Observed Specific Activity (nmoles $F_{420}H_2$ oxidized/min/mL) |
|---|---|
| $F_{420}H_2$ alone | <0.01 |
| $F_{420}H_2$ + MV | <0.01 |
| $F_{420}H_2$ + MTZ | <0.01 |
| $F_{420}H_2$ + MTZ + MV | <0.01 |
| $F_{420}H_2$ + extract | <0.01 |
| $F_{420}H_2$ + MTZ + extract | <0.01 |
| $F_{420}H_2$ + MTZ + MV + extract | 0.3 ± 0.03 |

Note: Assay buffer: 40 mM K-$PO_4$, 20% sucrose, pH 7.0.

The levels of this enzyme activity are logical when one takes into account that the enzyme in *Ml. tindarius* is required in the methanogenic pathway and hence has a bioenergetic role, while in *Msph. stadtmanae*, the role of the enzyme would be in the cell biosynthetic processes, where reduced $F_{420}$ is used as a source of electrons for biosynthesis through the enzyme, $NADP^+$:$F_{420}$ oxidoreductase. In addition, the levels of cofactor $F_{420}$ in *Msph. stadtmanae* are quite low with respect to the other methanogenic bacteria, therefore there would be no need for high levels of this enzyme activity since there is a very low level of electron donor/acceptor in the cell and its role in *Msph.*

**Table 5.4 - Assay for $F_{420}H_2$ Dehydrogenase Activity (in Crude Extract)**

| Organism | Specific Activity (nmoles $F_{420}H_2$ oxidized/min/mg) |
|---|---|
| *Methanosphaera stadtmanae* | 0.3 ± 0.03 |
| *Methanolobus tindarius* | 260 ± 30 |
| *Methanolobus tindarius* (Haase et al., 1992) | 200 |

Note: Assay buffer: 40 mM K-$PO_4$, 20% sucrose, pH 7.0.

*stadtmanae* would be to reduce cofactor $F_{420}$, not to oxidize $F_{420}H_2$ as the assay system used currently measures.

A valid question was asked early on whether this $F_{420}H_2$ dehydrogenase activity was an artifact, or a side reaction of another enzyme, the $NADP^+$:$F_{420}$ oxidoreductase. This is because the oxidoreductase could perform the oxidation reaction (ie., enzyme is reversible) that the dehydrogenase assay tests, and in crude systems, it is not known whether the electron acceptor used for the assay of the dehydrogenase, MV, could also serve as an electron acceptor in the oxidoreductase reaction.

This question may be answered in 3 ways: preliminary results on the latter activity indicate an enzyme that requires a high amount of ammonium sulphate in order to precipitate the protein, the enzyme can be studied aerobically, and the activity of the enzyme appears to increase on storage at 4°C (D. Elias, personal communication).

For comparison, the activity of the $F_{420}H_2$ dehydrogenase displays a differential ammonium sulphate precipitation pattern than the oxidoreductase (Table 5.5), the majority of the activity is precipitated prior to the point at which the oxidoreductase is precipitated.

In the same study, the activity of the enzyme was shown to be reduced on storage, while the oxidoreductase had actually increased in activity at the same time (more on the stability of the dehydrogenase activity later). As to the air-stability of the two enzymes,

**Table 5 - $(NH_4)_2SO_4$ Fractionation Distribution of $NADP^+$:$F_{420}$ Oxidoreductase and $F_{420}H_2$ Dehydrogenase**

| Fraction | $NADP^+$:$F_{420}$ Oxidoreductase (nmole $F_{420}H_2$/min/mg protein) | | $F_{420}H_2$ Dehydrogenase (nmole $F_{420}H_2$/min/mg protein) | |
|---|---|---|---|---|
| | Day 1 | Day 4 | Day 1 | Day 4 |
| **40% pellet** | 0.63 ± 0.06 | 5.0 ± 0.5 | <0.01 | <0.01 |
| **40-70% pellet** | 4.0 ± 0.2 | 13.2 ± 1.3 | 3.67 ± 0.4 | <0.01 |
| **70% supernatant** | 256 ± 15 | 346 ± 40 | 3.15 ± 0.3 | <0.01 |

Note: Both assays were performed in 40 mM K-$PO_4$ buffer, pH 7.0 and in the direction of $F_{420}H_2$ oxidation.

the $F_{420}H_2$ dehydrogenase is extremely $O_2$-sensitive, significantly less stable than the enzyme found in *Ml. tindarius*, with the activity declining to undetectable even after one day under aerobic conditions at 4°C (see Table 5.6), whereas the enzyme from *Ml. tindarius* could be handled aerobically for its purification (Haase et al., 1992).

**Table 5.6 - $O_2$-sensitivity of $F_{420}H_2$ dehydrogenase activity from *Msph. stadtmanae*.**

| Sample | Specific Activity Day 0 (nmoles $F_{420}H_2$/min/mg) | Specific Activity Day 1 (nmoles $F_{420}H_2$/min/mg) |
|---|---|---|
| **no additions** | 0.30 ± 0.05 | <0.01 |
| **Extract plus KCl (1.0 M)** | 0.30 ± 0.05 | <0.01 |
| **Extract plus $(NH_4)_2SO_4$ (1.0 M)** | 0.30 ± 0.05 | <0.01 |
| **Extract plus KCl (1.0 M) (assay w/ 0.15 M $(NH_4)_2SO_4$)** | 5.28 ± 0.5 | 0.19 |

The elevated activity in the presence of KCl in the extract was interesting, and this will be discussed further in the following section.

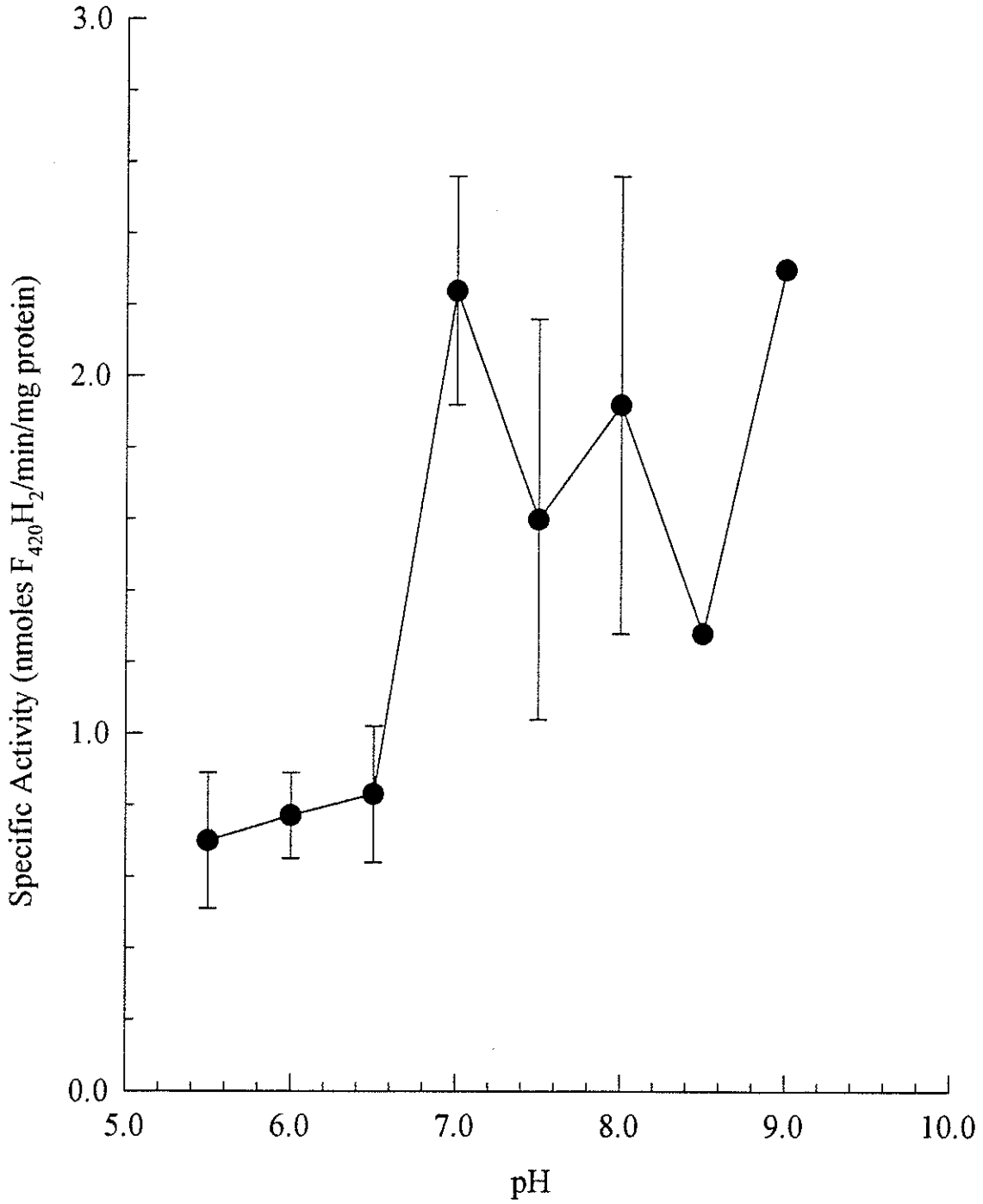
The reason for the general instability of the $F_{420}H_2$ dehydrogenase activity in this experiment is not known, later experiments demonstrated that the $F_{420}H_2$ dehydrogenase activity was stable under specific conditions (see later in Section 5.1.3.2.3.).

### 5.1.3.2. Characteristics of $F_{420}H_2$ Dehydrogenase Activity

#### 5.1.3.2.1. pH, Ion Effects

A preliminary study was performed on the $F_{420}H_2$ dehydrogenase activity of *Msph. stadtmanae* in terms of its properties and characteristics. A pH activity profile was performed early on, and based on its results, the activity did not have a precise maximum activity at a particular pH (figure 1). A possible problem may have been the fact that the buffers used were at low enough concentrations such that with the number of solutions added, the final pH was not the same as the initial pH of the buffer chosen (buffers were set at a concentration of 40 mM, which would have been reduced to a certain extent due

**Figure 1** - Preliminary pH activity profile for the $F_{420}H_2$ dehydrogenase activity from *Methanosphaera stadtmanae*. Assays were performed in 40 mM concentration buffers. Error bars represent the range of values obtained. (points are averages of triplicate assays). pH given is that of the assay tube prior to use.


3.0
2.0
1.0
0.0
Specific Activity (nmoles $F_{420}H_2$/min/mg protein)
5.0
6.0
7.0
8.0
9.0
10.0
pH

to the additions made) and this was found to be the case in testing the final pH's (ranging between pH 6.3-7.4) of the assay tubes after all of the components were added.

In order to correct this, the buffer salts were adjusted to a concentration of 150 mM in order to prevent any possible pH change caused by dilution and/or pH altering effects of the solutions or extracts being added to the assay tube. The buffers used were: piperazine at pH 5.0; 2-morpholino-ethanesulfonic acid (MES) at pH 6.0; potassium phosphate at pH 7.0; Tris-Cl at pH 8.0; and glycine at pH 9.0. It was during this survey that it was determined several chemical effects occurred with this $F_{420}H_2$ dehydrogenase assay system.

First of all, assay of this enzyme could not be performed at a pH greater than 8.5. At these values, the methyl viologen, used as the electron acceptor for the $F_{420}H_2$ dehydrogenase, would chemically be converted to an apparently reduced form in the absence of added extract, the assay tubes were found to have turned the characteristic blue colour, the colour of the MV when it is reduced. This property of the MV was previously discussed in Chapter 4. The pH profile determined was found to be erratic, with the maximum activity at the pH normally used to assay the enzyme (pH 7.0), but with low activity elsewhere except for a peak at pH 5.0 (see Figure 2) where it appears that there are two maxima in this pH profile. The reason for this activity at pH 5.0 is not known, since piperazine is not a buffer salt normally used in enzyme studies it may have other effects on the activity of the $F_{420}H_2$ dehydrogenase.

Another property, this time directly related to the $F_{420}H_2$ dehydrogenase activity, was discovered. The activity was found to be significantly affected by the concentration of the potassium phosphate used for the buffer in the assay tube. Several experiments were performed to study this effect.

The effect of potassium phosphate concentration on the activity of the $F_{420}H_2$ dehydrogenase was determined (see Figure 3). The activity of the enzyme appeared to reach a maximum at a concentration of 200 mM potassium phosphate, with an enhancement in detectable activity by almost thirty-fold compared to the standard assay concentration of potassium phosphate and fifty-fold compared to assay in the absence of

**Figure 2 -** pH Activity profile using higher concentration buffer systems. All buffers used at 150 mM concentration. Error bars represent the range of data obtained. (points are averages of triplicate assays).

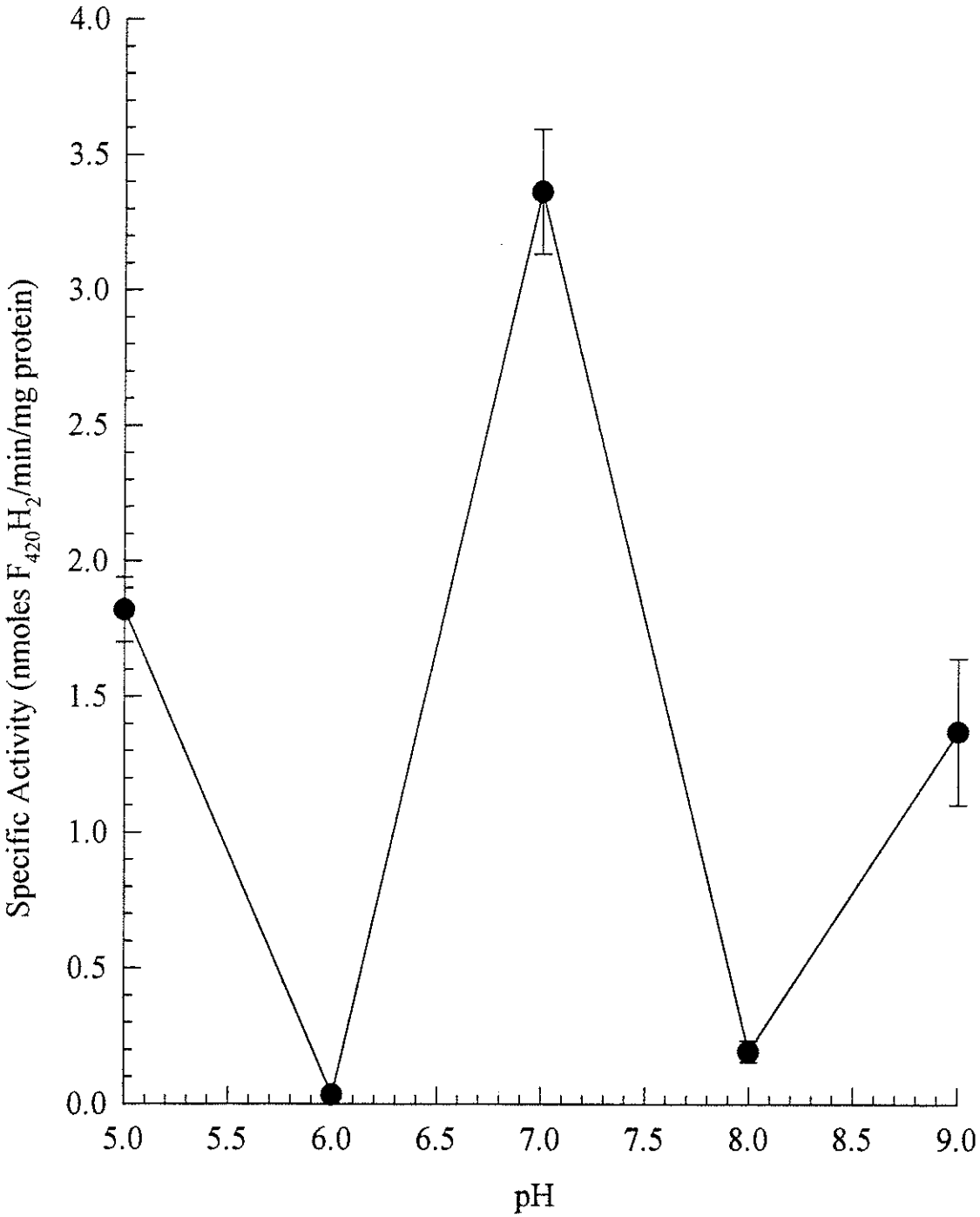
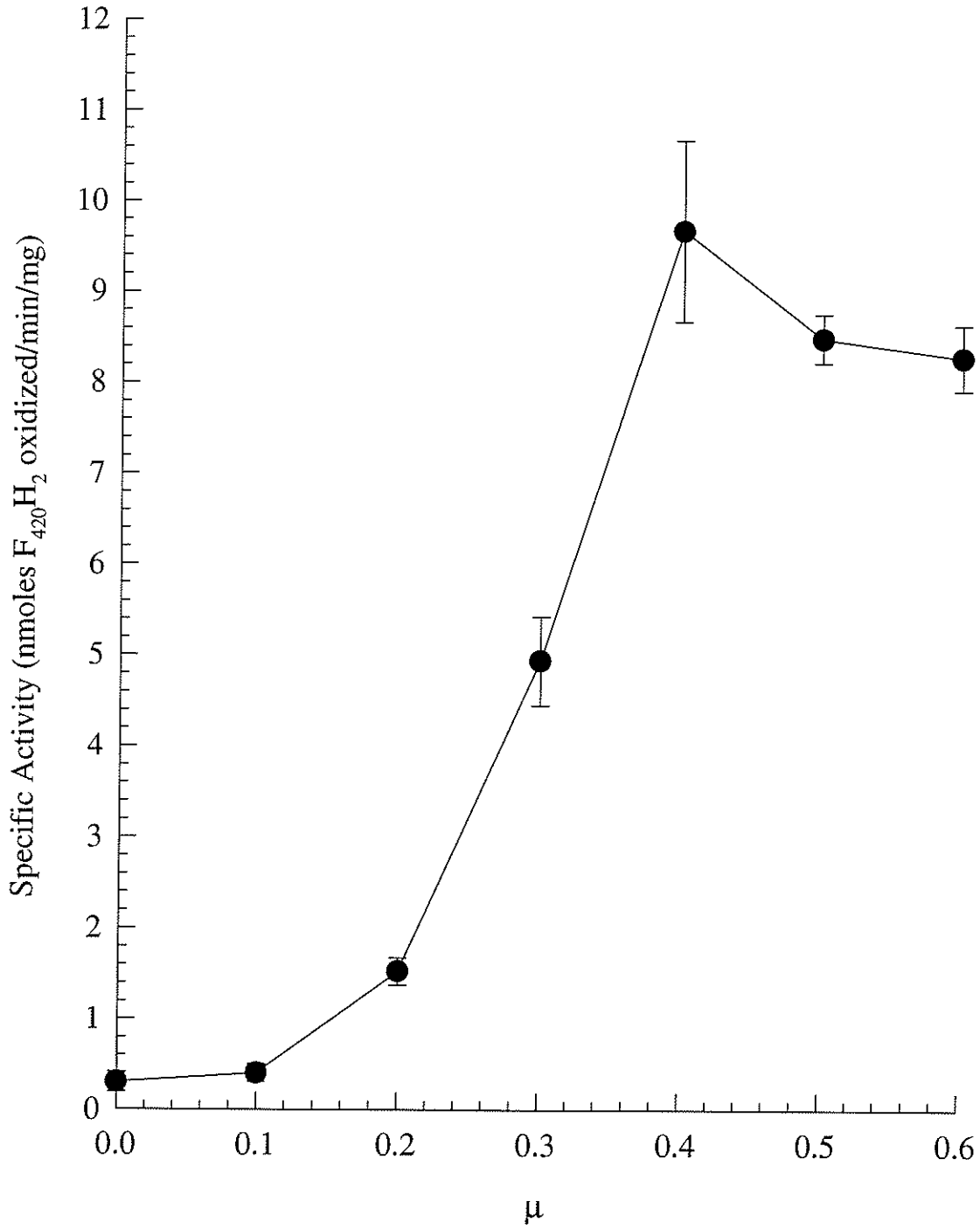

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Specific Activity (nmoles $F_{420}H_2$/min/mg protein)
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

**Figure 3 -** Effect of ionic strength of potassium phosphate on activity of $F_{420}H_2$ dehydrogenase from *Methanosphaera stadtmanae*. Error bars represent the range of data obtained. All assays performed at pH 7.0. (points are average of triplicate assays).


Specific Activity (nmoles $F_{420}H_2$ oxidized/min/mg)
12
11
10
9
8
7
6
5
4
3
2
1
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
μ

potassium phosphate (performed with 50 mM HEPES, pH 7.0).

To test for a general salt effect, other salt species were used. Other salts tested included sodium chloride, potassium chloride, ammonium sulphate, and sodium phosphate, all tested at 150 mM concentrations. The results are shown on Table 5.7.

**Table 5.7 - Effect of Salts on $F_{420}H_2$ Dehydrogenase Activity in *Methanosphaera stadtmanae***

| Salt Species | Concentration (mM) | Ionic Strength ($\mu$) | Specific Activity (nmoles $F_{420}H_2$/min/mg protein) |
|---|---|---|---|
| $K\text{-}PO_4$ | 40 | 0.12 | 0.30 ± 0.03 |
| $K\text{-}PO_4$ | 150 | 0.45 | 6.10 ± 0.22 |
| $(NH_4)_2SO_4$ | 150 | 0.57 | 5.28 ± 0.35 |
| KCl | 150 | 0.27 | 3.87 ± 0.16 |
| $Na\text{-}PO_4$ | 150 | 0.49 | 3.60 ± 0.18 |
| NaCl | 150 | 0.27 | 2.43 ± 0.08 |
| AMP | 1 | 0.12 | 0.30 ± 0.03 |
| ATP | 1 | 0.12 | 0.30 ± 0.03 |

- Note: all assays were performed in 40 mM $K\text{-}PO_4$ buffer (except in the 150 mM $K\text{-}PO_4$ and 150 mM $Na\text{-}PO_4$ assay), pH 7.0

There is a general salt effect, but to a smaller degree compared to the enhancement provided by potassium phosphate. Ammonium sulphate, at the same molar concentration as potassium phosphate, enhances the activity of the $F_{420}H_2$ dehydrogenase by almost the same amount, however, the ionic strength of the two assay solutions were not identical, and using ionic strength as a comparative guideline, there is no strong ionic strength effect (eg., with $Na\text{-}PO_4$ at $\mu = 0.47$: 3.6 nmoles/min/mg, with $K\text{-}PO_4$ at $\mu = 0.45$: 6.1 nmoles/min/mg). The effect of the same molar concentration of sodium phosphate is almost half the final activity determined for potassium phosphate, so the presence of specifically or solely phosphate does not enhance the activity. However, there does appear to be an effect by the addition of KCl, as judged by the higher activity

in the presence of this salt compared to even Na-$PO_4$. It is possible that the combined effect of the $PO_4^{3-}$ and $K^+$ are required for maximal activity of the dehydrogenase from *Msph. stadtmanae*.

Salts of ATP and AMP were also tested for their ability to stimulate $F_{420}H_2$ dehydrogenase activity, since there may be an effect relating to the phosphate levels in the cell. The results indicate that high levels of either of these molecules do not affect the activity of the dehydrogenase.

The response to increasing salt concentration in the assay buffer was performed also with KCl (figure 4) and $(NH_4)_2SO_4$ (figure 5). The response to these salts are similar to that shown for potassium phosphate in that increasing salt increases the level of activity, but differs by exhibiting an essentially linear profile throughout the concentration range tested (the activity peaks and plateaus after reaching 200 mM with K-$PO_4$).

A pH profile for the activity of the $F_{420}H_2$ dehydrogenase was finally determined with the findings concerning the potassium phosphate effect. All the buffers used in the pH profile included 200 mM potassium phosphate. However, a narrower range for the pH profile had to be used due to the methyl viologen problem, but the apparent values for the pH values 8.0 and 8.5 are included (Figure 6). The pH where maximum activity was found to occur was at pH 7.0. This is very similar to the reported value of the pH optimum of pH 6.8 for the enzyme in *Ml. tindarius* (Haase et al., 1992) while the pH optimum for the enzyme from *Ms. mazei* was reported at pH 8.5 (Abken and Deppenmeier, 1997).

Also noted is the increase in activity at pH 5.0 from the activities detected at either pH 5.5 or 6.0. The reason for this is not known, since there should be no interference by the methyl viologen at this pH value, but this increase was also observed in the previously performed pH profile using piperazine as the buffer agent. It is unlikely that the pH itself causes the increase in absorbance since at low pH, cofactor $F_{420}$ loses absorbance (ie., becomes colourless) as the pH is lowered (Cheeseman et al,1972). The buffer used for both pH 5.0 and 5.5 was citrate-phosphate in the profile in figure 6. There may be some effect by the phosphate, but the phosphate concentration in

**Figure 4 -** Effect of ionic strength of potassium chloride on $F_{420}H_2$ dehydrogenase activity from *Methanosphaera stadtmanae*. All assays performed at pH 7.0 in 40 mM potassium phosphate. (points are average of triplicate assays).

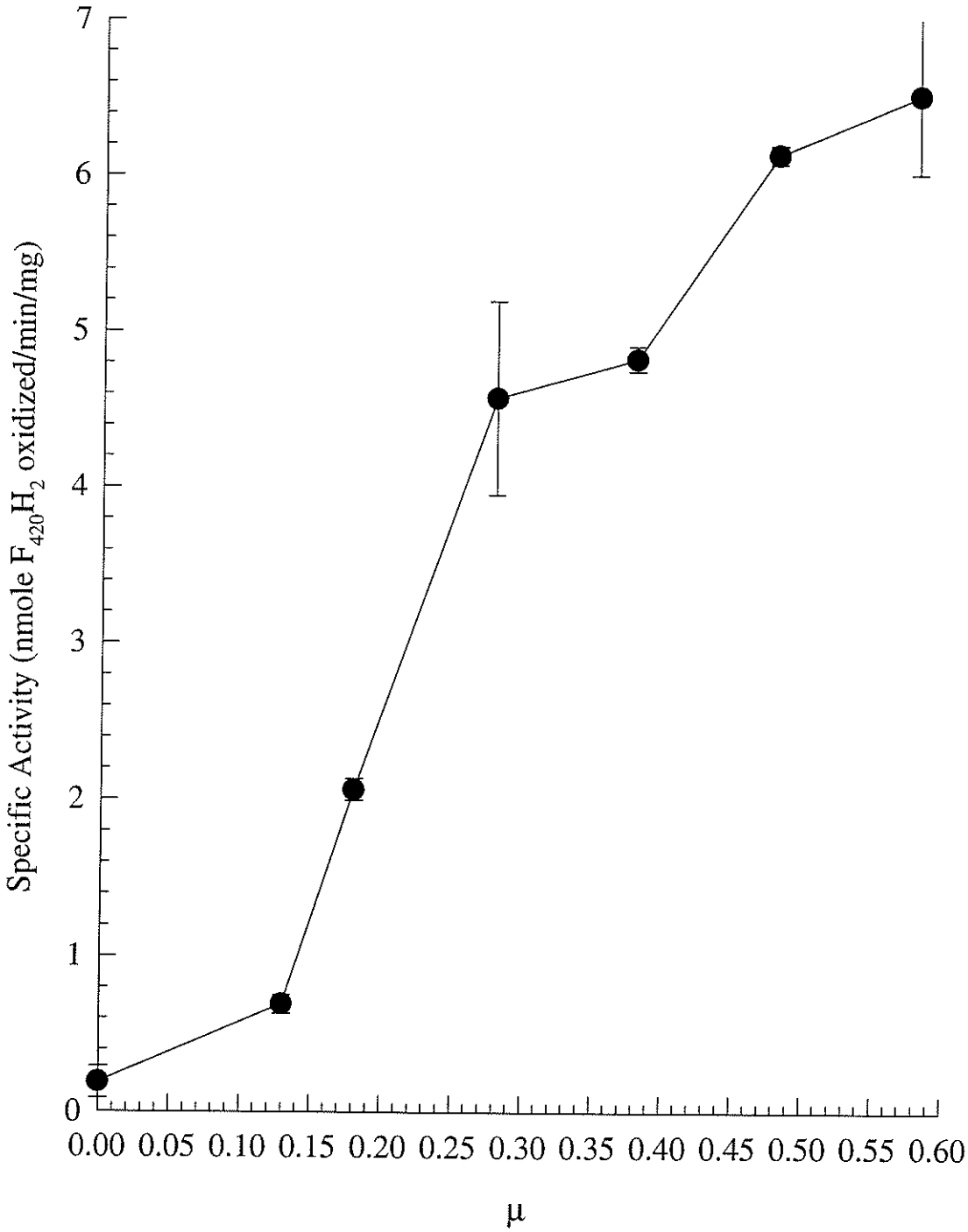

Specific Activity (nmole $F_{420}H_2$ oxidized/min/mg)
0
1
2
3
4
5
6
7
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35 0.40 0.45 0.50 0.55 0.60
μ

**Figure 5 -** Effect of ionic strength of ammonium sulphate on $F_{420}H_2$ dehydrogenase activity from *Methanosphaera stadtmanae*. All assays performed at pH 7.0 in 40 mM potassium phosphate. (points are average of triplicate assays).

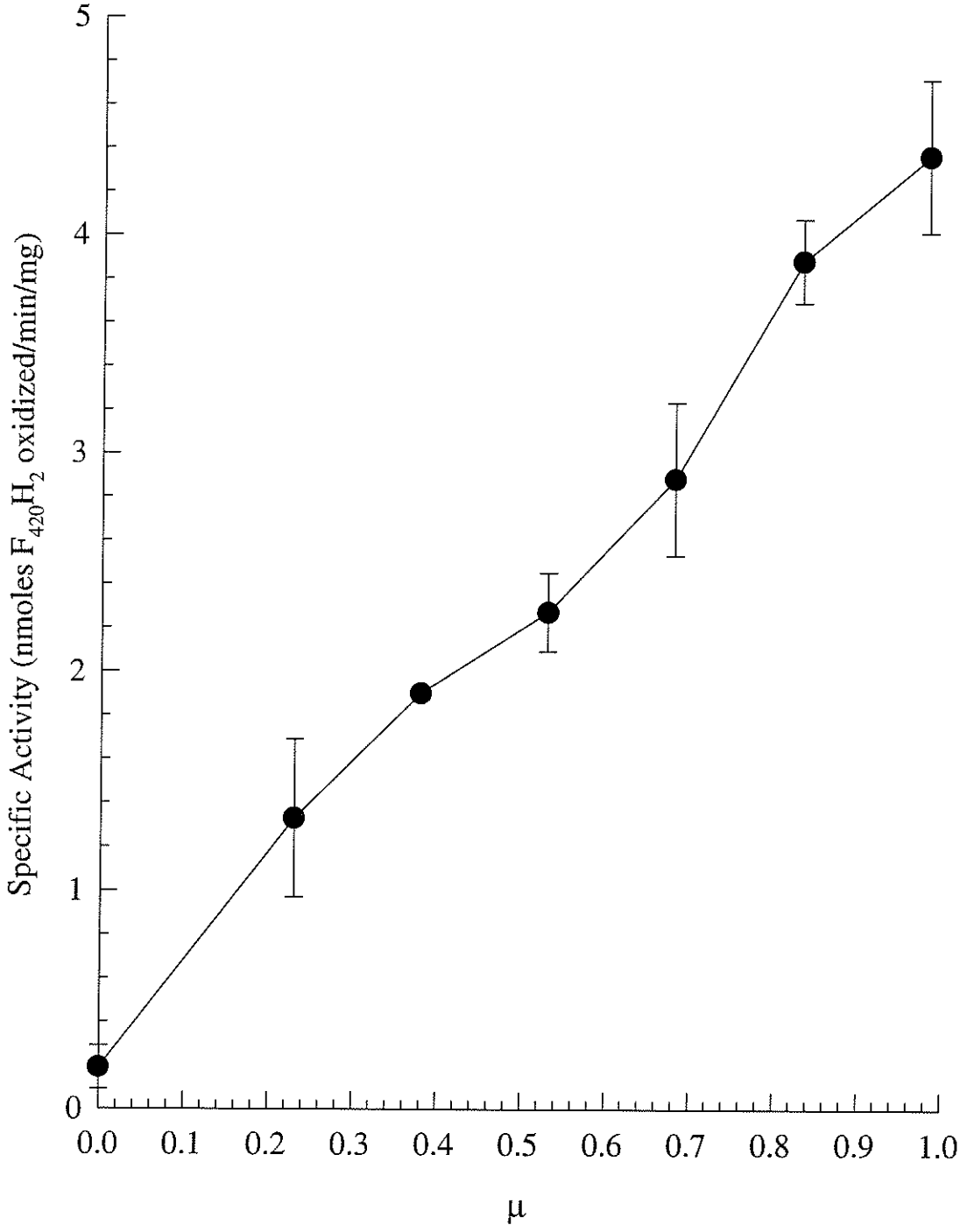
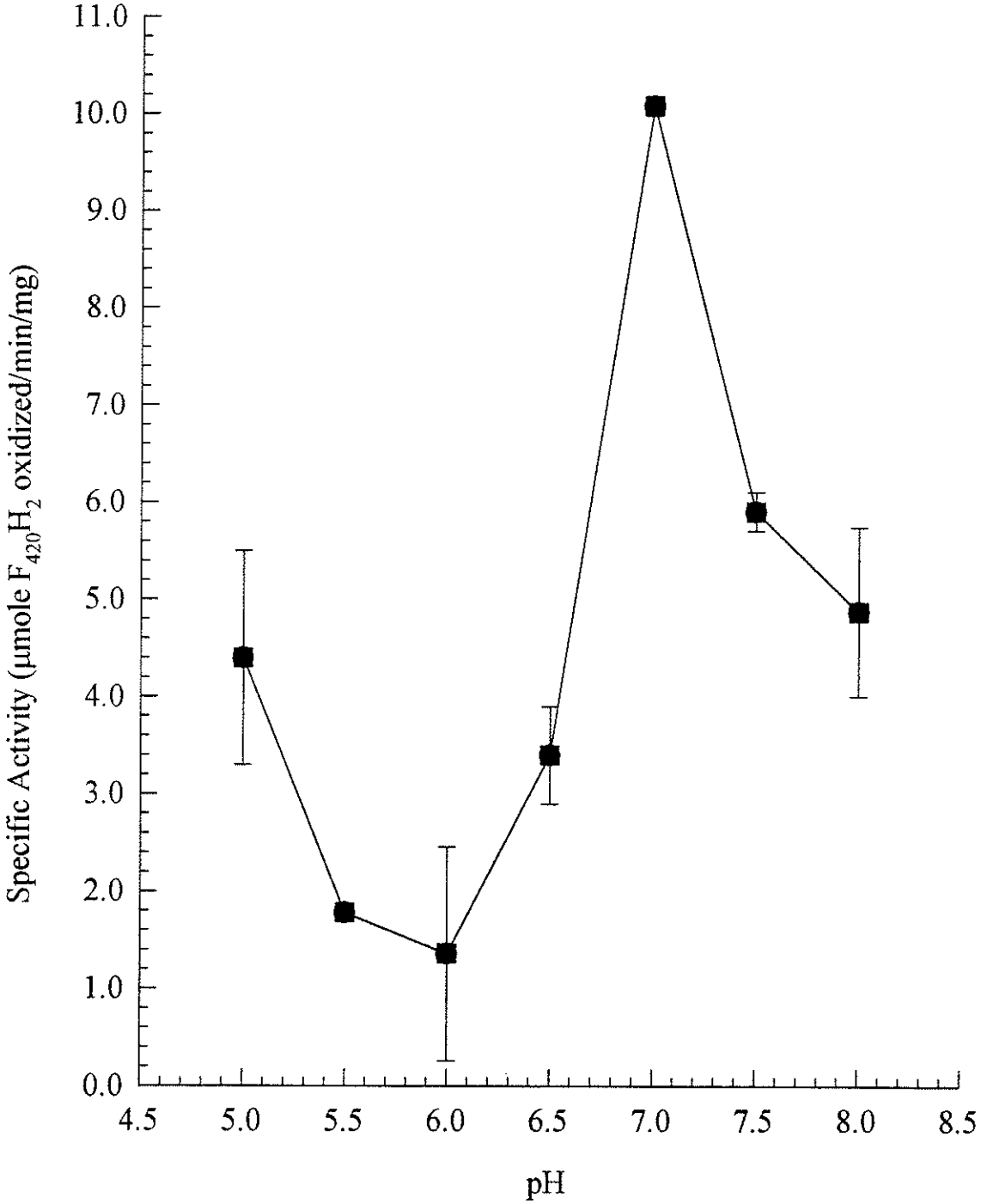

Specific Activity (nmoles $F_{420}H_2$ oxidized/min/mg)
5
4
3
2
1
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
μ

**Figure 6 -** pH Profile for the $F_{420}H_2$ dehydrogenase activity from *Methanosphaera stadtmanae*. All assays performed in the presence of 200 mM potassium phosphate. (points are average of triplicate assays).


11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Specific Activity (µmole $F_{420}H_2$ oxidized/min/mg)
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
pH

all buffers used was 200 mM and at higher concentrations it was shown that the enhancing effect levels off and the molar concentrations (150 mM) of the buffer for both pH 5.0 and 5.5 are the same. For most of the following studies, 150 to 200 mM K-$PO_4$ was used.

#### 5.1.3.2.2. Electron Acceptor Specificity of $F_{420}H_2$ Dehydrogenase Activity

In addition to methyl viologen, other electron acceptors have been used for the assay of the $F_{420}H_2$ dehydrogenase activity in other Archaea. These have included benzyl viologen in the assay of the $F_{420}$ hydrogenase of *Ms. barkeri* (Keltjens and Vogels, 1992) and various quinones for the activity purified from *A. fulgidus* (Kunow et al., 1994). For this study, several electron acceptors were tested in the assay of the $F_{420}H_2$ dehydrogenase activity from *Msph. stadtmanae*. These included benzyl viologen, FAD, FMN, NAD, ferredoxin, and cytochrome $c_3$. $NADP^+$ was considered, but since this is a crude system and there is a pre-existing $NADP^+$:$F_{420}$ oxidoreductase, this electron acceptor was omitted. The results of this survey are shown on Table 5.8.

The results indicate that benzyl viologen can replace MV in the assay, but the absorbance changes were not linear, while the others (eg., cytochrome, ferredoxin, $NAD^+$) do not appear to serve as electron acceptors for the $F_{420}H_2$ dehydrogenase. The flavins do both exhibit oxidation of $F_{420}H_2$, however it was found that $F_{420}H_2$ can donate electrons in the absence of cell extract to both of the flavins.

#### 5.1.3.2.3. Growth Phase and $F_{420}H_2$ Dehydrogenase Activity

A study on the effect of the growth phase on the activity of the $F_{420}H_2$ dehydrogenase may provide insight into the regulation of this activity and for future studies on this enzyme activity, the effect of the growth phase on the activity of the enzyme is necessary to determine an appropriate time to harvest the cells containing maximum activity. The results of this survey are shown on Figure 7. The figure shows a slight drop in the specific activity in mid- to late-logarithmic phase with the maxima at early logarithmic and stationary phase. Overall, there does not appear to be significant fluctuations in the

Table 8 - Electron Acceptor Specificity of $F_{420}H_2$ Dehydrogenase from *Msph. stadtmanae*.

| Electron Acceptor | Concentration | Specific Activity (nmoles $F_{420}H_2$/min/mg) |
|---|---|---|
| **Methyl Viologen** | 0.5 mM | 3.4 ± 0.3 |
| **Benzyl Viologen** | 0.5 mM | 6.8 ± 0.7 |
| **FMN (plus extract)** | 0.5 mM | *16.0 ± 0.9<br>$\Delta A = 0.019\ min^{-1}$ |
| **FMN (no extract)** | 0.5 mM | $\Delta A = 0.020\ min^{-1}$ |
| **FAD (plus extract)** | 0.5 mM | *30.4 ± 1.4<br>$\Delta A = 0.031\ min^{-1}$ |
| **FAD (no extract)** | 0.5 mM | $\Delta A = 0.037\ min^{-1}$ |
| **$NAD^+$** | 10 mM | <0.01 |
| **Ferredoxin** | 1 mg/mL | <0.01 |
| **Cytochrome $c_3$** | 1 mg/mL | <0.01 |

* - apparent specific activity before correction for rate of chemical electron transfer.

specific activity of the $F_{420}H_2$ dehydrogenase throughout the growth phase.

#### 5.1.3.2.4. Stability of Enzyme Activity

As a prelude to possible purification, a consideration about the stability of the activity must be addressed. It has already been stated that the activity is not stable when handled aerobically (See Section 4.1.3.1.). When handled under strictly anaerobic conditions, this activity may be very stable. Concern was expressed during the early studies on this activity when it had appeared that the activity was not stable during the differential ammonium sulphate precipitation patterns analysis. The stability of the activity under anaerobic conditions is shown (figure 8). This study was performed at room temperature since for future work, handling at room temperature would be easier in the anaerobic chamber. This study showed that there was a slight increase in activity after 24 hours, but the activity declined rapidly thereafter so that by the third day there was only 5% of the original activity detected.

**Figure 7 -** Growth phase dependence on the level of the $F_{420}H_2$ dehydrogenase activity from *Methanosphaera stadtmanae*. All assays were performed at pH 7.0 in 200 mM potassium phosphate. (points are average of triplicate assays).

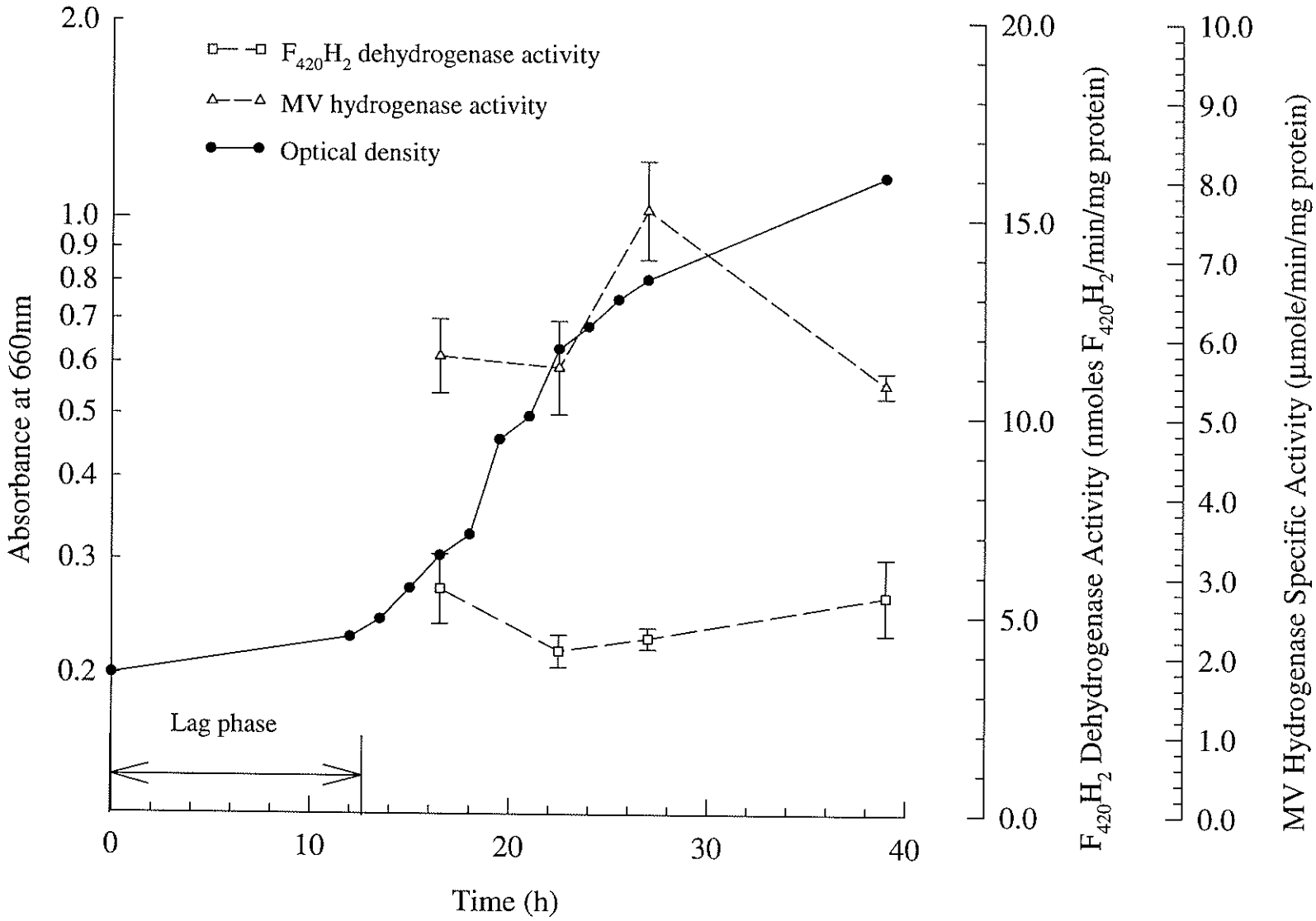
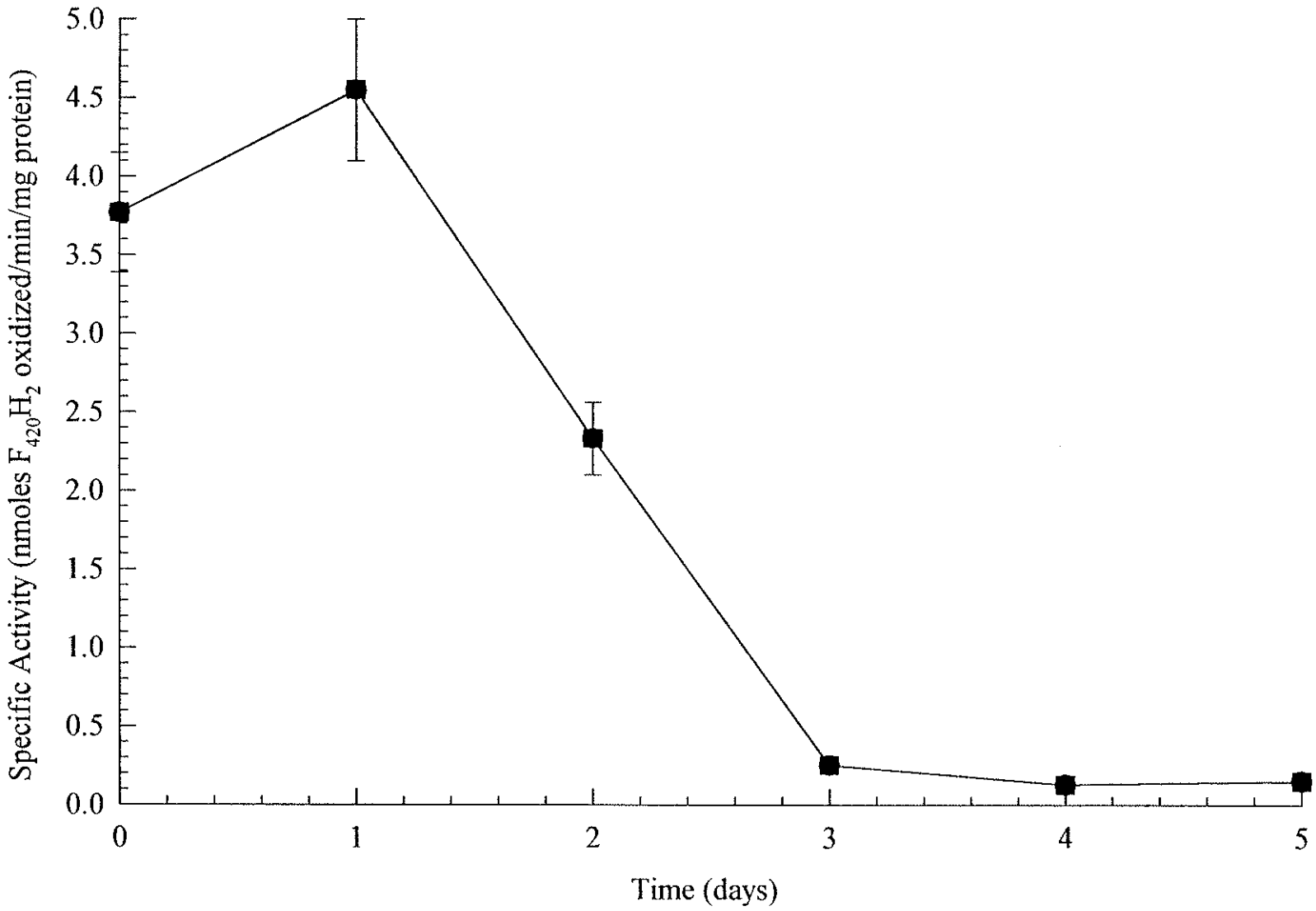

$F_{420}H_2$ dehydrogenase activity
MV hydrogenase activity
Optical density
Absorbance at 660nm
2.0
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
Lag phase
0
10
20
30
40
Time (h)
20.0
15.0
10.0
5.0
0.0
$F_{420}H_2$ Dehydrogenase Activity (nmoles $F_{420}H_2$/min/mg protein)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
MV Hydrogenase Specific Activity (µmole/min/mg protein)

**Figure 8 -** Stability of $F_{420}H_2$ dehydrogenase activity. Extract stored at room Temperature for the course of the survey. All assays were performed in 200 mM potassium phosphate, pH 7.0 under an atmosphere of $N_2$. (points are the average of triplicate assays).


Specific Activity (nmoles $F_{420}H_2$ oxidized/min/mg protein)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
Time (days)

A study was then devised to attempt stabilization of the activity with the additions of known enzyme stabilizers. This survey is summarized in Table 5.9. The extracts were stored at 4°C. The survey showed that the activity does not change when the dehydrogenase activity is stored at 4°C (as the control also did not decline in activity), however, the assay system used for this survey did not include the elevated potassium phosphate (as this survey was actually performed prior to that discovery). The presence of the salts potassium chloride and ammonium sulphate displayed elevated levels, as to be expected with the salts effect described earlier. It is interesting to note that the activity recovered in the third day and beyond is similar to the levels obtained in the absence of the elevated salts (approximately 0.3 nmoles/min/mg).

**Table 5.9 - $F_{420}H_2$ Dehydrogenase Activity Stability (Anaerobic)**

| Addition | Specific Activity Day 2 | Specific Activity Day 4 |
|---|---|---|
| **None** | 0.3 | 0.3 |
| **Protease Inhibitors (EDTA, PMSF, Pepstatin A)** | 0.3 | 0.3 |
| **Sucrose (10%)** | 0.3 | 0.3 |
| **Sucrose (20%)** | 0.3 | 0.3 |
| **Glycerol (10%)** | 0.3 | 0.3 |
| **Glycerol (20%)** | 0.3 | 0.3 |
| **Triton X-100 (0.5%)** | 0.3 | 0.3 |
| **Triton X-100 (1.0%)** | 0.4 | 0.3 |
| **KCl (1.0 M)** | 0.8 | 0.9 |
| **$(NH_4)_2SO_4$ (1.0 M)** | 0.8 | 0.7 |

Note: assay buffer: 40 mM potassium phosphate, 20% sucrose, pH 7.0. Specific activities are in nmoles $F_{420}H_2$ oxidized/min/mg protein. Starting specific activity was 0.3 U/mg with no additions.

### 5.1.3.2.5. Inhibitors of the $F_{420}H_2$ Dehydrogenase Activity

In the report of the enzyme purified from *Ml. tindarius* (Haase et al, 1992), there were no data reported concerning any inhibitors of the enzyme, thus it was of interest to find out what, if any, chemical additions to the assay system could impair the activity of the dehydrogenase. For this survey, the choice of the inhibitors were similar to those used in the inhibitor study performed on the MV hydrogenase in the previous chapter (Section 4.2.1.7.). The results of this survey are shown on Table 5.10.

The $F_{420}H_2$ dehydrogenase activity from *Msph. stadtmanae* was inhibited to a certain extent by many of the tested compounds. The most drastic effect was observed with the addition of p-hydroxymercuribenzoic acid (28.6% activity). This compound is a inhibitor for various dehydrogenases and transferases (Zollner, 1989). The addition of azide (47.6%) and the $Hg^{2+}$ (46.3%) produced similar inhibition levels. The addition of the methanogenic inhibitor, 2-bromoethanesulfonic acid, was found to slightly inhibit

**Table 5.10 - Inhibitors of $F_{420}H_2$ Dehydrogenase Activity from *Methanosphaera stadtmanae*.**

| Inhibitor | Concentration | % Relative Activity |
|---|---|---|
| none | - | 100.0[a] |
| $Cu^{2+}$ | 1 mM | 103.7 |
| $Mg^{2+}$ | 1 mM | 100.0 |
| $Cd^{2+}$ | 1 mM | 88.9 |
| $Hg^{2+}$ | 1 mM | 46.3 |
| $CN^-$ | 1 mM | 90.5 |
| $N_3^-$ | 3 mM | 47.6 |
| 2-BES | 5 mM | 76.2 |
| p-HMB | 25 µM | 28.6 |
| Acetylene | 1.0 % | 100.0 |

Note: assay system: 40 mM K-$PO_4$, pH 7.0. All assays were performed in triplicate.
[a] - 100% = 0.5 nmoles $F_{420}H_2$ oxidized/min/mg protein.

(89% activity) the activity of the dehydrogenase which cannot be explained, but does fall within limits of experimental error. The addition of acetylene into the atmosphere has been shown to inhibit the growth of methanogens (Sprott et al., 1982), but it does not affect the activity of the $F_{420}H_2$ dehydrogenase. Copper ions ($Cu^{2+}$) were found to have little effect on the activity of the dehydrogenase (103.7%).

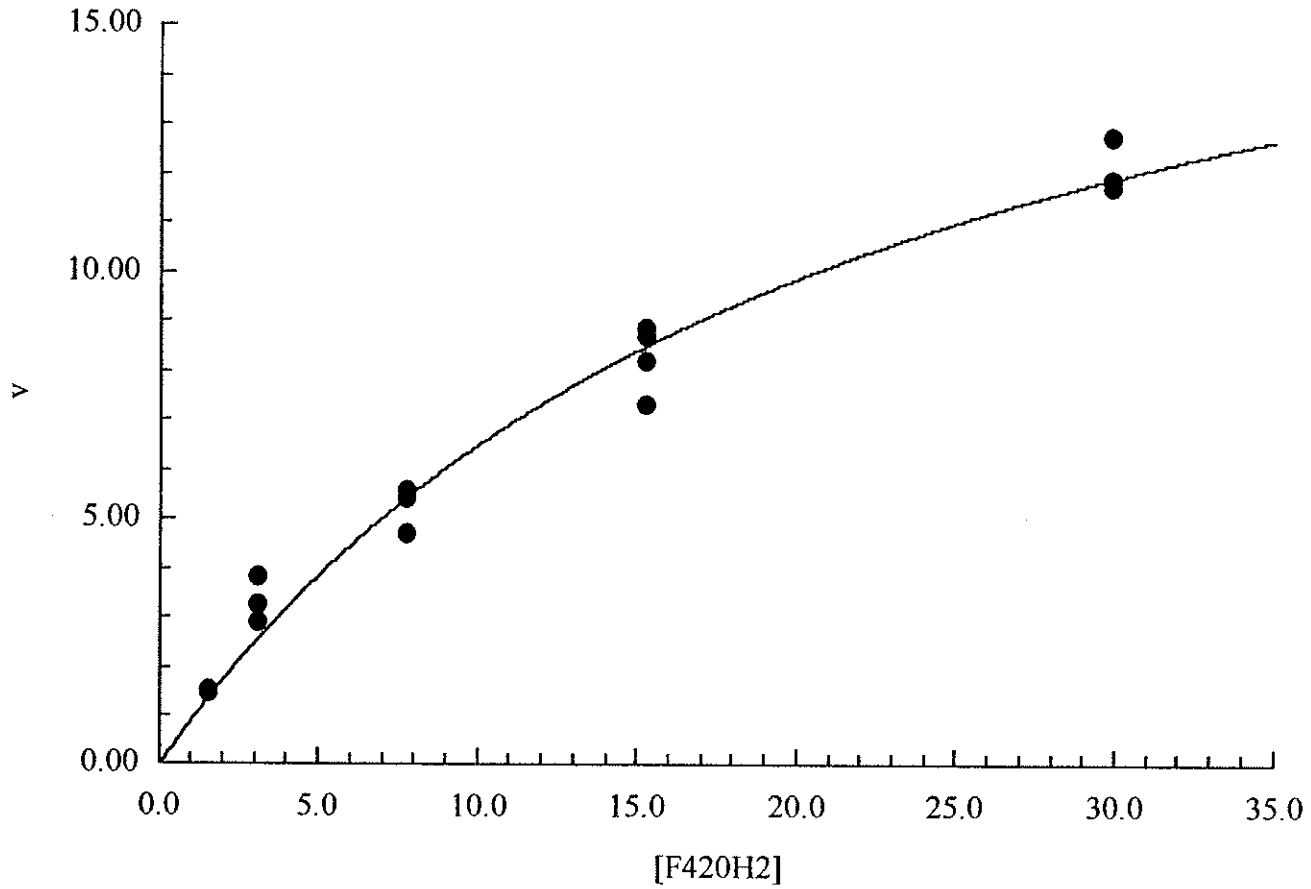
Some of the inhibitors for the dehydrogenase are shared with the hydrogenase (see 4.2.1.8), for example, $Hg^{2+}$ (21.4% hydrogenase activity) and $Cd^{2+}$ (57.1% hydrogenase activity) to a certain extent. Differences include stimulation of hydrogenase by p-HMB and $CN^-$ while the dehydrogenase is greatly inhibited by p-HMB but is not affected by $CN^-$.

#### 5.1.3.2.6. Kinetic Characteristics

The kinetic constants for the enzyme activity found in *Msph. stadtmanae* in crude extract were determined. The values for the $K_m$ and $V_{max}$ were determined after plotting using Michaelis-Menten (Michaelis and Menten, 1913), $K_m$ = 21.5 μM $F_{420}H_2$ , $V_{max}$ = 20.5 nmoles $F_{420}H_2$ oxidized/min/mg protein (figure 9), Lineweaver-Burk (Lineweaver and Burk, 1933), $K_m$ = 18.5 μM $F_{420}H_2$ , $V_{max}$ = 18.9 nmoles $F_{420}H_2$ oxidized/min/mg protein (Figure 10a), Woolf-Augustinsson-Hofstee or Eadie-Hofstee (Hofstee, 1959), $K_m$ = 20.0 μM $F_{420}H_2$ , $V_{max}$ = 18.5 nmoles $F_{420}H_2$ oxidized/min/mg protein (Figure 10b), Hanes-Woolf (Hanes, 1932), $K_m$ = 20.5 μM $F_{420}H_2$ , $V_{max}$ = 18.7 nmoles $F_{420}H_2$ oxidized/min/mg protein (Figure 11a) and Eadie-Scatchard (Scatchard, 1949), $K_m$ = 20.6 μM $F_{420}H_2$ , $V_{max}$ = 19.8 nmoles $F_{420}H_2$ oxidized/min/mg protein (Figure 11b) plots. These various mathematical manipulations of the data confirm the first-order nature of the data and the apparent $K_m$ for the dehydrogenase activity.

The reported value of the $K_m$s for the enzyme in *Ml. tindarius* is 5.4 μM for $F_{420}H_2$ (Haase et al., 1992) and in *Ms. mazei* is 7.0 μM (Abken and Deppenmeier, 1997). The experimentally determined (Michaelis-Menten value) value of 21.5 μM for *Msph. stadtmanae* is more than three times higher than the reported value for *Ml. tindarius*, and almost three times higher than that found in *Ms. mazei*, therefore the enzyme affinity for

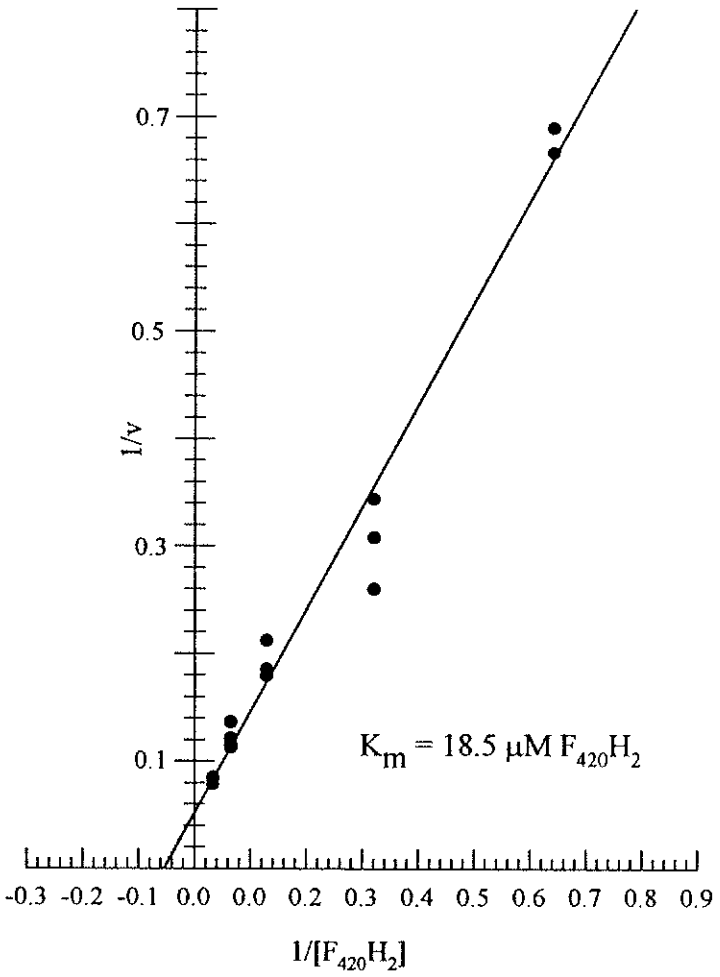
**Figure 9 -** Michaelis-Menten kinetic plot for $F_{420}H_2$ dehydrogenase activity from *Methanosphaera stadtmanae*. $r^2 = 0.998$.




$K_m$ = 21.5 μM $F_{420}H_2$
$V_{max}$ = 20.5 nmoles $F_{420}H_2$/min/mg protein

**Figure 10 -** Reciprocal kinetic replots for $F_{420}H_2$ dehydrogenase activity from *Methanosphaera stadtmanae*. (a) Lineweaver-Burk plot, $r^2 = 0.979$; (b) Woolf-Augustinsson-Hofstee plot, $r^2 = 0.973$.

a) Lineweaver-Burk Kinetic Plot




b) Woolf-Augustinsson-Hofstee Kinetic Plot

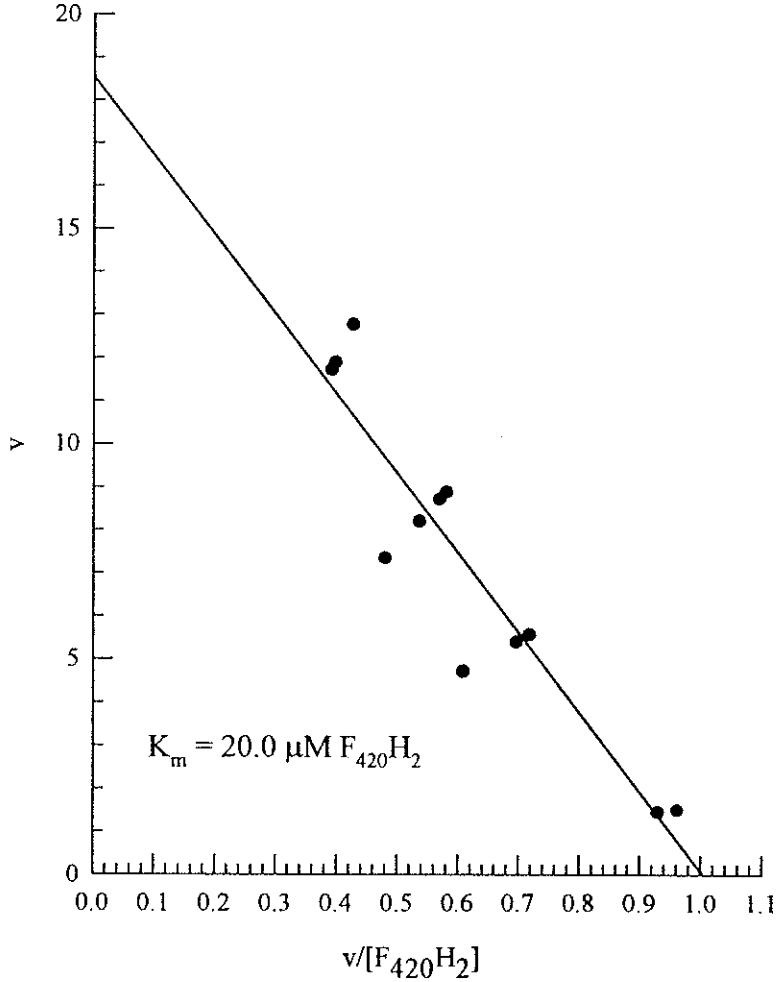
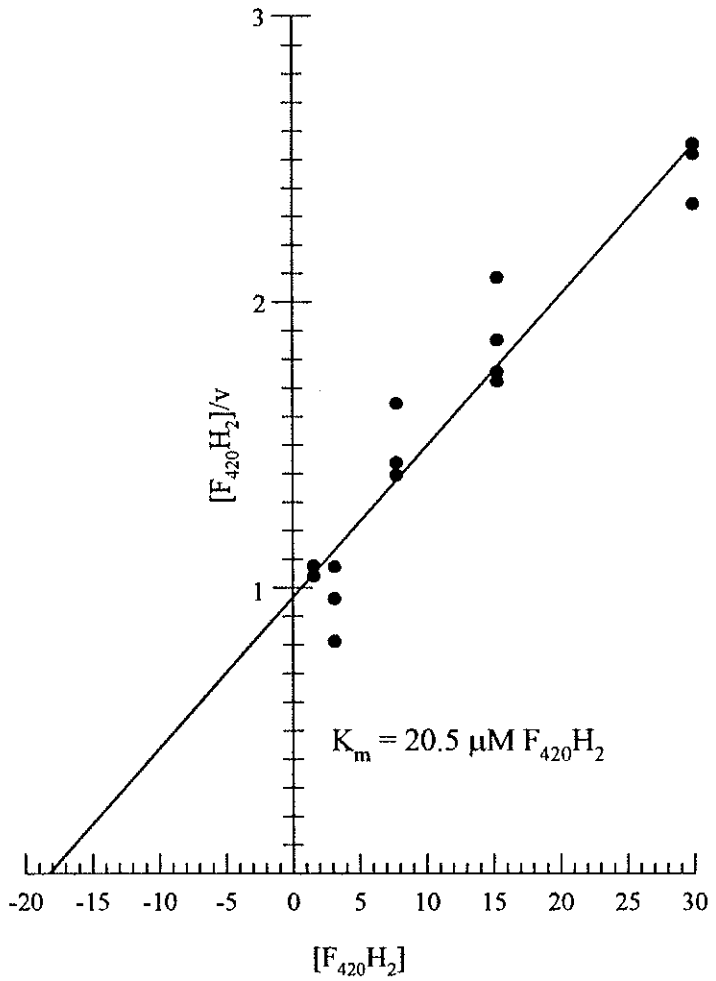
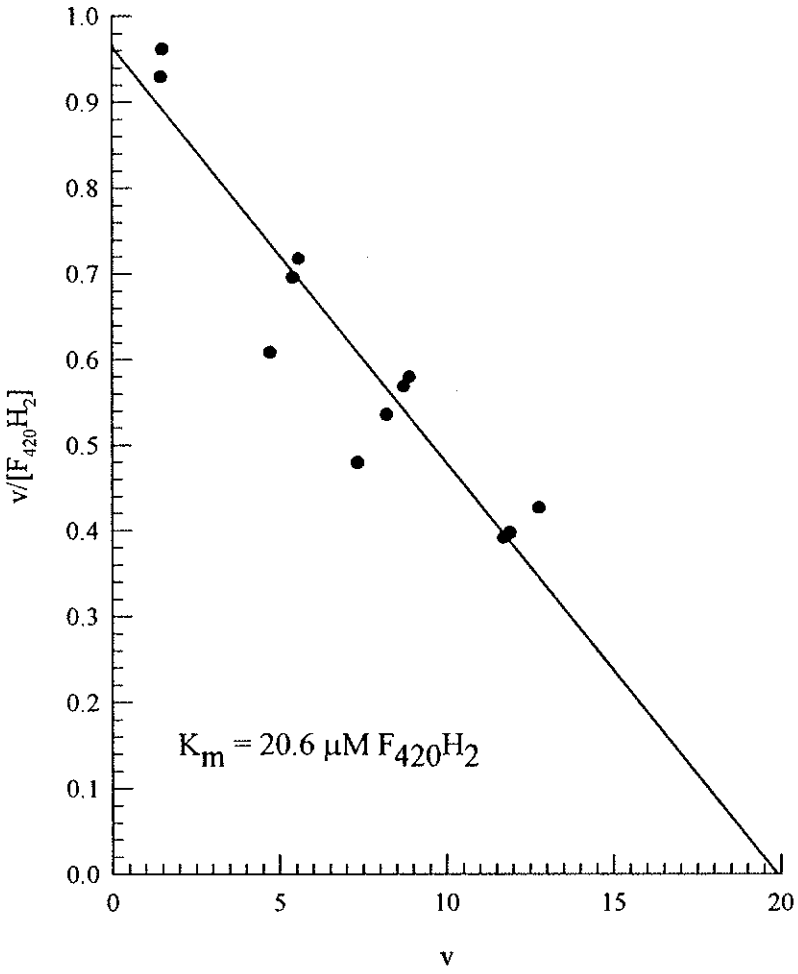


**Figure 11 -** Reciprocal kinetic replots for $F_{420}H_2$ dehydrogenase activity from *Methanosphaera stadtmanae*. (a) Hanes-Woolf plot, $r^2 = 0.966$; (b) Eadie-Scatchard plot, $r^2 = 0.973$.

a) Hanes-Woolf Kinetic Plot




b) Eadie-Scatchard Kinetic Plot



$F_{420}H_2$ in *Msph. stadtmanae* is lower than that in *Ml. tindarius* and *Ms. mazei*. It is possible that the affinity of the activity for the oxidized form of cofactor $F_{420}$ is higher in *Msph. stadtmanae*, since the proposed physiological role for this enzyme is actually in the opposite direction to that measured by this assay system (the reduction of $F_{420}$, not the oxidation of $F_{420}H_2$).

#### 5.1.3.2.7. Effect of 2-Propanol on $F_{420}H_2$ Dehydrogenase Activity

In previous work performed in this laboratory, it was discovered that the addition of secondary alcohols in the medium, like 2-propanol and 2-butanol, increased the growth densities of the cultures, and growth occurred at slightly faster rates (Z. Lin, personal communication). Methanogenesis could also occur with 2-propanol as the electron donor to methanol, though growth could not occur in the absence of hydrogen (Wong et al., 1994). A secondary alcohol dehydrogenase (ADH) has since been discovered in the cell extracts of *Msph. stadtmanae* and was found to be $NADP^+$-dependent.

With the discovery of the enzyme activities; $NADP^+$:$F_{420}$ oxidoreductase, $NADP^+$-dependent ADH, and the $F_{420}H_2$ dehydrogenase in *Msph. stadtmanae*, a proposal regarding a possible level of regulation could occur controlling the levels of the activities of these enzymes was made. A regulatory effect may occur where in the presence of a secondary alcohol, the activity of the $F_{420}H_2$ dehydrogenase may be depressed with the induction of the secondary ADH, since both would ultimately provide reduced $NADP^+$, the ADH directly and the $F_{420}H_2$ dehydrogenase through the cofactor $F_{420}$ and the oxidoreductase.

Enzyme activity levels of the $F_{420}H_2$ dehydrogenase in cells grown in the presence of 2-propanol in the medium was compared to the activity in cells grown in the absence of 2-propanol. The results are shown on Table 5.11 and indicate that the level of $F_{420}H_2$ dehydrogenase activity does not appear to change with the addition of 2-propanol into the growth medium.

**Table 5.11 - Effect of 2-Propanol on $F_{420}H_2$ Dehydrogenase Activity**

| Growth Medium Additions | Specific Activity (nmole $F_{420}H_2$/min/mg) |
|---|---|
| none | 0.75 ± 0.12 |
| 2-propanol | 0.71 ± 0.05 |

Note: assay buffer: 40 mM K-$PO_4$, 20% sucrose, pH 7.0. All assays performed in triplicate. Cells collected in late-log phase.

### 5.1.3.2.8. Activity With Phenazine as Electron Acceptor.

With the recent report of the discovery of the natural electron acceptor for the $F_{420}H_2$ dehydrogenase in *Ms. mazei* identified as a membrane associated phenazine derivative, methanophenazine (Abken et al., 1998), it was important that the $F_{420}H_2$ dehydrogenase activity of *Msph. stadtmanae* be tested for its ability to reduce this or related compounds.

For the purposes of this study, phenazine was used in place of MTZ and MV as the electron acceptor. This compound was reported to be only 18% as effective as the combination of MTZ and MV in *Ms. mazei* (Abken et al., 1998). The better derivatives, 2-hydroxyphenazine (88% effective) and 2-bromophenazine (84% effective) must both be chemically synthesized as neither are available commercially.

Phenazine was found to be able to replace the combination of metronidazole and methyl viologen as the electron acceptor for the oxidation of $F_{420}H_2$ (Table 5.12), although, it was not as effective (approximately 68%) as the MTZ + MV combination,

**Table 5.12 - Phenazine as electron acceptor for $F_{420}H_2$ dehydrogenase activity**

| Electron Acceptor System | Specific Activity (nmole $F_{420}H_2$/min/mg) |
|---|---|
| MV + MTZ | 6.4 ± 1.0 |
| Phenazine | 4.4 ± 0.5 |

Note: All assays performed in 200 mM K-$PO_4$, 20% sucrose, pH 7.0. All assays performed in triplicate.

similar to that reported for the *Ms. mazei* system (Abken et al., 1998).

The $K_m$ value for the $F_{420}H_2$ dehydrogenase activity from *Msph. stadtmanae* with respect to phenazine was determined (figure 12). The Michaelis-Menten kinetic plot gave a value of 148.6 μM, while the Lineweaver-Burk plot (figure 13) gives a value of 142.9 μM. These values are lower (ie. higher affinity) than that reported for the same electron acceptor in *Ms. mazei* (Abken et al., 1998) and fall between the values reported for phenazine ($K_m$ = 250 μM) and 2-hydroxyphenazine ($K_m$ = 35 μM) in *Ms. mazei*.

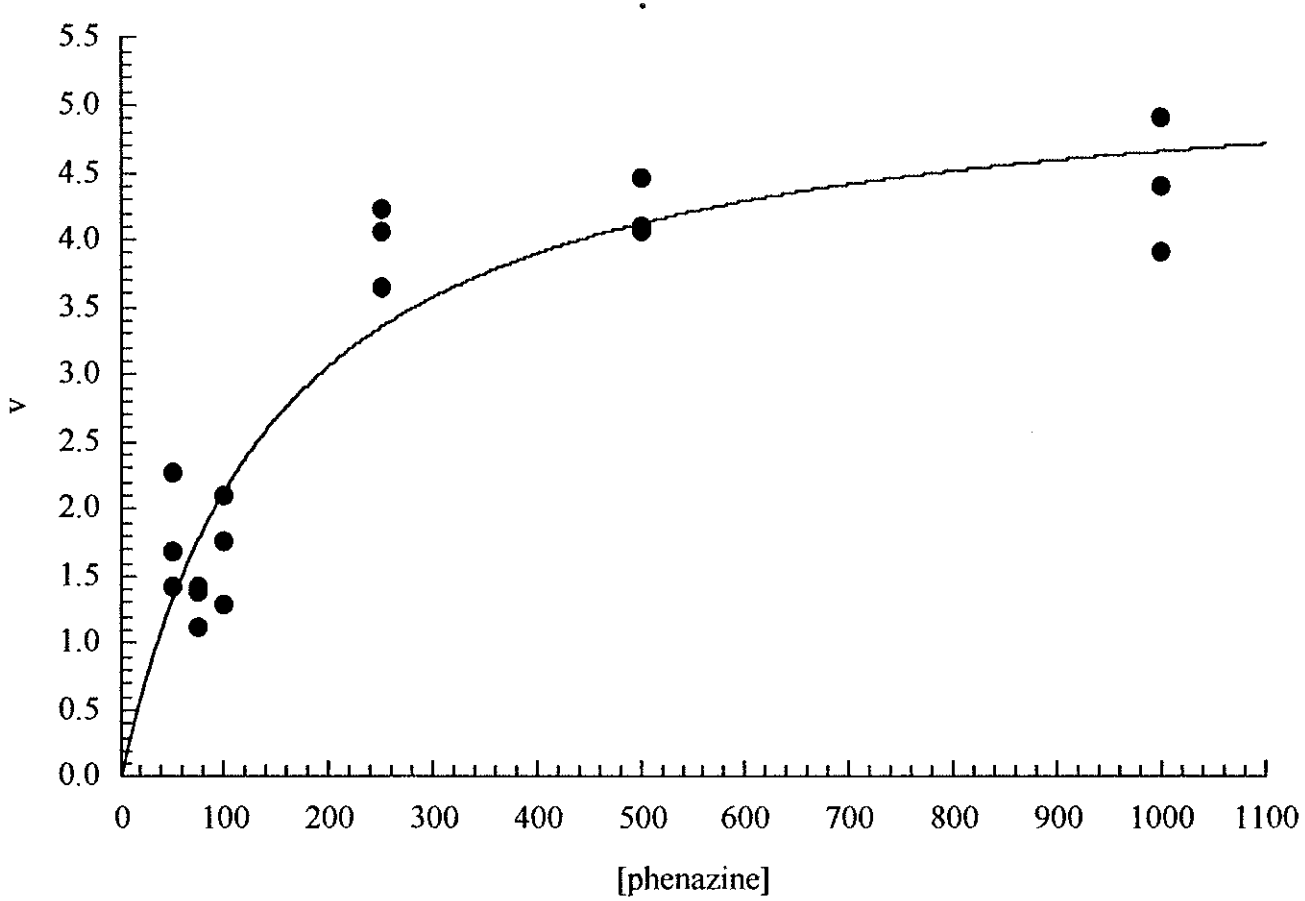
In crude extract, it was discovered that the addition of phenazine to an assay mixture of $F_{420}$ (oxidized), under $H_2$, which was shown previously not to reduce cofactor $F_{420}$, the phenazine could mediate the reduction of $F_{420}$ (Table 5.13), but only in the presence of high potassium phosphate (assayed at 200 mM and at pH 7.0), while using the standard assay buffer of 50 mM Tris-Cl, 20 mM $MgCl_2$, pH 8.0, no reduction of cofactor $F_{420}$ was measurable. It should also be noted that in an assay of the $F_{420}H_2$ dehydrogenase, using 50 mM Tris-Cl, pH 8.0, there was no detectable $F_{420}H_2$ oxidizing activity.

In the analysis of the $K_m$ for phenazine, it was determined that there was some effect by the solvent for the phenazine on the assay system. The solvent, dimethylformamide (DMF) was found to cause some erratic activity readings in the assay for the dehydrogenase. This is shown in the scatter of the low concentration values on both the Michaelis-Menten (figure 12) and Lineweaver-Burk (figure 13) kinetic plots. However, DMF was found to not have any chemical effect on the $F_{420}H_2$, ie., it did not

**Table 5.13 - $H_2$-dependent $F_{420}$ Reduction by *Methanosphaera stadtmanae*.**

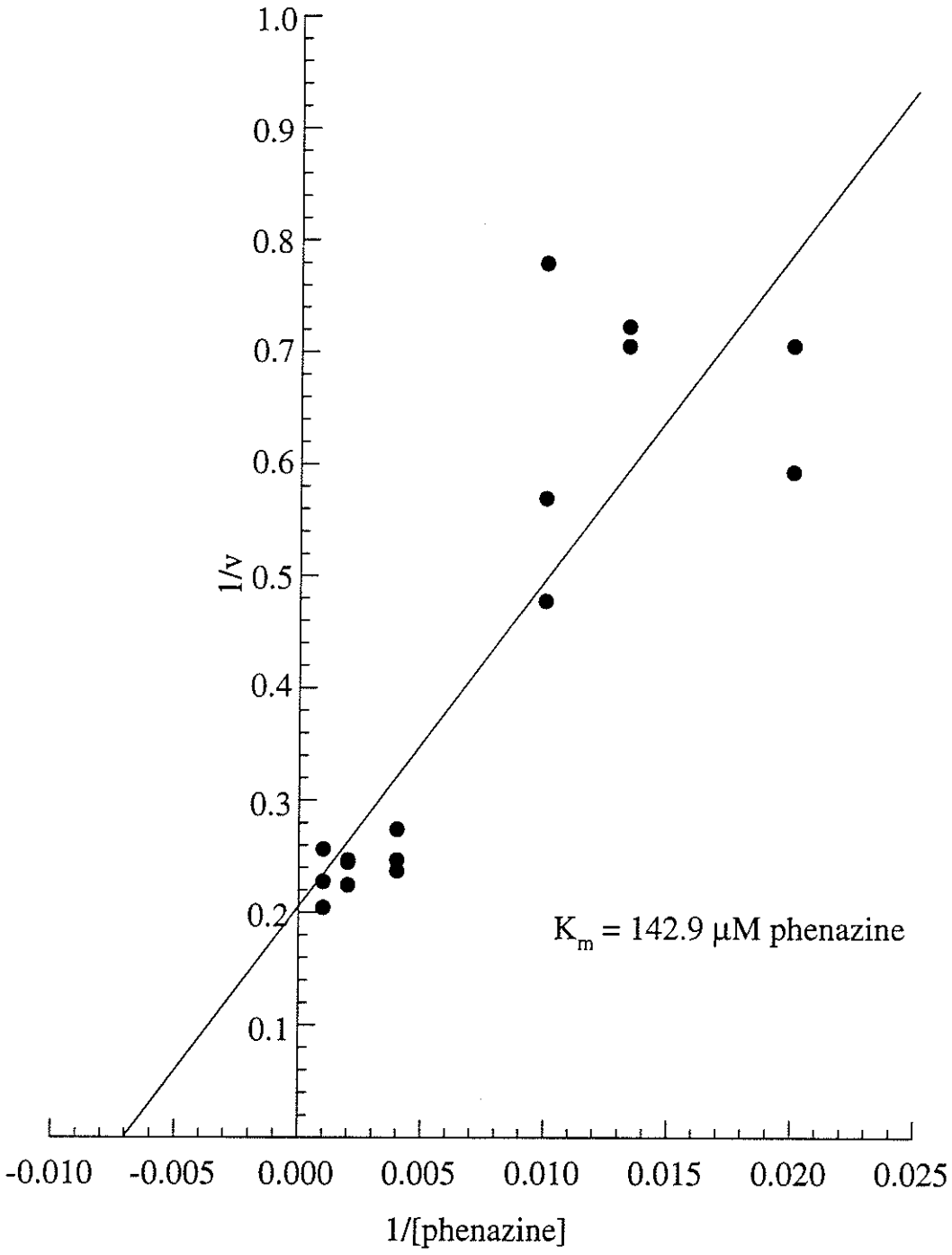
| Assay Buffer (under $H_2$) | Assay Components (electron acceptors) | Specific Activity (nmole $F_{420}$/min/mg) |
|---|---|---|
| **50 mM Tris-Cl, pH 8.0** | $F_{420}$ | < 0.01 |
| **50 mM Tris-Cl, pH 8.0** | $F_{420}$ + phenazine | < 0.01 |
| **200 mM K-$PO_4$, pH 7.0** | $F_{420}$ + phenazine | 0.35 ± 0.05 |

**Figure 12 -** Michaelis-Menten kinetic plot for phenazine for $F_{420}H_2$ dehydrogenase activity from *Methanosphaera stadtmanae*. $r^2 = 0.923$.




$K_m$ = 148.6 μM phenazine
$V_{max}$ = 5.4 nmole $F_{420}H_2$/min/mg protein

**Figure 13 -** Lineweaver-Burk kinetic plot for phenazine for $F_{420}H_2$ dehydrogenase activity from *Methanosphaera stadtmanae*. $r^2 = 0.757$.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1/v
-0.010
-0.005
0.000
0.005
0.010
0.015
0.020
0.025
1/[phenazine]
$K_m$ = 142.9 μM phenazine

cause the oxidation of the $F_{420}H_2$.

## 5.4. $F_{420}H_2$ Dehydrogenase Activity in Related Methanogens.

### 5.4.1. $F_{420}H_2$ Dehydrogenase in the *Methanobacteriales*.

While the absence of an $F_{420}$-dependent hydrogenase activity defines a role for the $F_{420}H_2$ dehydrogenase activity in *Msph. stadtmanae* (and *Msph. cuniculi*) for biosynthesis, it was important to determine whether other methanogens within the same family (the *Methanobacteriales*) also carried the enzyme activity, so two related methanogenic species, *Methanobacterium thermoautotrophicum* strain Marburg and *Methanobacterium bryantii* strain M.o.H. were studied for the presence of this activity in crude extract.

The results of this study are shown on Table 5.14. Both species of *Methanobacterium* were found to carry an $F_{420}H_2$ dehydrogenase activity at much higher levels than that found in both species of *Methanosphaera*. At this point it is difficult to say whether this activity is a true dehydrogenase activity, or is an artifact of the $F_{420}$-dependent hydrogenase that is present in both of these species (Braks et al, 1994).

Also in Table 5.14, the activity of the $F_{420}$-hydrogenase was assayed in the reverse direction (ie., $F_{420}H_2$-oxidizing direction) to obtain additional evidence that may support

**Table 5.14 - $F_{420}H_2$ oxidation activities in the *Methanobacteriaciae*.**

| Organism | $F_{420}H_2$ oxidation via $F_{420}H_2$ dehydrogenase (nmoles $F_{420}H_2$/min/mg) | $F_{420}H_2$ oxidation non-dehydrogenase (nmoles $F_{420}H_2$/min/mg) |
|---|---|---|
| *Methanosphaera cuniculi* | 2.0 ± 0.2 | - |
| *Methanobacterium bryantii* strain M.o.H. | 99 ± 10 | 10.0 ± 1.0 |
| *Mb. thermoautotrophicum* strain Marburg | 184 ± 20 | 8.0 ± 0.8 |

Note: Assay buffer: 40 mM K-PO4, 20% sucrose, pH 7.0. All assays performed in triplicate. All dehydrogenase assays used MV + MTZ as the electron acceptor system. The non-dehydrogenase assay contained no electron acceptor.

the presence of an $F_{420}H_2$ dehydrogenase. The relative affinities for both enzyme activities were determined (the $K_m$'s). The results of this survey are also shown in Table 5.15.

**Table 5.15 - Michaelis-Menten Kinetic values for $F_{420}H_2$ oxidation in the *Methanobacteriaciae*.**

| Organism | $F_{420}H_2$ dehydrogenase $K_m$ | $F_{420}$ hydrogenase $K_m$ |
|---|---|---|
| *Methanobacterium bryantii* strain M.o.H. | 2.5 µM | 29.6 µM |
| *Methanobacterium thermoautotrophicum* strain Marburg | 1.7 µM | 13.4 µM |
| *Methanolobus tindarius* | 5.4 µM | -[a] |
| *Methanosphaera cuniculi* | 6.2 µM | -[a] |
| *Methanosphaera stadtmanae* (included for comparison) | 21.5 µM | -[a] |

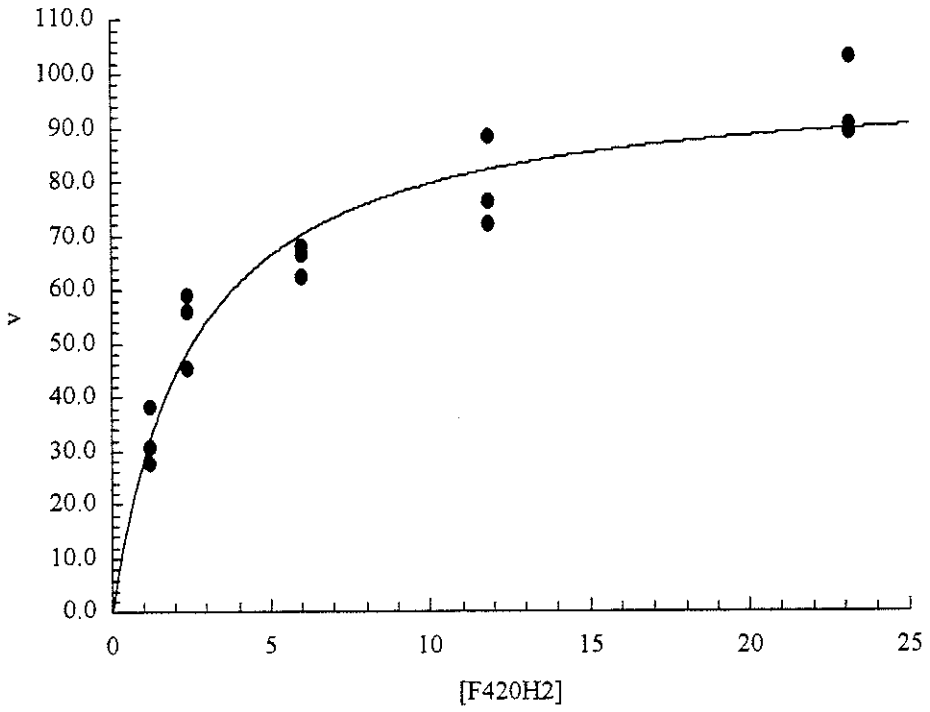
Note: all kinetic determinations were performed using MV + MTZ as the electron acceptor.
[a] - no $F_{420}$ hydrogenase was detected.

The relative activities for the dehydrogenase in both species of *Methanobacterium* are in the order of ten-fold higher than the counterpart reverse $F_{420}$-hydrogenase activity in the same organism. The apparent $K_m$'s of the dehydrogenase and the reverse hydrogenase are also significantly different, with the affinity of the dehydrogenase activity from *Mb. bryantii* (figure 14a) and *Mb. thermoautotrophicum* (figure 15a) being considerably lower than the value for the $F_{420}$-hydrogenase in *Mb. bryantii* (figure 14b) and *Mb. thermoautotrophicum* (figure 15b).

The $K_m$ value for the $F_{420}H_2$ dehydrogenase activity determined from *Msph. cuniculi* (figure 16) was found to be 6.2 µM $F_{420}H_2$, falling in the range of the reported values from the other methanogens.

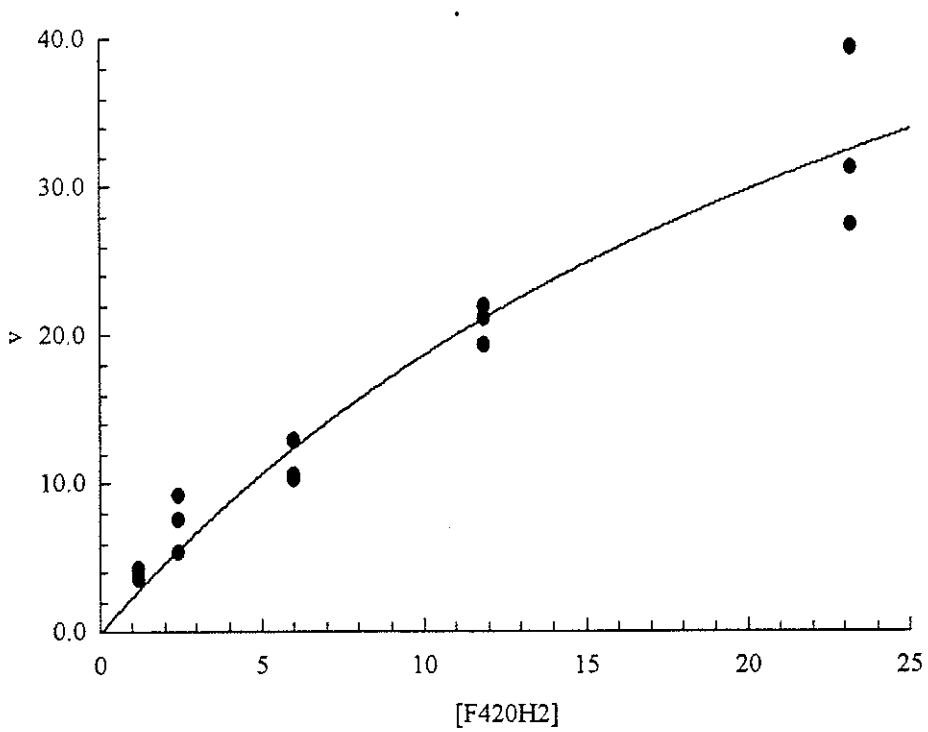
**Figure 14 -** Michaelis-Menten kinetic plots for (a) $F_{420}H_2$ oxidation activity ($F_{420}H_2$ dehydrogenase assay), $r^2 = 0.954$, and (b) $F_{420}H_2$ oxidation activity (reverse $F_{420}$ hydrogenase assay), $r^2 = 0.968$, in *Methanobacterium bryantii* strain M.o.H.

a)




$K_m$ = 2.5 μM $F_{420}H_2$
$V_{max}$ = 99.6 nmoles $F_{420}H_2$/min/mg protein

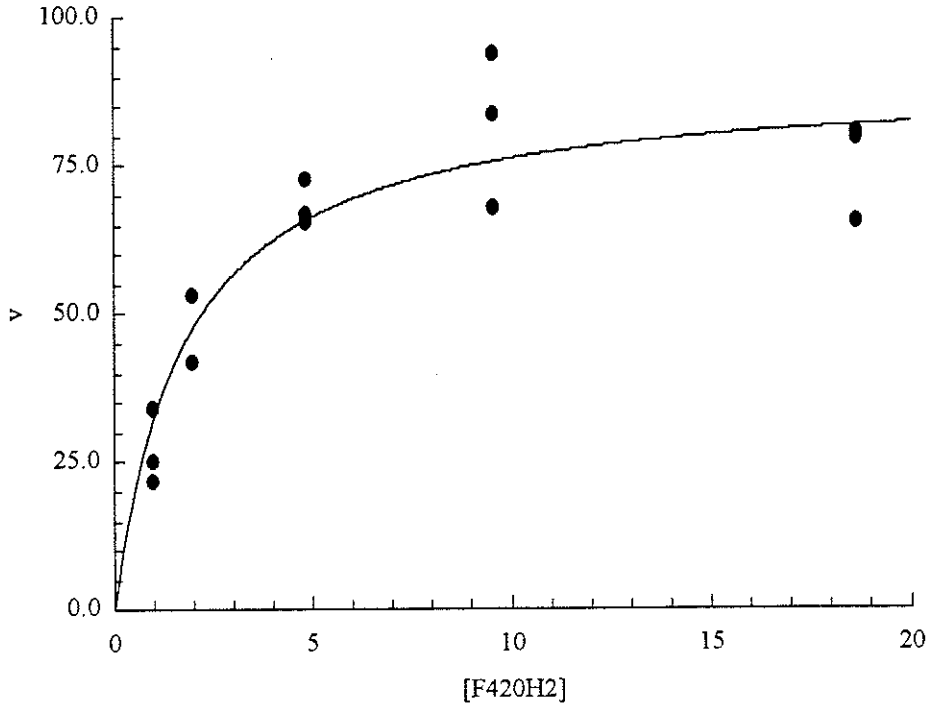
b)




$K_m$ = 29.6 μM $F_{420}H_2$
$V_{max}$ = 74.1 nmoles $F_{420}H_2$/min/mg protein

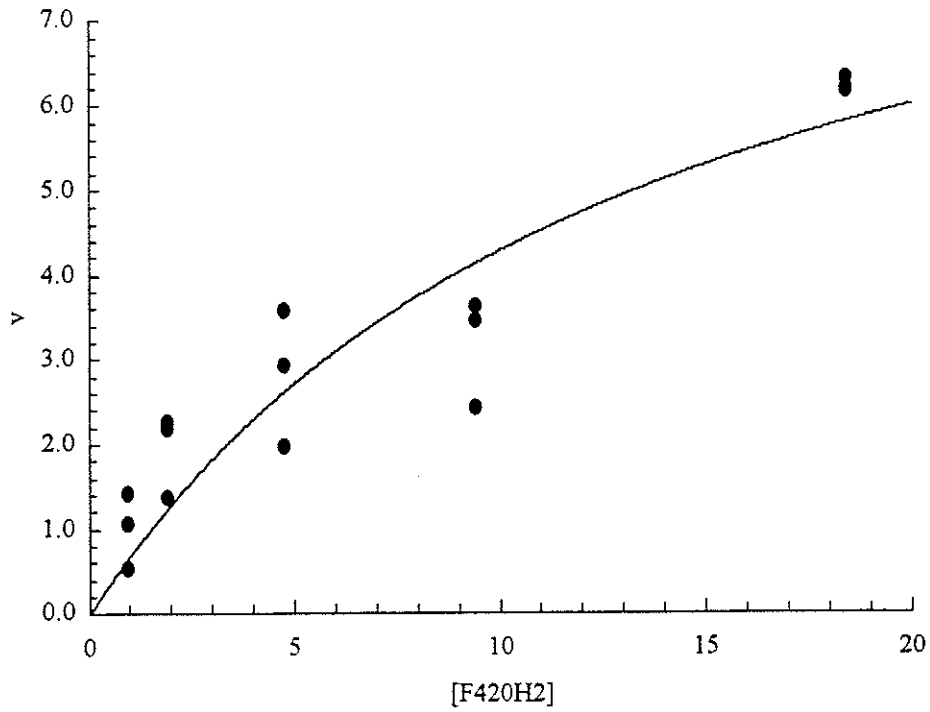
**Figure 15 -** Michaelis-Menten kinetic plots for (a) $F_{420}H_2$ oxidation activity ($F_{420}H_2$ dehydrogenase assay), $r^2 = 0.917$, and (b) $F_{420}H_2$ oxidation activity (reverse $F_{420}$ hydrogenase assay), $r^2 = 0.913$, in *Methanobacterium thermoautotrophicum* strain Marburg.

a)



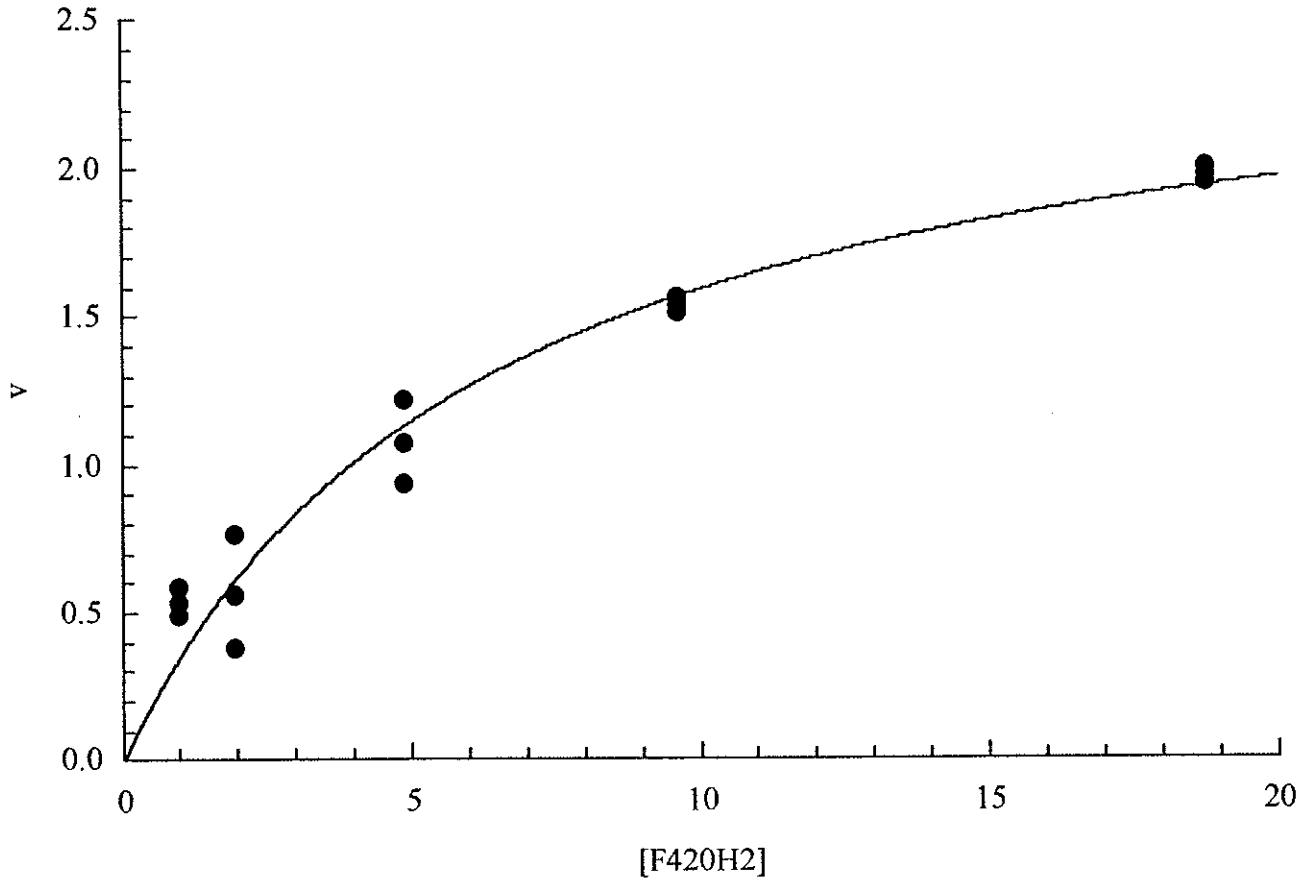

$K_m$ = 1.7 μM $F_{420}H_2$
$V_{max}$ = 89.4 nmoles $F_{420}H_2$/min/mg protein

b)




$K_m$ = 13.4 μM $F_{420}H_2$
$V_{max}$ = 10.0 nmoles $F_{420}H_2$/min/mg protein

**Figure 16 -** Michaelis-Menten kinetic plot for $F_{420}H_2$ dehydrogenase activity from *Methanosphaera cuniculi*. $r^2 = 0.973$.




$K_m$ = 6.2 μM $F_{420}H_2$
$V_{max}$ = 2.57 nmoles $F_{420}H_2$/min/mg protein

### 5.4.2. Effect of Phosphate on $F_{420}H_2$ Dehydrogenase Activity of the Related Methanogens.

As revealed in an earlier section, there was a salt effect, specifically potassium phosphate, on the activity measured of the $F_{420}H_2$ dehydrogenase discovered in *Msph. stadtmanae* (see Section 4.1.3.2.1.). It was shown that there was a stimulation of activity by increasing the concentration of the phosphate species (specifically the potassium phosphate salt), a stimulation of about thirty-fold was observed with maximum activity in crude extract occurring at approximately 200 mM potassium phosphate. Based on those results, it was interesting to see if there was a similar 'phosphate effect' with the $F_{420}H_2$ dehydrogenase activities from other methanogens that are closely related (ie. members of the *Methanobacteriales*).

For this survey, species from within the same family of methanogens were assayed for the presence and response to increasing phosphate concentrations on $F_{420}H_2$ dehydrogenase activity. Methanogenic species tested included *Msph. cuniculi*, *Mb. bryantii* strain M.o.H., and *Mb. thermoautotrophicum* strain Marburg.

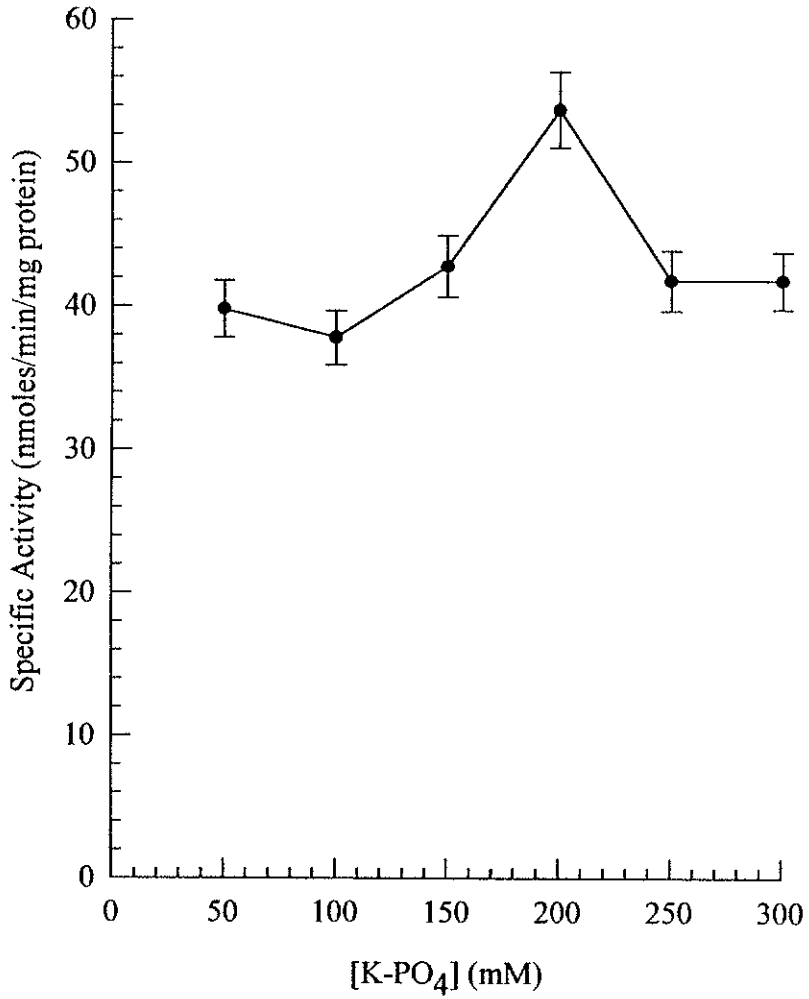
The effect of potassium phosphate on the activity of the dehydrogenase seemed to vary from species to species. In *Mb. bryantii*, there was little to no effect on the activity of the dehydrogenase over the concentration range that was tested (see figure 17a). On the other hand, in both *Mb. thermoautotrophicum* (figure 17b) and *Msph. cuniculi* (figure 18) there was a significant reduction of activity as the phosphate concentration increased, with a reduction by 50% at 300 mM potassium phosphate.

## 5.5. Purification Potential of the $F_{420}H_2$ Dehydrogenase Activity of *Methanosphaera stadtmanae*.

As a prelude for the purification of the $F_{420}H_2$ dehydrogenase activity of *Msph. stadtmanae*, it must be determined if the activity is stable for manipulations at room temperature (ambient temperature of the anaerobic chamber) since the activity is $O_2$-sensitive (Table 5.6). Some encouragement is shown with the recovery of a small amount (3.6%) of activity in the presence of salt (KCl) and assayed in the presence of high salt

**Figure 17 -** Effect of potassium phosphate on $F_{420}H_2$ dehydrogenase activity in members of the genus *Methanobacterium*. (a) *Methanobacterium bryantii* strain M.o.H.; (b) *Methanobacterium thermoautotrophicum* strain Marburg. (points are average of triplicate assays).

a) *Mb. bryantii* strain M.o.H.




b) *Mb. thermoautotrophicum* strain Marburg

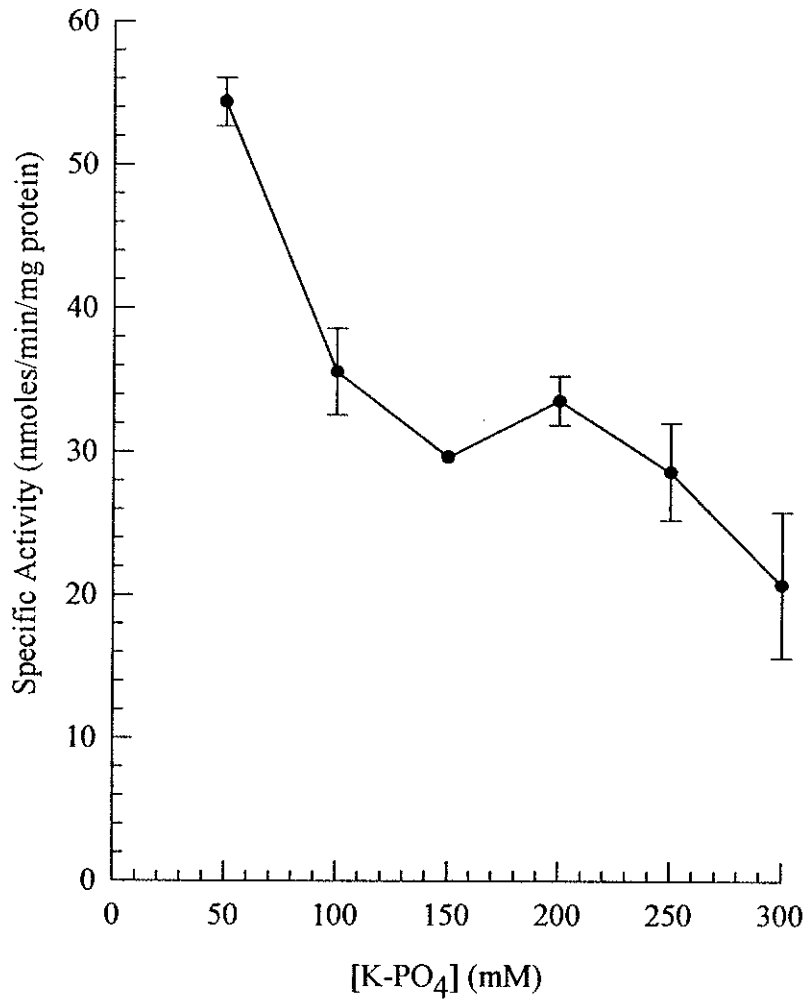
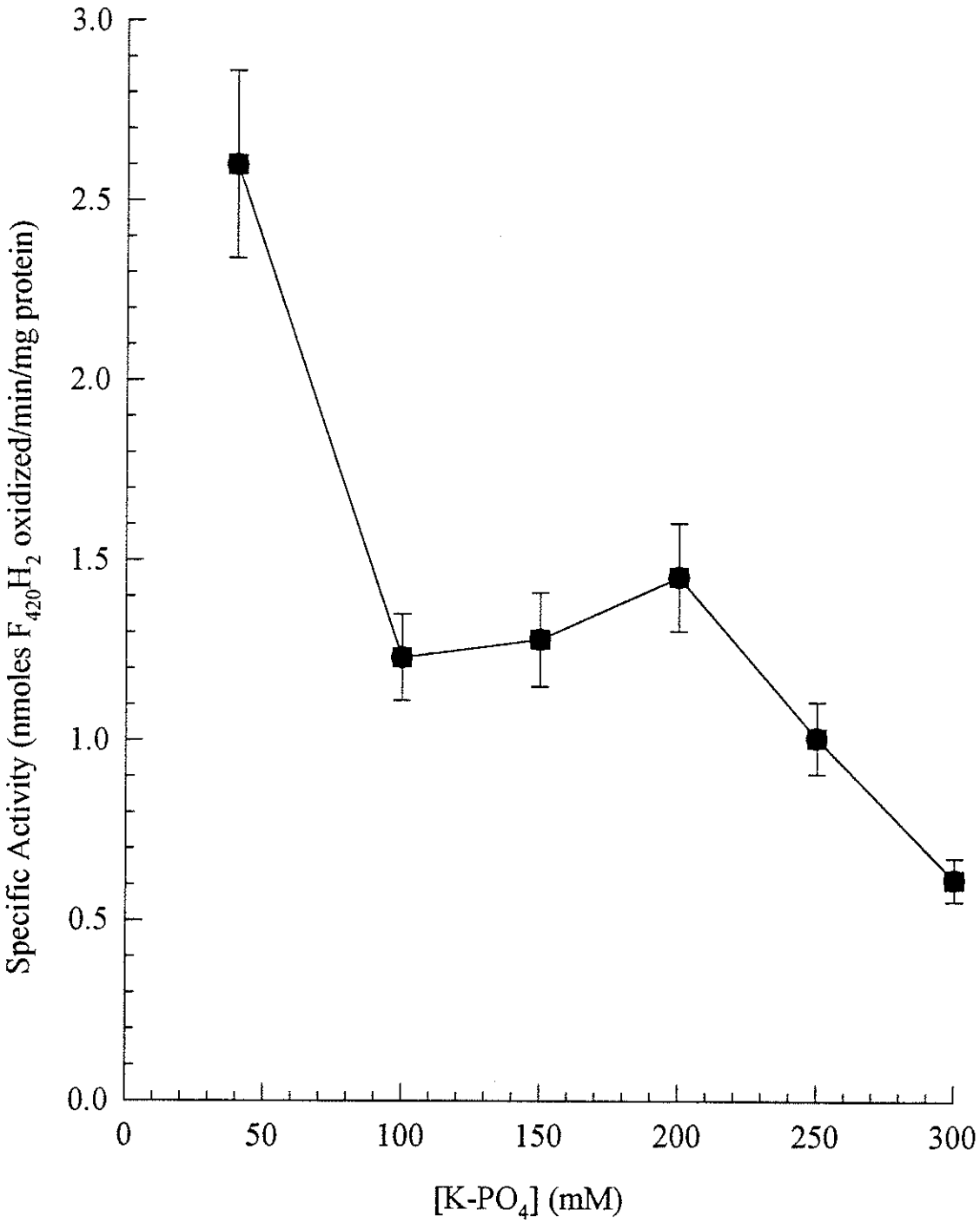


**Figure 18 -** Effect of potassium phosphate on $F_{420}H_2$ dehydrogenase activity from *Methanosphaera cuniculi*. (points are average of triplicate assays).


3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
Specific Activity (nmoles $F_{420}H_2$ oxidized/min/mg protein)
[K-$PO_4$] (mM)

(0.2 M $(NH_4)_2SO_4$).

A preliminary study was made to determine if the activity was stable at room temperature so as to allow for possible purification. In this study, the extract prepared was kept at room temperature for a period of one week under anaerobic conditions to see if the activity could be maintained. The results of this were shown on Figure 8. The results showed that the activity, without addition of any chemical stabilization agents, was stable only for a couple of days and declined rapidly by the fourth day onward. The reported stability of the dehydrogenase activity purified from *Ml. tindarius* was only a few days as well, but this latter enzyme was also found to be air stable.

**5.6. Summary**

A possible mechanism for the reduction of the methanogenic cofactor, $F_{420}$, in *Msph. stadtmanae* has been determined. The common method for the reduction of $F_{420}$ in hydrogenotrophic methanogens is typically through the hydrogenase, however, in *Msph. stadtmanae*, the hydrogenase is not $F_{420}$-linked, thus an alternate mechanism is required, in this case this may involve the $F_{420}H_2$ dehydrogenase. The enzyme activity has been found in the cell extracts of *Msph. stadtmanae* at low levels, not sufficient for a major role in methanogenesis, but in levels that are consistent with a role in biosynthesis (6.3 nmole $F_{420}H_2$ oxidized/min/mg protein in cell-free extracts with a $V_{max}$ of 19.3). This value is significantly lower than that found in *Ml. tindarius* in crude extract (0.26 µmoles $F_{420}H_2$ oxidized/min/mg protein), although this velocity value is made in what would be considered the opposite physiological direction to that suspected for *Msph. stadtmanae*.

A preliminary study into the properties of this enzyme activity has discovered that there is an enhancement of the activity levels of the enzyme by the increase in potassium phosphate concentration (up to 200 mM) of the assay buffer as well as the addition of some other salts like ammonium sulfate and sodium phosphate, but these other salts do not improve the activity to the same levels as provided by potassium phosphate. The pH optimum was found to be pH 7.0, compared to 6.8 for the enzyme found from *Ml. tindarius* (Haase et al., 1992) and 8.5 for *Ms. mazei* (Abken and Deppenmeier, 1997).

The enzyme activity was found to be very sensitive to $O_2$ and required strict anaerobic handling, in contrast to the activity found in *Ml. tindarius*, which could be handled aerobically (Haase et al., 1992). Kinetic parameters were determined in cell-free extract, the $K_m$ was found to be 21.5 μM for $F_{420}H_2$, and the $V_{max}$ was determined to be 19.3 nmoles $F_{420}H_2$ oxidized/minute/mg protein.

The study of the use of phenazine derivatives that were reported to be able to accept electrons from $F_{420}H_2$ via the $F_{420}H_2$ dehydrogenase from *Ms. mazei* has appeared to provide the last piece of the puzzle for the solution at the mechanism for the $H_2$-dependent reduction of $F_{420}$ in the absence of an $F_{420}$-hydrogenase activity in *Msph. stadtmanae*. It was shown that the addition of phenazine to an assay system that enhances $F_{420}H_2$ dehydrogenase activity (ie., 200 mM K-$PO_4$) in the presence of $F_{420}$ under $H_2$ was able to couple the reduction of $F_{420}$ to $H_2$ as the sole electron donor.

In a preliminary study, the enzyme activity known as the $F_{420}H_2$ dehydrogenase was found to be present in methanogenic species other than in *Msph. stadtmanae* and those previously reported and purified from (ie., *Ml. tindarius* and *Ms. mazei* strain Gö1). The species that were found to exhibit this activity included the closely related *Msph. cuniculi* (which was found also to be air sensitive and present at low levels in cell extracts), along with other related methanogenic species, *Mb. bryantii* and *Mb. thermoautotrophicum*. The discovery of this activity in species that do not utilize methanol as methanogenic substrate (as both the *Msph. spp*, *Ml. tindarius* and *Methanosarcina* all do) is surprising, and it would seem to be necessary to analyze other methanogenic species covering all three methanogenic orders for the presence of this activity.

# 6. $F_{420}H_2$ Dehydrogenase in the Methanogenic Archaea

## 6.1. Introduction

The discovery of the $F_{420}H_2$ dehydrogenase activity in *Msph. stadtmanae* (Wong et al., 1994), a member of the Order *Methanobacteriales*, is significant. With the enzymes purified from *Methanolobus tindarius* (Haase et al., 1992) and *Methanosarcina mazei* strain Gö1 (Abken and Deppenmeier, 1997), both from the Order *Methanomicrobiales* (Garcia, 1990), it was of interest first to determine whether this enzyme activity may be more widespread throughout all of the methanogenic bacteria. This is regardless of which methanogenic group they belong to, and regardless of the presence or absence of the $F_{420}$-reducing hydrogenase. Second, if an $F_{420}H_2$ dehydrogenase activity is detected, it would be important to determine that this activity does not represent an artifact of the $F_{420}$-linked hydrogenase activity, if present in that specific methanogen, since it has been reported that the purified $F_{420}$-dependent hydrogenase of *Ms. barkeri* strain Fusaro can couple $F_{420}$ oxidation to benzyl viologen (BV) reduction (Keltjens and Vogels, 1992).

The ability of this activity to link the oxidation of $F_{420}H_2$ to phenazine (or a derivative) reduction may be the key as the phenazine derivative, methanophenazine was determined to be the natural electron acceptor for the $F_{420}H_2$ dehydrogenase from *Ms. mazei* (Abken et al., 1998).

For this survey of the methanogenic Archaea, members from all three methanogenic Orders were studied: *Methanobacterium thermoautotrophicum* strain Marburg, *Methanobacterium bryantii* strain M.o.H., and *Methanobacterium* strain G2R from Order *Methanobacteriales*; *Methanococcus thermolithotrophicus* (grown on either $H_2$:$CO_2$ or on formate as methanogenic substrate) and *Methanococcus voltae* from Order *Methanococcales*; and *Methanolobus tindarius, Methanosaeta concilii, Methanosarcina barkeri* strain Fusaro, and *Methanospirillum hungatei* from Order *Methanomicrobiales*.

The substrates used for methanogenesis by these species for the experiments to be

described below range from methanol-only (*Methanolobus*), to $H_2$ plus $CO_2$ (*Methanobacterium* spp. and *Methanospirillum*), to acetate (*Methanosaeta*). *Ms. barkeri* strain Fusaro could grow and was tested on all three of the above substrates. *Mc. thermolithotrophicus* was grown on formate or on $H_2$ plus $CO_2$.

## 6.2. Results and Discussion

### 6.2.1. $F_{420}H_2$ Dehydrogenase Activities in Order *Methanobacteriales*

*Methanosphaera stadtmanae* and *Msph. cuniculi* are both members of the Order *Methanobacteriales*, so it was reasonable to analyze other members of this Order to determine whether other phylogenetically related species also possess this enzyme activity. Three hydrogenotrophic members of the genus *Methanobacterium*, *Mb. bryantii* strain M.o.H., *Mb. thermoautotrophicum* strain Marburg and *Methanobacterium* strain G2R were tested for the presence, or the absence of this activity. The results of this survey are shown on Table 1.

Activities were found for hydrogenase (both the MV and $F_{420}$-reducing forms), and the $F_{420}H_2$ dehydrogenase (although at this point it would be more correct to call this the MV-dependent $F_{420}H_2$ oxidation activity or as it will be referred to subsequently, the $F_{420}H_2$ dehydrogenase activity), and appears to indicate that there could be a distinct "$F_{420}H_2$ dehydrogenase" activity in all of the tested species of *Methanobacterium*. The $F_{420}H_2$ dehydrogenase activity could be handled aerobically, as were both the hydrogenase activities. This $F_{420}H_2$ dehydrogenase activity level in the crude cell extract of *Mb. thermoautotrophicum* was found to be actually quite high (0.184 µmoles $F_{420}H_2$ oxidized/minute/mg protein), even higher than the activity of the $F_{420}$-reducing hydrogenase (0.034 µmoles $F_{420}$/minute/mg protein) that was measured. *Mb. bryantii* strain M.o.H. carried activity levels that were similar in magnitude for both the $F_{420}H_2$ dehydrogenase (0.099 µmoles $F_{420}$/minute/mg protein) and the $F_{420}$-reducing hydrogenase (0.078 µmoles $F_{420}$/minute/mg protein).

In addition, an attempt was made to separate these enzyme activities via sucrose density gradient ultracentrifugation. The basis for this approach was the use of this

**Table 1 - Extract Activities for MV Hydrogenase, $F_{420}$ Hydrogenase and MV-dependent $F_{420}H_2$ Dehydrogenase from the *Methanobacteriales*.**

| Methanogenic species | MV Hydrogenase (µmoles MV/min/mg) | $F_{420}$ Hydrogenase (µmoles $F_{420}$/min/mg) | | $F_{420}H_2$ Dehydrogenase (µmoles $F_{420}$/min/mg) |
|---|---|---|---|---|
| | | Reducing | Oxidizing | |
| ***Methanobacterium bryantii* strain M.o.H.** | 1.255 | 0.078 | 0.010 | 0.099 |
| ***Methanobacterium thermoautotrophicum* strain Marburg** | 0.284 | 0.034 | 0.008 | 0.184 |
| ***Methanobacterium* strain G2R** | 1.745 | 0.046 | 0.022 | 0.052 |
| ***Methanosphaera cuniculi*** | 2.745 | not detected | - | 0.002* |
| ***Methanosphaera stadtmanae*** | 3.567 | not detected | - | 0.0003* |

* - note the levels presented here are lower than that reported in the previous chapter. This is due to the use of the 40 mM K-$PO_4$ buffer for these assays.

**Figure 1 -** Linear sucrose density gradient ultracentrifugation profile for *Methanobacterium bryantii* strain M.o.H. Cell-free extract was applied to a 0-20% sucrose gradient. (●) - $F_{420}$ hydrogenase; (○) - $F_{420}H_2$ dehydrogenase; (▲) - MV hydrogenase activity. The bars represent the absorbance at 280 nm.

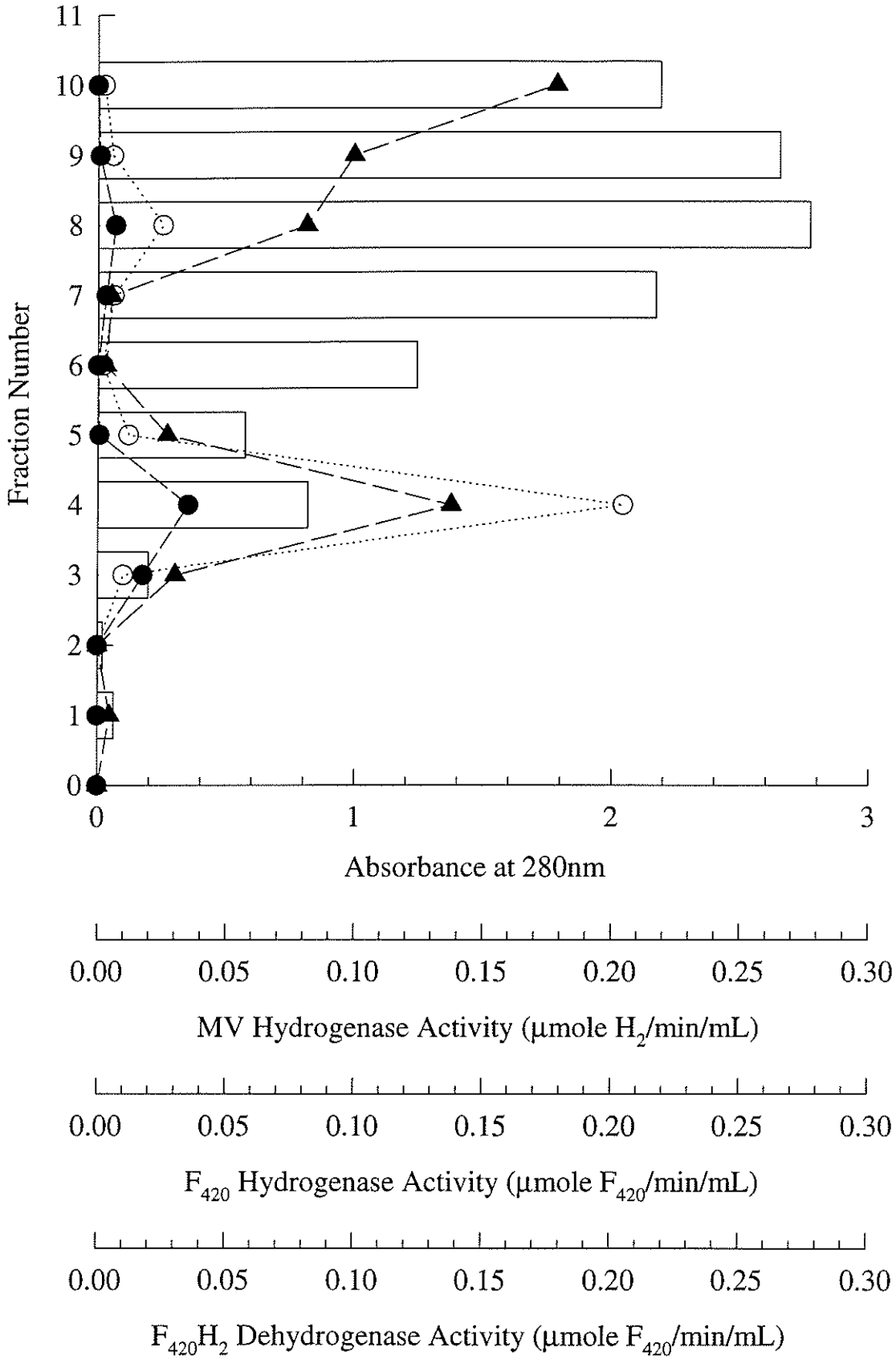

Fraction Number
11
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
Absorbance at 280nm
0.00
0.05
0.10
0.15
0.20
0.25
0.30
MV Hydrogenase Activity (µmole H2/min/mL)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
F420 Hydrogenase Activity (µmole F420/min/mL)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
F420H2 Dehydrogenase Activity (µmole F420/min/mL)

**Figure 2 -** Linear sucrose density gradient ultracentrifugation profile for *Methanobacterium thermoautotrophicum* strain Marburg. Cell-free extract was applied to a 0-20% sucrose gradient. (●) - $F_{420}$ hydrogenase;(○) - $F_{420}H_2$ dehydrogenase; (▲) - MV hydrogenase activity. The bars represent the absorbance at 280 nm.

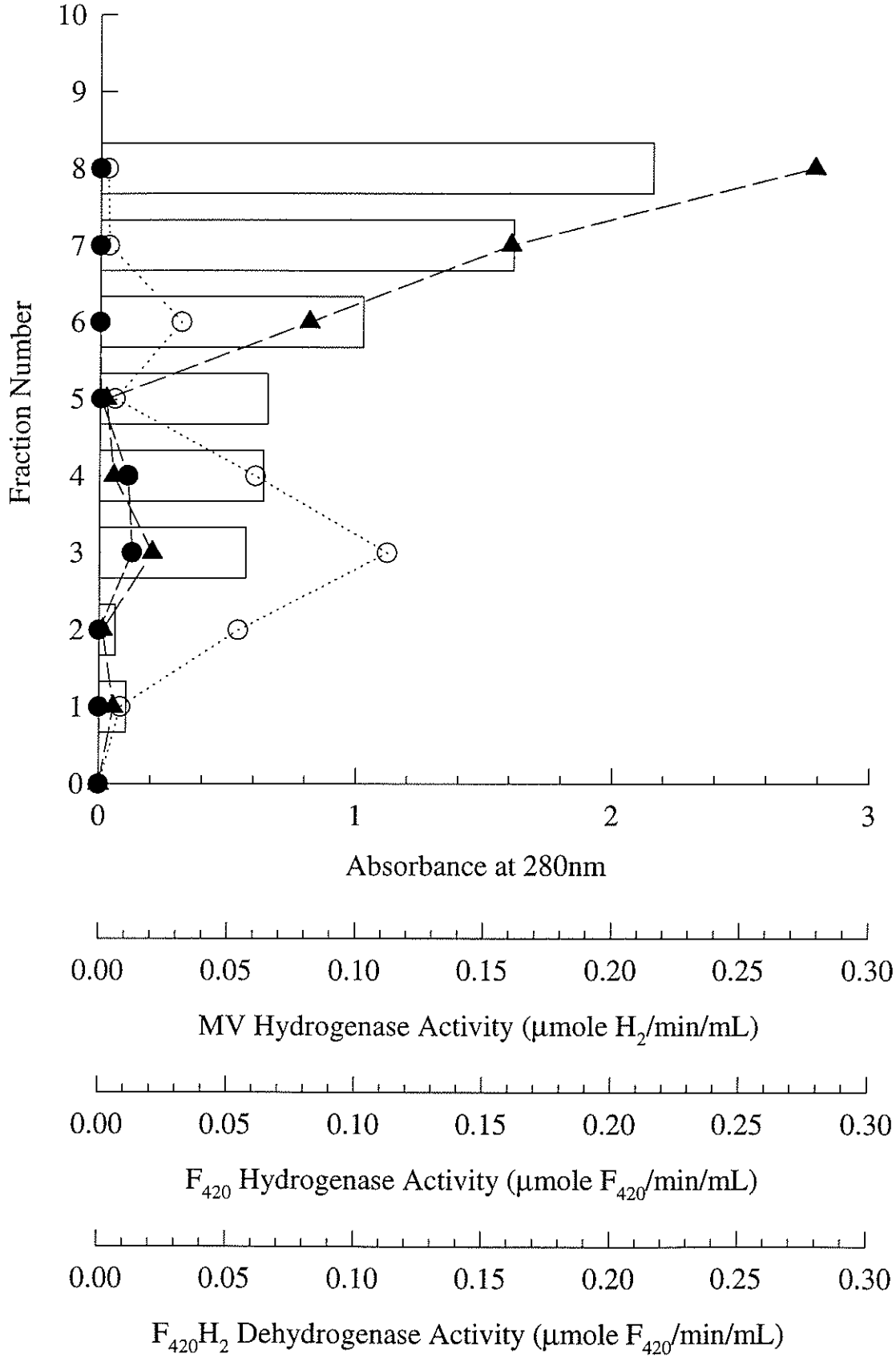
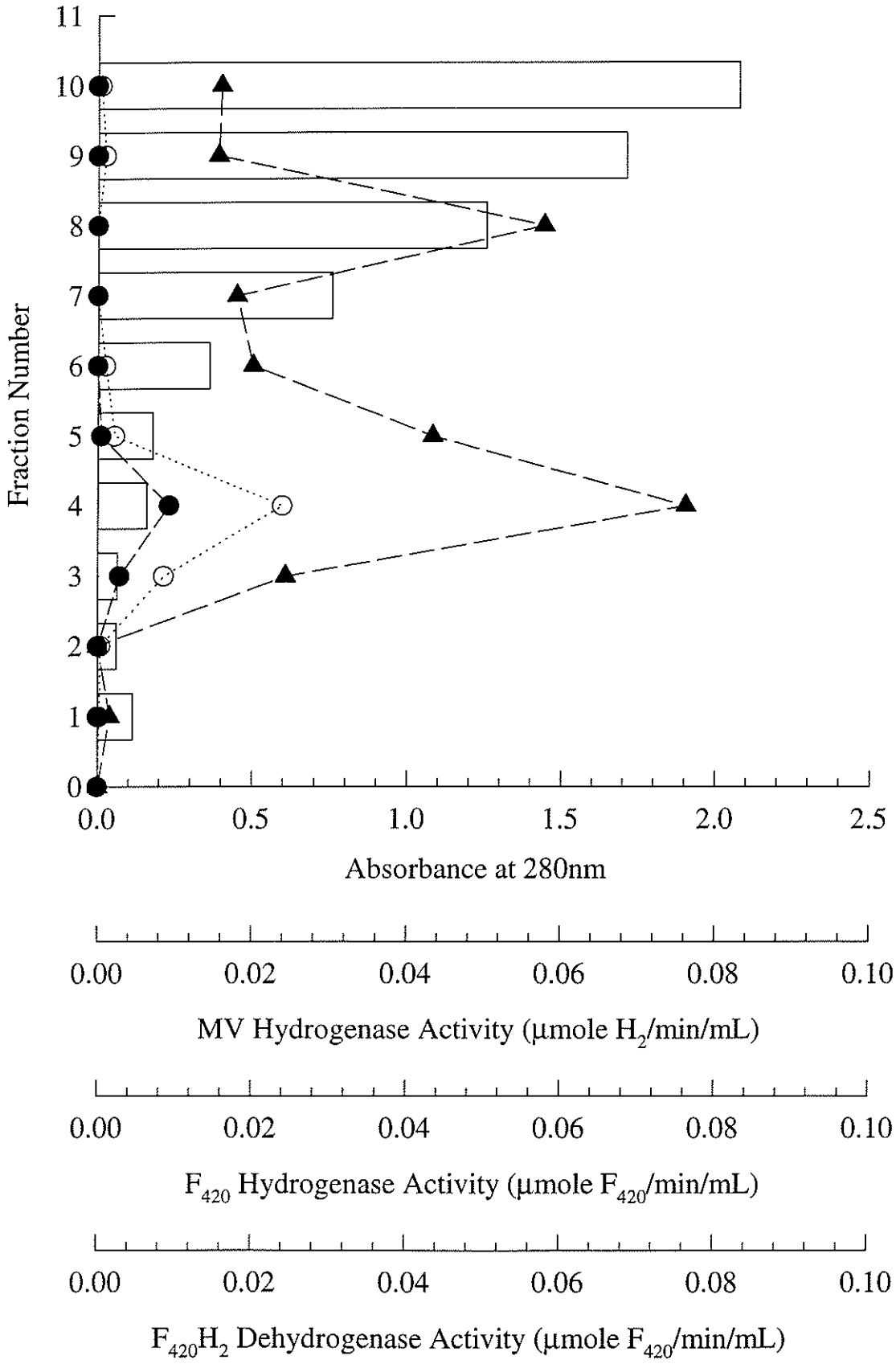

Fraction Number
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
Absorbance at 280nm
0.00
0.05
0.10
0.15
0.20
0.25
0.30
MV Hydrogenase Activity (µmole $H_2$/min/mL)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
$F_{420}$ Hydrogenase Activity (µmole $F_{420}$/min/mL)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
$F_{420}H_2$ Dehydrogenase Activity (µmole $F_{420}$/min/mL)

**Figure 3 -** Linear sucrose density gradient ultracentrifugation profile for *Methanobacterium* strain G2R. Cell-free extract was applied to a 0-20% sucrose gradient. (●) - $F_{420}$ hydrogenase; (○) - $F_{420}H_2$ dehydrogenase; (▲) - MV hydrogenase activity. The bars represent the absorbance at 280 nm.


Fraction Number
11
10
9
8
7
6
5
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
2.5
Absorbance at 280nm
0.00
0.02
0.04
0.06
0.08
0.10
MV Hydrogenase Activity (µmole H2/min/mL)
0.00
0.02
0.04
0.06
0.08
0.10
F420 Hydrogenase Activity (µmole F420/min/mL)
0.00
0.02
0.04
0.06
0.08
0.10
F420H2 Dehydrogenase Activity (µmole F420/min/mL)

method in the purification of the $F_{420}$ hydrogenase from *Msp. hungatei* (Sprott et al, 1987; Choquet and Sprott, 1990). The results of this analysis are shown on Figure 1 (*Mb. bryantii* strain M.o.H.), Figure 2 (*Mb. thermoautotrophicum* strain Marburg) and Figure 3 (*Methanobacterium* strain G2R). The activity profile along the sucrose density gradient for both *Mb. thermoautotrophicum* strain Marburg and *Methanobacterium* strain G2R did not conclusively indicate that the activities of the $F_{420}$-reducing hydrogenase were a distinct entity from the $F_{420}H_2$ dehydrogenase activity, but in a single fraction (number 6), a small amount of activity was detected independent of the $F_{420}$ hydrogenase.

In the profiles for both *Mb. thermoautotrophicum* and *Mb. bryantii*, there appeared to be a second smaller peak of $F_{420}H_2$ dehydrogenase activity in fraction 6 (figure 2), in which $F_{420}$-dependent hydrogenase activity was not detected. This could indicate the presence of the dehydrogenase as a separate entity in this species.

Thus the results obtained from the sucrose density gradient analysis were inconclusive as to the distinctiveness of the $F_{420}H_2$ dehydrogenase activity in these methanogens (except for the possibility in *Mb. thermoautotrophicum*). As a side note, $F_{420}$-linked hydrogenase activity was detected consistently from the extracts of *Methanobacterium* strain G2R, which was previously reported not to carry this activity (McKellar and Sprott, 1979), the reason for this discrepancy cannot be explained, this $F_{420}$ hydrogenase activity is in the same magnitude as detected from *Mb. thermoautotrophicum* strain Marburg.

In *Mb. bryantii*, there was a slight deviation in the activities of the dehydrogenase from the hydrogenase. In fraction tube number 3, the activity per mL of fraction was higher for the hydrogenase in the $F_{420}$-reducing assay (0.0176 U/mL) than the dehydrogenase (0.0099 U/mL), while the dehydrogenase activity (0.2043 U/mL) in the very next fraction (number 4) had a much higher activity than the $F_{420}$-reducing hydrogenase (0.0353 U/mL). One can analyze this by determining the ratios of the dehydrogenase versus the hydrogenase activity ($DH/H_2ase$) for the fractions where both activities are detected (see Table 2). For all of the fractions from all the tested species,

**Table 2 - Ratios of $F_{420}H_2$ DH:$F_{420}$ Hydrogenase activity in fractions of Sucrose Gradient.**

| Fraction # | *Methanobacterium* strain G2R | *Methanobacterium bryantii* | *Methanobacterium thermoautotrophicum* |
|---|---|---|---|
| **1** | ∞ | 0.0 | ∞ |
| **2** | ∞ | 0.0 | ∞ |
| **3** | 3.0 | 0.6 | 8.9 |
| **4** | 2.6 | 5.8 | 5.6 |
| **5** | 5.3 | 23.9 | 14.1 |
| **6** | ∞ | ∞ | ∞ |
| **7** | 0.0 | 1.9 | ∞ |
| **8** | 0.0 | 3.7 | ∞ |
| **9** | ∞ | 8.8 | - |
| **10** | ∞ | ∞ | - |

there is no constancy of the ratio DH/$H_2$ase, which would be expected if the same protein were performing both of the activities. The raw data for the activities detected for each fraction tested will be found in Appendix B.

In general, the $F_{420}$ hydrogenase may account for a portion of the $F_{420}H_2$-oxidizing activity in this group of hydrogenotrophic methanogens, mainly because of the similar, overlapping peaks for both $F_{420}$ hydrogenase and $F_{420}H_2$ dehydrogenase activities in the profile.

### 6.2.2. $F_{420}H_2$ Dehydrogenase Activities in Order *Methanococcales*

Two members of the Order *Methanococcales* were available for the determination of the presence of the $F_{420}H_2$ dehydrogenase activity, *Mc. thermolithotrophicus*, and *Mc. voltae*. Both are known to carry both the MV hydrogenase and $F_{420}$-reducing hydrogenase and *Mc. voltae* even carries multiple forms of each type of hydrogenase (Halboth and Klein, 1992). It was of definite interest to see whether they also carried an "$F_{420}H_2$

dehydrogenase" activity. In addition, *Mc. thermolithotrophicus* was grown with two different substrates, on formate and under $H_2$:$CO_2$, this was important since the formate dehydrogenase in *Mc. thermolithotrophicus* growing on formate acts as the sole electron source for cofactor $F_{420}$.

The results of this segment of the survey are shown in Table 3. The assay for the "$F_{420}H_2$ dehydrogenase" indicated the presence of this enzyme activity in this group of methanogens as well, and the specific activity of the dehydrogenase was found to be very similar in crude systems for both of the species tested. The "$F_{420}H_2$ dehydrogenase" activities from both species were found to be air-stable, like the purified enzymes from *Ml. tindarius* (Haase et al, 1992) and *Ms. mazei* (Abken and Deppenmeier, 1997).

A separation attempt was made using sucrose density gradient ultracentrifugation on these species as well, and the results are shown on Figures 4 (*Mc. thermolithotrophicus*, grown on formate), 5 (*Mc. thermolithotrophicus*, grown on $H_2$:$CO_2$), and 6 (*Mc. voltae*). *Mc. thermolithotrophicus* displayed maxima for the $F_{420}$-reducing hydrogenase and the "$F_{420}H_2$ dehydrogenase" that occured in different fractions (though side-by-side) in the sample grown on formate, with a similar profile for that grown on $H_2$:$CO_2$. *Mc. voltae* displays an interesting profile, there appeared to be two peaks for both the $F_{420}$ hydrogenase and the "$F_{420}H_2$ dehydrogenase", the lower density activities did not coincide precisely, although, the higher density activities did. Whether this dual peak of "$F_{420}H_2$ dehydrogenase" activity represents multiple forms of this activity is unknown, although it is probable that the $F_{420}$-hydrogenase activity may account for a portion of the $F_{420}H_2$-oxidizing activity in these methanogens, since MV independent $F_{420}H_2$ oxidation is also observed in these species (Table 3).

Like in the previous group of methanogens, the ratio of the activities DH/$H_2$ase were not constant in the fractions which held both activities (Table 4). The raw data for the activities detected in the fractions for all tested species will be found in Appendix B.

**Table 3 - Extract Activities for MV Hydrogenase, $F_{420}$ Hydrogenase and MV-dependent $F_{420}H_2$ Dehydrogenase from the *Methanococcales*.**

| Methanogenic species | MV Hydrogenase (µmoles MV/min/mg) | $F_{420}$ Hydrogenase (µmoles $F_{420}$/min/mg) | | $F_{420}H_2$ Dehydrogenase (µmoles $F_{420}$/min/mg) |
|---|---|---|---|---|
| | | Reducing | Oxidizing | |
| ***Methanococcus thermolithotrophicus*** **grown on $H_2$:$CO_2$** | 9.456 | 0.311 | 0.042 | 0.084 |
| ***Methanococcus thermolithotrophicus*** **grown on Formate** | 20.163 | 0.557 | 0.035 | 0.056 |
| ***Methanococcus voltae*** | 2.419 | 0.043 | 0.002 | 0.043 |

**Figure 4 -** Linear sucrose density gradient ultracentrifugation profile for *Methanococcus thermolithotrophicus* (formate). Cell-free extract was applied to a 0-20% sucrose gradient. (●) - $F_{420}$ hydrogenase; (○) - $F_{420}H_2$ dehydrogenase; (▲) - MV hydrogenase activity. The bars represent the absorbance at 280 nm.

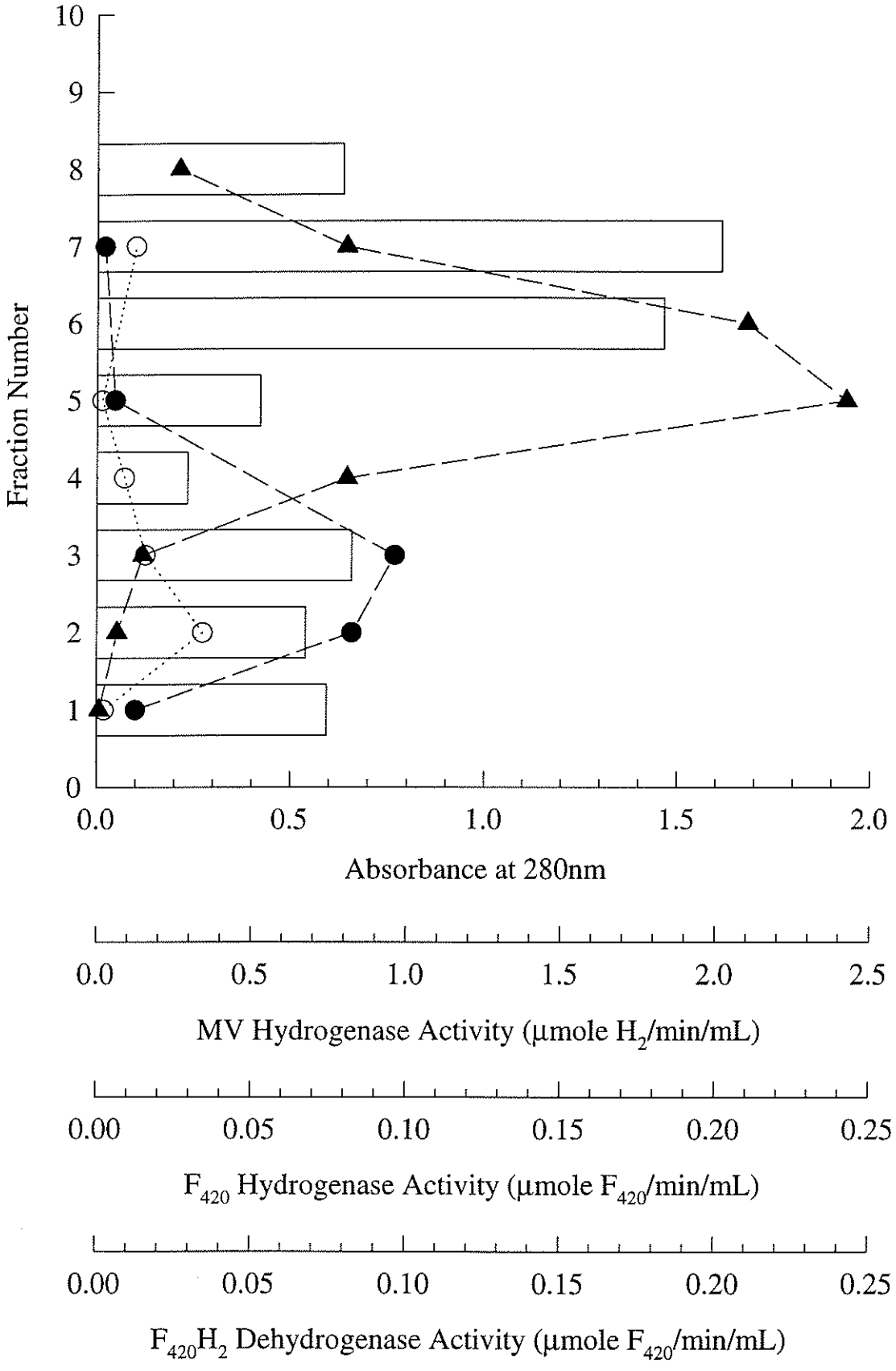

Fraction Number
10
9
8
7
6
5
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
Absorbance at 280nm
0.0
0.5
1.0
1.5
2.0
2.5
MV Hydrogenase Activity (µmole H2/min/mL)
0.00
0.05
0.10
0.15
0.20
0.25
F420 Hydrogenase Activity (µmole F420/min/mL)
0.00
0.05
0.10
0.15
0.20
0.25
F420H2 Dehydrogenase Activity (µmole F420/min/mL)

**Figure 5 -** Linear sucrose density gradient ultracentrifugation profile for *Methanococcus thermolithotrophicus* ($H_2$:$CO_2$). Cell-free extract was applied to a 0-20% sucrose gradient. (●) - $F_{420}$ hydrogenase; (○) - $F_{420}H_2$ dehydrogenase; (▲) - MV hydrogenase activity. The bars represent the absorbance at 280 nm.

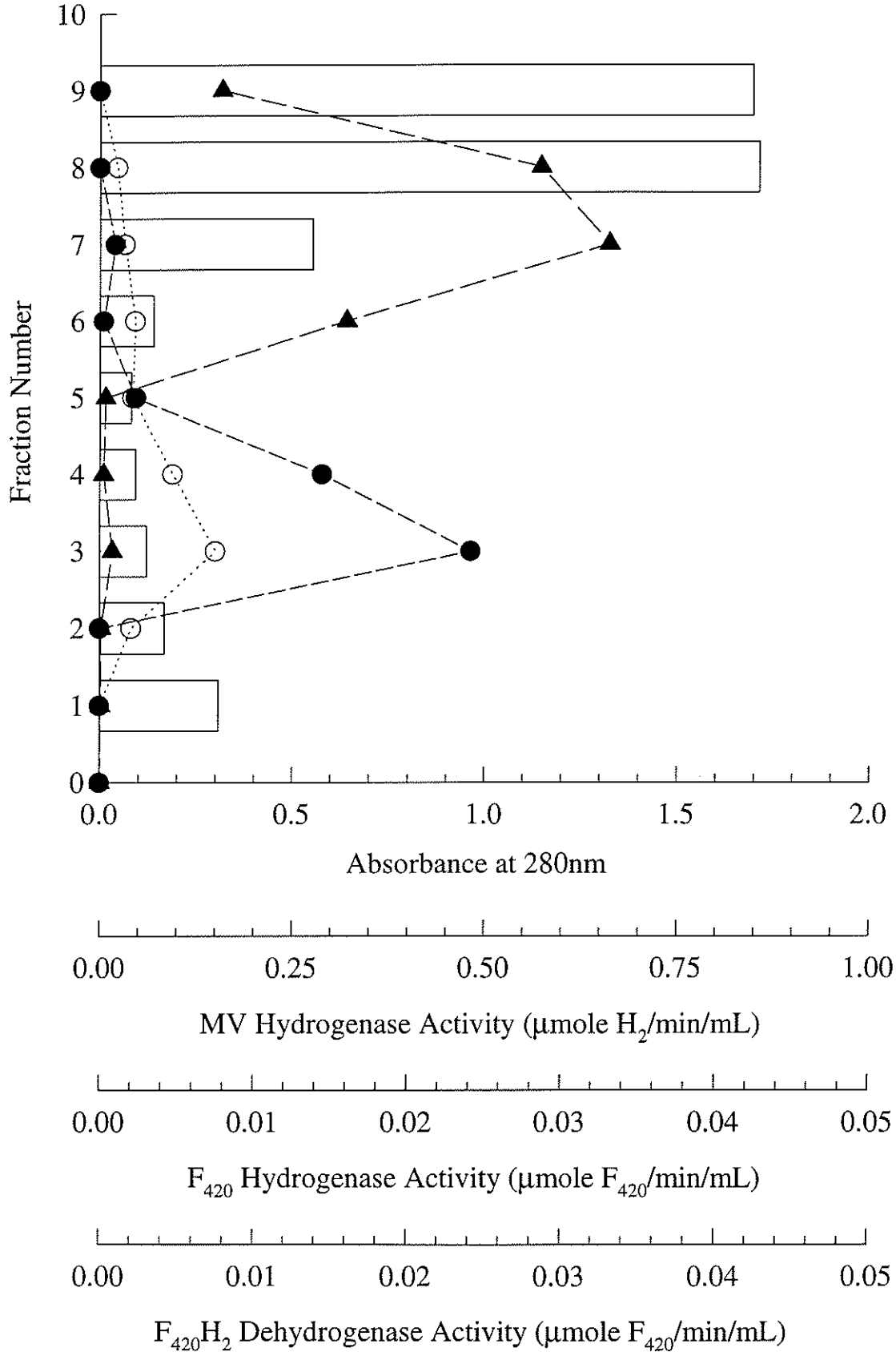

Fraction Number
10
9
8
7
6
5
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
Absorbance at 280nm
0.00
0.25
0.50
0.75
1.00
MV Hydrogenase Activity (µmole H2/min/mL)
0.00
0.01
0.02
0.03
0.04
0.05
F420 Hydrogenase Activity (µmole F420/min/mL)
0.00
0.01
0.02
0.03
0.04
0.05
F420H2 Dehydrogenase Activity (µmole F420/min/mL)

**Figure 6 -** Linear sucrose density gradient ultracentrifugation profile for *Methanococcus voltae*. Cell-free extract was applied to a 0-20% sucrose gradient. (●) - $F_{420}$ hydrogenase; (○) - $F_{420}H_2$ dehydrogenase; (▲) - MV hydrogenase activity. The bars represent the absorbance at 280 nm.

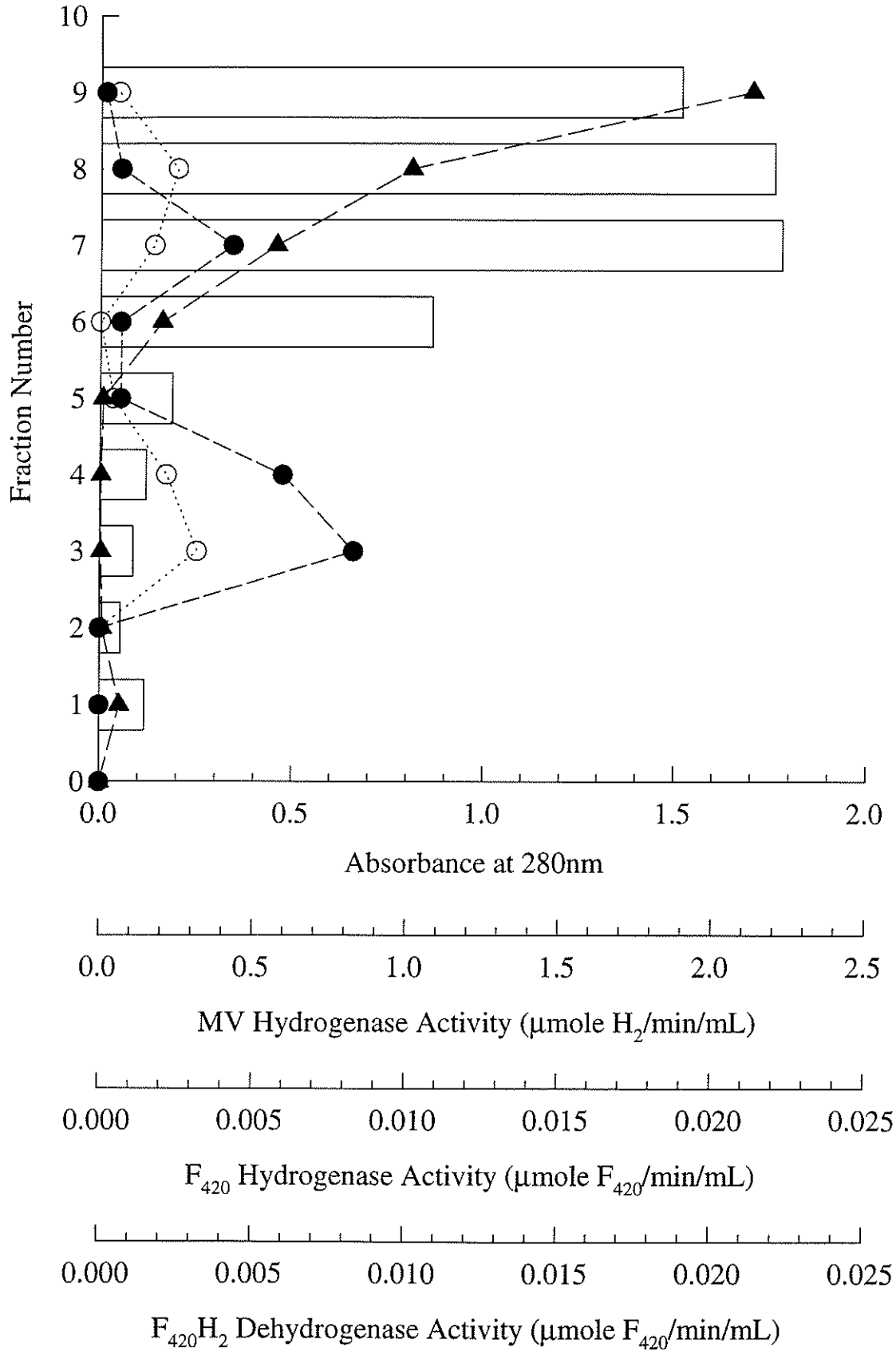

Fraction Number
10
9
8
7
6
5
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
Absorbance at 280nm
0.0
0.5
1.0
1.5
2.0
2.5
MV Hydrogenase Activity (µmole H2/min/mL)
0.000
0.005
0.010
0.015
0.020
0.025
F420 Hydrogenase Activity (µmole F420/min/mL)
0.000
0.005
0.010
0.015
0.020
0.025
F420H2 Dehydrogenase Activity (µmole F420/min/mL)

**Table 4 - Ratios of $F_{420}H_2$ DH:$F_{420}$ Hydrogenase activity in fractions of Sucrose Gradient.**

| Fraction # | *Methanococcus thermolithotrophicus* (Formate) | *Methanococcus thermolithotrophicus* ($H_2$:$CO_2$) | *Methancoccus voltae* |
|---|---|---|---|
| **1** | 0.2 | 0.0 | 0.0 |
| **2** | 0.4 | ∞ | 0.0 |
| **3** | 0.2 | 0.3 | 0.4 |
| **4** | - | 0.3 | 0.4 |
| **5** | 0.3 | 0.9 | 0.6 |
| **6** | - | 9.2 | 0.0 |
| **7** | 5.0 | 1.6 | 0.4 |
| **8** | - | ∞ | 3.8 |
| **9** | - | 0.0 | 3.5 |

Note: "-" indicates fraction not available or not tested.

### 6.2.3. $F_{420}H_2$ Dehydrogenase Activities in Order *Methanomicrobiales*

The presence of the $F_{420}H_2$ dehydrogenase in this Order is expected, since the original isolation of this activity was made on a member of this group (*Ml. tindarius*; Haase et al., 1992). In addition to *Ml. tindarius*, several other members of the *Methanomicrobiales* were tested for "$F_{420}H_2$ dehydrogenase" activity, including *Ms. barkeri* strain Fusaro, *Mst. concilii*, and *Msp. hungatei*.

The enzyme activity survey of these species is summarized in Table 5. Again, all tested organisms showed the presence of the "$F_{420}H_2$ dehydrogenase" activity in crude extract and all were found to be air-stable. The level of this activity is interestingly lower in *Ms. barkeri* grown on $H_2$:$CO_2$ compared to growth on methanol, at the same time, the level of the MV hydrogenase activity in the methanol grown cells is lower than in cells grown on $H_2$:$CO_2$. In general, the level of the "$F_{420}H_2$ dehydrogenase" activity in *Methanosarcina* is lower compared to those found in the other members of the *Methanomicrobiales* tested.

**Table 5 - Extract Activities of MV Hydrogenase, $F_{420}$ Hydrogenase and MV-dependent $F_{420}H_2$ Dehydrogenase from the *Methanomicrobiales*.**

| Organism | MV Hydrogenase (µmoles MV/min/mg) | $F_{420}$ Hydrogenase (µmoles $F_{420}$/min/mg) | | $F_{420}H_2$ Dehydrogenase (µmoles $F_{420}$/min/mg) |
|---|---|---|---|---|
| | | Reducing | Oxidizing | |
| *Methanolobus tindarius* | 0.059 | not detected | not detected | 0.263 |
| *Methanosaeta concilii* strain GP6 | 0.098 | 0.002 | not detected | 0.079 |
| *Methanosarcina barkeri* strain Fusaro grown on Acetate | 2.790 | 0.091 | 0.022 | 0.098 |
| *Methanosarcina barkeri* strain Fusaro grown on $H_2$:$CO_2$ | 0.933 | 0.081 | <0.001 | 0.011 |
| *Methanosarcina barkeri* strain Fusaro grown on Methanol | 0.723 | 0.166 | <0.001 | 0.036 |
| *Methanospirillum hungatei* strain GP1 | 0.908 | 0.100 | 0.013 | 0.084 |

All four species were analyzed via sucrose density gradient centrifugation as well, and the profiles are shown on Figures 7 (*Ml. tindarius*), 8 (*Mst. concilii*), 9 (*Msp. hungatei*), 10 (*Ms. barkeri* grown on acetate), 11 (*Ms. barkeri* grown on $H_2$:$CO_2$), and 12 (*Ms. barkeri* grown on methanol). The activities found in *Ml. tindarius* and *Mst. concilii* appear to have similar migration locations on sucrose gradients, and both have MV hydrogenase activity as has been previously reported (König and Stetter, 1982; Patel, 1984), while *Mst. concilii* has a very low level of $F_{420}$-reducing hydrogenase activity, which did coincide with the "$F_{420}H_2$ dehydrogenase" activity. In all of the profiles from *Ms. barkeri* and *Msp. hungatei*, the activities of the $F_{420}$ hydrogenase and the "$F_{420}H_2$ dehydrogenase" do appear to coincide on the sucrose gradients.

The ratios of $F_{420}$ DH/$H_2$ase (see Table 6 and 7) in the species which do carry detectable activities of both were also found to not be constant in the fractions in which both activities were detected. The data for all of the activities detected in the fractions for all species tested will be found in Appendix B.

It is interesting that the specific activity of the "$F_{420}H_2$ dehydrogenase" was found to be present and high when grown on acetate, compared to *Ms. barkeri* grown on $H_2$:$CO_2$. The activity of the MV hydrogenase on these profiles appear to vary based on the methanogenic substrate used. The activity (the low density MV-only form) is high when grown on acetate and higher than the $F_{420}$ hydrogenase activity in *Msp. hungatei.* There is a difference in the relative levels of both these enzyme activities in *Ms. barkeri* when grown on methanol compared to under $H_2$:$CO_2$. It is almost absent when grown on methanol (see figures 10-12). This variation in the MV hydrogenase levels between the methanol grown and the $H_2$:$CO_2$ grown forms follows the model of the methanogenic pathway as described by Deppenmeier et al (1994).

### 6.2.4. Interference of Assay System by Reverse $F_{420}$-Dependent Hydrogenase

An important consideration in assessing the presence of the enzyme activity, "$F_{420}H_2$ dehydrogenase", is the effect of the reverse activity of the $F_{420}$-dependent hydrogenase in order to ascertain whether the anaerobic $F_{420}H_2$-oxidizing activity that is

**Figure 7 -** Linear sucrose density gradient ultracentrifugation profile for *Methanolobus tindarius*. Cell-free extract was applied to a 0-20% sucrose gradient. (●) - $F_{420}$ hydrogenase; (○) - $F_{420}H_2$ dehydrogenase; (▲) - MV hydrogenase activity. The bars represent the absorbance at 280 nm.

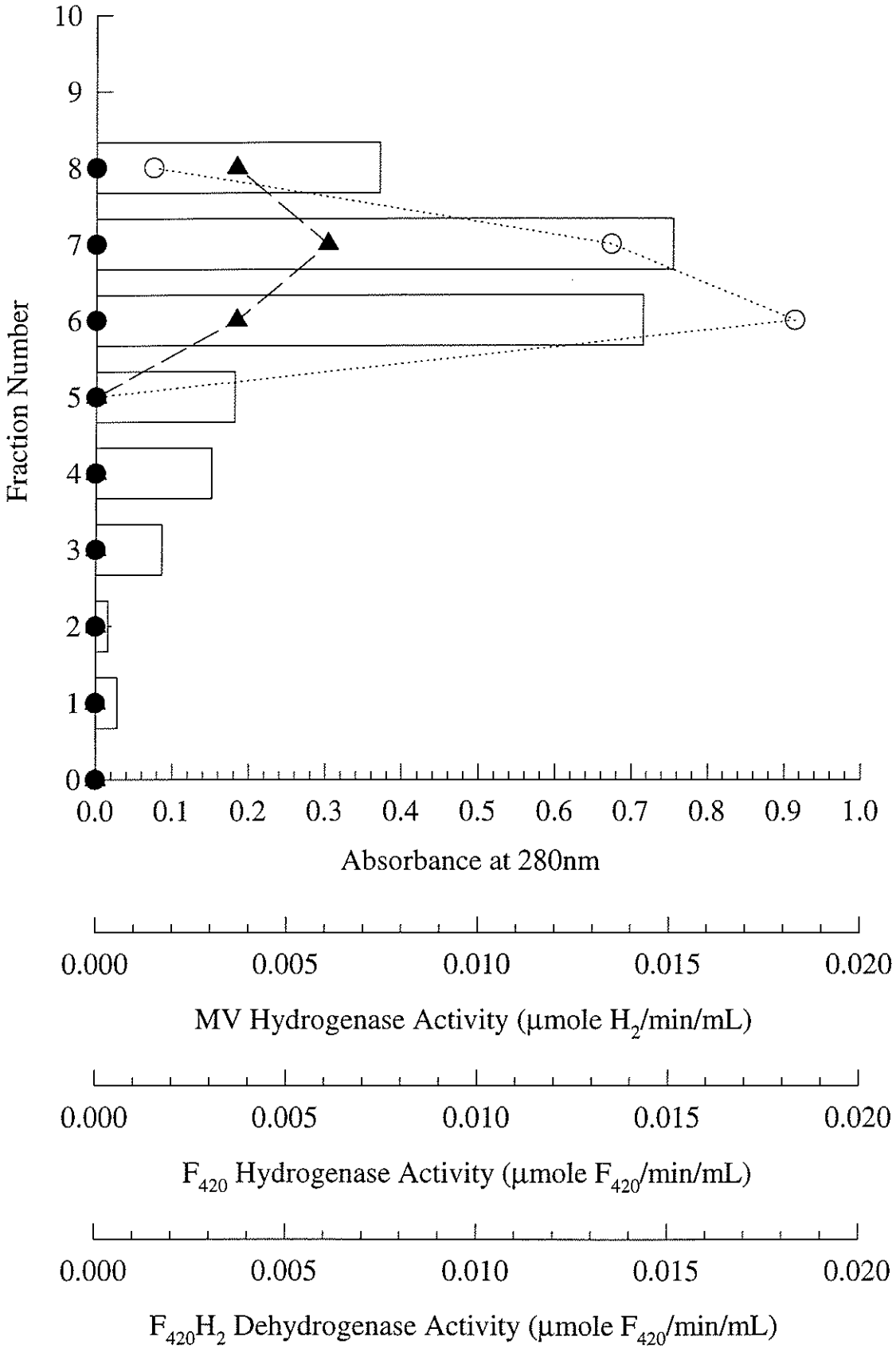

Fraction Number
10
9
8
7
6
5
4
3
2
1
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Absorbance at 280nm
0.000
0.005
0.010
0.015
0.020
MV Hydrogenase Activity (µmole $H_2$/min/mL)
0.000
0.005
0.010
0.015
0.020
$F_{420}$ Hydrogenase Activity (µmole $F_{420}$/min/mL)
0.000
0.005
0.010
0.015
0.020
$F_{420}H_2$ Dehydrogenase Activity (µmole $F_{420}$/min/mL)

**Figure 8 -** Linear sucrose density gradient ultracentrifugation profile for *Methanosaeta concilii*. Cell-free extract was applied to a 0-20% sucrose gradient. (●) - $F_{420}$ hydrogenase; (○) - $F_{420}H_2$ dehydrogenase; (▲) - MV hydrogenase activity. The bars represent the absorbance at 280 nm.

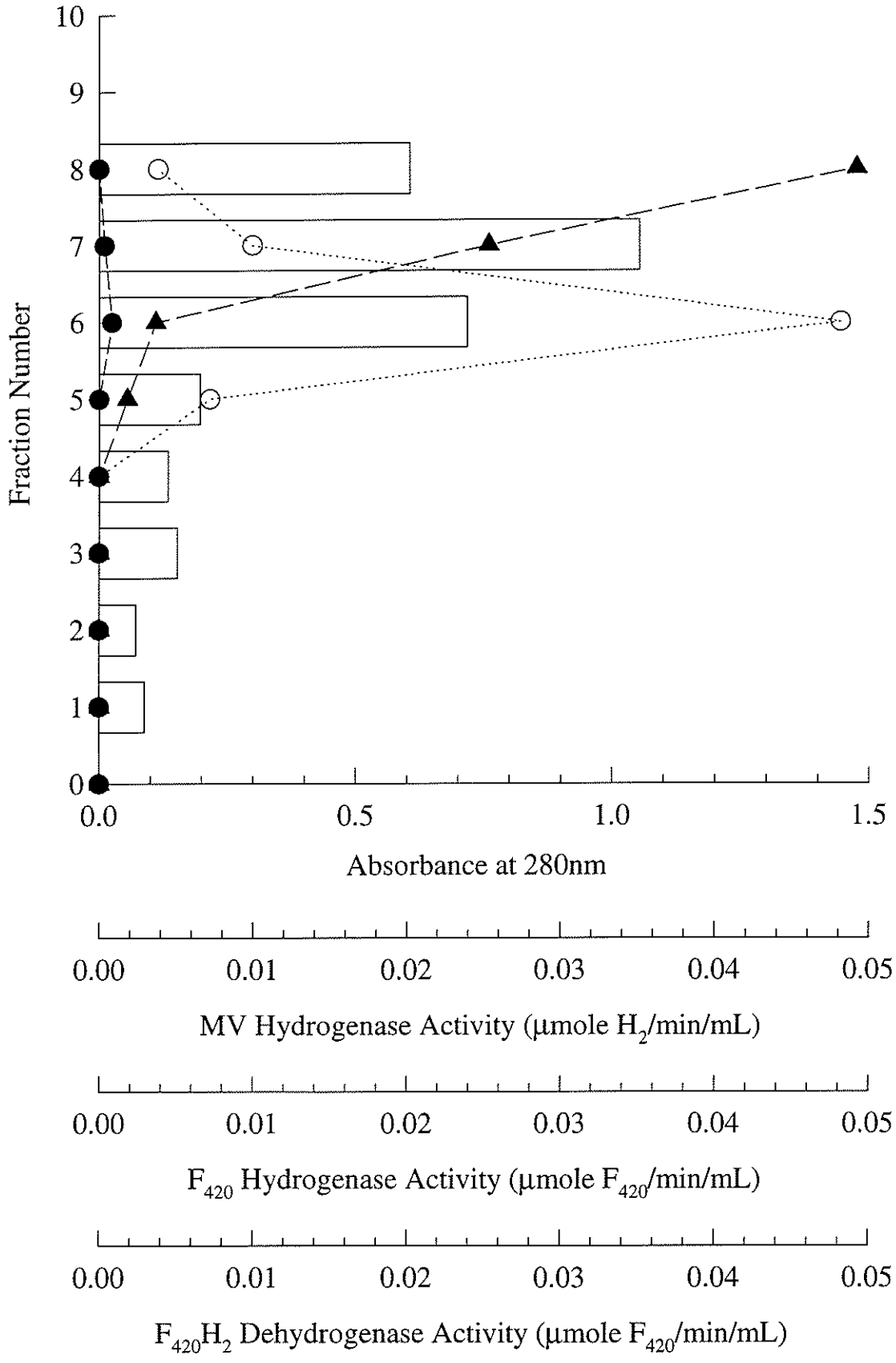

Fraction Number
10
9
8
7
6
5
4
3
2
1
0
0.0
0.5
1.0
1.5
Absorbance at 280nm
0.00
0.01
0.02
0.03
0.04
0.05
MV Hydrogenase Activity (µmole H2/min/mL)
0.00
0.01
0.02
0.03
0.04
0.05
F420 Hydrogenase Activity (µmole F420/min/mL)
0.00
0.01
0.02
0.03
0.04
0.05
F420H2 Dehydrogenase Activity (µmole F420/min/mL)

**Figure 9 -** Linear sucrose density gradient ultracentrifugation profile for *Methanospirillum hungatei*. Cell-free extract was applied to a 0-20% sucrose gradient. (●) - $F_{420}$ hydrogenase; (○) - $F_{420}H_2$ dehydrogenase; (▲) - MV hydrogenase activity. The bars represent the absorbance at 280 nm.

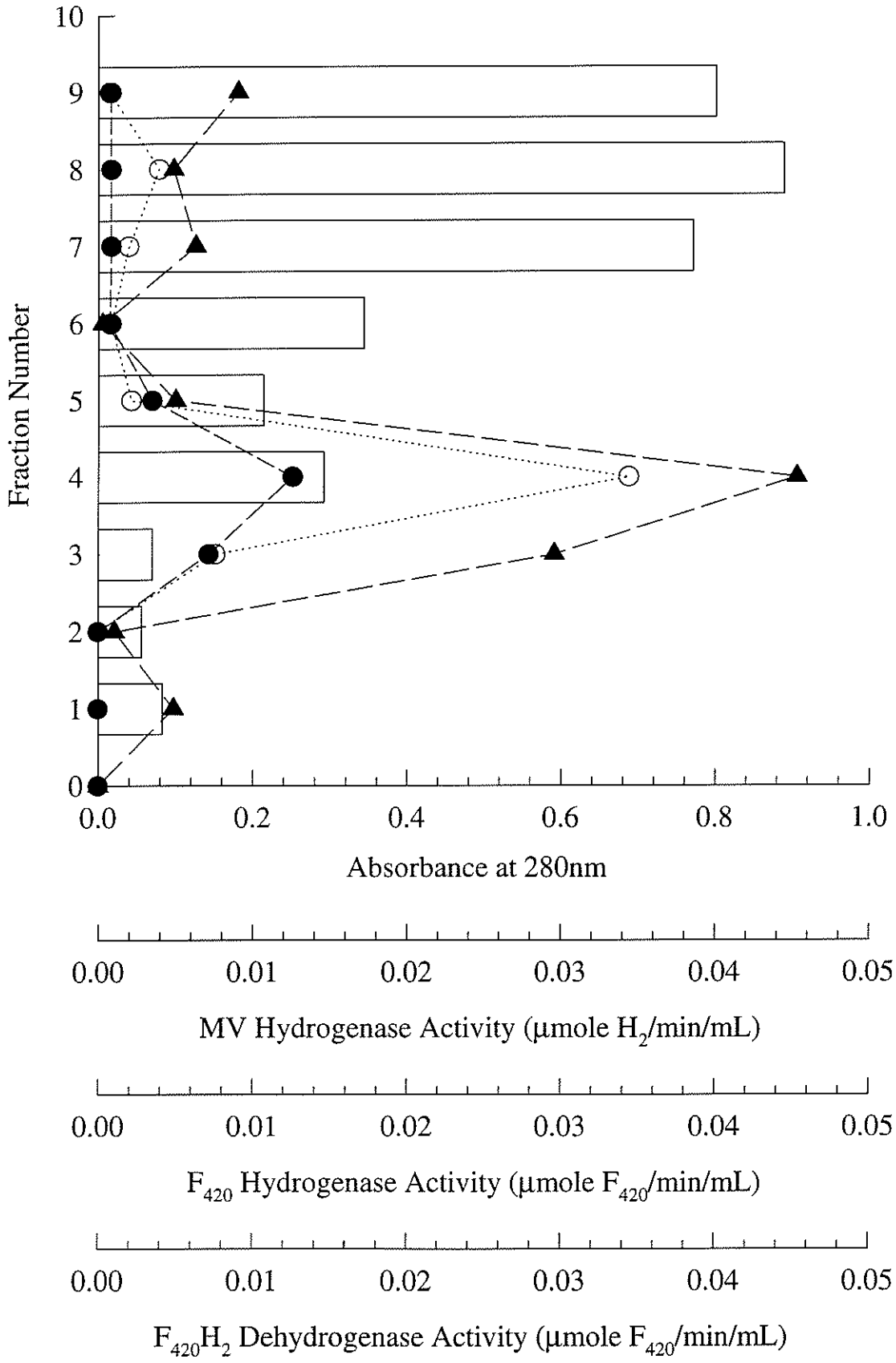

Fraction Number
10
9
8
7
6
5
4
3
2
1
0
0.0
0.2
0.4
0.6
0.8
1.0
Absorbance at 280nm
0.00
0.01
0.02
0.03
0.04
0.05
MV Hydrogenase Activity (µmole H2/min/mL)
0.00
0.01
0.02
0.03
0.04
0.05
F420 Hydrogenase Activity (µmole F420/min/mL)
0.00
0.01
0.02
0.03
0.04
0.05
F420H2 Dehydrogenase Activity (µmole F420/min/mL)

**Figure 10 -** Linear sucrose density gradient ultracentrifugation profile for *Methanosarcina barkeri* strain Fusaro (grown on acetate). Cell-free extract was applied to a 0-20% sucrose gradient. (●) - $F_{420}$ hydrogenase; (○) - $F_{420}H_2$ dehydrogenase; (▲) - MV hydrogenase activity. The bars represent the absorbance at 280 nm.

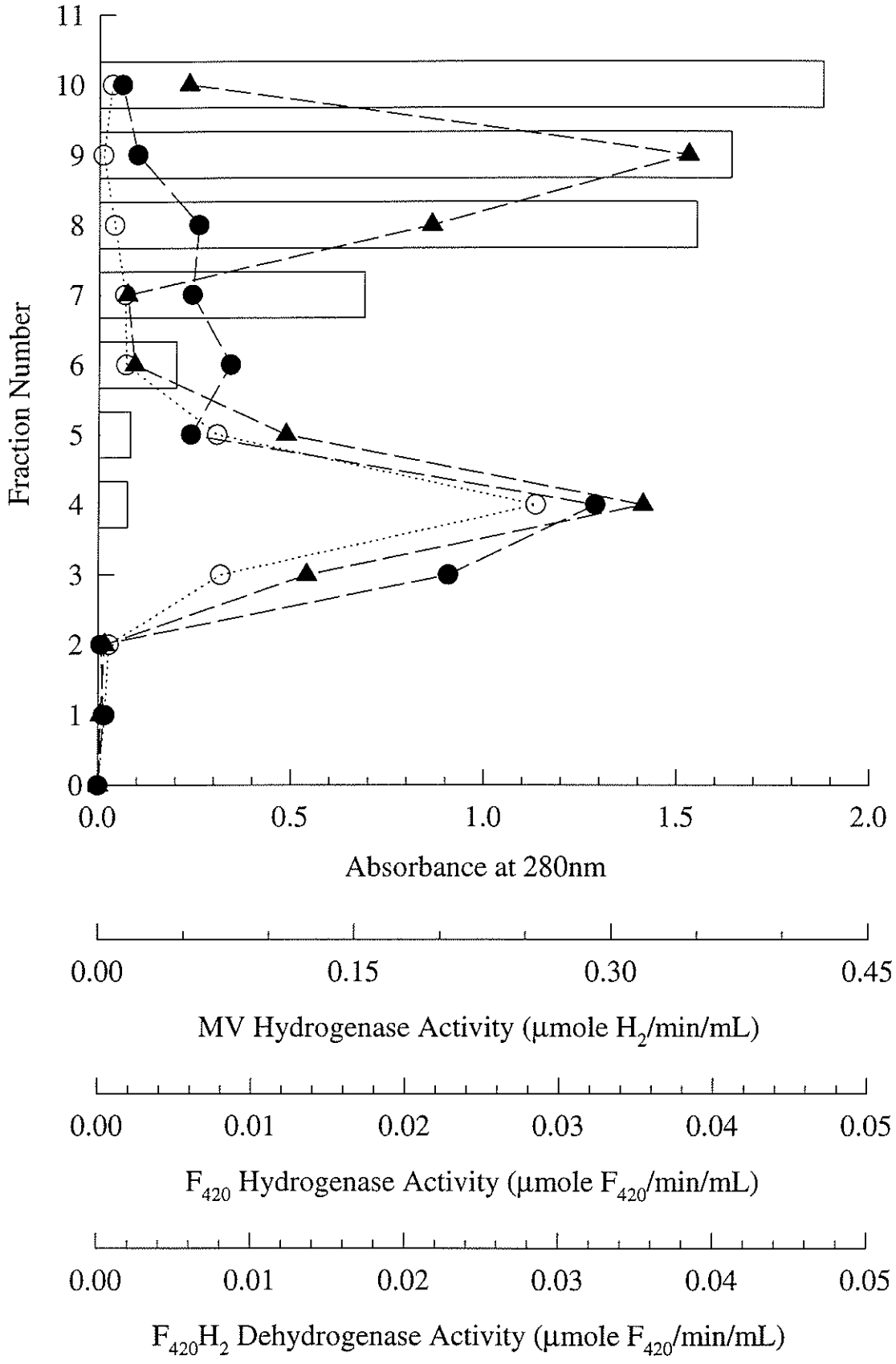

Fraction Number
11
10
9
8
7
6
5
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
Absorbance at 280nm
0.00
0.15
0.30
0.45
MV Hydrogenase Activity (µmole H2/min/mL)
0.00
0.01
0.02
0.03
0.04
0.05
F420 Hydrogenase Activity (µmole F420/min/mL)
0.00
0.01
0.02
0.03
0.04
0.05
F420H2 Dehydrogenase Activity (µmole F420/min/mL)

**Figure 11 -** Linear sucrose density gradient ultracentrifugation profile for *Methanosarcina barkeri* strain Fusaro (grown on $H_2$:$CO_2$). Cell-free extract was applied to a 0-20% sucrose gradient. (●) - $F_{420}$ hydrogenase; (○) - $F_{420}H_2$ dehydrogenase; (▲) - MV hydrogenase activity. The bars represent the absorbance at 280 nm.

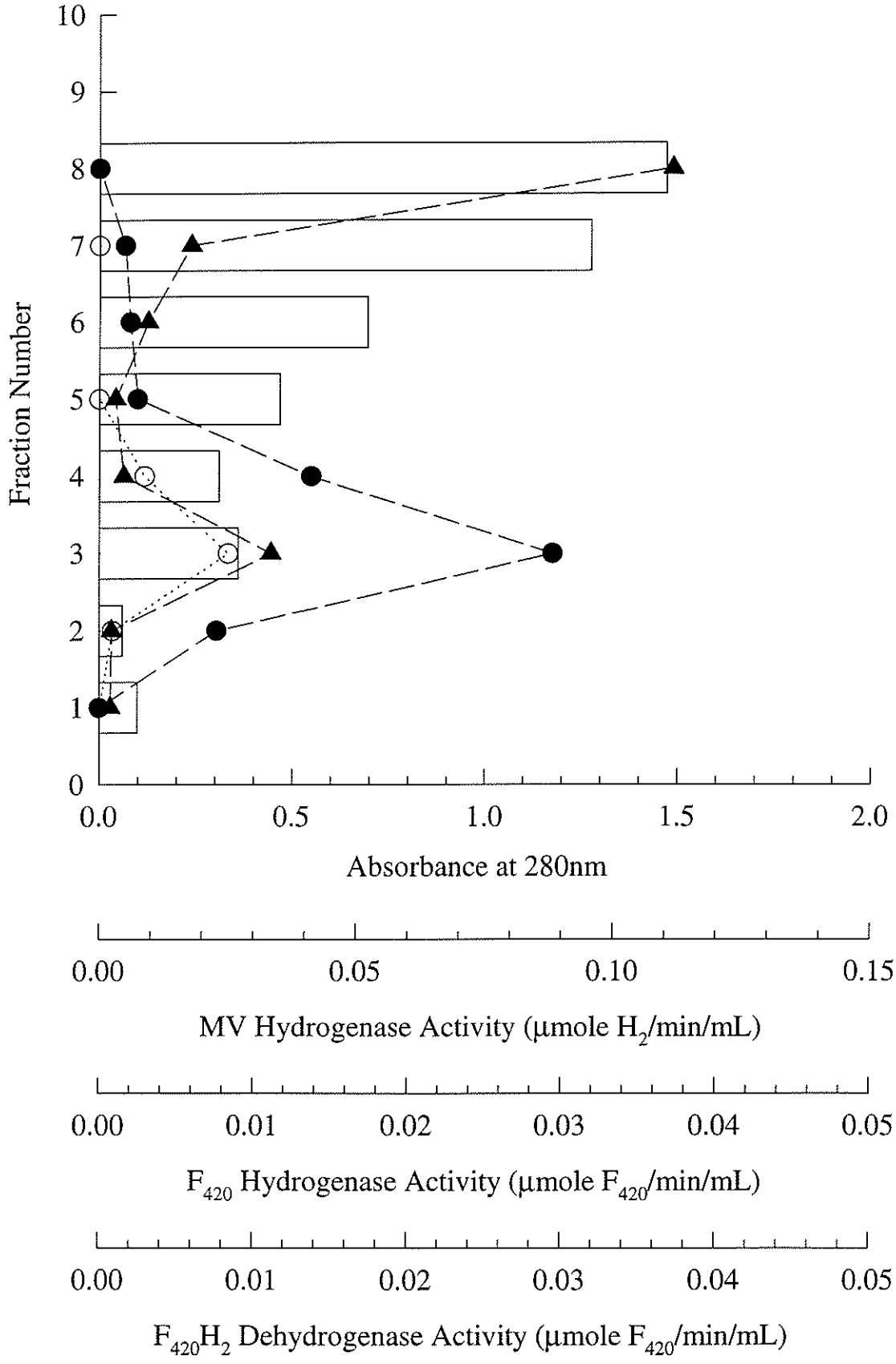

Fraction Number
10
9
8
7
6
5
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
Absorbance at 280nm
0.00
0.05
0.10
0.15
MV Hydrogenase Activity (µmole $H_2$/min/mL)
0.00
0.01
0.02
0.03
0.04
0.05
$F_{420}$ Hydrogenase Activity (µmole $F_{420}$/min/mL)
0.00
0.01
0.02
0.03
0.04
0.05
$F_{420}H_2$ Dehydrogenase Activity (µmole $F_{420}$/min/mL)

**Figure 12 -** Linear sucrose density gradient ultracentrifugation profile for *Methanosarcina barkeri* strain Fusaro (grown on methanol). Cell-free extract was applied to a 0-20% sucrose gradient. (●) - $F_{420}$ hydrogenase; (○) - $F_{420}H_2$ dehydrogenase; (▲) - MV hydrogenase activity. The bars represent the absorbance at 280 nm.

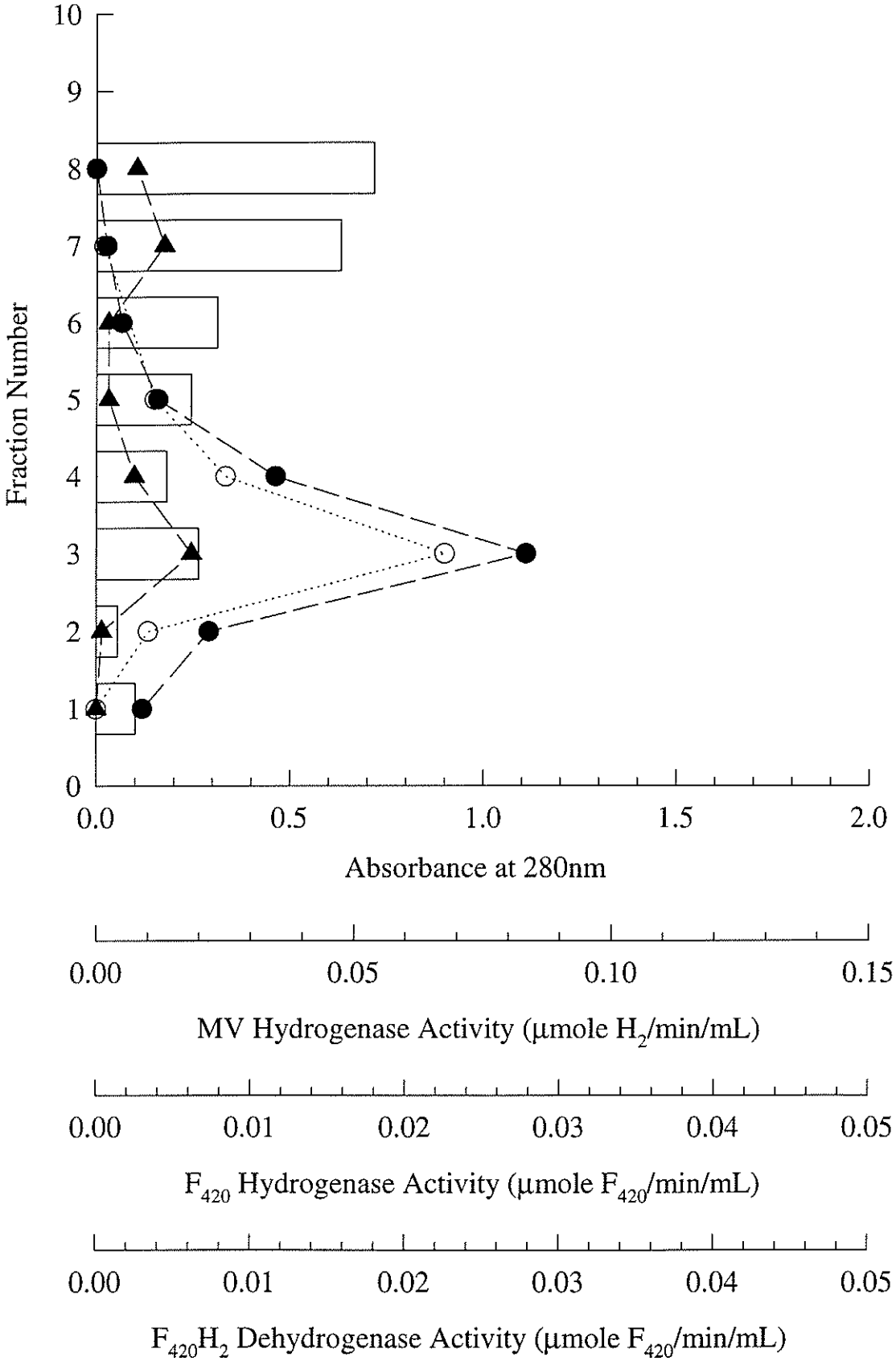

Fraction Number
10
9
8
7
6
5
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
Absorbance at 280nm
0.00
0.05
0.10
0.15
MV Hydrogenase Activity (µmole $H_2$/min/mL)
0.00
0.01
0.02
0.03
0.04
0.05
$F_{420}$ Hydrogenase Activity (µmole $F_{420}$/min/mL)
0.00
0.01
0.02
0.03
0.04
0.05
$F_{420}H_2$ Dehydrogenase Activity (µmole $F_{420}$/min/mL)

**Table 6 - Ratios of $F_{420}H_2$ DH:$F_{420}$ Hydrogenase activity in fractions of Sucrose Gradient.**

| Fraction # | *Methanolobus tindarius* | *Methanosaeta concilii* | *Methanospirillum hungatei* |
|---|---|---|---|
| **1** | 0.0 | 0.0 | 0.0 |
| **2** | 0.0 | 0.0 | 0.0 |
| **3** | 0.0 | 0.0 | 1.1 |
| **4** | 0.0 | 0.0 | 2.7 |
| **5** | 0.0 | ∞ | 0.6 |
| **6** | ∞ | 58.2 | 1.1 |
| **7** | ∞ | 121.0 | 2.4 |
| **8** | ∞ | ∞ | 4.8 |
| **9** | - | - | 0.9 |

Note: "-" indicates fraction not available or not tested.

**Table 7 - Ratios of $F_{420}H_2$ DH:$F_{420}$ Hydrogenase activity in fractions of Sucrose Gradient.**

| Fraction # | *Methanosarcina barkeri* (Acetate) | *Methanosarcina barkeri* ($H_2$:$CO_2$) | *Methanosarcina barkeri* (Methanol) |
|---|---|---|---|
| **1** | 1.3 | 0.0 | 0.0 |
| **2** | 4.0 | 0.1 | 0.5 |
| **3** | 0.3 | 0.3 | 0.8 |
| **4** | 0.9 | 0.2 | 0.7 |
| **5** | 1.3 | 0.0 | 0.9 |
| **6** | 0.2 | - | - |
| **7** | 0.3 | 0.0 | 0.8 |
| **8** | 0.2 | - | - |
| **9** | 0.1 | - | - |
| **10** | 0.6 | - | - |

Note: "-" indicates fraction not available or not tested.

measured is not just an artifact of the $F_{420}$-hydrogenase activity, which is found in many of the tested organisms used in the survey of the previous three sections. As another consideration, this reverse $F_{420}$-hydrogenase would be a background competitor (ie., the $F_{420}$ hydrogenase could cause activities of $F_{420}H_2$ oxidation to be higher than could be accounted for assuming that a $F_{420}H_2$ dehydrogenase were also present) for the $F_{420}H_2$ with the "$F_{420}H_2$ dehydrogenase" activity.

The extracts of the same tested organisms were also tested for $F_{420}H_2$ oxidation in the absence of added electron acceptors, in order to test the impact of the added cofactors on the rate of $F_{420}H_2$ oxidation in cell extracts and this oxidation is most likely attributed to the $F_{420}$ hydrogenase. The system used was similar to that used by Livingston et al. to assay the hydrogen-producing or the $F_{420}H_2$-oxidizing activity of the $F_{420}$ hydrogenase from *Mb. thermoautotrophicum* strain ΔH (Livingston et al, 1987), which was similar to the $F_{420}H_2$ dehydrogenase assay system with the exception that no other electron acceptors are added to the system (ie. methyl viologen and metronidazole), just anaerobic assay buffer under a non-hydrogen gas phase (Ar or $N_2$) and $F_{420}H_2$.

The results of this survey were summarized under the heading of the $F_{420}$ hydrogenase, oxidizing activity, on Tables 1, 3 and 5. The values for the $F_{420}H_2$ dehydrogenase activities can be compared in the column next to it. In general, it appears that the reverse $F_{420}$ hydrogenase only constitutes a small portion of the $F_{420}H_2$-oxidizing activity found in the extracts of the methanogens tested.

In the tested members of the *Methanobacteriales*, the fraction of the total $F_{420}H_2$-oxidizing activity attributed to the $F_{420}$ hydrogenase is 41.8% in *Methanobacterium* strain G2R, 9.9% in *Mb. bryantii*, and 4.2% in *Mb. thermoautotrophicum* strain Marburg. The value found in *Methanobacterium* strain G2R is fairly high and leads one to believe that the $F_{420}H_2$-oxidation activity is likely due to the $F_{420}$-dependent hydrogenase activity in this organism. In the *Methanococcales*, there appeared to be a wide range of the fraction of $F_{420}H_2$-oxidation attributed to the reverse hydrogenase activity under the conditions assayed, from a low of 4.9% in *Mc. voltae*, to 49.3% in *Mc. thermolithotrophicus* grown on $H_2$:$CO_2$ and 62.5% in *Mc. thermolithotrophicus* grown on formate. In the tested

members of the *Methanomicrobiales*, fairly low fractions of the total $F_{420}H_2$-oxidizing activity attributed to the $F_{420}$ hydrogenase are observed. In both growing conditions of methanol and under $H_2$:$CO_2$ for *Ms. barkeri*, there is barely detectable oxidation of $F_{420}H_2$ in the absence of added electron acceptors, while growth on acetate yields a fraction of 21.1%. In *Msp. hungatei*, the fraction of reverse hydrogenase activity compare to dehydrogenase activity was found to be 15%. In *Ml. tindarius* and *Mst. concilii*, there was no or low $F_{420}$ hydrogenase and none of its reverse activity detected.

The only methanogenic species with a significant fraction attributed to the $F_{420}$ hydrogenase was in *Mc. thermolithotrophicus*, grown either on formate or on $H_2$:$CO_2$ as methanogenic substrate, in both of these cases, approximately 50-60% of the $F_{420}H_2$-oxidizing activity may be associated with the $F_{420}$ hydrogenase activity acting in the reverse direction and in *Mb.* strain G2R, with around 40%. It appears that there may be an $F_{420}H_2$-oxidizing activity independent of the $F_{420}$ hydrogenase in these species, since in most cases only less than half of the observed $F_{420}H_2$-oxidizing activity can be attributed to the reverse hydrogenase activity, however, the difference in the assay concentrations of the electron acceptors for $F_{420}H_2$ in the two assay systems (0.5 mM MV versus approx. 0.01 μM $H^+$) would have a significant bearing on the levels of activity detected. In addition, some purified enzymes work better with artificial substrates, ie., have a higher detected activity with an artifical electron acceptor than with the perceived natural electron acceptor (eg., MV versus $H^+$).

### 6.2.5. Phenazine-mediated $F_{420}H_2$-oxidation.

The presence of an MV-dependent $F_{420}H_2$-oxidizing activity does not conclusively indicate that any of the tested organisms carry an $F_{420}H_2$ dehydrogenase. The recent discovery of a phenazine derived compound from the membranes of a methanogen, *Ms. mazei* strain Gö1 (Abken et al., 1998), and the shown linkage of the $F_{420}H_2$ dehydrogenase purified from this species (Abken and Deppenmeier, 1997) to this molecule as its natural electron acceptor may provide a more specific system to assay for the $F_{420}H_2$ dehydrogenase. In that study, it was also shown that the $F_{420}H_2$ dehydrogenase could also

use other phenazine derivatives, including the basic structural molecule, phenazine, as well as two substituted variants, 2-hydroxyphenazine and 2-bromophenazine. It was shown in the previous chapter that the $F_{420}H_2$ dehydrogenase activity from *Msph. stadtmanae* could oxidize $F_{420}H_2$ in the presence of phenazine. Several examples of methanogens were also tested for the presence of $F_{420}H_2$-oxidation in the presence of phenazine in the assay system. The results are shown in Table 8.

**Table 8 - Phenazine as Electron Acceptor for $F_{420}H_2$ Dehydrogenase Activity**

| Methanogenic Species | $F_{420}H_2$ Dehydrogenase (with MTZ and MV) | $F_{420}H_2$ Dehydrogenase (with phenazine) |
|---|---|---|
| *Methanobacterium bryantii* strain M.o.H. | 35 | 305 |
| *Methanococcus thermolithotrophicus* (grown on formate) | 30 | 388 |
| *Methanolobus tindarius* | 170 | 378 |
| *Methanosaeta concilii* | 85 | 84 |
| *Methanospirillum hungatei* | 284 | 169 |

Note: activity expressed in nmoles $F_{420}H_2$ oxidized/min/mg protein.

The selected methanogens include a representative from each Order of the methanogens. From Order *Methanobacteriales*, *Mb. bryantii* strain M.o.H. was selected since it is a well known methanogenic species and has been shown in the past to use alcohols as an electron souce for methanogenesis (Zellner and Winter, 1987). The assay with phenazine shows a significantly higher activity level (~10-fold) compared to using the artificial electron acceptor combination of MTZ and MV. The same activity proportions are shown from the Order *Methanococcales*, with *Mc. thermolithotrophicus* grown on formate. Several members from the Order *Methanomicrobiales* also showed activity with phenazine, though the levels of activity varied from species to species. There was no difference in the activity levels with either electron acceptor system in *Mst. concilii*, while in *Ml. tindarius*, there is a higher activity (~ 2-fold) with phenazine than

with MTZ and MV, and in *Msp. hungatei*, the activity is lower (~ 60% activity) with phenazine than with MTZ and MV. With phenazine as the electron acceptor for the purified enzyme from *Ms. mazei* strain Gö1, the activity with phenazine was only 18% of the activity measured with MTZ and MV (Abken et al, 1998).

### 6.2.6. Role of the $F_{420}H_2$ Dehydrogenase Activity?

If one takes the presence of the $F_{420}H_2$ dehydrogenase activity to be true and is a distinct activity separate from the $F_{420}$-hydrogenase, then a role for this activity must be considered, since it is apparently detected in all of the tested methanogens, regardless of the methanogenic substrate used.

Using the model described by Deppenmeier et al (Deppenmeier et al, 1994), the methanol-only methanogens use the $F_{420}H_2$ dehydrogenase activity to use the electrons carried by $F_{420}H_2$ for the heterodisulfide reductase reaction (through an intermediary electron carrier, cytochrome c, since the actual enzyme when purified only uses methyl viologen as electron acceptor) which is $F_{420}H_2$-linked when using inverted vesicles from two members of these methanogens, *Methanosarcina frisia* strain Gö1 and in *Methanolobus* (Deppenmeier et al, 1991).

Its role in acetate metabolizing methanogens may be similar to that found in the methanol methanogens, but this cannot be confirmed as the heterodisulfide reductase activity in acetate-grown methanogens has not been purified or studied. In the only acetate-grown methanogenic species tested, *Mst. concilii*, the CODH activity, an important enzyme in the metabolism of acetate for methanogenesis, was found to be cofactor $F_{420}$-dependent (see Section 4.1.2.) as well as the CODH enzyme that was purified from *Mtx. soehngenii* (Jetten et al, 1989). For acetate-metabolizing methanogens that do not carry an $F_{420}$-reducing CODH, an $F_{420}H_2$ dehydrogenase activity would still be required for the production of reduced $F_{420}$ for the purposes of biosynthesis.

In methanogens that use secondary alcohols (eg., 2-propanol) as the sole source of electrons, there is the same need for an $F_{420}H_2$ dehydrogenase where $F_{420}$ would be the electron carrier for methanogenesis.

The heterodisulfide reductase activity found in specifically *Mb. thermoautotrophicum* strain ΔH and in *Ms. mazei* strain Gö1 has not been demonstrated to have a cofactor $F_{420}$-dependence, therefore an $F_{420}H_2$ dehydrogenase activity may not be used for the same function as used by the methanol-only methanogens in the current model. This model of electron flow in the $H_2$:$CO_2$ methanogens involves the two different hydrogenases active at the two ends of the methanogenic pathway, the $F_{420}$-hydrogenase provides the electrons to $F_{420}$ for the methanogenic and the biosynthetic pathways and the MV hydrogenase provides the electrons for the heterodisulfide reduction step, which apparently in this model also requires the presence of cytochromes (Deppenmeier et al, 1994). The $F_{420}H_2$ dehydrogenase activity may then be used in these methanogens as an enzymatic regulator of the levels of electrons that are provided by the $F_{420}$-hydrogenase and the MV hydrogenase, assuming that this activity is also reversible. An exception to this two hydrogenase system is found in *Msp. hungatei* (Sprott et al, 1988), where there is only a single hydrogenase entity that is $F_{420}$-dependent and the presence of an $F_{420}H_2$ dehydrogenase may be required for the terminal heterodisulfide reduction step.

One may also envision that if the heterodisulfide reductase was $F_{420}H_2$-dependent, the $F_{420}H_2$ dehydrogenase activity would perform the function of providing the electrons from $F_{420}H_2$ for the purposes of heterodisulfide reduction, as in the case of the methanol and possibly the acetate methanogens as well.

It is interesting that both the $F_{420}H_2$ dehydrogenase and the MV hydrogenase activities share methyl viologen as the electron acceptor for their respective reactions, whether that indicates a similar or a shared natural electron acceptor is yet to be seen or determined.

### 6.3. Summary

In a survey of the methanogenic species available, all the tested cultures were found to be positive in containing an apparent $F_{420}H_2$ dehydrogenase activity, though not many could have the activity distinguished from the $F_{420}$ hydrogenase activity by using

sucrose density gradient ultracentrifugation. Assay for the reverse $F_{420}$ hydrogenase activity appeared to show that not all of the $F_{420}H_2$ dehydrogenase activity could be accounted for by the $F_{420}$ hydrogenase, though some of the activity displayed on assay for the $F_{420}H_2$ dehydrogenase may be attributed to the $F_{420}$-hydrogenase operating in the reverse direction (ie. $F_{420}H_2$-oxidizing or $H_2$-producing activity).

Several of these methanogens were also able to oxidize $F_{420}H_2$ in the presence of phenazine, the water soluble portion of the natural electron acceptor for the $F_{420}H_2$ dehydrogenase purified from *Ms. mazei* (Abken et al, 1998). The activity levels relative to the activities detected using the MTZ and MV system vary from species to species.

An attempt to purify the activity (it is unsure if MV or phenazine was used to track the activity during the purification) found in *Mc. voltae* has been made and was apparently found to be associated only with the $F_{420}$-hydrogenase (U. Deppenmeier, personal communication). Though this may rule out the presence of the $F_{420}H_2$ dehydrogenase in this particular species, it is still possible that this activity may be present in the other methanogens.

In a recent review (Keltjens and Vogels, 1993), the pure $F_{420}$ hydrogenase from *Ms. barkeri* strain Fusaro (the same strain as used in this thesis) was found to exhibit $F_{420}H_2$ dehydrogenase activity. However, no data was presented as to the magnitude of the dehydrogenase activity level compared to the activity level of the hydrogenase in the $F_{420}$-reducing direction.

An interesting feature that is shared by all of the tested methanogens is the fact that the $F_{420}H_2$ dehydrogenase activity was found to be $O_2$-stable, or can be handled aerobically, whereas the activity found specifically in *Msph. stadtmanae* (and *Msph. cuniculi*) was found to be extremely $O_2$-sensitive. Although the levels of the $F_{420}H_2$ dehydrogenase were not tested in this survey under strictly anaerobic conditions, it may be possible that a dehydrogenase could have escaped detection in any one of these methanogens, especially if the dehydrogenase activity shared the property of being extremely $O_2$-sensitive, like that detected in *Msph .stadtmanae.*

# 7. Model of Electron Transport

## 7.1. The Electron Transport Pathway of *Methanosphaera stadtmanae*.

The methanogen, *Msph. stadtmanae* is an obligate methanol plus $H_2$-utilizer, which means that the last reductive step of methanogenesis is not expected to require $F_{420}$ as the electron donor. This organism has been shown conclusively not to be able to reduce cofactor $F_{420}$ in the presence of $H_2$ through the enzyme, hydrogenase, directly. The levels of this cofactor are shown to be very low compared to other methanogens, as $F_{420}$ is not used as the electron donor in its methanogenic pathway.

*Msph. stadtmanae*, however, must be able to reduce this cofactor somehow, and this is where the $F_{420}H_2$ dehydrogenase may enter the picture, it has been found to be present in low activity levels (nmoles $F_{420}H_2$ oxidized/minute/mg protein), sufficient for its biosynthetic purposes. Other enzymes or enzyme activities (see Table 7.1) that have been discovered in this organism include an $NADP^+$:$F_{420}$ oxidoreductase (D. Elias, 1998, MSc thesis, U. of Manitoba) and a secondary alcohol dehydrogenase that is $NADP^+$-dependent (Z. Lin, 1996, PhD thesis, U. of Manitoba).

The most important discovery, next to the detection of the $F_{420}H_2$ dehydrogenase activity, was the apparent linkage between the hydrogenase and the dehydrogenase. It was found that the $H_2$-dependent reduction of $F_{420}$ could occur with the addition of

**Table 7.1 - Electron Transfer Enzyme Activities Detected/Purified from *Msph. stadtmanae*.**

| Enzyme | Purified | Electron Donor/Acceptor |
|---|---|---|
| **Hydrogenase** | Yes | $H_2$/MV (or phenazine?) |
| **$NADP^+$:$F_{420}$ Oxidoreductase** | Yes | $F_{420}H_2$/$NADP^+$<br>NADPH/$F_{420}$ |
| **$F_{420}H_2$ Dehydrogenase** | No | $F_{420}H_2$/MTZ +MV or Phenazine |
| **$NADP^+$-dependent Secondary Alcohol Dehydrogenase** | Yes | 2-propanol/$NADP^+$<br>NADPH/acetone |

phenazine (presumably substituting for a similar molecule that naturally occurs in *Msph. stadtmanae*) and under optimal conditions for the dehydrogenase activity (ie., pH 7.0 and in the presence of 200 mM K-$PO_4$). It has not been shown, yet, whether the hydrogenase from *Msph. stadtmanae* can reduce phenazine directly.

The $NADP^+$:$F_{420}$ oxidoreductase has been discovered in a number of methanogenic bacteria and provides a means of linking the biosynthetic cofactor, $NADP^+$, to the primary electron carrier in the methanogens, cofactor $F_{420}$. The enzyme detected in *Msph. stadtmanae* has been purified and characterized previously in this laboratory (D. Elias, unpublished results).

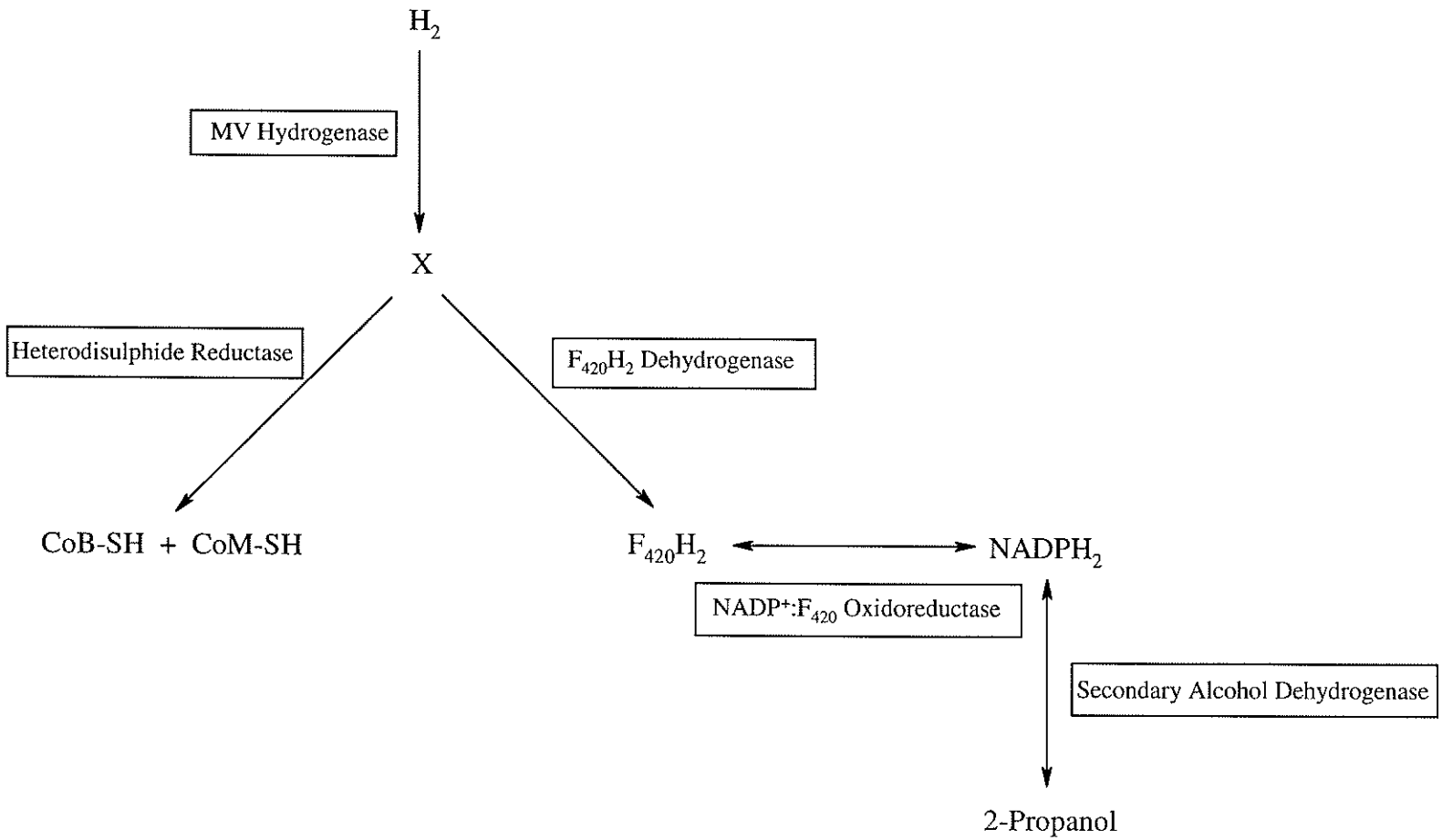
The secondary alcohol (or 2-propanol/2-butanol) dehydrogenase is an interesting finding, this enzyme has been purified recently (F. Lee, Honours Thesis, 1998), but the relevance of this activity is the fact that the activity is $NADP^+$-linked. The addition of a secondary alcohol like 2-propanol to the growth medium was found to increase the growth rate and final growth density of cultures of *Msph. stadtmanae* (Wong et al, 1994), and methanogenesis was found to occur with 2-propanol as the electron donor (ie. in the absence of $H_2$), so electrons from 2-propanol must have a means of entering the electron pathway in *Msph. stadtmanae*.

The presence of the secondary ADH along with the MV hydrogenase(s) and the $F_{420}H_2$ dehydrogenase provide the elements that are able to construct a preliminary model for the electron flow pathway that occurs in *Msph. stadtmanae* (figure 1).

There are two apparent forms of hydrogenase that have different overall charge or different properties on the different chromatographic methods. The structure of the two hydrogenase preparations appeared to be identical in subunit size. They are probably just charge isomers of the same enzyme.

The other findings in this thesis include the apparent existence of the $F_{420}H_2$ dehydrogenase activity in methanogens other than the methanol-utilizers. Based on the current models of methanogenesis from either acetate or $H_2$:$CO_2$, there is no requirement for this activity for either methanogenesis or biosynthesis. The levels detected were high in most of the methanogenic species tested, this leads one to wonder, assuming that this

**Figure 1** - Proposed electron flow pathway in *Msph. stadtmanae*. X = phenazine or a phenazine-like compound.


$H_2$
MV Hydrogenase
X
Heterodisulphide Reductase
$F_{420}H_2$ Dehydrogenase
CoB-SH + CoM-SH
$F_{420}H_2$
$NADPH_2$
$NADP^+$:$F_{420}$ Oxidoreductase
Secondary Alcohol Dehydrogenase
2-Propanol

activity is true and is not due to some other enzyme (eg., $F_{420}$ hydrogenase), what the purpose of this activity would be in these methanogens. A possible role could be to maintain the balance of the electron pools between $F_{420}$ and methanophenazine. The methanophenazine has been determined to be involved as an important electron carrier in the heterodisulphide reduction system in *Ms. mazei* strain Gö1 (Abken et al, 1998).

Although methanophenazine has not yet been isolated from other methanogens, it is possible that many methanogens carry this cofactor as a electron carrier for their heterodisulphide reductase system and a role for the detected $F_{420}H_2$ dehydrogenase activity may be proposed and is shown in figure 2. The presence of this system would allow for various sources of electrons for $F_{420}H_2$ to be used (formate, 2-propanol or via methanol oxidation).

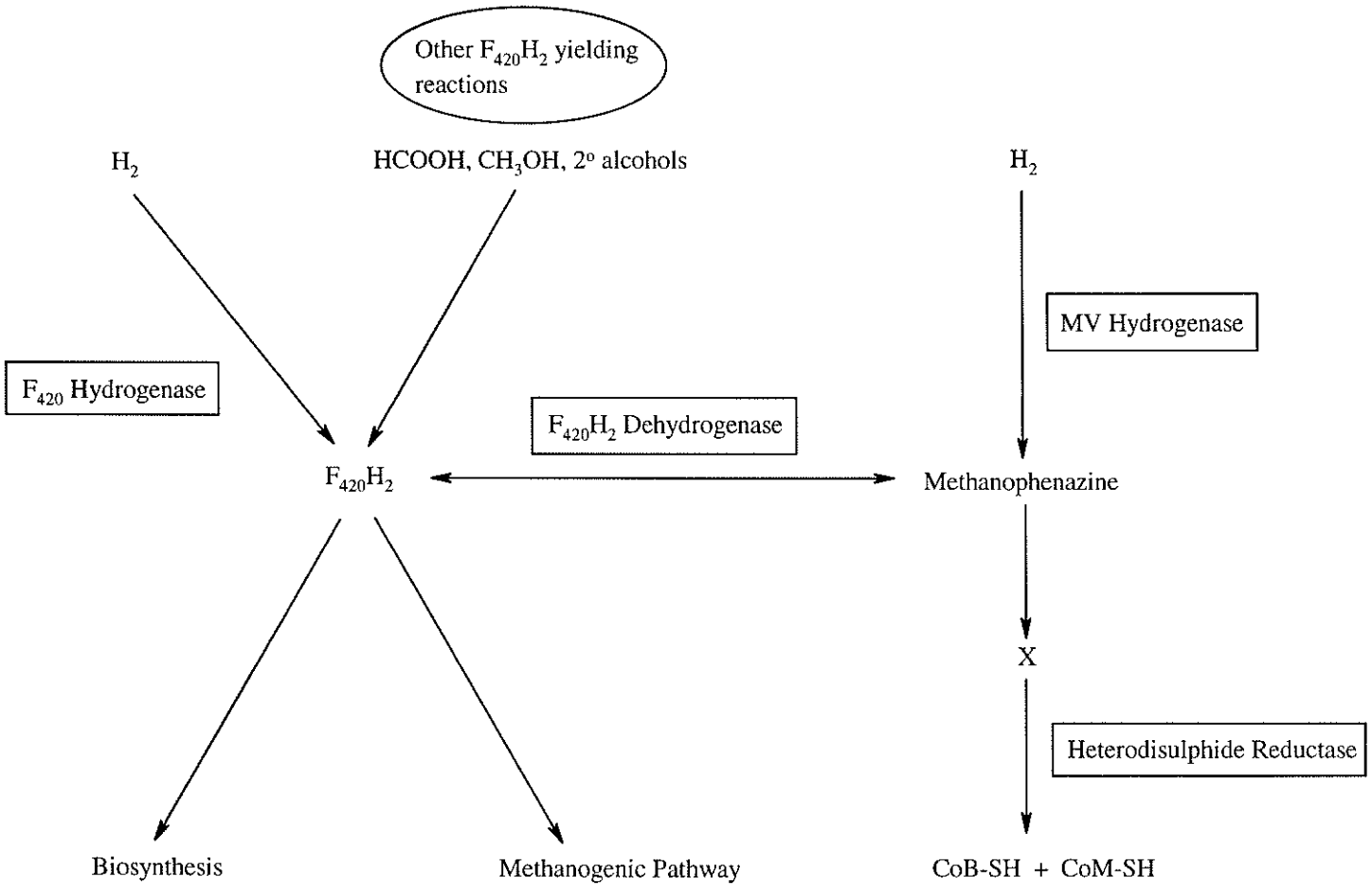
The $F_{420}H_2$ dehydrogenase would serve as a link between the two electron pools which would be important in situations where the $H_2$ levels fluctuate and may cut off one of the pools from their electron source. The relative affinities for $H_2$ by the different pools of hydrogenase ($F_{420}$- or MV-only) are the key.

**Future Work and Directions.**

First, the $F_{420}H_2$ dehydrogenase activity must be purified from *Msph. stadtmanae*. The initial step involves the stabilization of the activity to allow for the purification of this crucial enzyme for biosynthesis. The general properties observed for this activity was shown to be extremely $O_2$-sensitive, which is a departure from the purified enzyme from *Ml. tindarius*.

A second area of work is the purification and the establishment of $F_{420}H_2$ dehydrogenase as being present in a non-methanol-utilizing methanogen as a separate and distinct entity from the $F_{420}$-dependent hydrogenase activity. The likely candidate for this is *Msp. hungatei*. This methanogen does not have a MV-only hydrogenase and thus does not have a direct link to $H_2$ as source of reductant for the heterodisulphide reduction reaction. In addition, this methanogen is a member of the same Order of methanogens as both *Methanosarcina* and *Methanolobus*, the genera from where the dehydrogenase has

**Figure 2 -** Proposed pathways of electron flow and balance as regulated by the $F_{420}H_2$ dehydrogenase in methanogens that also do carry an $F_{420}$-dependent hydrogenase. X = cytochrome or other unknown cofactor.


Other $F_{420}H_2$ yielding reactions
$H_2$
HCOOH, $CH_3OH$, 2° alcohols
$H_2$
MV Hydrogenase
$F_{420}$ Hydrogenase
$F_{420}H_2$ Dehydrogenase
$F_{420}H_2$
Methanophenazine
X
Heterodisulphide Reductase
Biosynthesis
Methanogenic Pathway
CoB-SH + CoM-SH

been purified.

The findings in this thesis, in particular, chapters 5 and 6, suggest that we do not know all that is occurring in the methanogens with respect to the electron flow and balance systems. Based on these results, and those reported by Deppenmeier, indicate that further work be done in this area involving the $F_{420}H_2$ dehydrogenase, the electron carrier, methanophenazine and both of the major hydrogenases, the $F_{420}$- and the MV-dependent and possibly the other enzymes that have been found to be involved in the production of reduced $F_{420}$ or as alternative enzymes used to provide electrons for methanogenesis and for biosynthesis (e.g., the two malate dehydrogenases in *Mb. thermoautotrophicum*, Thompson et al., 1998; the $H_2$-forming methylene-$H_4$MPT dehydrogenase, Afting et al., 1998). Alternative electron sources are important, and their mechanism of entering these pathways are important to consider as well ($NADP^+$-dependent alcohol dehydrogenases and $NADP^+$:$F_{420}$ oxidoreductases, Berk and Thauer, 1997). The combination of all of these elements will provide a complete view on the electron flow system in the methanogens.

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## Appendix A - Methanogen Media Formulations

1. *Methanobacterium* strain G2R

| | |
|---|---|
| $Na_2CO_3$ | 0.48 g/L |
| $(NH_4)_2SO_4$ | 0.45 |
| $K_2HPO_4$ | 0.29 |
| $KH_2PO_4$ | 0.18 |
| $MgSO_4 \cdot 7\ H_2O$ | 0.12 |
| $CaCl_2 \cdot 2\ H_2O$ | 0.06 |
| NaCl | 0.054 |
| $FeSO_4 \cdot 7\ H_2O$ | 0.021 |
| Na Acetate | 0.42 |
| Resazurin | 1.00 mL/L from 0.025% stock solution |
| $Na_2S \cdot 9\ H_2O$ | add @ 1% (v/v) from 200mM stock after gassing |
| Mineral Elixer (see Recipe #13) | 10.0 mL/L |
| Vitamin Supplement (see Recipe #14) | 10.0 mL/L |

- Growth pH 6.5 under $H_2$:$CO_2$ (pH 7.5 prior to gassing)
- Growth temperature: 35 °C

2. *Methanobacterium bryantii* strain M.o.H. (DSM 2133)

| | |
|---|---|
| $KH_2PO_4$ | 0.42 g/L |
| $K_2HPO_4$ | 0.23 |
| $MgCl_2 \cdot 6\ H_2O$ | 0.04 |
| $CaCl_2 \cdot 2\ H_2O$ | 0.03 |
| NaCl | 0.595 |
| $NH_4Cl$ | 0.70 |
| $Na_2CO_3$ | 0.16 |
| Resazurin | 1.00 mL/L from 0.025% stock solution |
| $Na_2S \cdot 9\ H_2O$ | add @ 1% (v/v) from 200mM stock after gassing |
| Mineral Elixer (see Recipe #13) | 10.0 mL/L |

- Growth pH 7.0 under $H_2$:$CO_2$ (pH 8.5 prior to gassing)
- Growth temperature: 35 °C

3. *Methanobacterium thermoautotrophicum* strain Marburg (DSM 2133)

| | |
|---|---|
| $KH_2PO_4$ | 0.42 g/L |
| $K_2HPO_4$ | 0.23 |
| $MgCl_2 \cdot 6\ H_2O$ | 0.04 |
| $CaCl_2 \cdot 2\ H_2O$ | 0.03 |
| NaCl | 0.595 |
| $NH_4Cl$ | 0.70 |
| $Na_2CO_3$ | 0.16 |
| Resazurin | 1.00 mL/L from 0.025% stock solution |
| $Na_2S \cdot 9\ H_2O$ | add @ 1% (v/v) from 200mM stock after gassing |
| Mineral Elixer (see Recipe #13) | 10.0 mL/L |

- Growth pH 7.0 under $H_2$:$CO_2$ (pH 8.5 prior to gassing)
- Growth temperature: 63°C

4. *Methanococcus thermolithotrophicus* (DSM 2095) - $H_2$:$CO_2$ medium

| | |
|---|---|
| NaCl | 29.2 g/L |
| $MgCl_2 \cdot 6\ H_2O$ | add @ 1% from 2.5M sterile stock after autoclaving |
| $NH_4Cl$ | 1.00 |
| KCl | 0.34 |
| $KH_2PO_4$ | 0.28 |
| $K_2HPO_4$ | 0.28 |
| $CaCl_2 \cdot 2\ H_2O$ | 0.10 |
| Resazurin | 1.00 mL/L from 0.025% stock solution |
| $NaWO_4 \cdot 2\ H_2O$ | 1.00 mL/L from 1mM stock solution |
| $NaSeO_4$ | 0.50 mL/L from 1mM stock solution |
| $Na_2CO_3$ | 0.20 |
| Mineral Elixer (see Recipe #13) | 10.0 mL/L |
| $Na_2S \cdot 9\ H_2O$ | add @ 1% (v/v) from 200mM stock after gassing |

- Growth pH 5.8-6.2 under $H_2$:$CO_2$ (pH 7.4 prior to gassing)
- Growth temperature: 62-65°C

5. *Methanococcus thermolithotrophicus* (DSM 2095) - Formate Triple Buffer medium

| | |
|---|---|
| KCl | 0.34 g/L |
| $NH_4Cl$ | 1.00 |
| $MgCl_2 \cdot 6\ H_2O$ | add @ 1% from 2.5M sterile stock after autoclaving |
| $CaCl_2 \cdot 2\ H_2O$ | 0.10 |
| $K_2HPO_4$ | 0.25 |
| $Na_2S \cdot 9\ H_2O$ | add @ 1% (v/v) from 200mM stock after gassing |
| 2-(N-morpholino)-ethanesulfonic acid (MES) | 9.75 |
| Piperazine-N,N'-bis-2-ethanesulfonic acid (PIPES) | 15.12 |
| N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) | 11.90 |
| Reazurin | 1.00 mL/L from 0.025% stock solution |
| $NaWO_4 \cdot 2\ H_2O$ | 1.00 mL/L from 1mM stock solution |
| $NaSeO_4$ | 0.50 mL/L from 1mM stock solution |
| Mineral Elixer (see Recipe #13) | 10.0 mL/L |
| NaCl | 11.68 |
| Na Formate | 20.40 |

- Growth pH 5.8-6.2 under $N_2$:$CO_2$ (pH 6.5 prior to gassing)
- Growth temperature: 63 °C

6. *Methanococcus voltae* (DSM 1537)

| | |
|---|---|
| KCl | 0.335 g/L |
| $MgCl_2 \cdot 6\ H_2O$ | 4.00 |
| $MgSO_4 \cdot 7\ H_2O$ | 3.45 |
| $NH_4Cl$ | 0.25 |
| $CaCl_2 \cdot 2\ H_2O$ | 0.14 |
| $K_2HPO_4$ | 0.14 |
| Yeast Extract | 2.00 |
| Tryticase/Tryptone | 2.00 |
| NaCl | 18.00 |
| $NaHCO_3$ | 5.00 |
| $Fe(NH_4)_2(SO_4)_2 \cdot 7\ H_2O$ | 0.002 |
| Resazurin | 1.00 mL/L from 0.025% stock solution |
| $Na_2S \cdot 9\ H_2O$ | add @ 1% (v/v) from 200mM stock after gassing |
| Na Acetate | 1.00 |
| Mineral Elixer (see Recipe #13) | 10.00 mL/L |
| Vitamin Supplement (see Recipe #14) | 10.00 mL/L |

- Growth pH 7.0 under $H_2$:$CO_2$ (pH 7.5 prior to gassing)
- Growth temperature: 37°C

7. *Methanolobus tindarius* (DSM 2278)

| | |
|---|---|
| KCl | 0.335 g/L |
| $MgCl_2 \cdot 6\ H_2O$ | 4.00 |
| $MgSO_4 \cdot 7\ H_2O$ | 3.45 |
| $NH_4Cl$ | 0.25 |
| $CaCl_2 \cdot 2\ H_2O$ | 0.14 |
| $K_2HPO_4$ | 0.14 |
| NaCl | 18.00 |
| $NaHCO_3$ | 1.00 |
| $Fe(NH_4)_2(SO_4)_2 \cdot 7\ H_2O$ | 0.002 |
| Resazurin | 1.00 mL/L from 0.025% stock solution |
| $Na_2S \cdot 9\ H_2O$ | add @ 1% (v/v) from 200mM stock after gassing |
| Methanol | add @ 1% (v/v) from sterile stock of 40% after autoclaving |
| Mineral Elixer (see Recipe #13) | 10.00 mL/L |
| Vitamin Supplement (see Recipe #14) | 10.00 mL/L |

- Growth pH 6.5 under $N_2$:$CO_2$ (pH 8.3 prior to gassing)
- Growth temperature: 25°C

8. *Methanosaeta concilii* (DSM 3671)

| | |
|---|---|
| $KH_2PO_4$ | 0.30 g/L |
| NaCl | 0.60 |
| $MgCl_2 \cdot 6\ H_2O$ | 0.10 |
| $CaCl_2 \cdot 2\ H_2O$ | 0.08 |
| $NH_4Cl$ | 1.00 |
| $KCO_3$ | 4.00 |
| Mineral Elixer (see Recipe #13) | 10.00 mL/L |
| Vitamin Supplement (see Recipe #14) | 10.00 mL/L |
| $Na_2S \cdot 9\ H_2O$ | add @ 1% (v/v) from 200mM stock after gassing |
| Resazurin | 1.00 mL/L from 0.025% stock solution |
| Na Acetate | 6.80 |

- Growth pH 7.0 under $N_2$:$CO_2$ (pH 7.7 prior to gassing)
- Growth temperature: 35°C

9. *Methanosarcina barkeri* strain Fusaro (DSM 804)

| | |
|---|---|
| $K_2HPO_4$ | 0.35 g/L |
| $KH_2PO_4$ | 0.23 |
| $NH_4Cl$ | 0.50 |
| Yeast Extract | 2.00 |
| $MgSO_4 \cdot 7\ H_2O$ | 0.50 |
| $CaCl_2 \cdot 2\ H_2O$ | 0.25 |
| NaCl | 2.25 |
| $NaHCO_3$ | 0.85 |
| $Na_2CO_3$ | 0.50 |
| $Na_2S \cdot 9\ H_2O$ | add @ 1% (v/v) from 200mM stock after gassing |
| Resazurin | 1.00 mL/L from 0.025% stock solution |
| Mineral Elixer (see Recipe #13) | 10.00 mL/L |
| Vitamin Supplement (see Recipe #14) | 10.00 mL/L |
| Methanol | add @ 1% (v/v) from sterile stock of 40% after autoclaving |

- Growth pH 6.5-6.7 under $N_2$:$CO_2$ (pH 7.5 prior to gassing)
- alternate growing condition under $H_2$:$CO_2$ +/- methanol (pH 7.5 prior to gassing)
- Growth temperature: 37°C
- for growth on acetate, Na acetate is added to final concentration of 200 mM (27.216g/L of trihydrate) and grown under $N_2$:$CO_2$.

10. *Methanosphaera cuniculi* (DSM 4103)

| | |
|---|---|
| Trypticase/Tryptone | 1.00 g/L |
| Yeast Extract | 1.00 |
| $K_2HPO_4$ | 0.30 |
| $KH_2PO_4$ | 0.30 |
| $(NH_4)_2SO_4$ | 0.30 |
| NaCl | 0.61 |
| $MgSO_4 \cdot 7\ H_2O$ | 0.13 |
| $CaCl_2 \cdot 2\ H_2O$ | 0.08 |
| Na Acetate | 2.50 |
| $NaSeO_4$ | 0.50 mL/L from 1mM stock solution |
| $NaHCO_3$ | 3.00 |
| Resazurin | 1.00 mL/L from 0.025% stock solution |
| Mineral Elixer (see Recipe #13) | 10.00 mL/L |
| Vitamin Supplement (see Recipe #14) | 10.00 mL/L |
| $Na_2S \cdot 9\ H_2O$ | add @ 1% (v/v) from 200mM stock after gassing |
| Methanol | add @ 1% (v/v) from sterile stock of 40% after autoclaving |

- Growth pH 6.8-7.0 under $H_2$:$CO_2$ (pH 7.4 prior to gassing)
- Growth temperature: 35°C

11. *Methanosphaera stadtmanae* (DSM 3091)

| | |
|---|---|
| Trypticase/Tryptone | 2.00 g/L |
| Yeast Extract | 2.00 |
| $K_2HPO_4$ | 0.30 |
| $KH_2PO_4$ | 2.80 |
| $(NH_4)_2SO_4$ | 0.30 |
| $MgSO_4 \cdot 7\ H_2O$ | 0.13 |
| $CaCl_2 \cdot 2\ H_2O$ | 0.08 |
| NaCl | 0.61 |
| Na Acetate | 0.50 |
| $NaSeO_4$ | 0.50 mL/L from 1mM stock solution |
| Resazurin | 1.00 mL/L from 0.025% stock solution |
| Mineral Elixer (see Recipe #13) | 15.00 mL/L |
| Vitamin Supplement (see Recipe #14) | 15.00 mL/L |
| $Na_2CO_3$ | 1.70 |
| $NH_4Cl$ | 1.00 |
| $Na_2S \cdot 9\ H_2O$ | add @ 1% (v/v) from 200mM stock after gassing |
| Methanol | add @ 1% (v/v) from sterile stock of 40% after autoclaving |

- Growth pH 6.8-7.0 under $H_2$:$CO_2$ (pH 7.4 prior to gassing)
- Growth temperature: 37°C
- Note: for minimal medium, omit both yeast extract and trypticase, and add 0.1812 g/L of each of L-leucine and L-isoleucine.

12. *Methanospirillum hungatei* strain GP1 (DSM 1101)

| | |
|---|---|
| NaCl | 0.30 g/L |
| $MgCl_2 \cdot 6\ H_2O$ | 0.064 |
| $NH_4Cl$ | 0.40 |
| Na Acetate | 0.42 |
| $K_2HPO_4$ | 0.82 |
| $CaCl_2 \cdot 2\ H_2O$ | 0.064 |
| Resazurin | 1.00 mL/L from 0.025% stock solution |
| $Na_2CO_3$ | 1.00 |
| Mineral Elixer (see Recipe #13) | 10.00 mL/L |
| Vitamin Supplement (see Recipe #14) | 10.00 mL/L |
| $Na_2S \cdot 9\ H_2O$ | add @ 1% (v/v) from 200mM stock after gassing |

- Growth pH 7.0 under $H_2$:$CO_2$ (pH 8.5 prior to gassing)
- Growth temperature: 37°C

13. Mineral Elixer Stock Solution

| | |
|---|---|
| Trisodium Nitrilacetate | 2.02 g/L |
| $FeCl_3 \cdot 6\ H_2O$ | 0.21 |
| $CoCl_2 \cdot 6\ H_2O$ | 0.20 |
| $MnCl_2 \cdot 4\ H_2O$ | 0.10 |
| $ZnCl_2$ | 0.10 |
| $NiCl_2 \cdot 6\ H_2O$ | 0.10 |
| $CaCl_2 \cdot 2\ H_2O$ | 0.05 |
| $CuSO_4 \cdot 2\ H_2O$ | 0.05 |
| $Na_2MoO_4 \cdot 2\ H_2O$ | 0.05 |

14. Vitamin Supplement

| | |
|---|---|
| Pyridoxine-HCl | 10 mg/L |
| Riboflavin | 5 |
| Thiamine | 5 |
| Nicotinic Acid | 5 |
| p-Aminobenzoic Acid | 5 |
| Lipoic Acid (Thioctic Acid) | 5 |
| Biotin | 2 |
| Folic Acid | 2 |
| Cyanocobalamin | 1 |

15. Heterotroph Test Medium (Test for Contaminants)

| | |
|---|---|
| Yeast Extract | 10.00 g/L |
| Trypticase/Tryptone | 10.00 |
| NaCl | 0.50 |
| Resazurin | 1.00 mL/L from 0.025% stock solution |

- pH 6.8-7.2 under $N_2$:$CO_2$

## Appendix B - $F_{420}H_2$ Dehydrogenase Sucrose Gradient Data

- **note:** fractions in direction from the bottom of gradient to the top
- **note:** all activities are represented in µmoles electron acceptor per mL of fraction.

*Methanobacterium* strain G2R

| Fraction # | $A_{280}$ Absorbance | MV Hydrogenase | $F_{420}$ Hydrogenase | $F_{420}H_2$ Dehydrogenase |
|---|---|---|---|---|
| 1 | 0.113 | 0.0015 | 0.0000 | 0.0002 |
| 2 | 0.060 | 0.0000 | 0.0000 | 0.0003 |
| 3 | 0.063 | 0.0243 | 0.0028 | 0.0086 |
| 4 | 0.159 | 0.0761 | 0.0093 | 0.0239 |
| 5 | 0.178 | 0.0433 | 0.0004 | 0.0022 |
| 6 | 0.362 | 0.0201 | 0.0000 | 0.0010 |
| 7 | 0.756 | 0.0180 | 0.0000 | 0.0000 |
| 8 | 1.255 | 0.0578 | 0.0000 | 0.0000 |
| 9 | 1.709 | 0.0156 | 0.0000 | 0.0009 |
| 10 | 2.077 | 0.0106 | 0.0000 | 0.0004 |

*Mb. bryantii*

| Fraction # | $A_{280}$ Absorbance | MV Hydrogenase | $F_{420}$ Hydrogenase | $F_{420}H_2$ Dehydrogenase |
|---|---|---|---|---|
| 1 | 0.063 | 0.0047 | 0.0000 | 0.0000 |
| 2 | 0.020 | 0.0000 | 0.0000 | 0.0000 |
| 3 | 0.197 | 0.0302 | 0.0176 | 0.0099 |
| 4 | 0.817 | 0.1376 | 0.0353 | 0.2043 |
| 5 | 0.573 | 0.0270 | 0.0005 | 0.0119 |
| 6 | 1.242 | 0.0032 | 0.0000 | 0.0020 |
| 7 | 2.169 | 0.0053 | 0.0032 | 0.0061 |
| 8 | 2.769 | 0.0814 | 0.0068 | 0.0254 |
| 9 | 2.651 | 0.0998 | 0.0007 | 0.0058 |
| 10 | 2.186 | 0.1785 | 0.0000 | 0.0025 |

*Mb. thermoautotrophicum* strain Marburg

| Fraction # | $A_{280}$ Absorbance | MV Hydrogenase | $F_{420}$ Hydrogenase | $F_{420}H_2$ Dehydrogenase |
|---|---|---|---|---|
| 1 | 0.104 | 0.0055 | 0.0000 | 0.0083 |
| 2 | 0.062 | 0.0013 | 0.0000 | 0.0540 |
| 3 | 0.568 | 0.0205 | 0.0126 | 0.1121 |
| 4 | 0.636 | 0.0055 | 0.0109 | 0.0606 |
| 5 | 0.652 | 0.0025 | 0.0004 | 0.0058 |
| 6 | 1.023 | 0.0814 | 0.0000 | 0.0315 |
| 7 | 1.161 | 0.1602 | 0.0000 | 0.0033 |
| 8 | 2.153 | 0.2783 | 0.0000 | 0.0027 |

*Mc. thermolithotrophicus* ($H_2$:$CO_2$)

| Fraction # | $A_{280}$ Absorbance | MV Hydrogenase | $F_{420}$ Hydrogenase | $F_{420}H_2$ Dehydrogenase |
|---|---|---|---|---|
| 1 | 0.307 | 0.0009 | 0.0000 | 0.0000 |
| 2 | 0.167 | 0.0016 | 0.0000 | 0.0020 |
| 3 | 0.121 | 0.0161 | 0.0241 | 0.0075 |
| 4 | 0.092 | 0.0050 | 0.0145 | 0.0047 |
| 5 | 0.081 | 0.0075 | 0.0023 | 0.0021 |
| 6 | 0.140 | 0.3217 | 0.0002 | 0.0023 |
| 7 | 0.554 | 0.6628 | 0.0010 | 0.0016 |
| 8 | 1.713 | 0.5740 | 0.0000 | 0.0011 |
| 9 | 1.697 | 0.1589 | 0.0000 | 0.0000 |

*Mc. thermolithotrophicus* (Formate)

| Fraction # | $A_{280}$ Absorbance | MV Hydrogenase | $F_{420}$ Hydrogenase | $F_{420}H_2$ Dehydrogenase |
|---|---|---|---|---|
| 1 | 0.594 | 0.0079 | 0.0125 | 0.0023 |
| 2 | 0.539 | 0.0651 | 0.0824 | 0.0342 |
| 3 | 0.658 | 0.1486 | 0.0961 | 0.0157 |
| 4 | 0.234 | 0.8071 | - | 0.0088 |
| 5 | 0.421 | 2.4215 | 0.0054 | 0.0017 |
| 6 | 1.464 | 2.1000 | - | - |
| 7 | 1.613 | 0.8060 | 0.0025 | 0.0125 |
| 8 | 0.634 | 0.2652 | - | - |

*Mc. voltae*

| Fraction # | $A_{280}$ Absorbance | MV Hydrogenase | $F_{420}$ Hydrogenase | $F_{420}H_2$ Dehydrogenase |
|---|---|---|---|---|
| 1 | 0.116 | 0.0643 | 0.0000 | 0.0000 |
| 2 | 0.054 | 0.0084 | 0.0000 | 0.0000 |
| 3 | 0.086 | 0.0035 | 0.0083 | 0.0032 |
| 4 | 0.120 | 0.0042 | 0.0059 | 0.0022 |
| 5 | 0.187 | 0.0088 | 0.0007 | 0.0004 |
| 6 | 0.866 | 0.2008 | 0.0007 | 0.0000 |
| 7 | 1.778 | 0.5723 | 0.0043 | 0.0017 |
| 8 | 1.759 | 1.0148 | 0.0007 | 0.0025 |
| 9 | 1.517 | 2.1269 | 0.0002 | 0.0006 |

*Ml. tindarius*

| Fraction # | $A_{280}$ Absorbance | MV Hydrogenase | $F_{420}$ Hydrogenase | $F_{420}H_2$ Dehydrogenase |
|---|---|---|---|---|
| 1 | 0.028 | 0.0000 | 0.0000 | 0.0000 |
| 2 | 0.016 | 0.0000 | 0.0000 | 0.0000 |
| 3 | 0.086 | 0.0000 | 0.0000 | 0.0000 |
| 4 | 0.151 | 0.0000 | 0.0000 | 0.0000 |
| 5 | 0.181 | 0.0000 | 0.0000 | 0.0000 |
| 6 | 0.714 | 0.0037 | 0.0000 | 0.0183 |
| 7 | 0.753 | 0.0061 | 0.0000 | 0.0134 |
| 8 | 0.370 | 0.0037 | 0.0000 | 0.0015 |

*Ms. barkeri* strain Fusaro (Acetate)

| Fraction # | $A_{280}$ Absorbance | MV Hydrogenase | $F_{420}$ Hydrogenase | $F_{420}H_2$ Dehydrogenase |
|---|---|---|---|---|
| 1 | 0.000 | 0.0013 | 0.0003 | 0.0004 |
| 2 | 0.000 | 0.0037 | 0.0002 | 0.0007 |
| 3 | 0.000 | 0.1215 | 0.0227 | 0.0079 |
| 4 | 0.074 | 0.3176 | 0.0322 | 0.0284 |
| 5 | 0.081 | 0.1095 | 0.0060 | 0.0077 |
| 6 | 0.201 | 0.0207 | 0.0086 | 0.0018 |
| 7 | 0.689 | 0.0165 | 0.0061 | 0.0017 |
| 8 | 1.550 | 0.1943 | 0.0065 | 0.0010 |
| 9 | 1.639 | 0.3439 | 0.0025 | 0.0003 |
| 10 | 1.877 | 0.0525 | 0.0015 | 0.0008 |

*Ms. barkeri* strain Fusaro ($H_2$:$CO_2$)

| Fraction # | $A_{280}$ Absorbance | MV Hydrogenase | $F_{420}$ Hydrogenase | $F_{420}H_2$ Dehydrogenase |
|---|---|---|---|---|
| 1 | 0.098 | 0.0021 | 0.0000 | 0.0000 |
| 2 | 0.059 | 0.0024 | 0.0076 | 0.0008 |
| 3 | 0.359 | 0.0334 | 0.0294 | 0.0083 |
| 4 | 0.309 | 0.0047 | 0.0137 | 0.0029 |
| 5 | 0.468 | 0.0032 | 0.0025 | 0.0000 |
| 6 | 0.695 | 0.0095 | 0.0020 | - |
| 7 | 1.276 | 0.0179 | 0.0017 | 0.0134 |
| 8 | 1.470 | 0.1116 | 0.0000 | - |

*Ms. barkeri* strain Fusaro (Methanol)

| Fraction # | $A_{280}$ Absorbance | MV Hydrogenase | $F_{420}$ Hydrogenase | $F_{420}H_2$ Dehydrogenase |
|---|---|---|---|---|
| 1 | 0.100 | 0.0000 | 0.0030 | 0.0000 |
| 2 | 0.054 | 0.0011 | 0.0073 | 0.0034 |
| 3 | 0.263 | 0.0184 | 0.0278 | 0.0225 |
| 4 | 0.181 | 0.0074 | 0.0116 | 0.0083 |
| 5 | 0.244 | 0.0024 | 0.0040 | 0.0034 |
| 6 | 0.312 | 0.0024 | 0.0017 | - |
| 7 | 0.631 | 0.0132 | 0.0007 | 0.0005 |
| 8 | 0.716 | 0.0079 | 0.0000 | - |

*Mst. concilii*

| Fraction # | $A_{280}$ Absorbance | MV Hydrogenase | $F_{420}$ Hydrogenase | $F_{420}H_2$ Dehydrogenase |
|---|---|---|---|---|
| 1 | 0.088 | 0.0000 | 0.0000 | 0.0000 |
| 2 | 0.071 | 0.0000 | 0.0000 | 0.0000 |
| 3 | 0.153 | 0.0000 | 0.0000 | 0.0000 |
| 4 | 0.135 | 0.0000 | 0.0000 | 0.0000 |
| 5 | 0.197 | 0.0018 | 0.0000 | 0.0072 |
| 6 | 0.718 | 0.0037 | 0.0008 | 0.0481 |
| 7 | 1.053 | 0.0254 | 0.0003 | 0.0100 |
| 8 | 0.606 | 0.0492 | 0.0000 | 0.0038 |

*Msp. hungatei*

| Fraction # | $A_{280}$ Absorbance | MV Hydrogenase | $F_{420}$ Hydrogenase | $F_{420}H_2$ Dehydrogenase |
|---|---|---|---|---|
| 1 | 0.083 | 0.0049 | 0.0000 | 0.0000 |
| 2 | 0.056 | 0.0011 | 0.0000 | 0.0000 |
| 3 | 0.070 | 0.0296 | 0.0071 | 0.0076 |
| 4 | 0.291 | 0.0453 | 0.0126 | 0.0344 |
| 5 | 0.214 | 0.0050 | 0.0035 | 0.0021 |
| 6 | 0.343 | 0.0002 | 0.0007 | 0.0008 |
| 7 | 0.771 | 0.0063 | 0.0008 | 0.0020 |
| 8 | 0.889 | 0.0049 | 0.0008 | 0.0039 |
| 9 | 0.801 | 0.0091 | 0.0008 | 0.0007 |