β-HYDROXYBUTYRATE DEHYDROGENASE FROM *THIOBACILLUS NOVELLUS*

BY

BILL POHAJDAK

A Thesis

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MASTER OF SCIENCE

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ABSTRACT

D-3-hydroxybutyrate: NAD oxidoreductase E.C.1.1.1.30 a soluble, reversible, NAD dependent enzyme was purified approximately 100-fold from the facultative chemoautotroph *Thiobacillus novellus* grown on 1% glucose at 28°C. The enzyme was purified by a series of steps involving $(NH_4)_2SO_4$ fractionation, DEAE-Sephadex A50 chromatography and Blue dextran affinity chromatography. The purified enzyme contained 2 protein contaminants as determined by 5% polyacrylamide disc gel electrophoresis. The enzyme stored frozen (-20°C) in 0.1 M phosphate buffer pH 8.0 containing 1 M $(NH_4)_2SO_4$ was stable for months. The enzyme was determined to have an optimal pH of 8.6 for the forward oxidation reaction. By the use of various sulfhydryl inhibitors, the enzyme was found to contain sensitive sulfhydryl groups which were required for catalytic activity. No direct requirement for metal ions for enzyme activity could be demonstrated. Steady-state kinetics were performed and the initial velocity plots were linear and indicated a non-allosteric, sequential or ordered mechanism. Michaelis constants were 0.77 mM for D-3-hydroxybutyrate and 45.5 μM for NAD. The enzyme was competitively inhibited by the structural analogue D-lactate. Product inhibition analysis was performed and the data obtained are consistent with an ordered Bi Bi mechanism.

To my family.

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ABBREVIATIONS

ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
ATP	Adenosine-5'-triphosphate
DEAE	Diethylaminoethane
DTNB	5,5'-Dithiobis-(2-Nitrobenzoic Acid)
EDTA	Ethylenediamine tetraacetic acid
3-HB	D-3-hydroxybutyrate
3-HBDH	D-3-hydroxybutyrate dehydrogenase (D-3-hydroxybutyrate: NAD oxidoreductase)
NAD, NADH	Oxidized or reduced nicotinamide adenine dinucleotide
NADP, NADPH	Oxidized or reduced nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium
NEM	N-ethyl maleimide
PHB	Poly-β-hydroxybutyrate
PMS	Phenazine methosulfate
SDS	Sodium dodecyl sulfate
Tris	Tris(hydroxymethyl) amino methane

INTRODUCTION

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Thiobacillus novellus is a facultative chemolithotroph that can undergo two modes of life depending on the substrates available in its environment. The autotroph growing on sodium thiosulfate as its energy source and CO_2 as its carbon source can switch its metabolism to a heterotrophic mode of life when place into a medium containing a suitable reduced organic compound. Much work has been reported on the mechanism of thiosulfate oxidation in autotrophically grown *T. novellus* while less has been done on the heterotrophic energy metabolism in this microorganism. In 1967, LéJohn *et al.* demonstrated that autotrophically grown *T. novellus* undergoes catabolite repression of the thiosulfate oxidizing enzymes in the presence of several organic compounds. In 1969, Van Caeseele and Lees during a study of the ultrastructure of autotrophically and heterotrophically grown *T. novellus* first observed that only the heterotrophic microorganism contained poly-β-hydroxybutyrate (PHB). However, they identified the large electron opaque granules in heterotrophically grown *T. novellus* as polysaccharide rather than PHB. The problem of the inability of *T. novellus* to switch quickly from heterotrophic to autotrophic growth (although the reverse occurs rather rapidly) which was first demonstrated by Charles and Suzuki (1965) could be due to the presence of the storage material, PHB.

The goal of this study was to provide some evidence for the ability

of *T. novellus* to undergo metabolic and enzymatic changes when grown heterotrophically on 1% glucose. The enzyme 3-hydroxybutyrate dehydrogenase (3-HBDH) a degradative enzyme in PHB metabolism was investigated as a possible key enzyme in the regulation of PHB metabolism. Understanding the kinetic mechanism, regulation and physical and chemical properties of 3-HBDH may give further insight to the switch from the heterotrophic to the autotrophic mode of life. This study is a continuation of the work done on *T. novellus* and may answer some of the questions which are not yet well understood.

HISTORY

HISTORY

Chemolithotrophs are microorganisms capable of oxidizing exogenous reduced inorganic compounds as a sole source of metabolic energy. The chemolithotrophs can fix atmospheric CO_2 as their carbon source and may also be referred to as autotrophs or chemoautotrophs. The concept of autotrophy was first discovered by Winogradsky in 1887 working with respiration in a *Beggiatoa* species. The chemoautotrophs are subdivided into threee major groups. The first group, the obligate chemoautotrophs, cannot grow on organic nutrients and depend on CO_2 as their principal carbon source. These microorganisms derive energy only from inorganic compounds such as reduced nitrogen compounds (NH_3, NO_2^-) or reduced sulfur compounds (H_2S, S, $S_2O_3^=$). The second group, the facultative chemoautotrophs or facultative chemolithotrophs can undergo two modes of life depending upon their nutritional environment. The facultative chemoautotroph can exist autotrophically obtaining its carbon from CO_2 and its energy from oxidation of inorganic compounds or can exist heterotrophically by obtaining both its carbon and energy from a suitable organic compound. This group should not be confused with chemoheterotrophs which always utilize organic compounds as both carbon and energy sources. The last group in the chemoautotrophs are the mixotrophs which can utilize inorganic compounds as a source of energy while simultaneously utilizing an organic compound as both a carbon and energy source.

Thiobacilli

Gram negative chemolithotrophic rods capable of obtaining energy from reduced sulfur compounds are placed into the genus *Thiobacillus* (Buchanan and Gibbons, 1974). The thiobacilli are divided into three groups based on their carbon and energy requirements. The first group, the obligate autotrophs includes *T. thioparus, T. neapolitanus, T. thioxidans, T. dentitrificans* and *T. ferrooxidans*. The second group, the facultative autotrophs, includes *T. novellus* and *T. intermedius*. The third group which can grow heterotrophically but require sulfur for optimal growth (mixotroph) contain only one member, *T. perometabolis* (Buchanan and Gibbons, 1974). All the thiobacilli are found in the sea, fresh water, soil, acid mines, sewage, sulfur springs and sulfur deposits (Buchanan and Gibbons, 1974). Reviews on the sulfur metabolism in microorganisms can be found in articles by Peck (1962) and Trudinger (1967). The mechanisms by which chemolithotrophic organisms obtain energy are discussed in a review article by Suzuki (1974).

Thiobacillus novellus ATCC 8093

Thiobacillus novellus was first isolated by Starkey (1935) from a soil sample during an investigation of thiosulfate utilizing bacteria. Starkey (1935) and Santer *et al.* (1959) describe *T. novellus* as a gram negative, non-motile rod measuring 0.5 to 1.0 μ long, growing best at pH 7.8 to 9.0 on either inorganic sulfur or organic media. The mechanism of thiosulfate oxidation by *T. novellus* has been investigated by Charles and Suzuki (1966), Charles (1966) and Oh and

Suzuki (1977a, 1977b).

When first studied by Starkey (1935) this microorganism grew autotrophically on thiosulfate and heterotrophically only on glutamic or aspartic acid, and other carbohydrates did not support growth. The growth on glutamate is in agreement with several workers, and glutamate uptake (Hoban and Lyric, 1977) and NAD and NADP glutamate dehydrogenases (LéJohn *et al.* 1968) have been described from *T. novellus*. There are, however, conflicting reports about the ability of *T. novellus* to grow on other organic compounds. Santer *et al.* (1959) reported that various organic compounds such as sucrose, glucose, lactose, acetate, succinate and malate did not support the growth of *T. novellus*. Charles and Suzuki (1965) found that *T. novellus* grown heterotrophically on glucose, required repeated culture transfers (4 times) with decreasing concentrations of glucose and increasing concentrations of thiosulfate before the organism would convert back to autotrophic metabolism. Charles (1971) was able to grow *T. novellus* on a variety of substrates including glucose, pyruvate, succinate, acetate, glutamate, malate and citrate. Under mixotrophic growth conditions LéJohn *et al.* (1967) were able to show that fermentable carbon sources such as glucose, glycerol, lactate, ribose and pyruvate were able to catabolically repress the thiosulfate oxidizing mechanism. Amino acids and organic acids which are aerobically metabolized did not repress the thiosulfate oxidizing system.

An interesting review article recently published by Matin (1978)

discusses organic nutrition in all three groups of chemolithotrophs. This author states that *T. novellus* cannot grow on glucose unless the medium is supplemented with a small amount of yeast extract. When growing mixotrophically (glucose, yeast extract and thiosulfate), thiosulfate was utilized along with glucose indicating that *T. novellus* is a mixotroph. Matin has suggested that the various discrepancies in results concerning heterotrophic growth of *T. novellus* could have resulted from use of different strains, a different inoculum history or even poor culture purity.

Storage Reserves

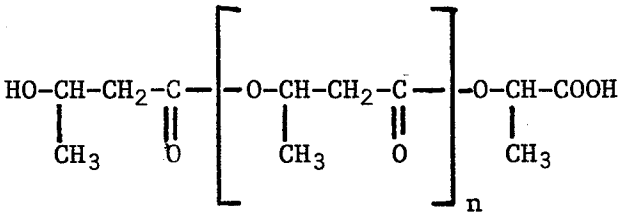
Some prokaryotes, like eukaryotes, have the ability to store intracellular energy reserve polymers. Most of the storage polymers serve both as a carbon and energy source during deprived conditions. In the absence of growth in microorganisms, energy is required for pH maintenance, osmotic regulation, motility, encystment, sporulation, luminescence, and turnover of proteins and nucleic acids (Dawes and Senior, 1973). Three criteria have been proposed by Macrae and Wilkinson (1958) for energy storage compounds. The first is that the compound should be accumulated when exogenous energy exceeds that which is required for growth and other energy related processes. The second criterion is that during starvation the compound should be re-utilized so the cell can maintain its energy requirements. The final criterion is that the compound when re-utilized should provide a suitable energy source for the microorganism.

To date, three types of storage materials have been found in microorganisms, these being polysaccharides, which include glycogen and glycogen-like compounds; lipids, including poly-β-hydroxybutyrate, and polyphosphates (Dawes and Senior, 1973). All three storage materials are high molecular weight polymers and exert low osmotic pressure. Poly-β-hydroxybutyrate is unique in that it is only found in microorganisms. A comprehensive review of all three types of storage compounds can be found in an article by Senior and Dawes (1973).

Poly-β-hydroxybutyrate (PHB)

In 1926 Lemoigne isolated two compounds from *Bacillus megaterium*. One was crystalline 3-hydroxybutyrate and the other amorphous compound was poly-β-hydroxybutyrate (PHB).

PHB is a common storage material in a variety of microorganisms. It exists as a straight chain homopolymer of D-3-hydroxybutyrate (3-HB) having the empirical formula $(C_4H_6O_2)_n$:



Molecular weight of the polymer was determined to be proportional to viscosity (Lundgren *et al.* 1965) giving a minimum molecular weight of 1,000 and a maximum of 250,000 for the microorganisms under study by these investigators. These investigators showed that PHB from 11 genera had similar molecular weights, infrared-absorption spectra, X-ray diffraction patterns and precipitate structures. The molecule

is osmotically inert and exists in a crystalline state in the organism making it an ideal storage material for microorganisms. The molecule is soluble in some organic solvents such as chloroform, acetic acid and phenol but is insoluble in water, methanol, acetone, ether and other compounds. A complete description of the chemical and physical properties of PHB can be found in the review by Dawes and Senior (1973).

PHB is found in a variety of microorganisms. It is found in both gram negative and gram positive photoheterotrophs, chemoautotrophs and chemoheterotrophs. The occurrence of PHB in different microorganisms has been the subject of many reviews (Dawes and Ribbons, 1964; Dawes and Senior, 1973; Lundgren *et al.* 1965). PHB has been isolated and characterized from the photolithotroph *Rhodospirillum rubrum* (Lundgren *et al.* 1965; Stanier *et al.* 1959). This storage material has also been reported in the photoautotrophic blue-green algae *Chlorogloea fritschii* (Carr, 1966) grown heterotrophically on acetate.

PHB has been isolated from chemolithotrophic microorganisms. The storage material was found in *Ferrobacillus ferrooxidans* when the culture medium was supplied with 0.5% glucose rather than iron (Wang and Lundgren, 1969).

An unusual finding was that *Hydrogenomonas,* a chemolithotroph growing autotrophically (H_2, CO_2, O_2), accumulated the storage material (Schlegel *et al.* 1961). These authors showed that when the medium was depleted of nitrogen, causing the cells to stop growing, there

was an increase in dry weight and turbidity due to an increase in PHB synthesis. The culture when incubated with 3-HB or crotonate as a substrate, increased both the rate and amount of PHB synthesis as compared to the autotrophically grown cells. Schlegel *et al.* (1970), have also been able to isolate mutants of *Hydrogenomonas* H16 that do not accumulate PHB.

Van Gool *et al.* (1971), demonstrated that *Nitrobacter winogradsky* accumulated PHB when CO_2 fixation and nitrite oxidation were occurring and that the PHB levels decreased rapidly during nitrite depletion. When the medium was supplemented with acetate or 3-HB the organism increased its content of PHB and showed decreased levels of CO_2 fixation, bicarbonate consumption and nitrite oxidation.

Preliminary results by VanCaeseele and Lees (1969) indicated that heterotrophically grown *T. novellus* contained PHB (4.6% by weight). Autotrophically grown cells did not contain any PHB.

Synthesis of PHB

The accumulation of PHB in bacteria as a storage material is under physiological and enzymatic control. The physiological growth conditions ideal for PHB accumulation vary from one genus to another. Synthesis occurs when carbon and energy sources are in excess of that required for normal cellular metabolism and growth. The majority of workers have found that the microorganisms under investigation store PHB when one compound is limiting while another is in excess.

The enzymatic pathways for PHB synthesis vary slightly from one

microorganism to another. The pathways have been determined for *Azotobacter beijernckii* (Senior and Dawes, 1973), *Hydrogenomonas eutropha* H16 (Oeding and Schlegel, 1973), *Pseudomonas* AM1 (Taylor and Anthony, 1976) and *Rhodospirillum rubrum* (Stanier *et al.* 1959). These pathways are quite similar and for this discussion the pathway of PHB synthesis and degradation in *Azotobacter beijernckii* will serve as an example (Fig. 1).

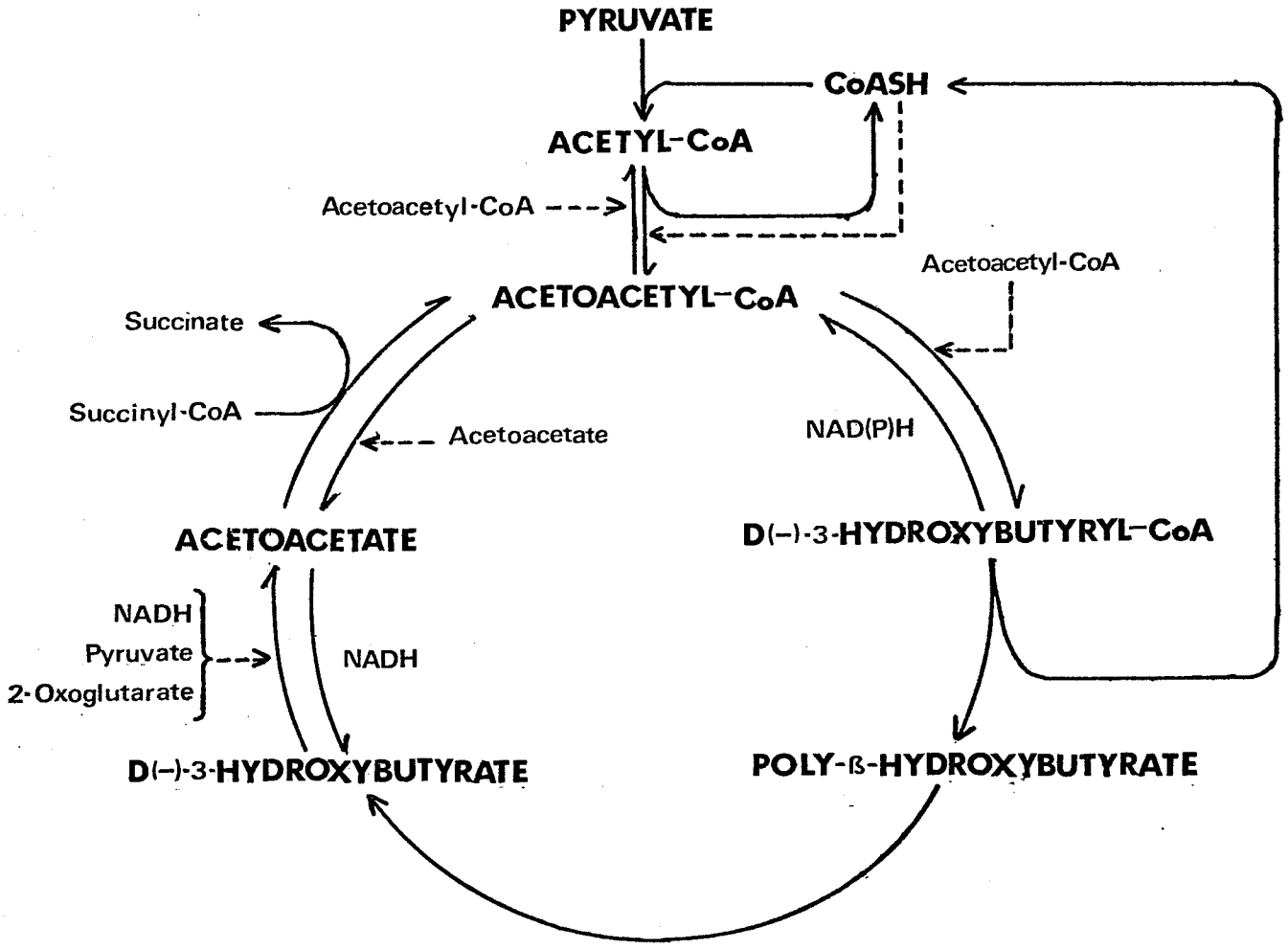
PHB Degradation

Although PHB can be degraded extracellularly by depolymerase enzyme from a variety of *Pseudomonads* (Delafield *et al.* 1965; Chowdhury, 1963) most microorganisms which store this material degrade it intracellularly. Delafield *et al.* (1965) were able to show that the *Pseudomonads* were able to produce extracellular enzymes which digested the PHB to 3-HB and a dimeric ester (3-D-(3'-D-hydroxybutanoyloxy) butanoic acid) were then taken into the cell and further metabolized. The dimeric ester is then further hydrolyzed by a constitutive intracellular dimer hydrolase to form 3-HB (Delafield *et al.* 1965).

Most work has been done on the intracellular PHB depolymerase. Hippe and Schlegel (1967) have studied a soluble depolymerase from the chemolithotrophic bacterium *Hydrogenomonas* H16. This enzyme produced 3-HB as the only end product of hydrolysis of native PHB granules. The depolymerization activity could be increased by adding trypsin which is believed to be an artificial activator for the enzyme's attack. Merrick and Doudoroff (1964) have been able

Figure 1. The cyclical synthesis and degradation of PHB in *Azotobacter beijerinckii* (Senior and Dawes, 1973).

---Inhibition

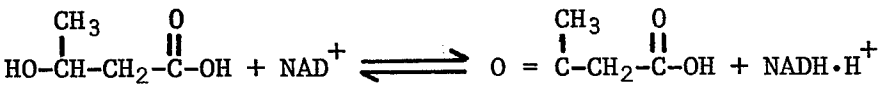

PYRUVATE
CoASH
ACETYL-CoA
Acetoacetyl-CoA
ACETOACETYL-CoA
Acetoacetyl-CoA
Succinate
Succinyl-CoA
Acetoacetate
NAD(P)H
ACETOACETATE
D(−)-3-HYDROXYBUTYRYL-CoA
NADH
Pyruvate
2-Oxoglutarate
NADH
D(−)-3-HYDROXYBUTYRATE
POLY-ß-HYDROXYBUTYRATE

to isolate from the soluble extracts of *Rhodospirillum rubrum* a three component system capable of depolymerizing PHB granules from *Bacillus megaterium*. The system consisted of a thermolabile depolymerase, an esterase and a thermostable activator. The depolymerase has little activity on the PHB granules unless the activator is present. This activator which does not show any proteolytic or hydrolytic activity by itself can be replaced by trypsin. The mechanism of PHB degradation by this enzyme system is still unknown.

Merrick (1965) has shown that a variety of antibiotics including polymixin B and tyrocidine which cause cell membrane damage and disorganization, strongly inhibit the action of the depolymerase enzyme.

3-Hydroxybutyrate dehydrogenase (3-HBDH)

3-Hydroxybutyrate dehydrogenase E.C.1.1.1.30 occurs both in bacterial and mammalian cells. It is found in all microorganisms which accumulate PHB. A partial list of the bacterial and mammalian sources and properties of 3-HBDH is presented in Table 1. The enzyme catalyzes the reversible NAD linked oxiation of 3-HB to acetoacetate:



Substrate Specificity

To date all reports indicate that 3-HBDH is a NAD dependent oxidoreductase. NADP in the forward oxidation reaction or NADPH in the reverse reduction reaction cannot replace NAD or NADH (Bergmeyer

TABLE 1

SOURCE	Purity Specific Activity	Optimum pH Oxidation	Optimum pH Reduction	Km D-3-Hydroxy-butyrate	Km Acetoacetate	Km_{NAD^+}	$Km_{NADH \cdot H^+}$	Molecular Weight	Ions	Effects of Various Reagents	Reference
Mycobacterium phlei	14.0 u/mg	8.4	6.4-7.0	7.4 mM		0.66 mM			Ca^{+2} Mn^{+2}	Sensitive to p-chloro-mercuribenzoate $HgCl_2$	Dhariwal *et al.* (1978)
Bacillus cereus	21.0 u/mg	8.0		2.3 mM		0.95 mM		93,000	$MgCl_2$ Ca^{+2} Ba^{+2} Mn^{+2}	EDTA inhibits	Thompson and Nakada (1973)
Rhodopseudomonas spheriodes	17.2 u/mg	7.0-9.0	6.2-6.9	0.41 mM	0.28 mM	0.88 mM	.054 mM	85,000			Bergmeyer *et al.* (1967)
Azotobacter vinelandii	6.4 u/mg										Jurtshuk *et al.* (1968)
Pseudomonas legmoignei	189 u/mg	8.0		0.6 mM			.083 mM		Mg^{+2}	EDTA inhibits	Delafield *et al.* (1965)
Rhodospirillum rubrum	15.3 u/mg	6.8-8.5	6.2-6.9	0.84 mM	0.071 mM	0.07 mM			Mg^{+2}	EDTA inhibits	Shuster and Doudoroff (1962)
Azotobacter beirjerinckii	15.3 u/mg	8.4-8.5		0.877 mM		0.07 mM					Senior and Dawes (1973)
Beef Heart	100-fold	8.0-8.5	7.0	0.09 mM		0.11 mM				Thiol inhibitors lecithin	Sekuzu *et al.* (1963)
Pig Kidney	100-fold	8.5	6.3	5.3 mM	6.4 mM					Thiol inhibitors	Smiley and Ashwell (1961)
Rat Liver		8.0-8.2		.47-.53 mM		0.25 mM				Thiol inhibitors	Lehninger *et al.* (1960)

et al. 1967; Jurtshuk *et al.* 1968; Dhariwal and Venkitasubramanian, 1978; Delafield *et al.* 1965). The enzyme is specific for the D(-) Stereoisomer of D,L-3-hydroxybutyrate and will not oxidize the L(-) stereoisomer (Dhariwal and Venkitasubramanian, 1978; Bergmeyer *et al.* 1967; Delafield *et al.* 1965; Shuster and Doudoroff, 1962). The enzyme from *Rhodospirillum rubrum* (Shuster and Doudoroff, 1962) was able to reduce β-ketovalerate at a rate of 6% of that of acetoacetate. In addition to acetoacetate and 3-HB the enzyme from *Rhodopseudomonas spheroides* (Bergmeyer *et al.* 1967) was shown to react slowly with 3-hydroxypentanoate, 3-hydroxyhexanoate, 3-oxopentanoate and 3-oxohexanoate. Similar results were obtained with the enzyme and 3-oxopentanoate from *Pseudomonas lemoignei* (Delafield *et al.* 1965).

Km Values

From Table 1, the Km values for 3-HB vary slightly from 0.41 to 0.877 mM for *R. spheroides, P. lemoignei, R. rubrum* and *A. beijerinckii*. The value of 7.4 mM for *M. phlei* and 2.3 mM for *B. cereus* enzymes are considerably higher. The Km values for NAD range from 0.07 to 0.11 mM for *R. spheroides, R. rubrum* and *A. beijerinckii*. Again the Km values for NAD from the enzymes from *B. cereus* and *M. phlei* are considerably higher (10-fold).

Stability

Most investigators found that 3-HBDH is stable if frozen at -20 to -40^{o}C (Dhariwal and Venkitasubramanian, 1978; Delafield *et al.* 1965;

Senior and Dawes, 1973). From *Bacillus cereus* T, Thompson and Nakata (1973) have shown that the enzyme is stable frozen but at $4^{o}C$ loses activity unless incubated with $MgCl_2$ or Mn^{+2}, BA^{+2}, Ca^{+2} ions. The requirement of Mg^{+}, Mn^{+} or Ca^{+} for stability was also shown by the enzyme from *Pseudomonas lemoignei* (Delafield *et al.* 1965). The requirement for Ca^{+} was shown when 3-HBDH from *R. spheroides* was incubated at $37^{o}C$ (Bergmeyer *et al.* 1967). An unusual finding was that 3-HBDH from *R. rubrum* was cold sensitive as the enzyme at $0^{o}C$ was inactive unless warmed to room temperature (Shuster and Doudoroff, 1962).

Metal Ion Requirement

No direct requirement for metal ions has been shown for 3-HBDH activity. As stated earlier several investigators have shown that divalent metal ions stabilize the enzyme but are not required for catalytic activity. The enzymes from *R. spheroides* (Bergmeyer *et al.* 1967) and *M. phlei* (Dhariwal and Venkitasubramanian, 1978) are not inactivated by EDTA. Loss of enzyme activity through dialysis has been reported for *R. spheroides* (Preuveneers *et al.* 1973), *R. rubrum* (Shuster and Doudoroff, 1962) and *A. vinelandii* (Jurtshuk *et al.* 1968). The activity could be regained by addition of Mg^{+2} and NAD to the dialysis buffer for the enzyme from *R. rubrum*, but only Mg^{+2} was required in the dialysis buffer for the enzyme from *A. vinelandii*.

Sulfhydryl Reagents

Numerous investigators have shown that the addition of sulfhydryl

reducing agents such as dithiothreitol, β-mercaptoethanol, glutathione or cysteine do not lead to any increase in enzyme activity (Dhariwal and Venkitasubramanian, 1978; Shuster and Doudoroff, 1962; Senior and Dawes, 1973; Delafield *et al.* 1965). These results however, do not necessarily indicate that the enzyme did not have sensitive thiol groups required for activity, but only that the sulfhydryl groups required were already in the reduced form. The enzymes from *M. phlei* (Dhariwal and Venkitasubramanian, 1978) and *R. spheroides* (Bergmeyer *et al.* 1967) were sensitive to thiol inhibiting reagents such as p-hydroxymercuribenzoate and mercuric ions. This inactivation could be prevented by incubation with NADH or Ca^{+2}. The enzyme from *B. cereus* T was also inhibited by p-hydroxymercuribenzoate (Thompson and Nakata, 1973). Delafield *et al.* (1965) found that freshly prepared 3-HBDH from *P. lemoignei* was not inhibited by p-hydroxymercuribenzoate or iodoacetamide but that during storage at $-20^{o}C$ the enzyme became more sensitive to inhibition with these reagents. The enzymes from *R. rubrum* (Shuster and Doudoroff, 1962) and *A. beijerinckii* (Senior and Dawes, 1973) appear to have no sensitive thiol groups when incubated with sulfhydryl reagents.

Molecular Weight

The molecular weight of 3-HBDH from *R. spheroides* (Bergmeyer *et al.* 1967) was calculated to be 85,000 by ultracentrifugation using the sedimentation equilibrium method. The enzyme from *B. cereus* (Thompson and Nakata, 1973) was found to have a molecular weight of 93,000 as determined by gel filtration.