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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TABLE OF CONTENTS

TABLE OF CONTENTS CONT'D

LIST OF FIGURES

LIST OF FIGURES CONTINUED... Page

LIST OF TABLES

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

INTRODUCTION

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

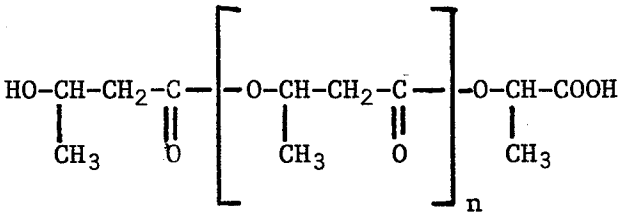
Some prokaryotes, like eukaryotes, have the ability to store intracellular energy reserve polymers. Most of the storage polymers serve both as a carbon and energy source during deprived conditions. In the absence of growth in microorganisms, energy is required for pH maintenance, osmotic regulation, 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).

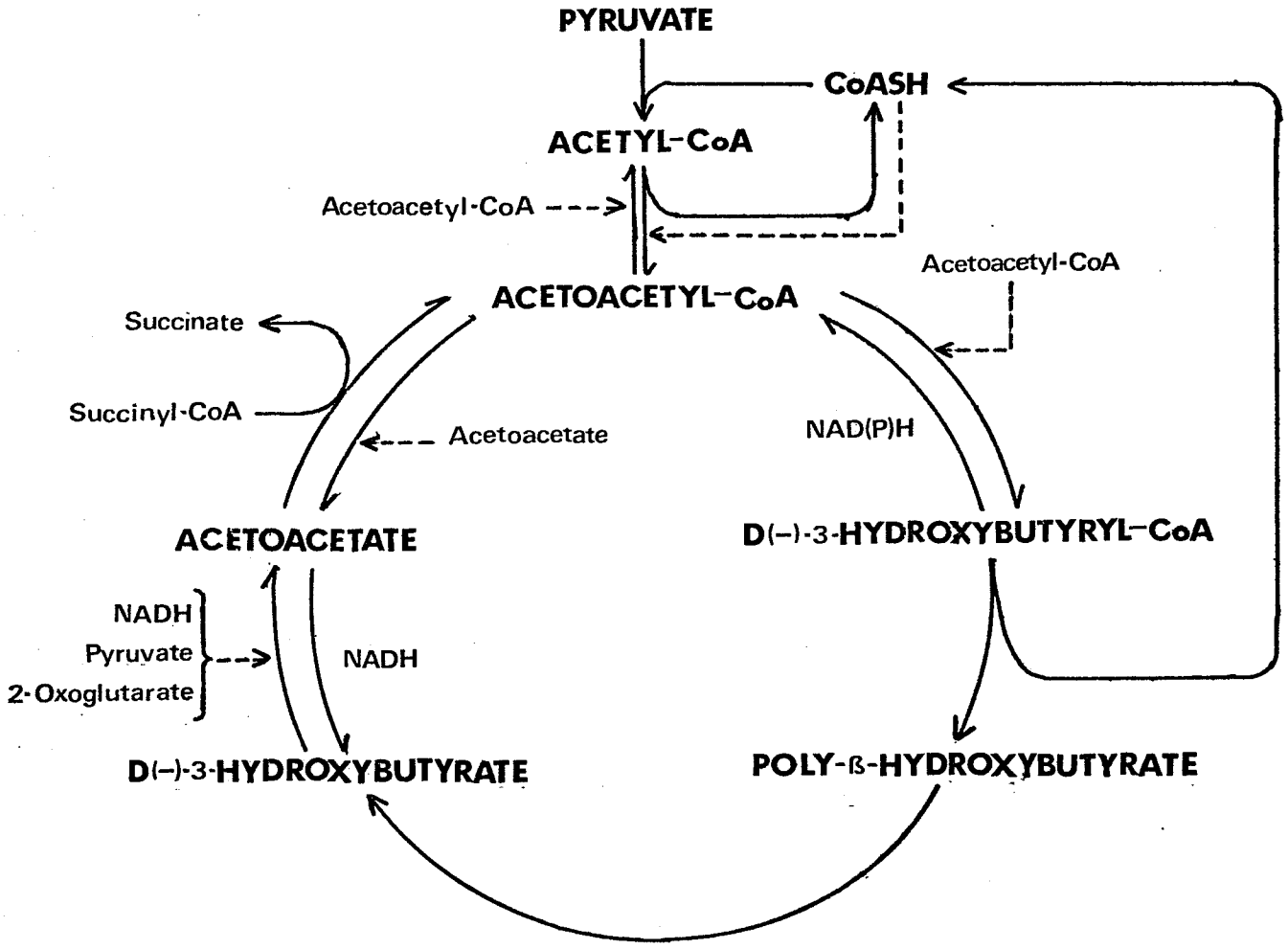
PHB Degradation

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

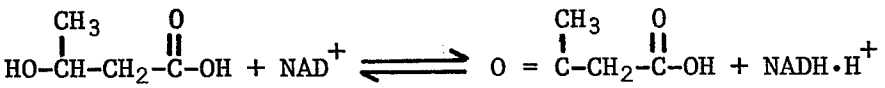

PYRUVATE
CoASH
ACETYL-CoA
Acetoacetyl-CoA
ACETOACETYL-CoA
Acetoacetyl-CoA
Succinate
Succinyl-CoA
Acetoacetate
NAD(P)H
ACETOACETATE
D(−)-3-HYDROXYBUTYRYL-CoA
NADH
Pyruvate
2-Oxoglutarate
NADH
D(−)-3-HYDROXYBUTYRATE
POLY-ß-HYDROXYBUTYRATE

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.

Kinetic Mechanism

The steady state kinetic mechanism of 3-HBDH has been determined for the enzyme from *R. spheroides* (Preuveneers *et al.* 1973; Hurst *et al.* 1973). Using initial velocity, dead-end inhibition and product inhibition analysis, Preuveneers *et al.* (1973) determined that the enzyme follows an ordered Bi Bi mechanism (Cleland, 1963a,b,c) with possible multiple enzyme-nicotinamide nucleotide complexes. These authors also covalently attached the enzyme to DEAE-cellulose and showed that the mechanism still follows an ordered Bi Bi. The results indicated that NAD was bound first by the enzyme followed by 3-HB. The first product to be released was acetoacetate followed by the release of NADH.

Senior and Dawes (1973) determined that the enzyme from *A. beijerinckii* was inhibited by NADH (competitive), pyruvate (competitive) and α-oxoglutarate (competitive) while L-malate or oxaloacetate showed no inhibition. Delafield *et al.* (1965) demonstrated that the enzyme from *P. lemoignei* was inhibited by D-lactate and 2-D, L-hydroxybutanoate while pyruvate was a weak inhibitor. Bergmeyer *et al.* (1967) showed that the enzyme from *R. spheroides* was inhibited by malonate (complex type), succinate (complex type) D,L-lactate (competitive) and 2-hydroxybutyrate (competitive). An unusual finding was that the enzyme from *R. rubrum* (Shuster and Doudoroff, 1962) did not show any inhibition with D,L-lactate, β-ketocaproate or pyruvate.

Regulation of PHB Degradation Through 3-HBDH

From the previous discussion on the kinetics of 3-HBDH it is

apparent that all investigators have reported that NADH is a competitive inhibitor of the enzyme. During growth with a suitable carbon and energy source the microorganism would be capable of producing large amounts of NADH which would compete with NAD, inhibiting 3-HBDH. The result would be a suppression of utilization of PHB until lower levels of reducing power were present in the cell. Senior and Dawes (1973) suggested that the inhibition of 3-HBDH by pyruvate and 2-oxoglutarate was also important in the regulation of PHB degradation. The levels of these two compounds would increase during glucose catabolism and PHB as a carbon or energy source would not be required.

Mammalian 3-HBDH

During β-oxidation of fatty acids during fasting or in people with diabetes mellitus, there is an increase in the blood of ketone bodies such as acetoacetate and 3-HB. THese are formed during an excess of fatty acid degradation, forming excessive acetyl-CoA which is condensed to form acetoacetyl-CoA by acetyl-CoA acetyltransferase (Lehninger, 1975). Acetoacetyl-CoA then undergoes deacylation to yield acetoacetate. The enzyme 3-HBDH which is found in the liver mitochondrial membrane then converts acetoacetate to 3-HB. Both acetoacetate and 3-HB can diffuse from the liver and enter the blood stream where they then enter the peripheral tissues. Here 3-HB is converted to acetoacetate which is then converted to succinic acid and finally to acetoacetyl-CoA where it can then undergo cleavage and enter the TCA cycle (Lehninger, 1975). 3-HBDH activity has been found in the mito-

chondrial fraction from a variety of organs and tissues in the rat (Lehninger, 1960). Sekuzu *et al.* (1961) and Jurtshuk *et al.* (1961) were able to show that the enzyme had a specific and absolute requirement for lecithin (phosphatidylcholine).

To date all 3-HBDHs from mammalian sources have been shown to contain a very sensitive thiol group and activity could only be demonstrated after incubation of the enzyme with sulfhydryl reducing agents such as thioglycerol, β-mercaptoethanol, glutathione or cysteine (Gotterer, 1967; Sekuzu, 1961; Sekuzu, 1963).

The enzyme is similar to the bacterial enzyme in that it shows specificity for the D(-) stereoisomer of D,L-hydroxybutyrate and is NAD dependent (Lehninger, 1960).

Nielsen *et al.* (1972) were able to purify 3-HBDH from bovine heart mitochondria 150-fold and examined the kinetic mechanism. They determined the mechanism to be an ordered Bi Bi where NAD is the first substrate to bind to the enzyme and NADH is the last product. These results are similar to the ordered Bi Bi mechanism proposed for the enzyme from *R. spheroides* (Preuveneers *et al.* 1973; Hurst *et al.* 1973). Berry (1964) was able to demonstrate inhibition of 3-HBDH by fatty acids (hexanoate, octanoate), dicarboxylic acids (malonate, succinate, glutamate) and also by adenine nucleotides (AMP, ADP, ATP). One unusual finding was that pyruvate and 2-oxoglutarate did not show any inhibition in contrast to the enzyme from *Azotobacter beijerinckii* (Senior and Dawes, 1973) which was inhibited competitively by both compounds. Gotterer (1969) using rat liver mitochondrial 3-HBDH was

able to demonstrate competitive inhibition with a variety of topical anaethetics such as propanol, benzimidazole and tetracaine. These compounds show no structural similarity to either 3-HB or acetoacetate.

MATERIALS AND METHODS

MATERIALS

All chemicals used were of analytical grade and obtained commercially from the following companies:

Sigma Chemical Co. (St. Louis, Missouri, U.S.A.)

Acetoacetate (Na salt)
AMP
ATP
Cis-aconitate
Cysteine
Coomassie Brilliant Blue R
Dithiothreitol
DTNB
EDTA
L-Glutamate
Glutathione (reduced)
D,L-β-Hydroxybutyrate(Na Salt)
3-HBDH *R. spheroides*
α-Ketoglutarate
α-Ketobutyrate
D-lactate
D,L-Malate
NAD (98%)
NADH (98%)
NADP (98%)
NEM
NBT
Pyruvate
PMS
Protamine sulfate
Tris

Pharmacia (Uppsala, Sweden)

DEAE-Sephadex A50
G-200 Sephadex superfine
PD-10 G-25 Sephadex

Fischer Scientific Co. (Fair Lawn, N.J., U.S.A.)

$(NH_4)_2SO_4$ (primary standard) $ZnCl_2$

$MnCl_2 \cdot 4H_2O$ NaCl

$MgCl_2 \cdot 6H_2O$ K_2HPO_4

$FeCl_3 \cdot 6H_2O$ $Na_2S_2O_3$

$FeSo_4 \cdot 7H_2O$ $CoCl_2 \cdot 6H_2O$

$CuCl_2 \cdot 2H_2O$ $Ni(OH)_3$

$CaCl_2$

Eastman Kodak Co. (Rochester, N.Y., U.S.A.)

Succinate

Acrylamide

N,N'-Methylene bisacrylamide

Matheson Coleman & Bell Co. (Cincinnatti, Ohio, U.S.A.)

Malonate

McArthur Chemical Co. (Montreal, Canada)

Citrate

Blue Dextran-Sepharose 4B was kindly donated by Dr. I. Suzuki (Department of Microbiology, University of Manitoba).

ORGANISM AND GROWTH CONDITIONS

Thiobacillus novellus (ATCC 8093) originally from Dr. R.L. Starkey was used in this investigation. The organism was grown on a modified Starkey (1934) No. 2 minimal salt medium supplemented with either 1% sodium thiosulfate for autotrophic growth or 1% glucose for heterotrophic growth. The minimal salt medium contained (per L of glass distilled H_2O):

K_2HPO_4	4.0 g
KH_2PO_4	1.5 g
$CaCl_2 \cdot 2H_2O$	0.1 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$(NH_4)_2SO_4$	0.3 g
$FeCl_3 \cdot 6H_2O$	0.02 g
$MnSO_4$	0.02 g

The medium was then adjusted to pH 8.0 with 10% KOH and sterilized by autoclaving for 20 min at 121°C. Glucose (10%) or sodium thiosulfate (10%) were made separately in glass distilled H_2O and autoclaved for 15 min at 121°C.

The stock culture was maintained autotrophically in cotton-gauze plugged 250 ml flasks containing 100 mL of the minimal salt medium, 10 mL of inoculum and 10 mL of 10% sodium thiosulfate. The culture was grown at either room temperature or 28°C on a rotary shaker. Every 7 days the culture was transferred to fresh medium. At this time samples were streaked onto trypticase soy and nutrient agar plates to check for purity.

For heterotrophic growth the culture was grown in the same manner as the autotroph except that thiosulfate was replaced with glucose. The heterotroph required transfer into fresh medium every day (or second day) as compared to the autotroph (every 7 days). For high cell yields (i.e. batch culture), *T. novellus* was grown heterotrophically on 1% glucose in 15 L glass carboys. The carboys contained 12 L of sterile minimal salt medium, 1.2 L of sterile 10% glucose, and were inoculated with 1.2 L of inoculum. The carboys were incubated at 28°C and were given forced sterile atmospheric aeration through sintered glass spargers. After 22 h the cells were harvested by centrifuging at 50,000 r.p.m. in a water cooled (7°C) Sharples super centrifuge. The cells were then washed twice with 500 mL of 0.1 M phosphate buffer pH 8.0 and collected by centrifugation at 4,000 X g. The cell pellet was stored at -20°C.

PREPARATION OF CELL FREE EXTRACT

The frozen cell paste was allowed to thaw at room temperature. To this wet cell past, 0.1 M phosphate buffer pH 8.0 was added in a ratio of 1 g per 3 mL of buffer and suspended evenly by stirring. The resulting suspension (maintained on ice) was passed through an Aminco French Pressure Cell, twice, using 1,100 p.s.i., and the extract was centrifuged at 12,000 X g at 4°C for 30 min. The supernatant fluid was centrifuged in a Beckman L2-65B ultracentrifuge using a 60 Ti rotor at 150,000 X g for 2 h.

3-HYDROXYBUTYRATE DEHYDROGENASE PURIFICATION PROCEDURE

All enzyme purification procedures (where possible) were conducted at 4°C. The ultracentrifuged cell extract was divided into 50 mL quantities which were individually treated to the following procedure. Protamine sulfate (dissolved in 0.1 M phosphate buffer pH 8.0) was added to a concentration of 2 mg/mL and the mixture was stirred for 5-10 min. The resulting precipitation was removed by centrifugation at 4,000 X g for 10 min and discarded. Solid $(NH_4)_2SO_4$ (primary standard grade) was added to 40% saturation and stirred for 10 min. The suspension was centrifuged at 15,000 X g for 30 min, and the pellet discarded. Solid $(NH_4)_2SO_4$ was again added to 60% saturation to the previous supernatant and stirred for 10 min. The suspension was centrifuged at 15,000 X g for 30 min. The resulting active pellet was redissolved in 6.0 mL of 0.1 M phosphate buffer pH 8.0. The suspension was then desalted using a PD-10 Sephadex G-25 column (Pharmacia). The 10 mL of desalted enzyme suspension was applied to a DEAE-Sephadex A50 column (2.6 X 25 cm bed) equilibriated with 0.1 M phosphate buffer pH 8.0. The enzyme was eluted with 0.1 M phosphate buffer pH 8.0 containing a 0.1 - 0.6 M NaCl linear gradient. Five mL fractions were collected using a Gilson fraction collector. The most active enzyme fractions (33-42) were pooled and solid $(NH_4)_2SO_4$ was added to 60% saturation and stirred for 10 min. The suspension was then centrifuged at 15,000 X g for 30 min.

The resulting active pellet was resuspended in 5 mL of 0.1 M phosphate buffer pH 8.0 and applied to a Sephadex G-200 superfine column (2.6 X 35 cm bed) equilibriated with 0.1 M phosphate buffer pH 8.0 containing 1 M $(NH_4)_2SO_4$. The enzyme was eluted (0.75 mL/min) using a 0.1 M phosphate buffer pH 8.0 containing 1 M $(NH_4)_2SO_4$. The fractions (5 mL) were collected by a fraction collector. The fractions containing the highest enzyme activity (fractions 17 - 24) were pooled and again desalted in a PD-10 Sephadex G-25 column. The sample was then divided into 5 mL aliquots and this amount was applied to a Blue Dextran Sepharose 4B affinity column (1.4 X 4.0 cm bed) which had been previously equilibriated with 10 mM Tris-HCl buffer pH 8.0. The column was then washed with 5 mL of 10 mM Tris-HCl buffer pH 8.0 and the solution coming through discarded. The enzyme was eluted from the column by 5 mL of a 10 mM Tris-HCl buffer pH 8.0 containing 1 mM NAD. The column was washed with 3 M KCl (Ryan and Vesting, 1974) and again equilibriated with a large volume (20 - 30 mL) of 10 mM Tris-HCl buffer pH 8.0 prior to reuse.

To the purified enzyme, solid $(NH_4)_2SO_4$ was added to 60% saturation and stirred for 10 min. The enzyme solution was centrifuged at 15,000 X g for 30 min. The resulting minute precipitate was redissolved in 0.1 M phosphate buffer pH 8.0 containing 1 M $(NH_4)_2SO_4$ and was then frozen at $-20^{o}C$.

ENZYME ASSAY

The enzyme was assayed routinely at room temperature in the forward oxidation direction by the method of Krebs *et al.* (1969). The initial

velocity was measured using a Gilford Model 2000 multiple recording spectrophotometer and 3 mL quartz cells with a 1 cm light path. The assay curvette contained (final concentration): 2 mM NAD, 10 mM D,L-3-Hydroxybutyrate, 33 mM Tris-HCl buffer pH 8.4. The enzyme sample (0.1 mL) was added to the reaction curvette and the blank (containing no 3-HB). The enzyme activity was determined by measuring the increase in absorbance at 340 nm. One unit of enzyme activity was defined as the amount of enzyme which catalyzes the conversion of 1 micromole of substrate per min at room temperature (Krebs *et al.* 1969) where:

$$c[\mu mole/mL] = \frac{\Delta E \times V}{10^{-6} \times \varepsilon \times d \times v}$$

ΔE = O.D. change at 340 nm, V = volume of solution in cuvette (3.0 mL), ε = Extinction coefficient of NADH (6.22×10^{6}), d = light path length (1 cm) and v = volume of sample (0.1 mL).

The enzyme is also capable of catalyzing the reverse or reduction reaction. The assay cuvette contained (final concentration): 3.33 mM acetoacetate (lithium or sodium salt), 0.33 mM NADH and 33 mM phosphate buffer pH 6.8 (Krebs *et al.* 1969). The enzyme (0.1 mL) was added to the reaction cuvette and also to the blank which contained no acetoacetate. Enzyme activity was measured by following the decrease in absorbance at 340 nm.

KINETIC ANALYSIS

For the kinetic analysis only the forward oxidation reaction was measured. Initial velocities were measured by an increase in absorbance

at 340 nm in 0.1 M Tris-HCL buffer pH 8.6.

D,L-3-Hydroxybutyrate (sodium salt) was found to contain 41% of the D(-) stereoisomer enzymatically. All concentrations were adjusted so that the D(-) stereoisomer would be present in the appropriate amount. The concentration of NAD was determined spectrophotometrically $E^{mM}_{260nm} = 17.9$. The concentration of NADH as an inhibitor for the reaction was also determined spectrophotometrically $E^{mM}_{340nm} = 6.22$. The concentration of all reactants are stated in the RESULTS.

One pooled batch of purified enzyme was used for the entire kinetic study. The activity was determined to be 18.9 units/mg protein.

Two trials were conducted for each assay and the average velocity was determined. All points that did not fall close to the preliminary line on reciprocal plots ($\frac{1}{[S]}$ vs $\frac{1}{v}$) were immediately repeated. All reciprocal plots were drawn and fitted by eye.

The two products of the reaction, acetoacetate and NADH were used inihibit the forward oxidation reaction. One substrate (NAD or 3-HB) was varied while the other was held at a constant concentration, either saturated or non-saturated. Cleland's rules (1963 a,b,c) were used to determine the kinetic mechanism.

PH OPTIMUM

The pH optimum of the oxidation reaction was determined by the following procedure. The enzyme (0.2 mL) was incubated with the

appropriate pH buffer (0.2 mL) at room temperature for 5 - 10 min. From this mixture 0.1 mL was removed and assayed in a curvette containing 1.0 mL of the same incubation buffer. The enzyme activity was measured twice for each pH value and the average initial velocity determined.

MOLECULAR WEIGHT DETERMINATION

Molecular weight determination was performed by the gel filtration method of Pharmacia. The gel Sephadex G-200 superfine had a 2.6 X 36 cm bed (Vt = 190 mL) and was equilibriated with 0.1 M phosphate buffer pH 8.0. The eluant was also 0.1 M phosphate buffer pH 8.0. Three molecular weight standards were used: aldolase MW 158,000, ovalbumin MW 45,000, chymotrypsinogen A MW 25,000 (Pharmacia). Blue dextran 2000 (Pharmacia) was used to determine the void volume (Vo=68 mL).Kav values were determined for the standard curve by the following calculation (Pharmacia):

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

where, Ve = elution volume for protein, Vo = elution volume for Blue dextran and Vt = total bed volume.

POLY-β-HYDROXYBUTYRATE ANALYSIS

T. novellus was grown heterotrophically in a series of 250 mL flasks containing 100 mL of minimal salts media, 10 mL of sterile 10% glucose and 10 mL of inoculum. The culture was incubated at 28°C on a rotary shaker (200 r.p.m.). At 2 h intervals up to 24 h, a flask

was removed. From this flask samples were removed for PHB, protein and enzyme analysis. PHB analysis was performed as described by Ward and Dawes (1973). The concentration of PHB was determined from a standard curve of 3-HB which was linear in the range of 1 to 10 µg/mL of 3-HB.

DISC POLYACRYLAMIDE GEL ELECTROPHORESIS

Disc gel electrophoresis was performed using the method of Weber *et al.* (1972). The electrophoresis buffer contained 0.05 M Tris and 0.4 M glycine at a pH of 8.5. Gels (5 and 10% polyacrylamide) were 8 - 9 cm long and were run at 4 m.a./tube until the tracking dye (0.005% Bromphenol blue) reached the end of the tube. The gels were fixed in 12% trichloroacetic acid for 20 min and were then stained for protein (90 min) in a 0.2% Coomassie brilliant blue R, 45% ethanol and 10% acetic acid solution made in glass distilled H_2O. Destaining was done overnight in a 25% ethanol and 10% acetic acid solution. The destained gels were placed in a 10% acetic acid storage solution in a screw-capped test tube.

ACTIVITY STAIN

The location of 3-HBDH in the polyacrylamide gel was determined by an activity stain which was a modification of the methods of Lawrence *et al.* (1960) and Goldberg (1963). The activity stain contained (final concentration): 0.1 M D,L-3-Hydroxybutyrate, 0.3 mg/mL NAD, 1.0 mg/mL nitro blue tetrazolium and 0.02 mg/mL phenazine methosulfate

in a 0.1 M Tris-HCl buffer pH 8.5. The gels after electrophoresis were washed quickly in 0.1 M Tris-HCL buffer pH 8.5 and incubated with the activity stain, in the dark at room temperature. The gels were observed every 10 min for the appearance of blue bands.

PROTEIN DETERMINATION

The protein determinations were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

RESULTS

RESULTS

3-HBDH and PHB Levels in Heterotrophically Grown *T. novellus*

Levels of 3-HBDH, PHB, protein and cell density (A_{660}nm) of heterotrophically (1% glucose) grown *T. novellus* are presented in Figure 2. The protein concentration begins to level off at 18 h, while the cell density (A_{660}nm) is still increasing slightly. Levels of both 3-HBDH and PHB begin to increase simultaneously following a 10 h lag after inoculation. The maximum level of PHB occurs at 20 h followed by a gradual decrease. Highest levels (specific activity) of 3-HBDH were obtained after 22-24 h of growth. Cells harvested after 48 h of growth had levels of 3-HBDH which were close to the levels found in the 22 or 24 h cultures. Cells grown heterotrophically with 0.5% glutamate replacing 1% glucose, showed identical levels of PHB, although cell density (A_{660} nm) and protein concentration increased more rapidly in the glutamate grown cells.

Purification of 3-HBDH

The purification procedure for 3-HBDH from heterotrophically (1% glucose) grown *T. novellus* is summarized in Table 2. The initial enzyme activity was measured after ultracentrifugation because only very low levels could be detected in the crude supernatant. Protamine sulfate was efficient in removing large amounts of nucleic acid, but did not remove a substantial amount of protein or reduce

Figure 2. Levels of 3-HBDH, PHB, cell density (A_{660}nm) and protein in heterotrophically (1% glucose) grown *T. novellus*. 3-HBDH and PHB were measured as described in *Materials and Methods*. Cell density was measured by using a Klett-Summerson photoelectric colorimeter with a red filter (A_{660}nm). Cells were disrupted by 60 second sonication using an Insonator with a micro-tip. Total cellular protein was determined as described in *Materials and Methods*.

○ 3-HBDH enzyme activity (units/mg protein)

▽ PHB (μg/mL)

□ Protein (mg/mL)

△ Cell density (Klett units)

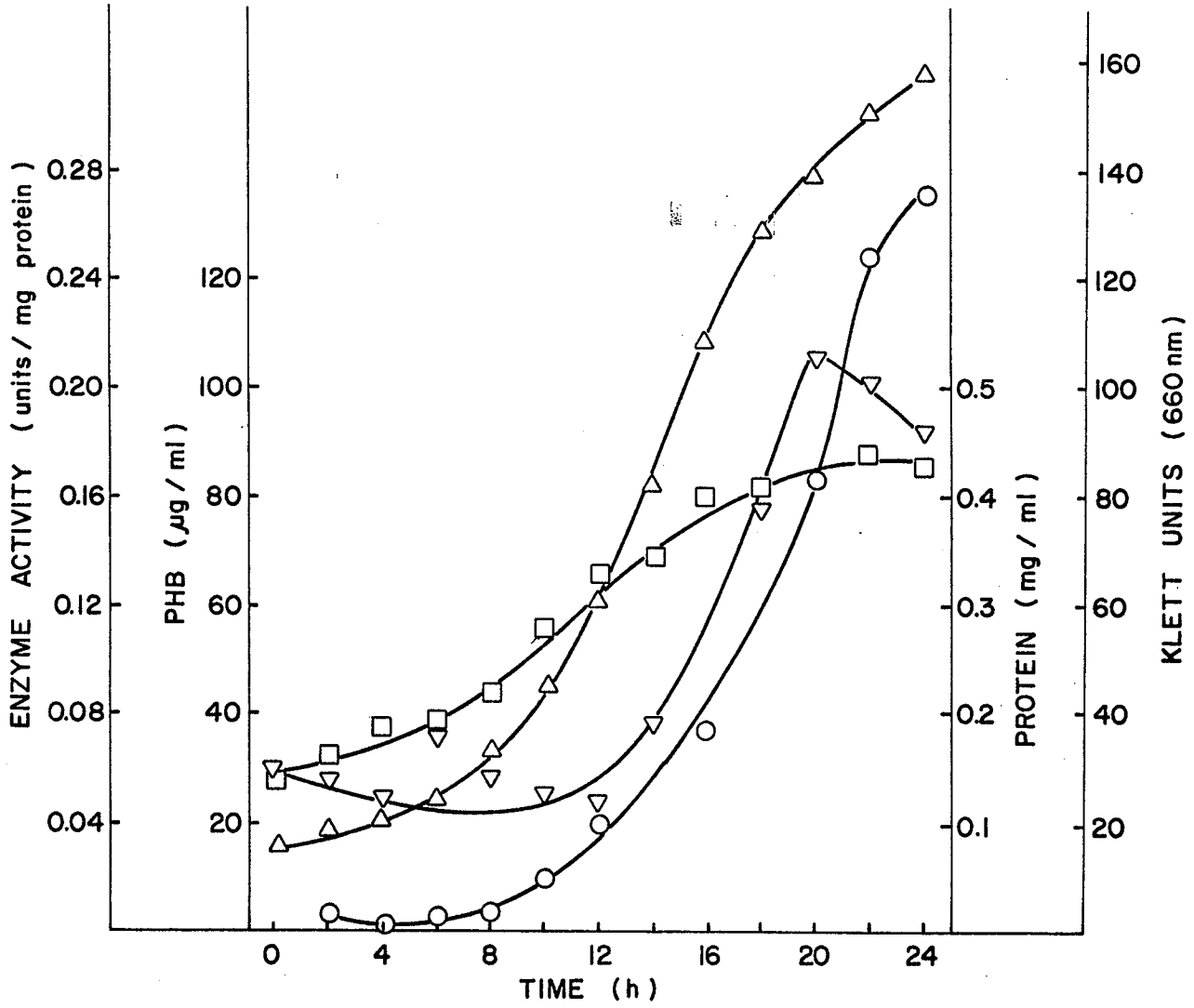

ENZYME ACTIVITY (units / mg protein)
0.28
0.24
0.20
0.16
0.12
0.08
0.04
PHB (µg / ml)
120
100
80
60
40
20
PROTEIN (mg / ml)
0.5
0.4
0.3
0.2
0.1
KLETT UNITS (660 nm)
160
140
120
100
80
60
40
20
0
4
8
12
16
20
24
TIME (h)

TABLE 2

PROCEDURE	Fraction No.	Volume (mL)	Units/mL	Total Units	Protein mg/mL	Units/mg	Yield %	Fold Purification
Ultracentrifugation	1	50	2.75	137.5	9.5	0.29	100	
Protamine Sulphate	2	50	2.75	137.5	8.7	0.32	100	1.1
$(NH_4)_2SO_4$ Precipitation	3	7.5	14.5	108.8	26.6	0.545	79	1.9
DEAE-Sephadex	33-42	50	1.78	89.0	0.61	2.92	64.7	10.1
G-200-Sephadex	17-24	40	3.05	122.0	0.31	9.84	88.7	33.9
Blue Dextran Affinity Column	6	20	2.24	44.9	0.08	28.0	32.7	96.6

enzyme activity. $(NH_4)_2SO_4$ precipitation at 40% saturation removed a considerable amount of contaminating protein, leaving the enzyme in the supernatant. $(NH_4)_2SO_4$ at 60% saturation caused the enzyme to precipitate from the solution. The enzyme was desalted on a PD-10 G-25 Sephadex column rather than by dialysis due to large decreases in enzyme activity during dialysis in distilled H_2O or 0.1 M phosphate buffer pH 8.0 both in the cold (4^oC) and at room temperature. There was a slight loss of enzyme activity during the desalting procedure.

The enzyme was eluted as a single peak (fractions 33 - 42) on DEAE-Sephadex A50 corresponding to a concentration of 0.2 - 0.3 M NaCl as presented in Figure 3.

The enzyme was eluted from the G-200 Sephadex superfine column as a single peak (fractions 17 - 24) as presented in Figure 4.

Approximately a 3-fold purification occurred using the Blue Dextran-Sepharose 4B affinity column. The enzyme was eluted from the column with 1 mM NAD in 10 mM Tris-HCl pH 8.0. It was found that high levels of 3-HB or any salt could elute the enzyme although this would result in lower specific activities. NADH (1 mM) was not as effective as NAD.

The enzyme was purified approximately 100-fold by the procedure presented. After precipitation with 60% saturated $(NH_4)_2SO_4$ the active redissolved enzyme pellet was found enzymatically to be free of the NAD which was used in the affinity column elution buffer.

Figure 3. The elution profile of 3-HBDH activity from DEAE-Sephadex A50 using a 0.1 - 0.6 M NaCl linear gradient. The procedure is described in *Materials and Methods*. Protein was determined as the absorbance at 280 nm. Enzyme activity was determined as described in *Materials and Methods*.

Fraction volume = 5 mL

○ Absorbance at 280 nm

□ Enzyme activity (units/mL)

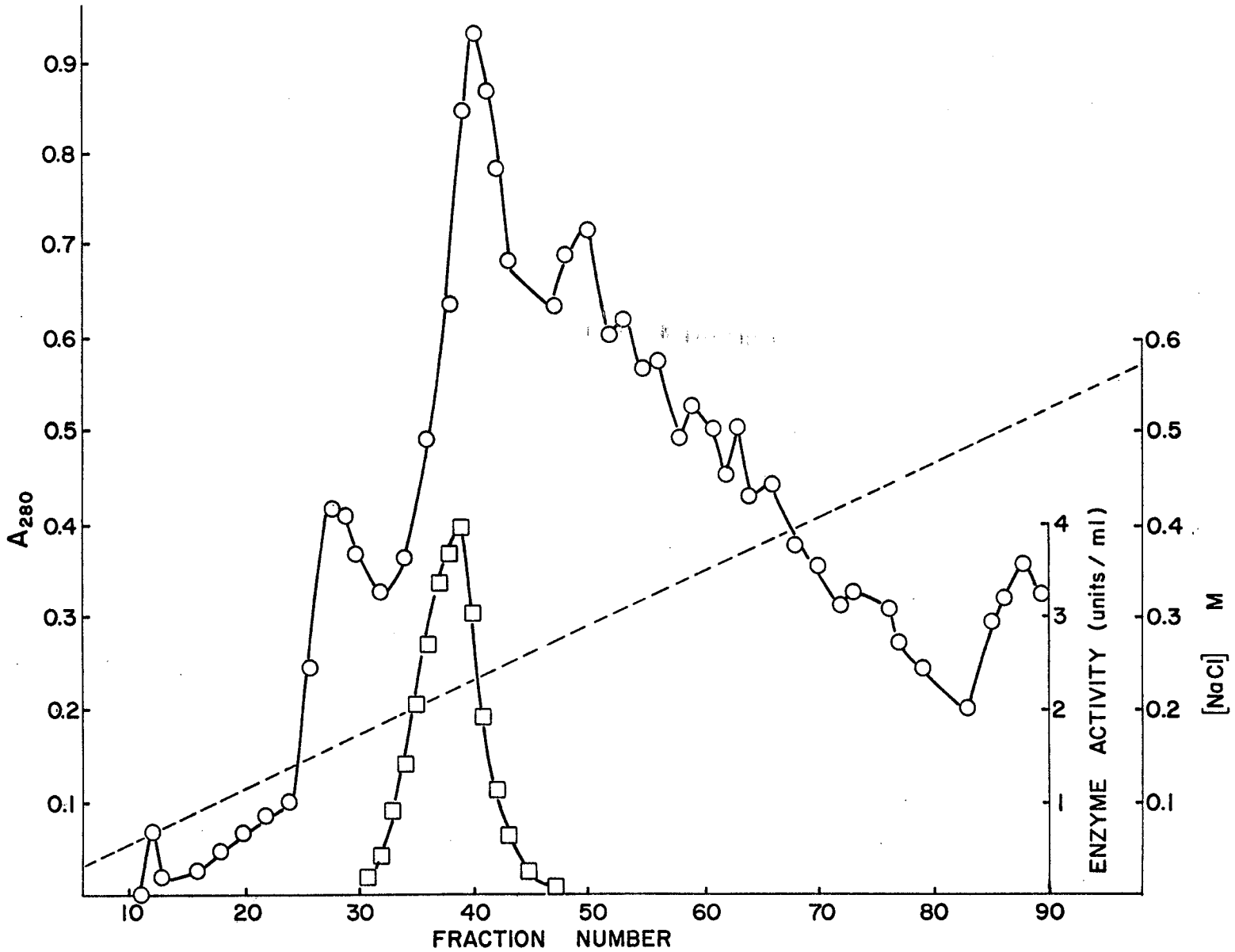
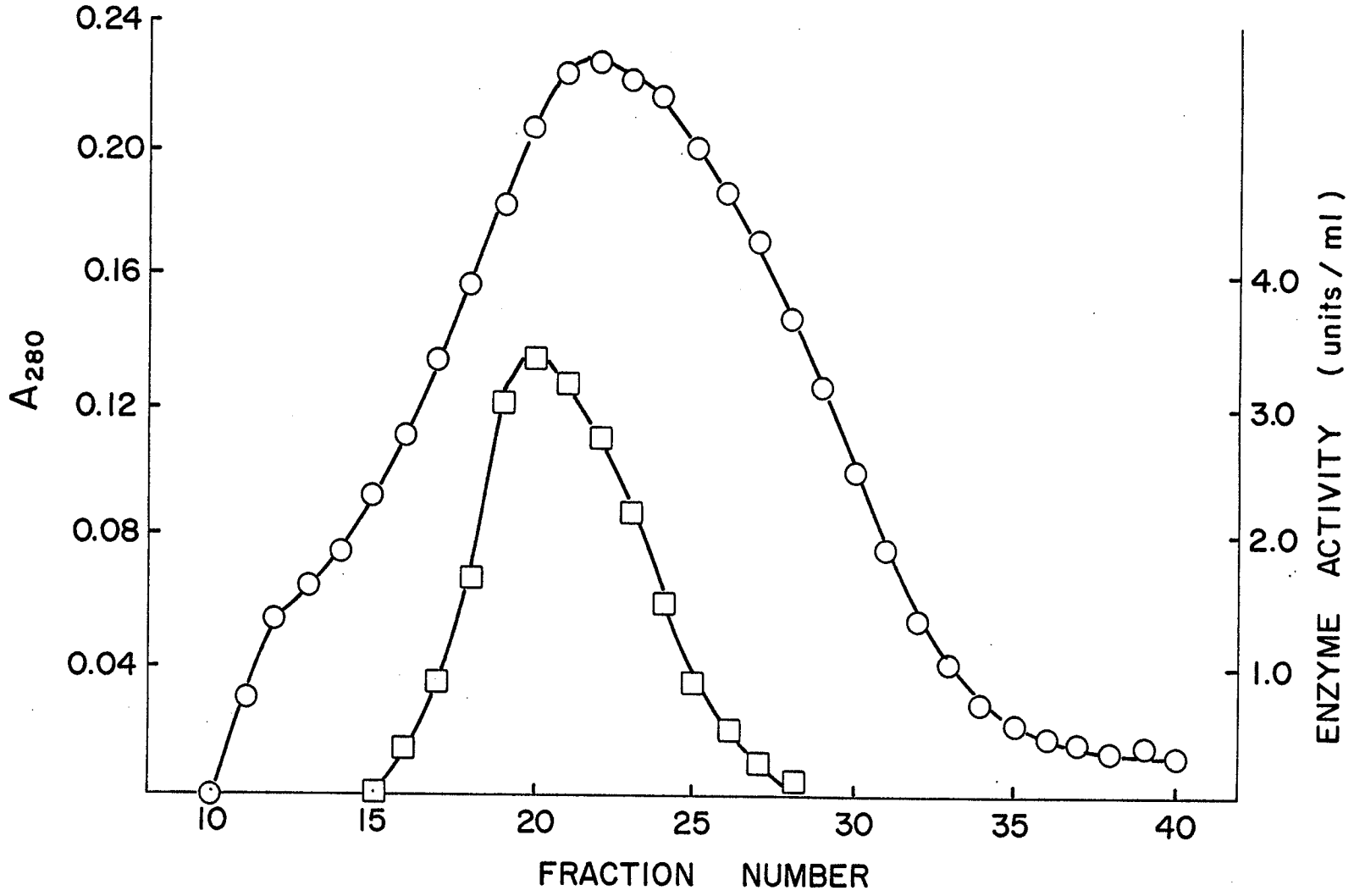

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A280
10
20
30
40
50
60
70
80
90
FRACTION NUMBER
4
3
2
1
ENZYME ACTIVITY (units / ml)
0.6
0.5
0.4
0.3
0.2
0.1
[NaCl] M

Figure 4. The elution profile of 3-HBDH activity from G-200 Sephadex superfine. The procedure was described in *Materials and Methods*. 3-HBDH activity (units/mL) was determined as described in *Materials and Methods*. Protein was determined as the absorbance at 280 nm.

Fraction volume = 5 mL

○ Absorbance at 280 nm

□ Enzyme Activity (units/mL)


0.24
0.20
0.16
0.12
0.08
0.04
A_{280}
4.0
3.0
2.0
1.0
ENZYME ACTIVITY (units / ml)
10
15
20
25
30
35
40
FRACTION NUMBER

Repeating the affinity column procedure or using a 0 - 0.1 mM NAD linear gradient did not increase the specific activity of the enzyme.

Disc Polyacrylamide Gel Electrophoresis

The purified enzyme preparation following disc electrophoresis contained three protein bands (2 major, 1 minor) on 5% polyacrylamide gels as shown in Figure 5a. Utilizing an activity stain the enzyme was localized on the 5% polyacrylamide gel as a single band corresponding to the furthest protein band (P_1) from the tracking dye as shown in Figure 5b. Controls such as omitting the substrate 3-HB or boiling the enzyme at 100°C resulted in no activity band formation indicating specificity for 3-HBDH.

Stability of 3-HBDH

To determine the optimal storage conditions and effect of freezing and thawing of 3-HBDH from *T. novellus* a series of samples were frozen (-20°C) or kept in the cold (4°C). From Figure 6 it can be seen that the enzyme loses activity in 16 d if kept frozen or in 0.1 M phosphate buffer pH 8.0. The addition of 1 mM NAD confers some protection to the enzyme stored at 4°C, but does not protect against freezing. The enzyme was stabilized greatly in the presence of either 1 or 2 M $(NH_4)_2SO_4$. After 54 d the enzyme stored frozen in 1 or 2 M $(NH_4)_2SO_4$ experienced no detectable loss in activity. The enzyme stored in the cold showed a decrease in activity of 40% after 54 d in 1 or 2 M $(NH_4)_2SO_4$. The addition of $MgCl_2$ (1 mM) did

Figure 5a. Electrophoretic protein analysis of purified 3-HBDH on 5% polyachrylamide. Electrophoresis was performed and stained for protein as described in *Materials and Methods*. (Approximately 5ug protein was applied to each tube)

P_1 3-HBDH protein band

P_2 Major protein contaminant

NOTE: Minor protein contaminant is not visible on this reproduction but is located slightly above P_2.

Figure 5b. Electrophoretic activity analysis of purified 3-HBDH on 5% polyacrylamide. Electrophoresis was performed and stained for 3-HBDH activity as described in *Materials and Methods*.

NOTE: Gels vary in size due to shrinking and swelling in the appropriate buffers used in the activity and protein stain.

T Tracking dye

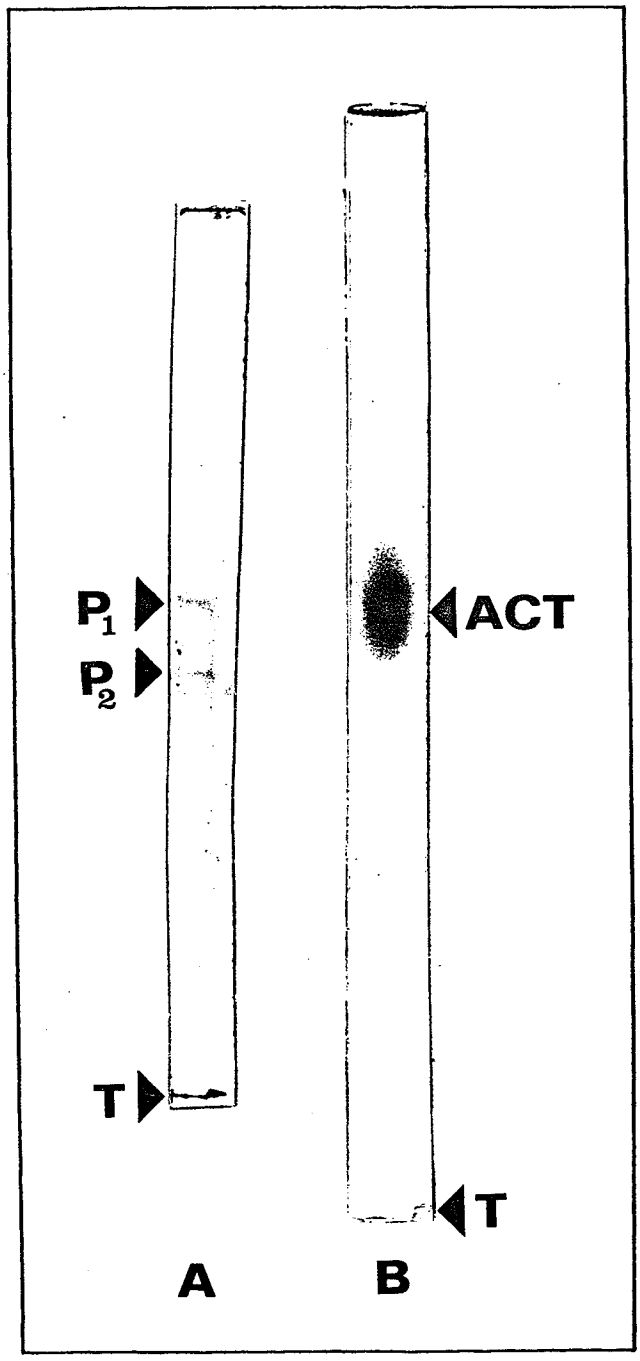
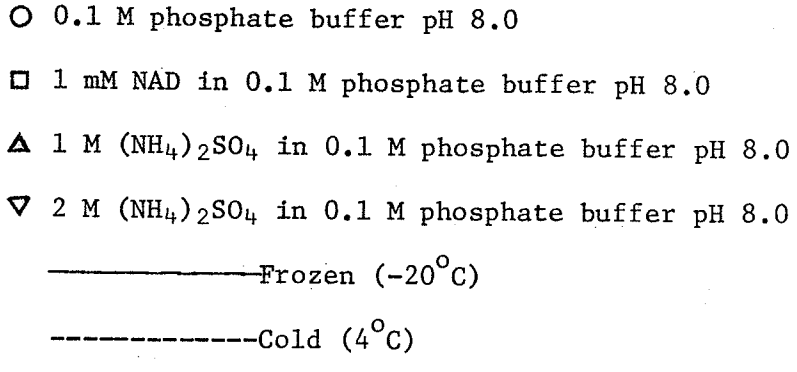
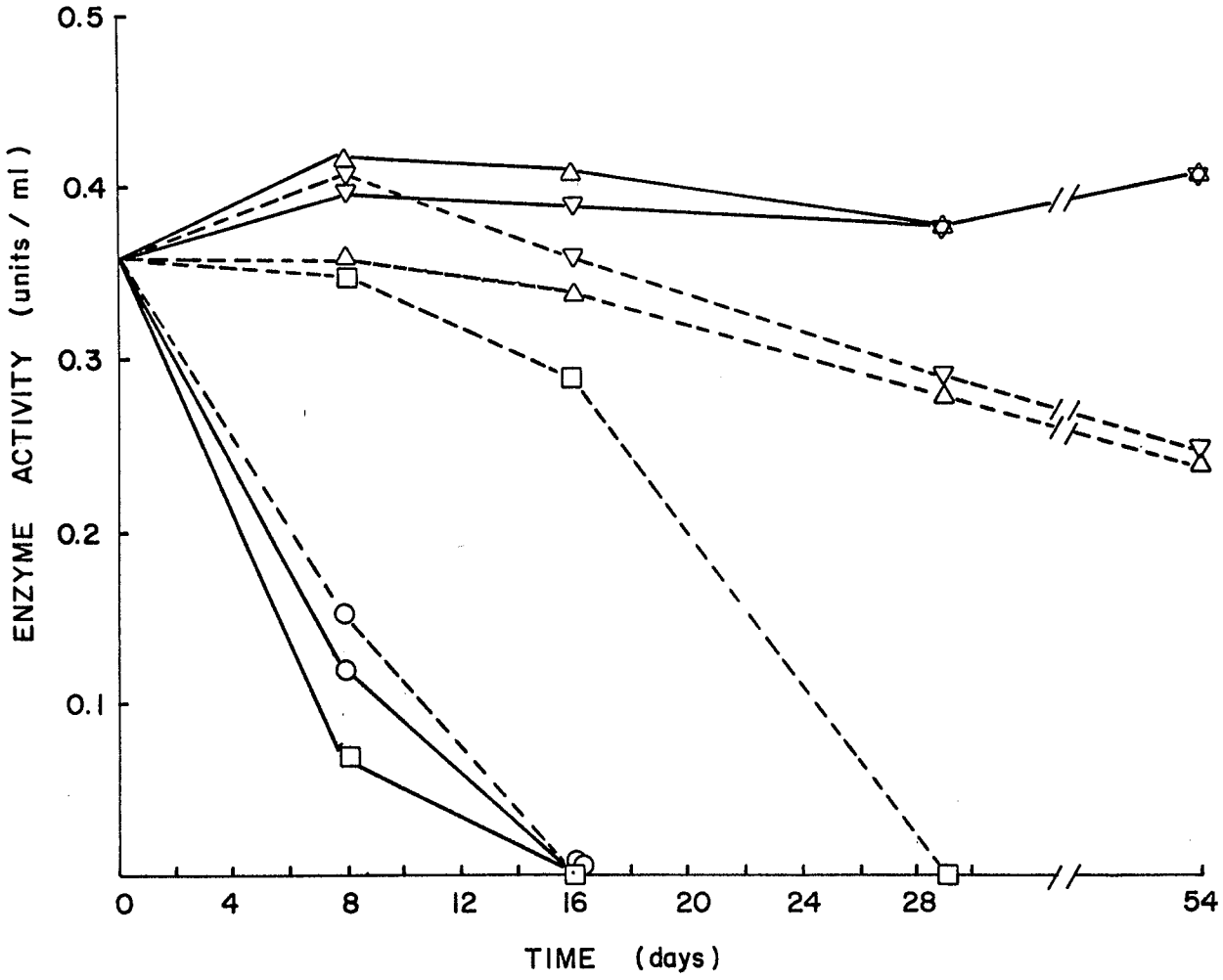

P1
P2
T
ACT
T
A
B

Figure 6. Effect of storage conditions and freezing and thawing of 3-HBDH over a 54 d time period. The enzyme was assayed as described in *Materials and Methods*. Enzyme specific activity was 0.28 units/mg protein.




0.5
0.4
0.3
0.2
0.1
ENZYME ACTIVITY (units / ml)
0
4
8
12
16
20
24
28
54
TIME (days)

not increase the stability as compared to the enzyme stored frozen in 0.1 M phosphate buffer pH 8.0.

Optimal pH

The pH profile for the forward oxidation reaction is presented in Figure 7. An optimal pH of 8.6 - 9.0 was determined from the curve. Enzyme activity decreased to 50% of maximal activity at pH 7.0 or pH 10.4.

Effect of Metal Ions on 3-HBDH Activity

The effect of various metal ions (and some non-metal ions) on the enzyme activity is presented in Table 3. The most potent inhibitors were: $HgCl_2$, $ZnCl_2$, $CoCl_2$ and $CuCl_2$. The other ions tested did not significantly inhibit the enzyme or stimulate its activity. Figure 8 presents the effect of various concentrations of $HgCl_2$ on enzyme activity. Dithiothreitol (1 mM) competely reversed $HgCl_2$ inhibition. Inhibition by 0.01 mM $HgCl_2$ could also be relieved by 0.1 mM glutathione (reduced form), 0.1 mM β-mercapto-ethanol and 0.1 mM cysteine. EDTA (0.1 and 1.0 mM) did not relieve inhibition by 0.01 mM $HgCl_2$.

Effect of Sulfhydryl Reducing Agents on 3-HBDH Activity

The effect of various sulfhydryl reducing agents on the activity of 3-HBDH is presented in Table 4. Glutathione (reduced form) and β-mercaptoethanol at 0.1 and 1.0 mM caused no observable increase

Figure 7. The pH profile of 3-HBDH activity. Enzyme assay and procedure for pH profile determination were as described in *Materials and Methods*.

△ 0.1 M phosphate buffer

○ 0.1 M Tris-HCl buffer

▽ 0.1 M Glycine-NaOH buffer

□ 0.1 M cyclohexylaminopropane Sulfonic Acid (CAPS) buffer

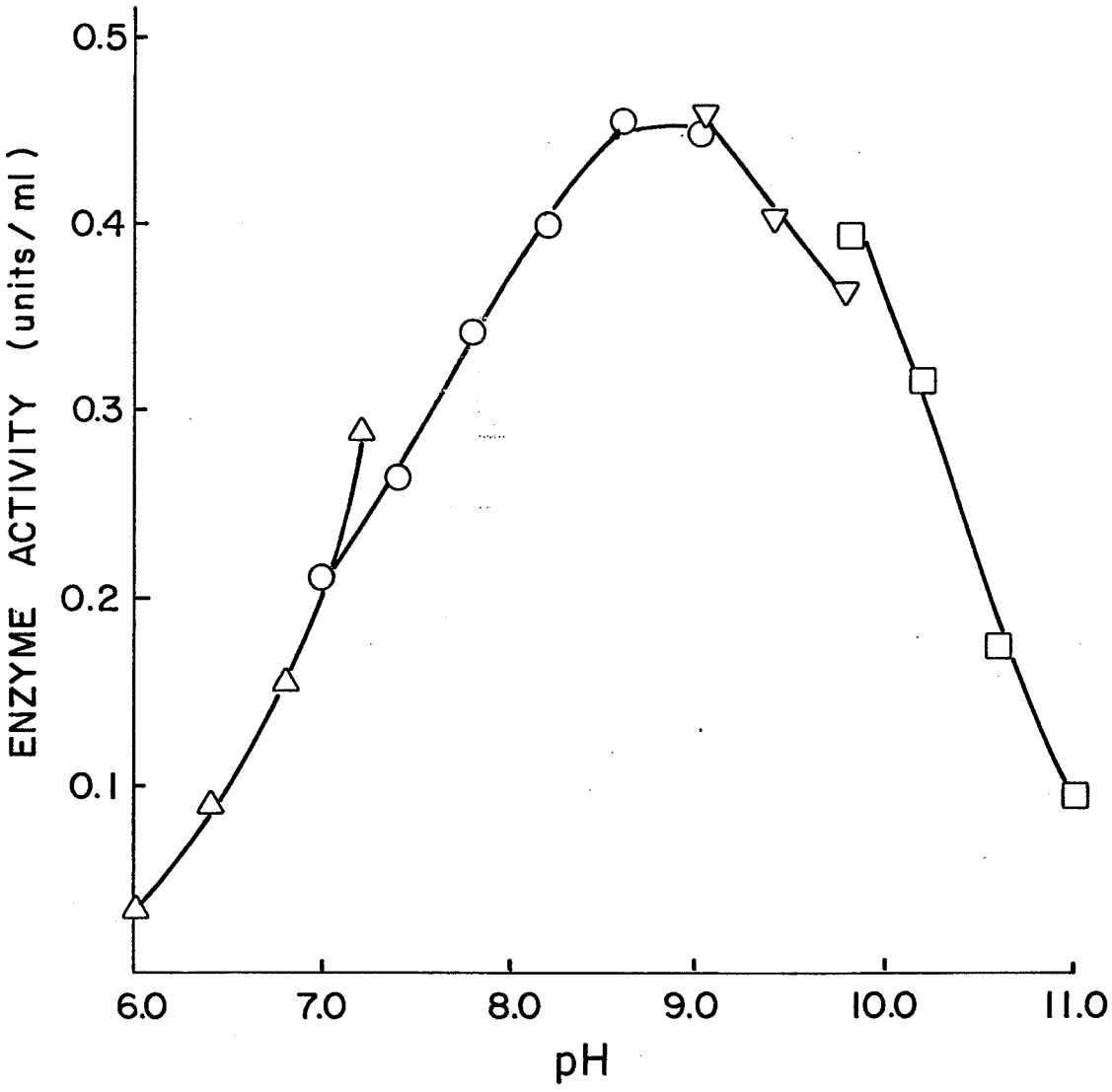

ENZYME ACTIVITY (units / ml)
0.5
0.4
0.3
0.2
0.1
6.0
7.0
8.0
9.0
10.0
11.0
pH

TABLE 3

Effect of metal ions on 3-HBDH activity.[1]

Ion	Concentration (mM)	Inhibition (%)
$HgCl_2$	0.33	100
	1.0	100
$ZnCl_2$	0.33	53
	1.0	93
$CoCl_2$	0.33	17
	1.0	23
$CuCl_2$	0.33	5
	1.0	18
$MnCl_2$	0.33	0
	1.0	0
$MgCl_2$	0.33	0
	1.0	0
$FeCl_3$	0.33	0
	1.0	ND[2]
$FeSO_4$	0.33	0
	1.0	ND
$CaCl_2$	0.33	0
	1.0	0
$Ni(OH)_3$	0.33	0
	1.0	0
NaCl	0.33	0
	1.0	0
K_2HPO_4	0.33	0
	1.0	0
$(NH_4)_2SO_4$	0.33	0
	1.0	0
$Na_2S_2O_3$	0.33	0
	1.0	0

[1]The enzyme, buffer, distilled H_2O and metal ions were incubated for 5 min at room temp. prior to addition of both 3-HB and NAD. Concentrations of enzyme, buffer and substrate were as described in *Materials and Methods* for enzyme assay.

[2]ND - Not Determined (due to strong absorbance at 340 nm).

Figure 8. The effect various concentrations of $HgCl_2$ on 3-HBDH activity with and without 1.0 mM dithiothreitol. Enzyme and buffer were incubated for 5 min at room temperature in the reaction cuvette containing the appropriate amount of $HgCl_2$ prior to the addition of dithiothreitol. Following this the substrates (NAD and 3-HB) were added and the enzyme was assayed as described in *Materials and Methods*.

○ Without dithiothreitol

□ 1.0 mM dithiothreitol added

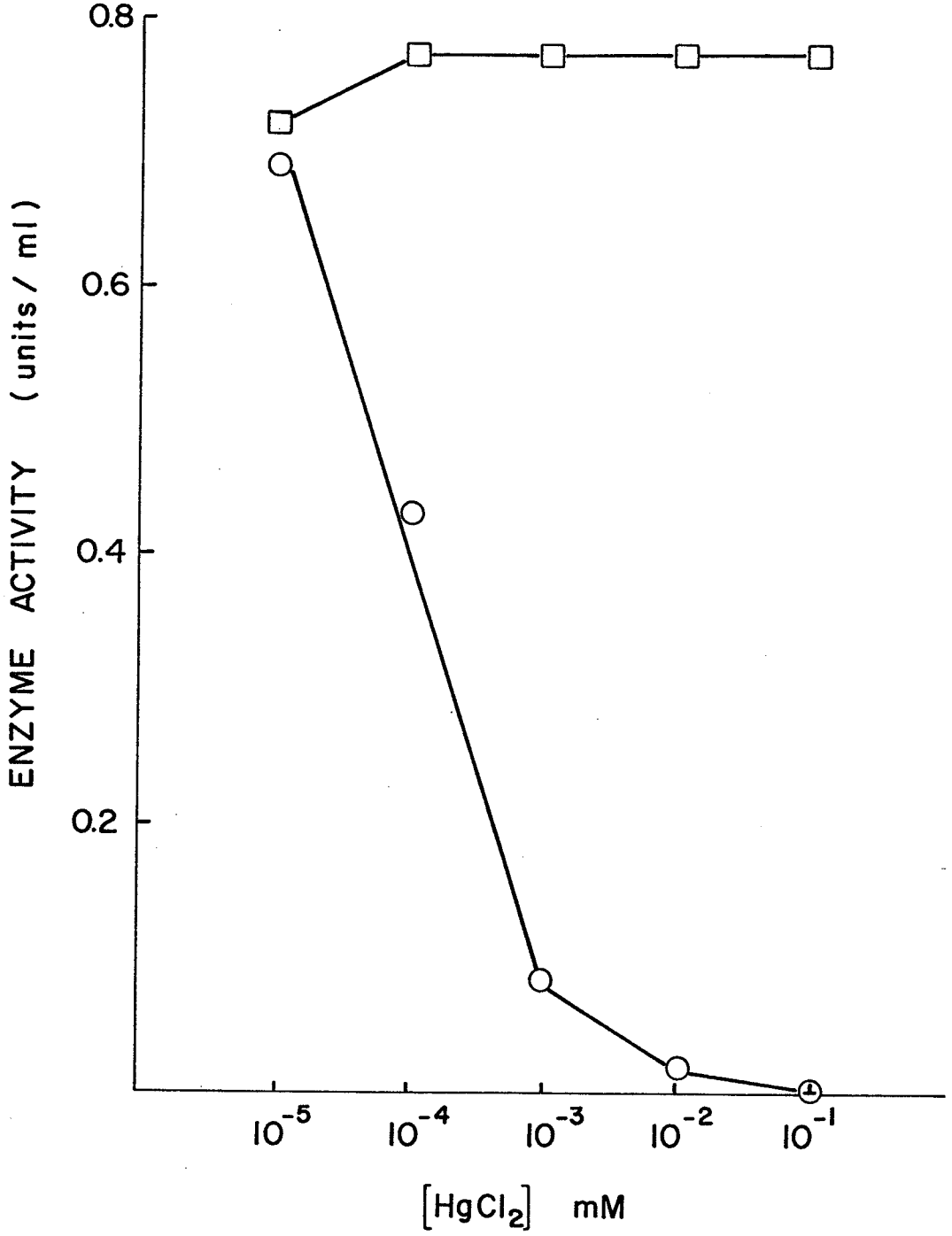

0.8
0.6
0.4
0.2
ENZYME ACTIVITY (units / ml)
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
$[HgCl_2]$ mM

TABLE 4

Effect of sulfhydryl reducing agents.[1]

Reagent	Concentration (mM)	Activity Increase (%)
Glutathione	0.1	0
	1.0	0
2-Mercaptoethanol	0.1	0
	1.0	0
Dithiothreitol	0.1	0
	1.0	4.1
Cysteine	0.1	2.8
	1.0	6.0

[1]The enzyme, buffer, distilled H_2O, NAD and sulfhydryl reducing agent were incubated for 5 min at room temperature prior to addition of 3-HB. Concentrations of enzyme, buffer and substrates were as described in *Materials and Methods* for enzyme study.

in enzyme activity. Dithiothreitol (1.0 mM) and cysteine (0.1 and 1.0 mM) caused only a slight stimulation of 3-HBDH activity

Effect of Sulfhydryl Inhibitors on 3-HBDH Activity

All sulfhydryl inhibitors cause inhibition of 3-HBDH activity from *T. novellus*. The most effective (Table 5) are NEM and DTNB, while 0.1 and 1.0 mM iodoacetamide was a poor inhibitor. Inhibition by DTNB could be relieved by addition of dithiothreitol. Since NEM was an effective inhibitor of 3-HBDH activity, a time course study of this inactivation was performed as illustrated in Figure 9. Addition of either 1.0 mM dithiothreitol, 1.0 mM β-mercaptoethanol, 1.0 mM cysteine or 1.0 mM glutathione did not relieve the inhibition caused by NEM.

Molecular Weight of 3-HBDH

A molecular weight determination of 3-HBDH from *T. novellus* and *R. spheroides* was performed by the G-200 Sephadex superfine gel filtration method using Aldolase MW 158,000 (ve=88 mL), ovalbumin MW 45,000 (ve=122 mL) and chymotrypsinogen A MW 25,000 (ve=140 mL) as the standards. The results shown in Figure 10 indicate a MW of 110,000 (ve=99 mL) for 3-HBDH from *T. novellus* and a MW of 120,000 (ve=96 mL) for 3-HBDH from *R. spheroides*.

Structural Analogues

The enzyme was determined to be specific for NAD as NADP (1.0 mM) could not replace NAD and did not inhibit enzyme activity.

TABLE 5

Effect of sulfhydryl inhibitors on 3-HBDH activity.[1]

Inhibitor	Concentration (mM)	Incubation Time (min)	Inhibition (%)
NEM	0.1	5	61
	1.0	10	100
Iodoacetamide	0.1	5	0
	1.0	10	12
DTNB[2]	1.0	15	93

[1]The enzyme, buffer, distilled H_2O and sulfhydryl inhibitor were incubated at room temperature prior to addition of both 3-HB and NAD. Concentrations of enzyme, buffer and substrates were as described in *Materials and Methods* for enzyme assay.

[2]10 mM DTNB (0.1 mL) was incubated at room temperature with 0.9 mL Enzyme and 0.1 mL of this solution was assayed using concentrations of buffer and substrates as described in *Materials and Methods* for enzyme assay.

Figure 9. Effect of NEM on enzyme activity. The enzyme and 0.33 mM (final concentration) NEM were incubated at room temperature and assayed at various times as described in *Materials and Methods*.

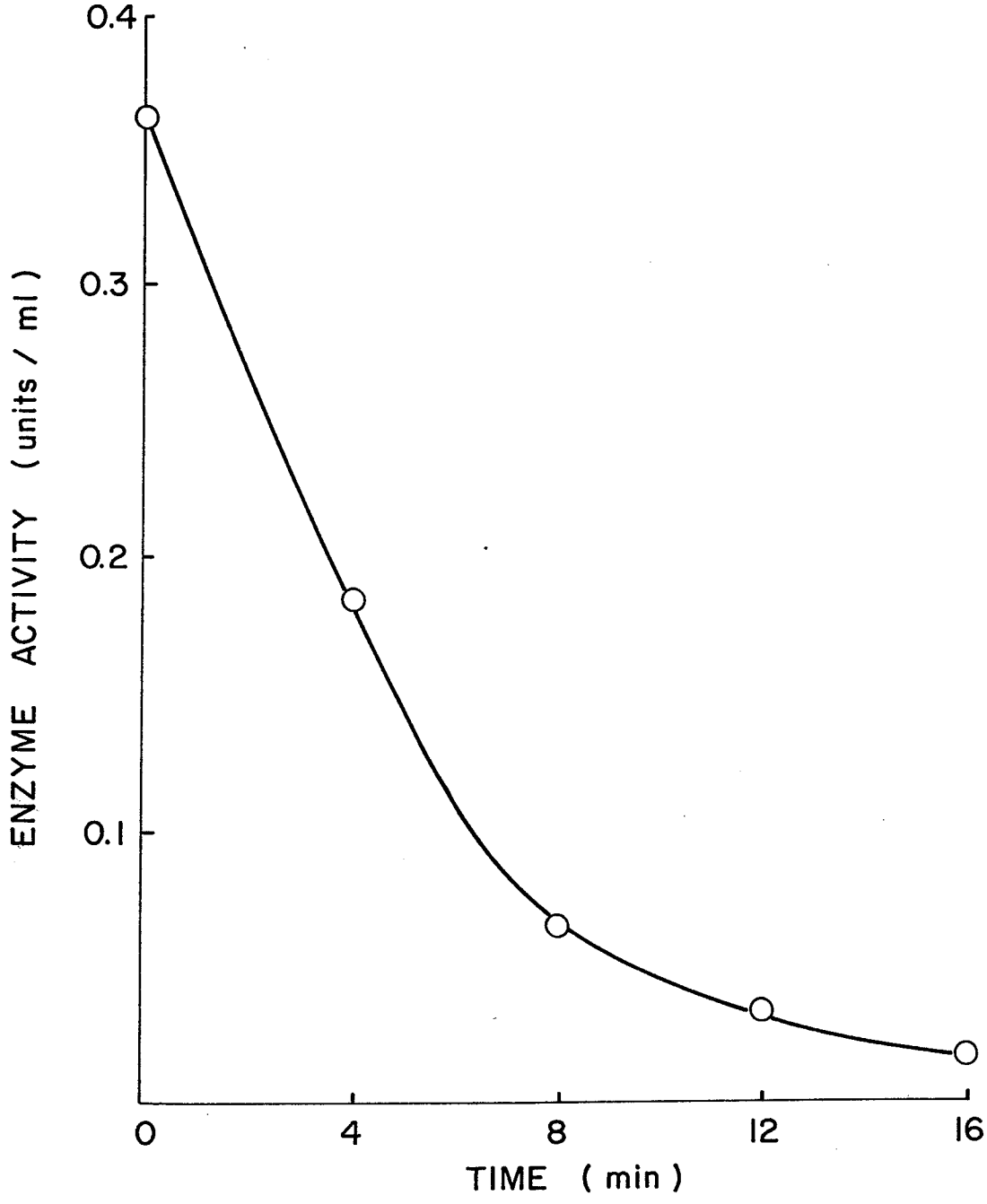
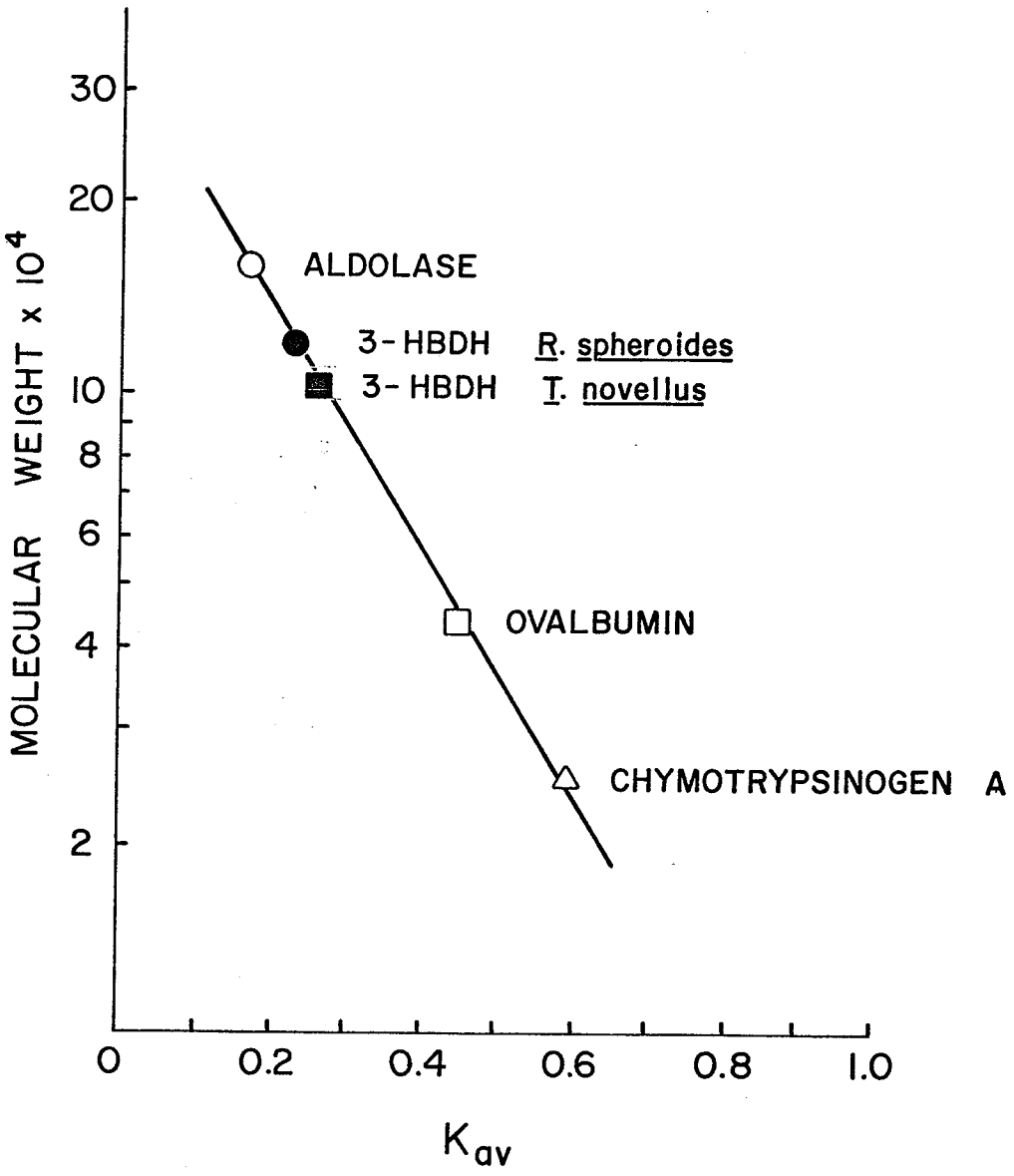

0.4
0.3
0.2
0.1
ENZYME ACTIVITY (units / ml)
0
4
8
12
16
TIME (min)

Figure 10. The molecular weight determination of 3-HBDH from *T. novellus* and *R. spheroides* (Sigma). Aldolase, ovalbumin and chymotrypsinogen A elution volumes were determined by protein absorption at 280 nm. The elution of 3-HBDH was determined enzymatically as described in *Materials and Methods*.


MOLECULAR WEIGHT x 10^4
30
20
10
8
6
4
2
ALDOLASE
3-HBDH R. spheroides
3-HBDH T. novellus
OVALBUMIN
CHYMOTRYPSINOGEN A
0
0.2
0.4
0.6
0.8
1.0
K_{av}

The following compounds at a final concentration of 1.0 mM did not cause NAD reduction or inhibition of 3-HBDH activity when assayed with 1.0 mM 3-HB and 100 μM NAD: pyruvate, butyrate, acetone, succinate, oxaloacetate, α-ketobutyrate, α-ketoglutarate, α-ketoisovalerate, oxalate, L-malate, D,L-malate, malonate, L-glutamate, L-threoine, citrate, cis-aconitate, isocitrate, AMP and ATP.

D(-) lactate was found to be a potent inhibitor resulting in 39% inhibition at 1.0 mM and 85% inhibition at 10 mM. As illustrated in Figure 11, D-lactate was determined to be a competitive inhibitor when 3-HB concentrations were varied with a constant amount of NAD.

Kinetics of 3-HBDH

A. Initial Velocity Data

The initial velocity data for varying concentrations of NAD and fixed concentrations of 3-HB in the absence of products, is presented as a Lineweaver-Burk plot in Figure 12. This plot consisted of straight lines with an intersecting point below the χ-axis. The intercept replot, Figure 14a was a straight line yielding a K_m of 0.77 mM for 3-HB. The slope replot, Figure 15a gave a slope value of 1.5.

The initial velocity data for varying concentrations of 3-HB and fixed concentrations of NAD is presented as a Lineweaver-Burk plot in Figure 13. This plot yielded straight lines with an intersecting point below the χ-axis. The intercept replot, Figure 14b gave a K_m of 45.5 μM for NAD. The slope replot, Figure 15b gave a slope value

Figure 11. Inhibition of 3-HBDH by D-lactate. 3-HB was varied in the presence of a constant amount of NAD (100 μM). Enzyme assays were performed as described in Kinetic Analysis in *Materials and Methods*.

○ Normal velocity curve

□ 1 mM D-lactate

△ 2 mM D-lactate

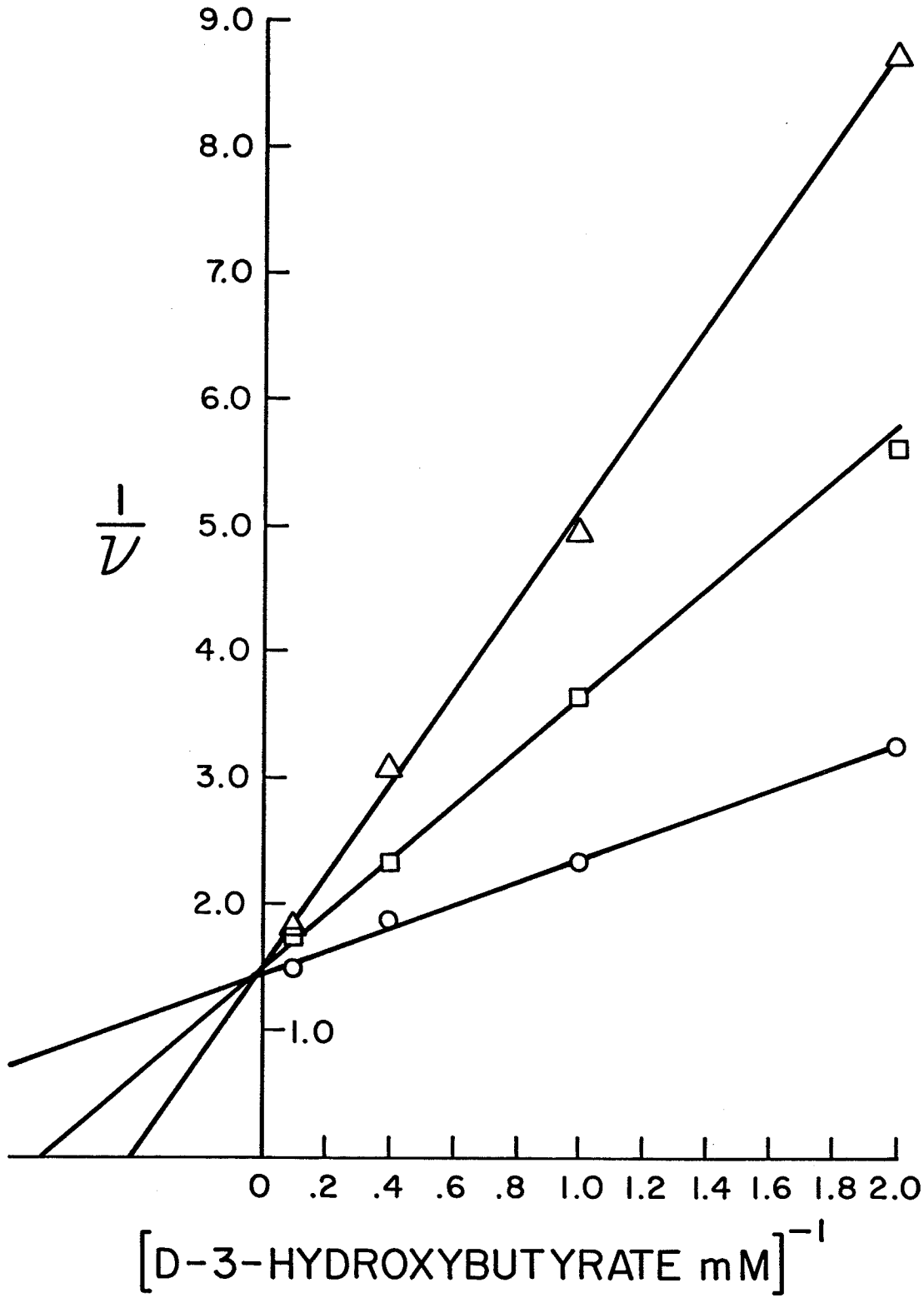

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1/v
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
[D-3-HYDROXYBUTYRATE mM]⁻¹

Figure 12. The initial velocity data (double reciprocal form) in the absence of products with varying concentrations of NAD and fixed concentrations of 3-HB. Kinetic assays were performed as described in *Materials and Methods*.

● 10.0 mM 3-HB

■ 2.5 mM 3-HB

▲ 1.0 mM 3-HB

▼ 0.5 mM 3-HB

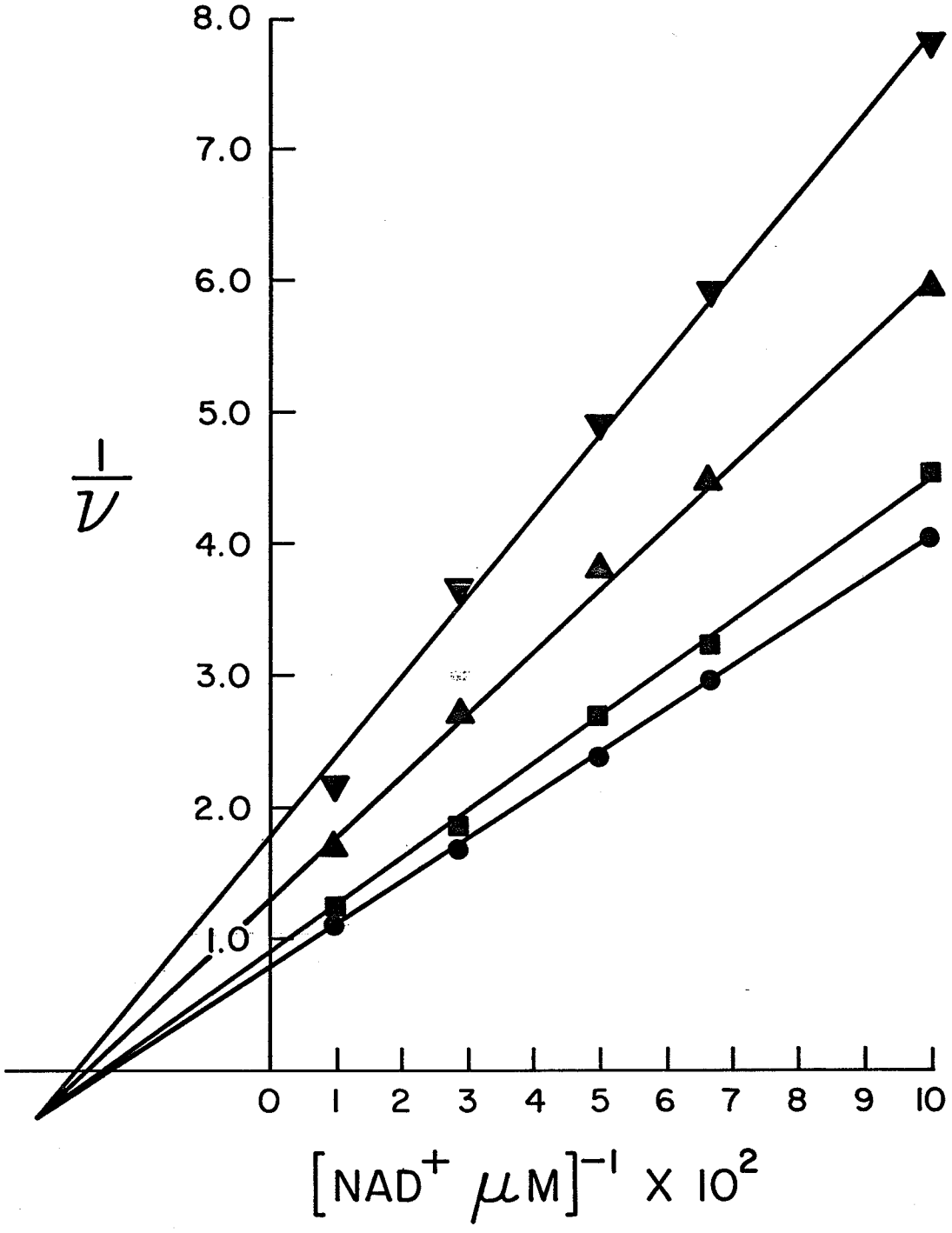

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1/v
0 1 2 3 4 5 6 7 8 9 10
[NAD+ μM]-1 X 10²

Figure 13. The initial velocity data (double reciprocal form) for varying concentrations of 3-HB and fixed concentrations of NAD. Kinetic assays were performed as described in *Materials and Methods*.

● 100.0 μM NAD

■ 35.0 μM NAD

▲ 20.0 μM NAD

▼ 15.0 μM NAD

○ 10 μM NAD

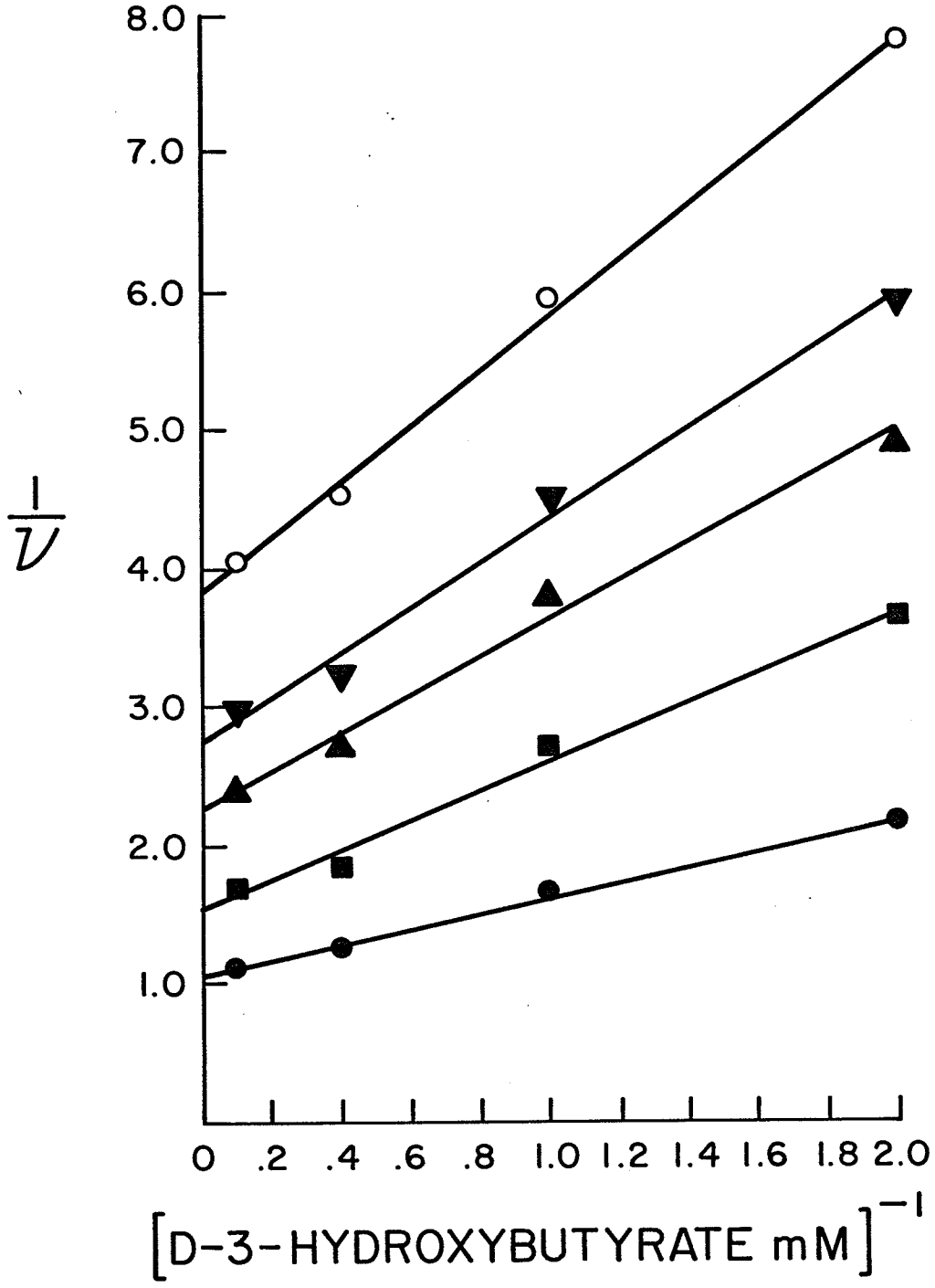

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1/v
0 .2 .4 .6 .8 1.0 1.2 1.4 1.6 1.8 2.0
[D-3-HYDROXYBUTYRATE mM]⁻¹

Figure 14a. Intercept replot from Figure 12.

Figure 14b. Intercept replot from Figure 13.

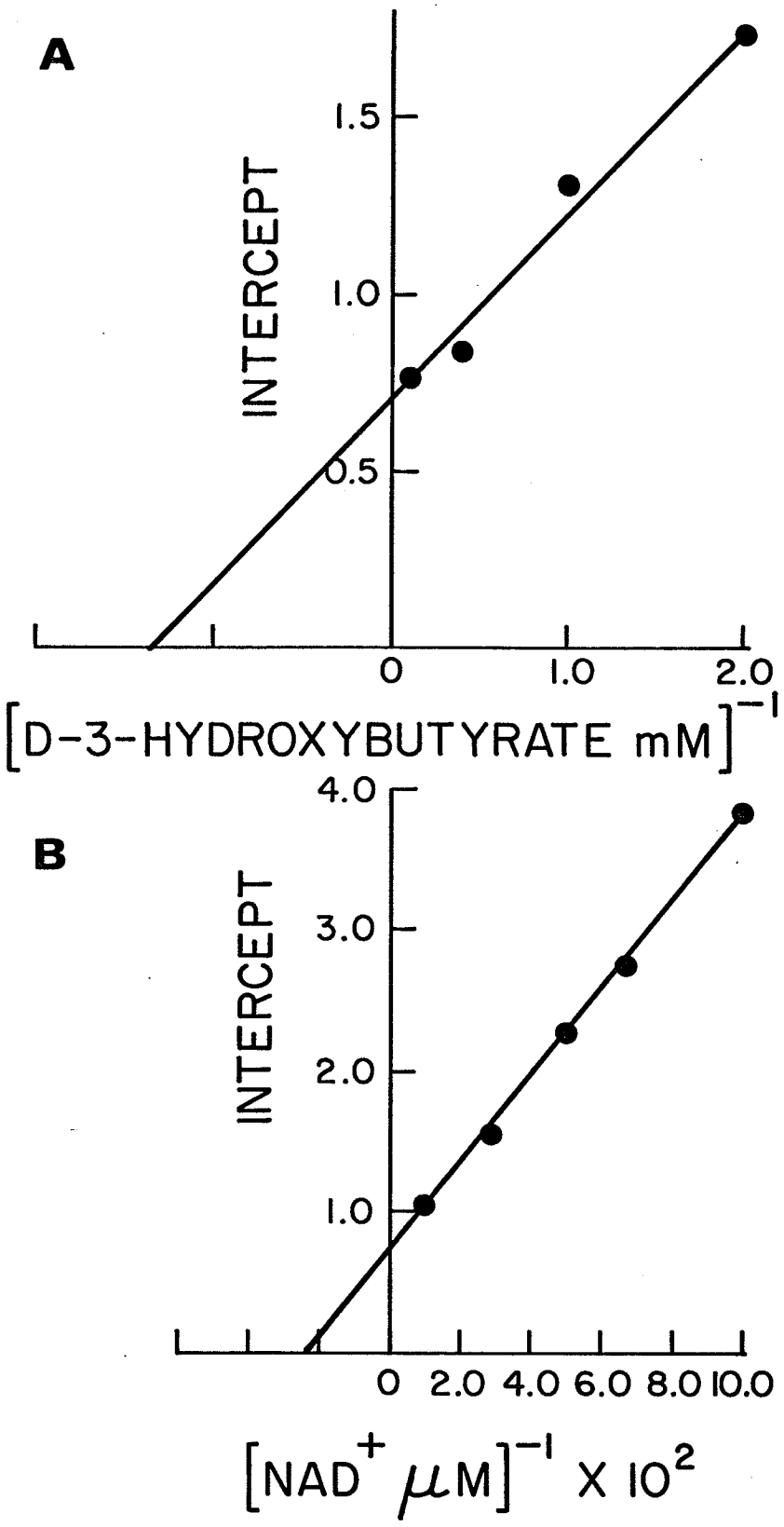
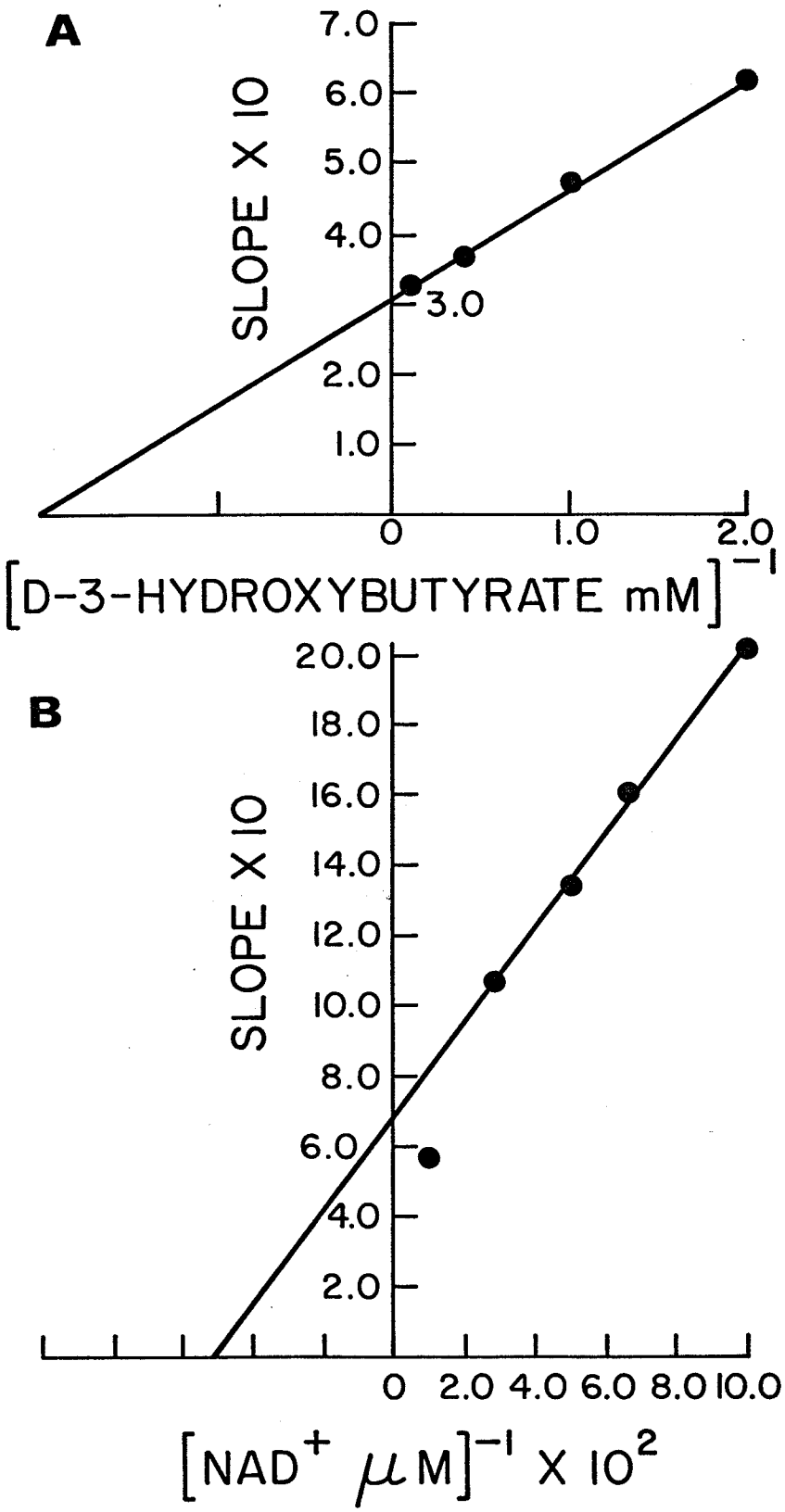

A
INTERCEPT
1.5
1.0
0.5
0
1.0
2.0
[D-3-HYDROXYBUTYRATE mM]$^{-1}$
B
INTERCEPT
4.0
3.0
2.0
1.0
0
2.0
4.0
6.0
8.0
10.0
[NAD$^+$ μM]$^{-1}$ X 10^2

Figure 15a. Slope replot of Figure 12.

Figure 15b. Slope replot of Figure 13.


A
SLOPE X 10
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
1.0
2.0
[D-3-HYDROXYBUTYRATE mM]$^{-1}$
B
SLOPE X 10
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0 2.0 4.0 6.0 8.0 10.0
[NAD$^+$ μM]$^{-1}$ X 10^2

of 1.4. Both intercept and slope replots were straight lines.

B. Product Inhibition

Inhibition by the product acetoacetate with NAD as the variable substrate with a fixed unsaturated concentration of 3-HB (1.0 mM) is presented in Figure 16. This Lineweaver-Burk plot is characteristic of a noncompetitive inhibitor (slope and intercept change). The intercept replot, Figure 17a is linear with a Kip value of 1.8 mM acetoacetate. The Ki slope value of 1.9 mM acetoacetate was determined from the slope replot Figure 17b.

Inhibition by the product acetoacetate with NAD as the variable substrate with a fixed saturated concentration of 3-HB (20 mM) is presented in Figure 18. This Lineweaver-Burk plot is of the uncompetitive type (only intercept changes). Initially 10 mM 3-HB (13 times Km) was thought to be saturating, although non-competitive plots were obtained. It was not until 20 mM 3-HB (26 times Km) was used that definite uncompetitive inhibition plots were obtained. Figure 19, the intercept replot is linear yielding a Kip value of 2.0 mM acetoacetate.

Inhibition by the product acetoacetate, with 3-HB as the variable substrate with a fixed non-saturated concentration of NAD (100 μM) is presented in the Lineweaver-Burk plot, Figure 20. This double reciprocal plot is of the non-competitive inhibition type. The intercept and slope replots are linear as presented in Figure 21a and Figure 21b respectively.

Figure 16. Inhibition by the product acetoacetate with NAD varying at a fixed unsaturated concentration of 3-HB (1.0 mM). Kinetic assays were performed as described in *Materials and Methods*.

● Normal velocity curve (1.0 mM 3-HB)

■ 1.0 mM acetoacetate

▲ 2.0 mM acetoacetate

▼ 5.0 mM acetoacetate

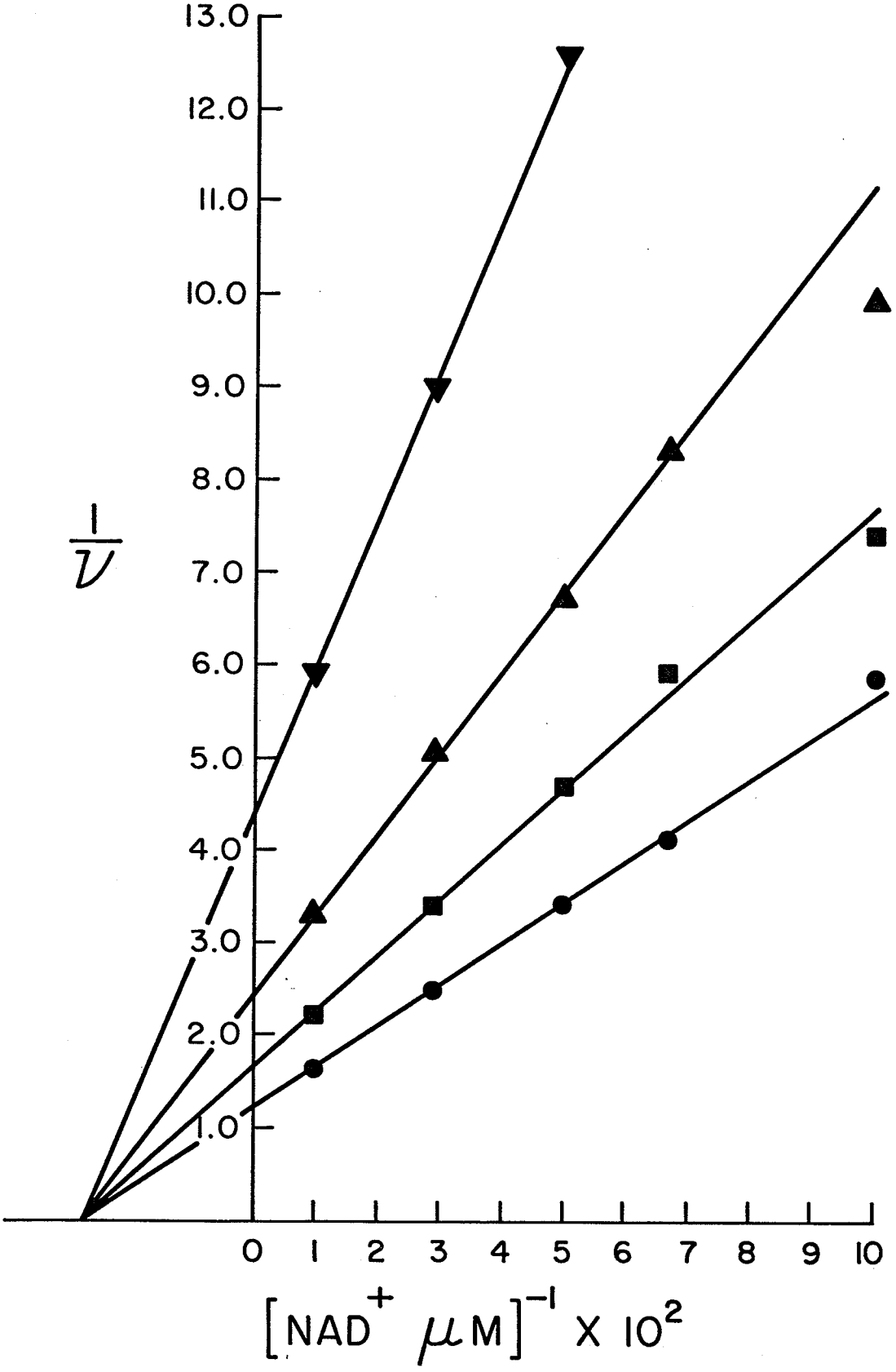
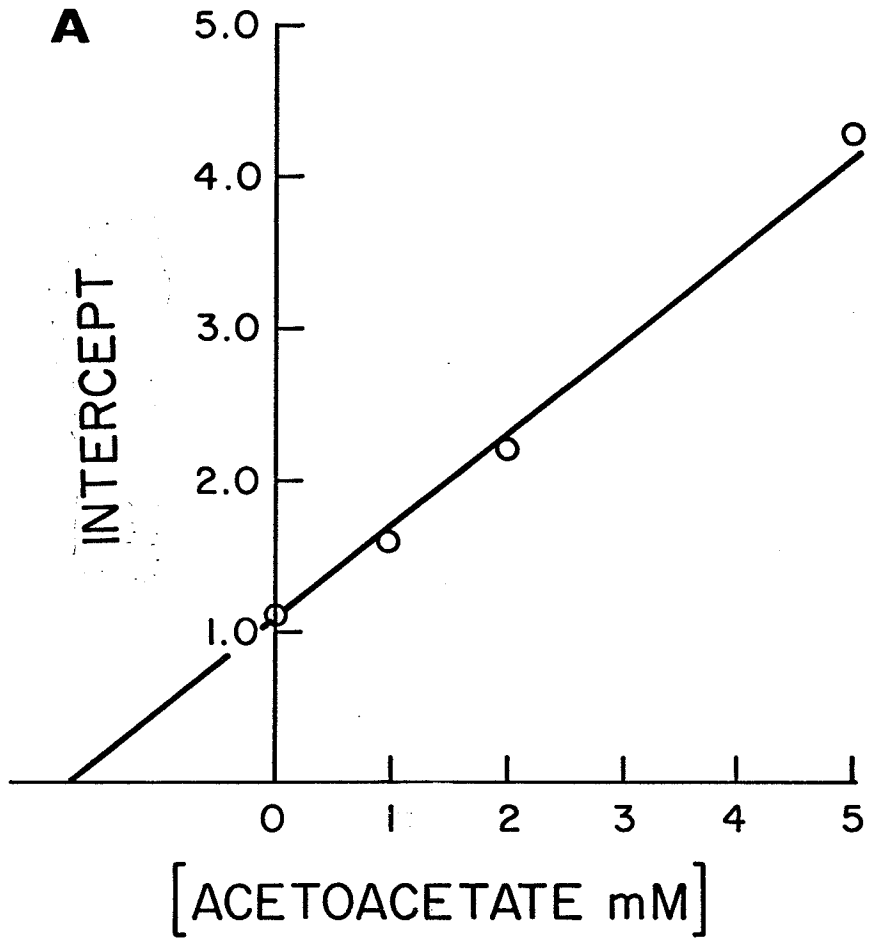

13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1/v
0 1 2 3 4 5 6 7 8 9 10
[NAD+ μM]-1 X 10²

Figure 17a. Intercept replot of Figure 16.

Figure 17b. Slope replot of Figure 16.


A
5.0
4.0
3.0
2.0
1.0
INTERCEPT
0
1
2
3
4
5
[ACETOACETATE mM]

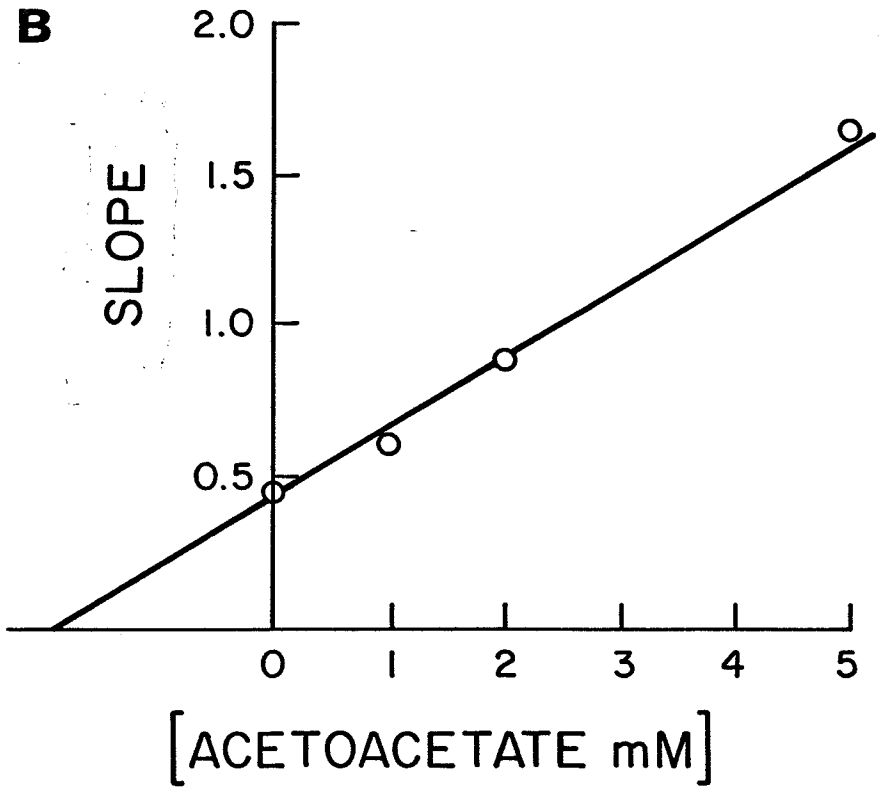

B
2.0
1.5
1.0
0.5
SLOPE
0
1
2
3
4
5
[ACETOACETATE mM]

Figure 18. Product inhibition by acetoacetate with varying concentrations of NAD and a fixed saturated concentration of 3-HB (20 mM). Kinetic assays were performed in *Materials and Methods*.

● Normal velocity curve (20 mM 3-HB)

■ 1.0 mM acetoacetate

▲ 5.0 mM acetoacetate

▼ 10.0 mM acetoacetate

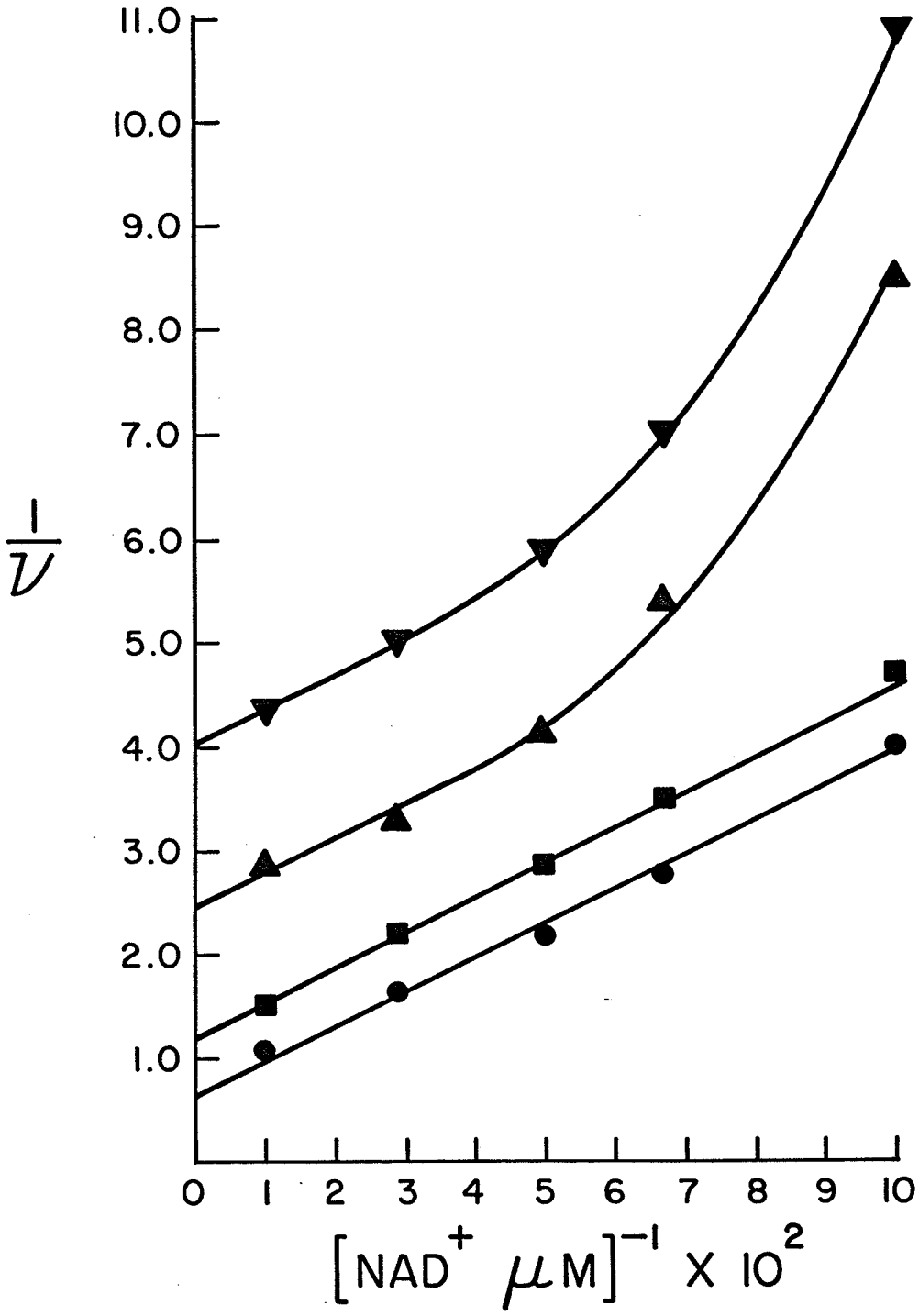

11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1/v
0 1 2 3 4 5 6 7 8 9 10
[NAD+ μM]−1 X 10^2

Figure 19. Intercept replot of Figure 18.

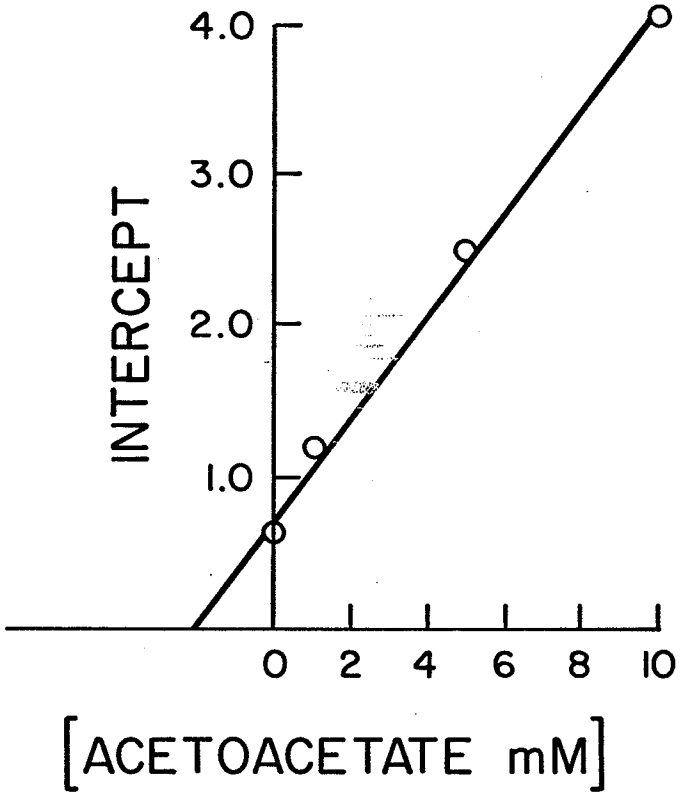

4.0
3.0
2.0
1.0
INTERCEPT
0
2
4
6
8
10
[ACETOACETATE mM]

Figure 20. Product inhibition by acetoacetate with varying concentration of 3-HB and a fixed non-saturated concentration of NAD (100 μM). Kinetic assays were performed as described in *Materials and Methods*.

● Normal velocity curve (100 μM NAD)

■ 1.0 mM acetoacetate

▲ 2.0 mM acetoacetate

▼ 3.5 mM acetoacetate

○ 5.0 mM acetoacetate

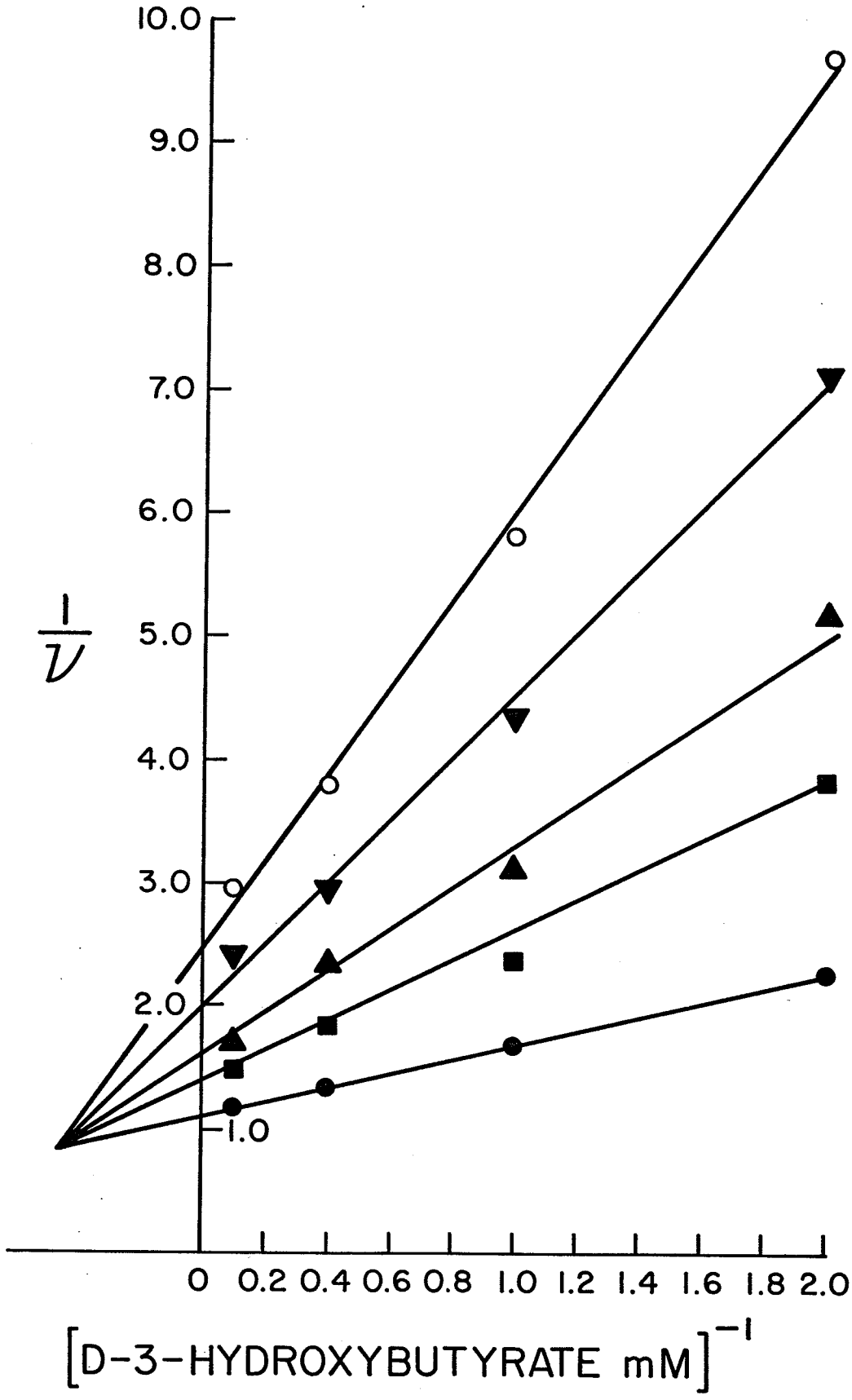
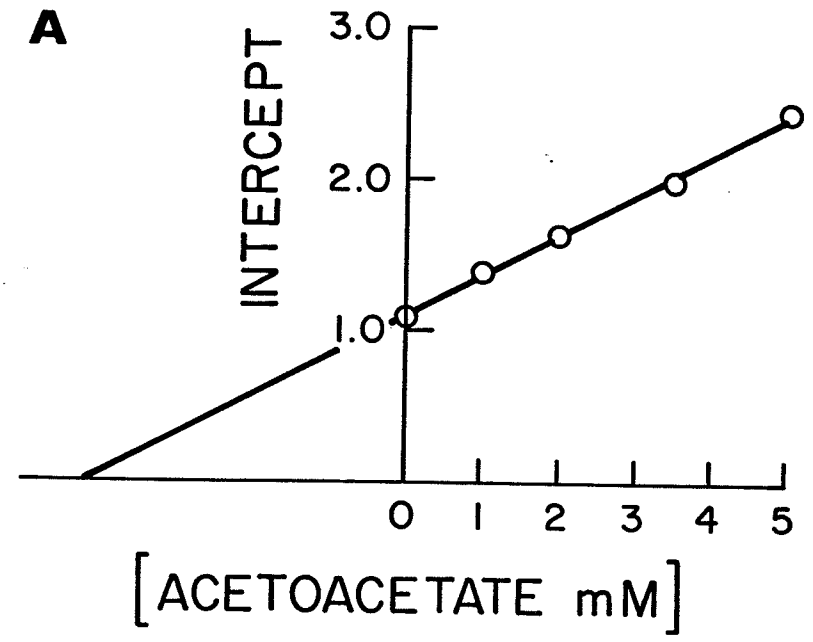
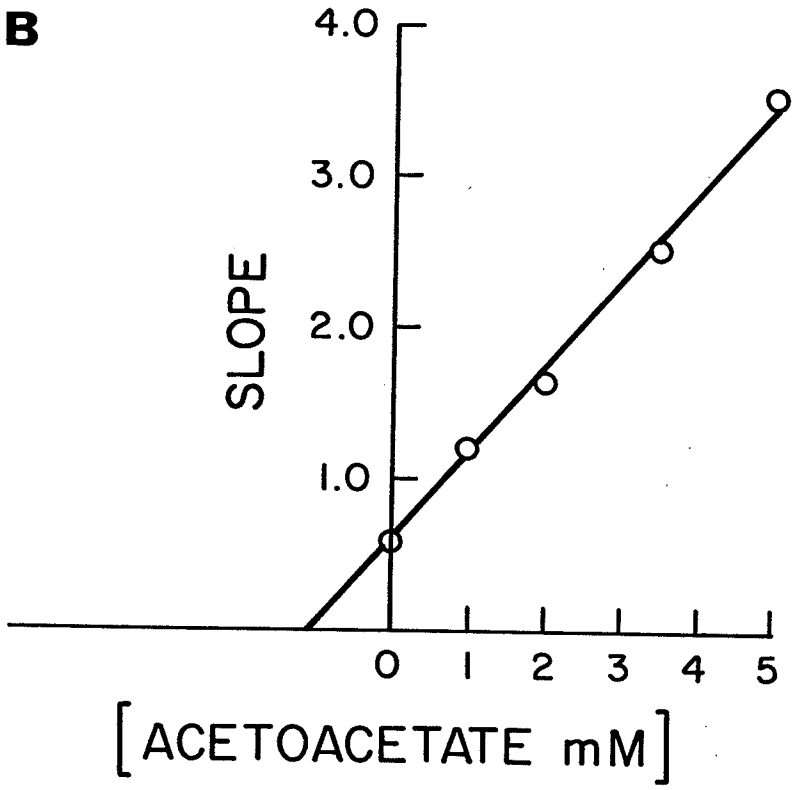

10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1/v
0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
[D-3-HYDROXYBUTYRATE mM]^-1

Figure 21a. Intercept replot of Figure 20.

Figure 21b. Slope replot of Figure 20.


A
INTERCEPT
3.0
2.0
1.0
0
1
2
3
4
5
[ACETOACETATE mM]


B
SLOPE
4.0
3.0
2.0
1.0
0
1
2
3
4
5
[ACETOACETATE mM]

Inhibition by the product acetoacetate with 3-HB as the variable substrate with a fixed saturated concentration of NAD (500 μM) is presented in Figure 22. This double reciprocal plot is also of the noncompetitive type. Figure 23a and Figure 23b are the linear intercept and slope replots respectively.

Inhibition by the product NADH with NAD as the variable substrate with a fixed non-saturated concentration of 3-HB (1.0 mM) is presented in Figure 24. This product inhibition plot is of the competitive inhibitor type (only slope change). Figure 25a is the linear slope replot giving a Kiq of 9.0 μM NADH with non-saturating 3-HB.

Inhibition by the product NADH with NAD as the variable substrate with a fixed saturated concentration of 3-HB (20 mM) is presented in Figure 26. This plot is also of the competitive inhibitor type. The concentration of 3-HB (20.0 mM) was 26 times the Km value of 3-HB. The slope replot, Figure 25b was a linear plot and yielded a Kiq value of 19.0 μM NADH at saturating 3-HB.

Inhibition by the product NADH with 3-HB as the variable substrate with a fixed non-saturating concentration of NAD (100 μM) is presented in Figure 27. This plot was of the noncompetitive inhibitor type. Figures 28a and 28b are the linear intercept and slope replots respectively.

Inhibition by the product NADH with 3-HB as the variable substrate with a fixed saturated concentration of NAD (1.0 mM) is presented in Figure 29. This plot indicates slight inhibition of

Figure 22. Production inhibition by acetoacetate with varying concentrations of 3-HB and a fixed saturated concentration of NAD (500 μM). Kinetic assays were performed as described in *Materials and Methods*.

● Normal velocity curve (500 μM NAD)

■ 1.0 mM acetoacetate

▲ 3.5 mM acetoacetate

▼ 5.0 mM acetoacetate

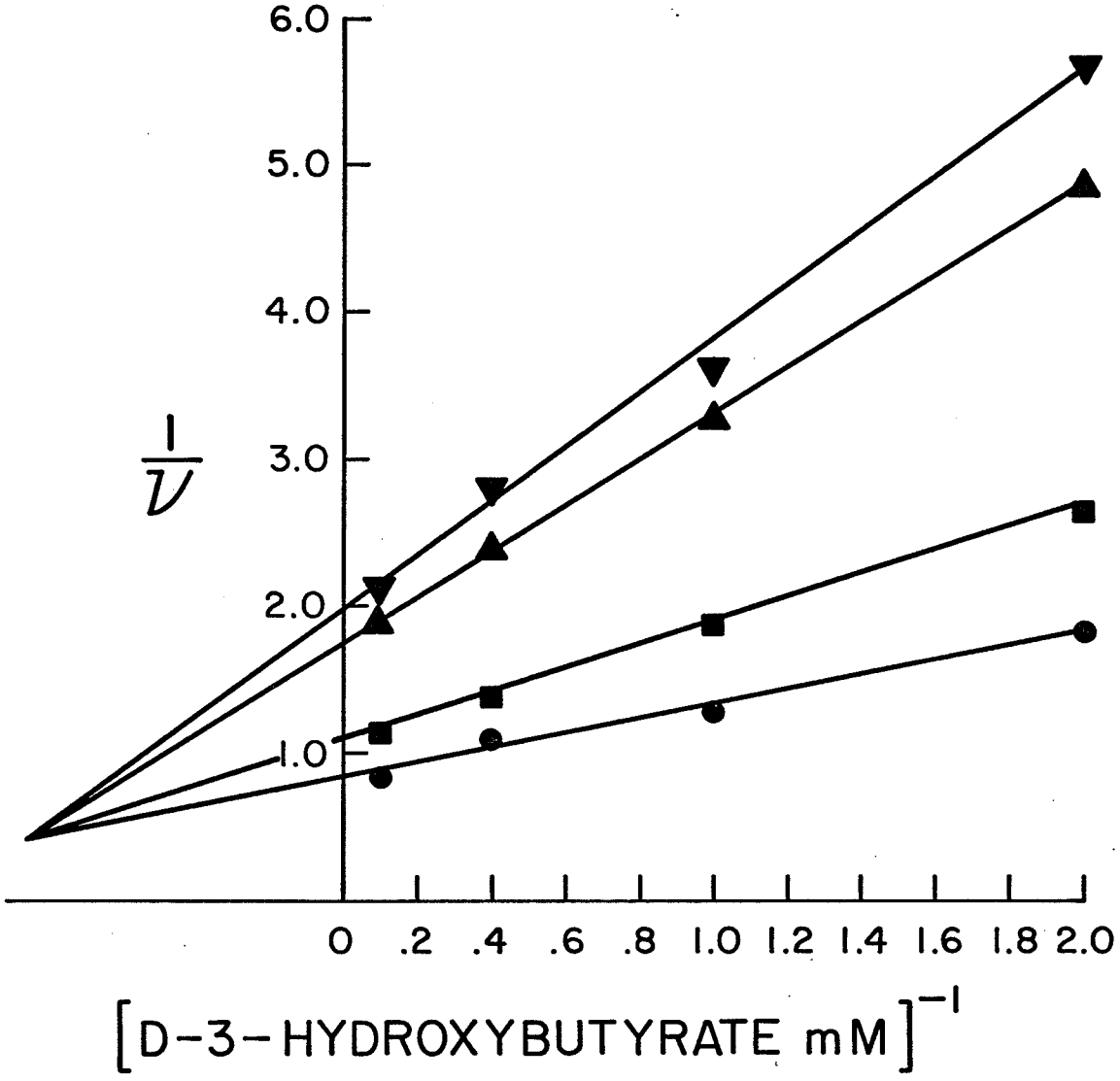
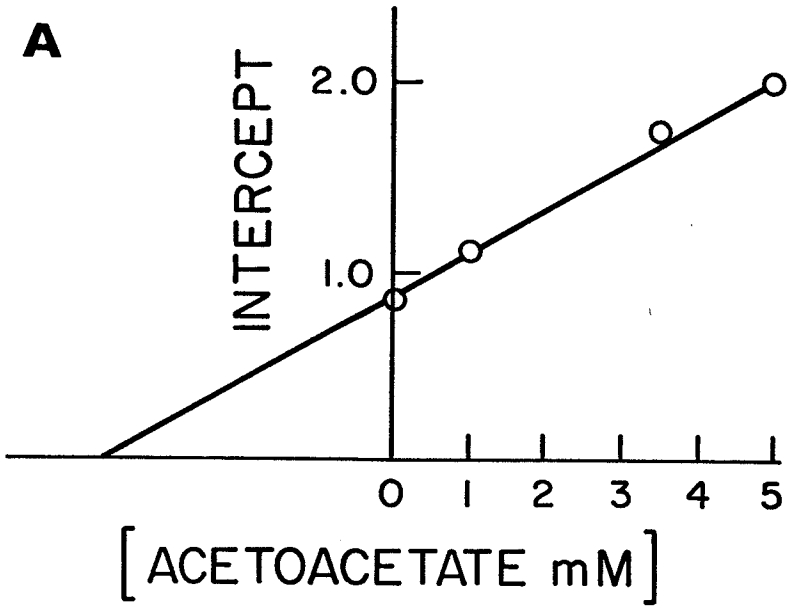

6.0
5.0
4.0
3.0
2.0
1.0
1/V
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
[D-3-HYDROXYBUTYRATE mM]^-1

Figure 23a. Intercept replot of Figure 22.

Figure 23b. Slope replot of Figure 22.


A
INTERCEPT
2.0
1.0
0 1 2 3 4 5
[ACETOACETATE mM]

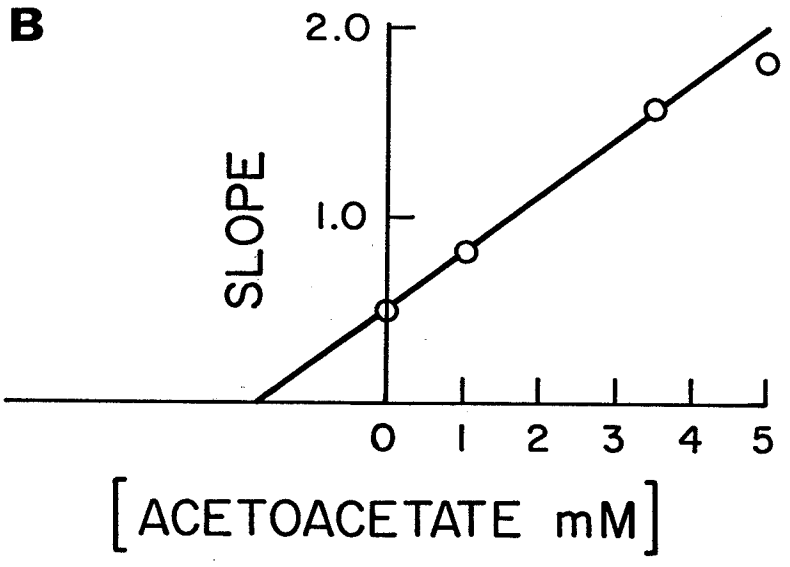

B
SLOPE
2.0
1.0
0 1 2 3 4 5
[ACETOACETATE mM]

Figure 24. Product inhibition by NADH with NAD as the variable substrate with a fixed non-saturated concentration of 3-HB (1.0 mM). Kinetic assays were performed as described in *Materials and Methods*.

● Normal velocity curve (1.0 mM 3-HB)

■ 5.0 µM NADH

▲ 10.0 µM NADH

▼ 20.0 µM NADH

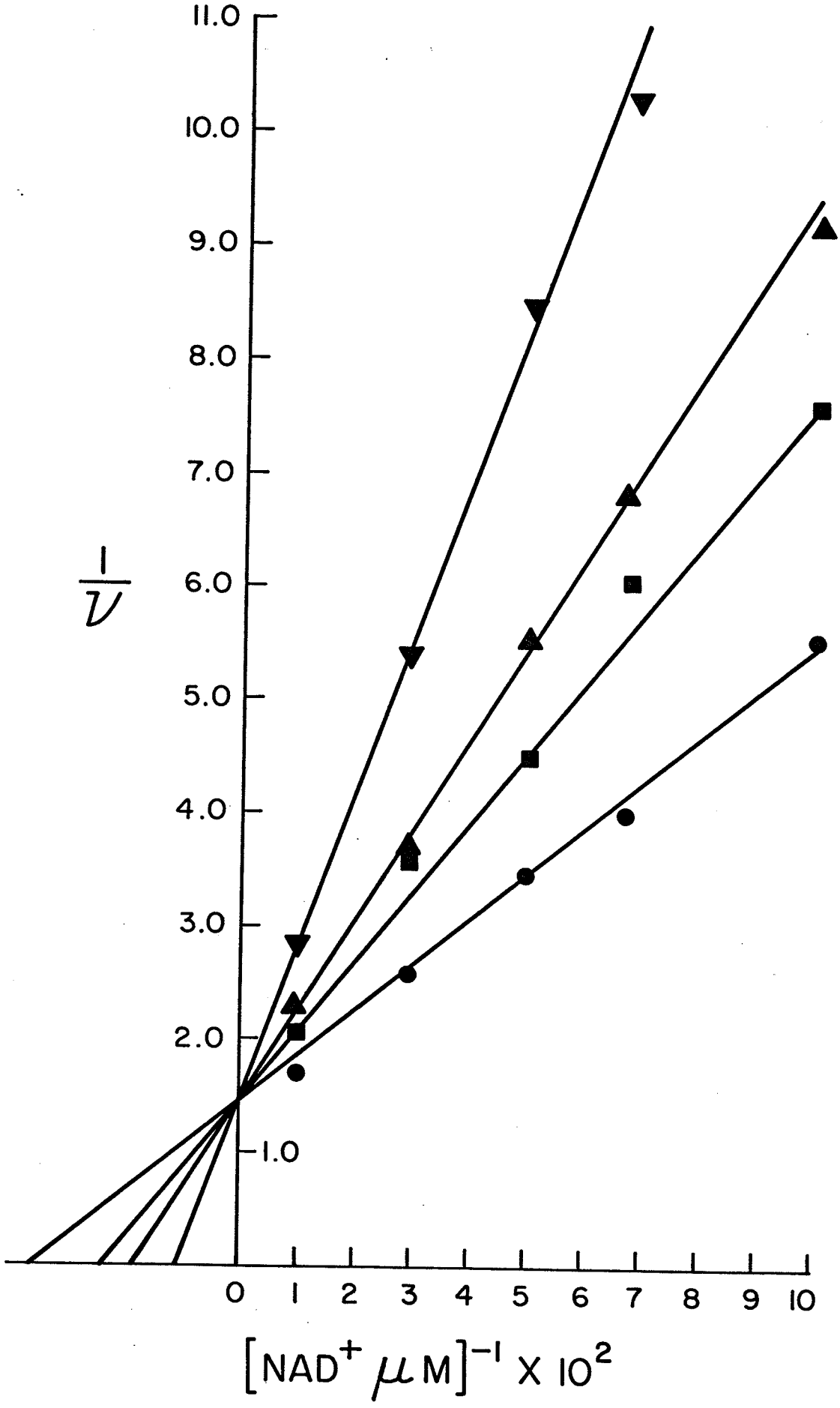

11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1/v
0 1 2 3 4 5 6 7 8 9 10
[NAD+ μM]-1 X 10²

Figure 25a. Slope replot of Figure 24.

Figure 25b. Slope replot of Figure 26.

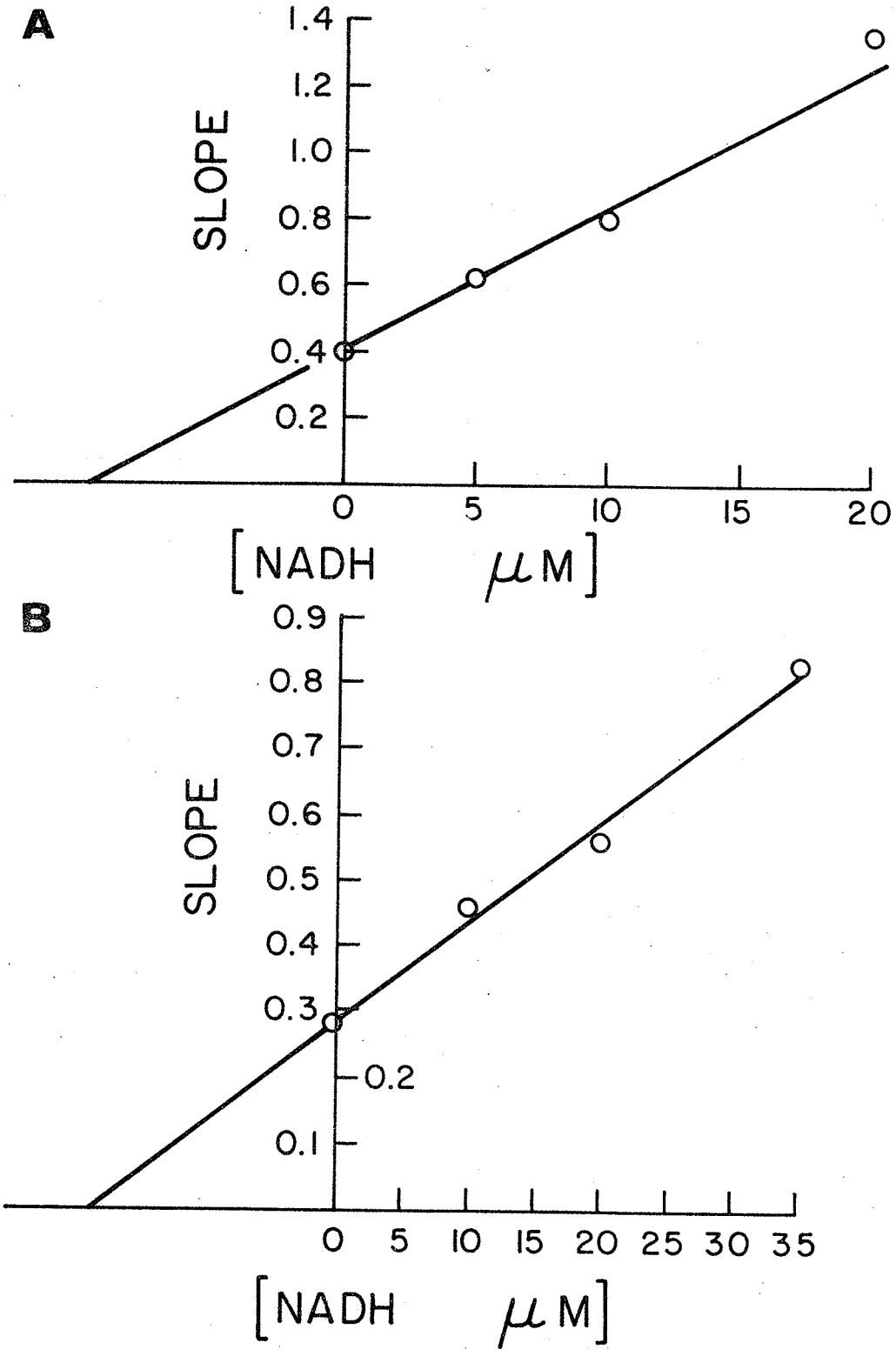

A
SLOPE
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
[NADH μM]
B
SLOPE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25
30
35
[NADH μM]

Figure 26. Product inhibition by NADH with varying concentrations of NAD and a fixed saturated concentration of 3-HB (20.0 mM). Kinetic assays were performed as described in *Materials and Methods*.

● Normal velocity curve (20.0 mM 3-HB)

■ 10.0 μM NADH

▲ 20.0 μM NADH

▼ 35.0 μM NADH

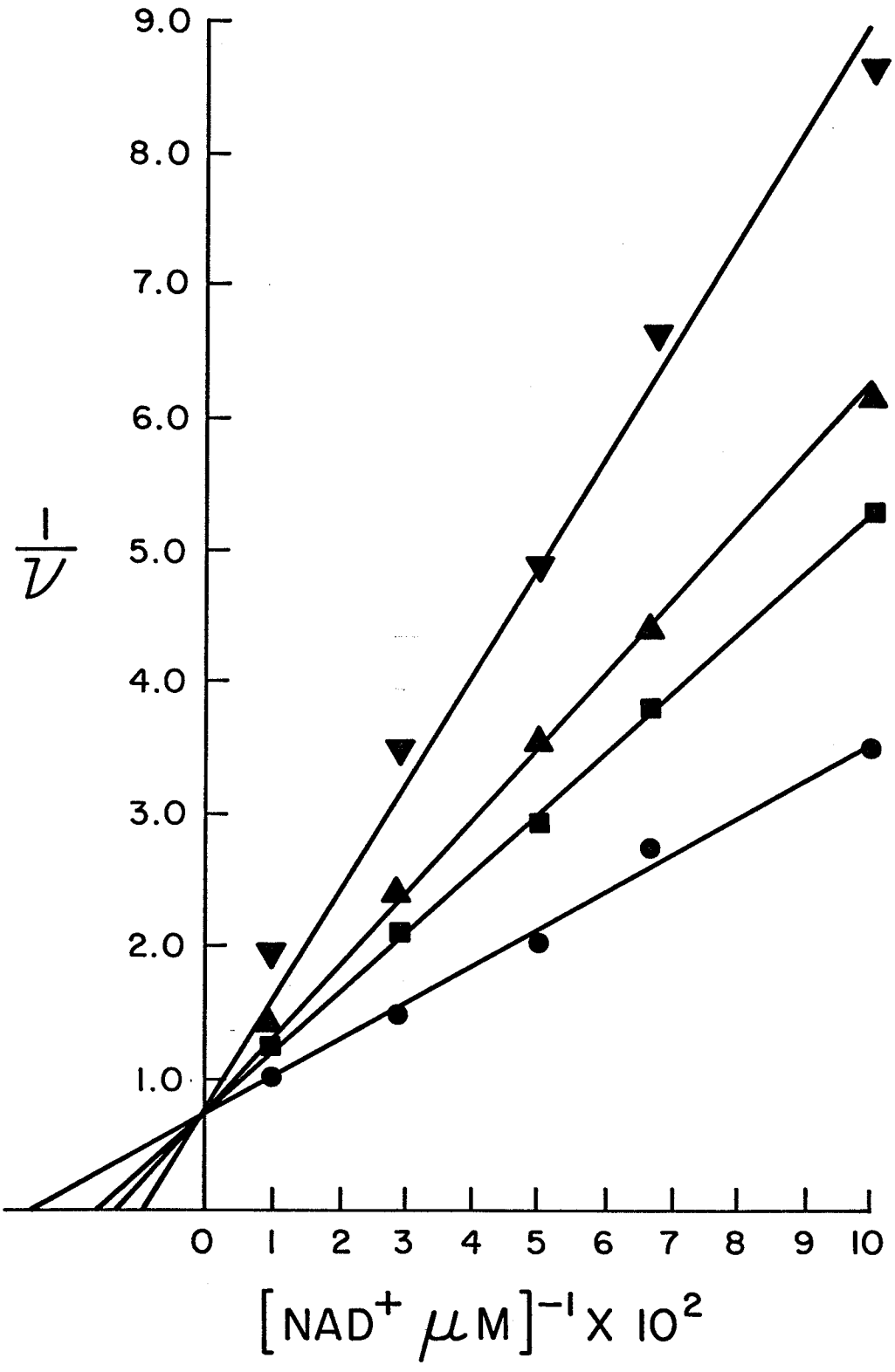

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1/v
0 1 2 3 4 5 6 7 8 9 10
[NAD+ μM]-1 X 10²

Figure 27. Product inhibition by NADH with varying concentrations of 3-HB and a fixed non-saturating concentration of NAD (100 μM). Kinetic assays were performed as described in *Materials and Methods*.

● Normal velocity curve (100 μM NAD)

■ 10.0 μM NADH

▲ 35.0 μM NADH

▼ 100.0 μM NADH

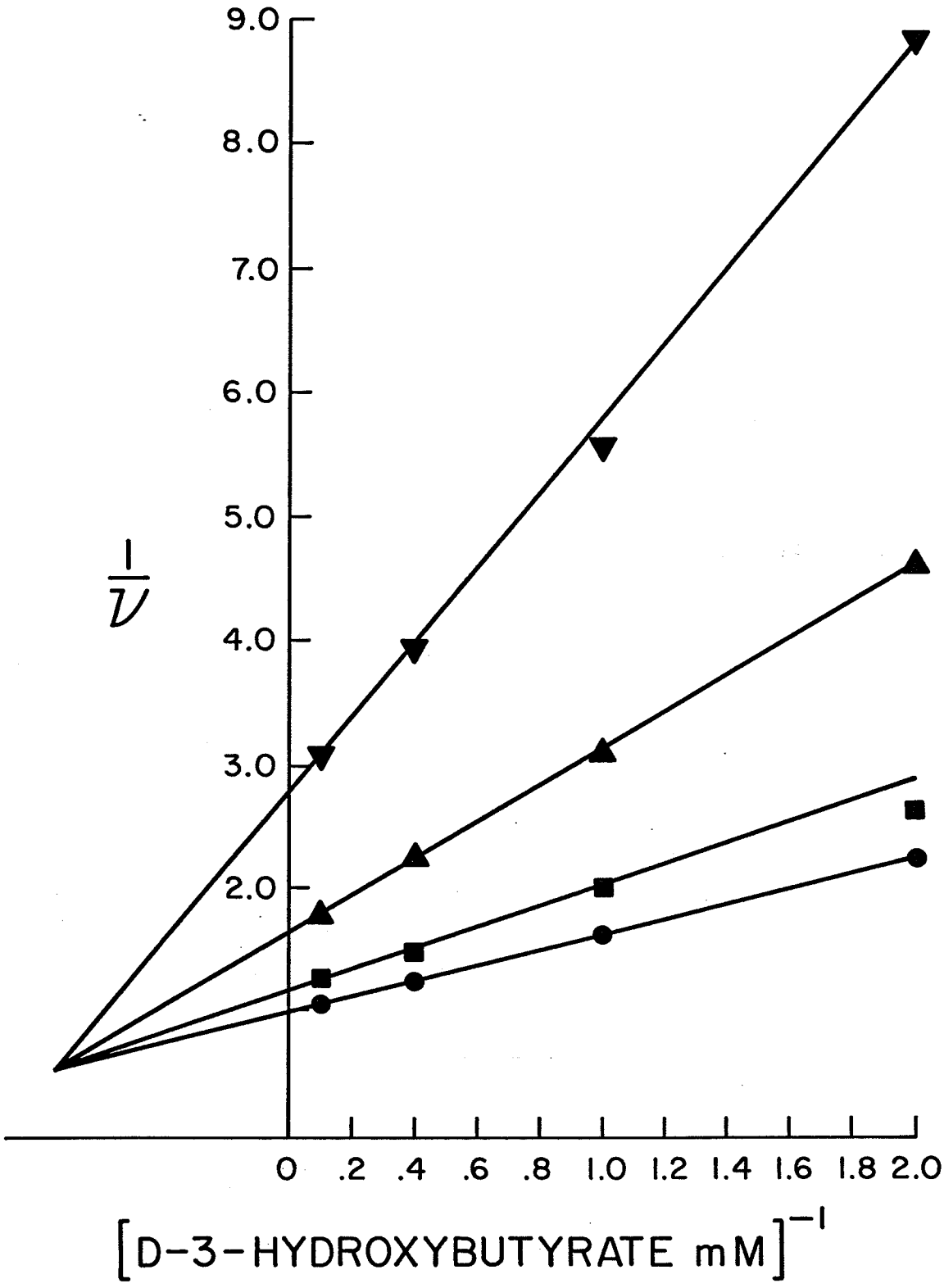
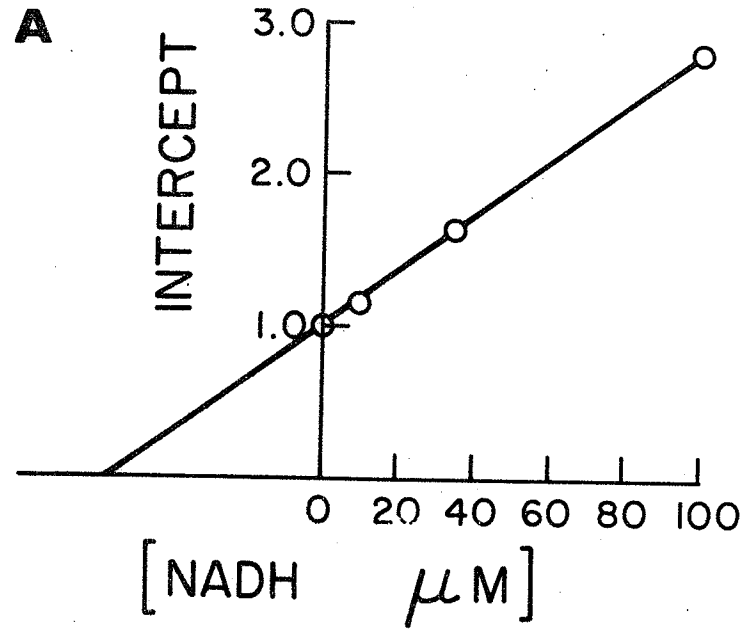

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1/v
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
[D-3-HYDROXYBUTYRATE mM]⁻¹

Figure 28a. Intercept replot of Figure 27.

Figure 28b. Slope replot of Figure 27.


A
INTERCEPT
3.0
2.0
1.0
0 20 40 60 80 100
[NADH μM]

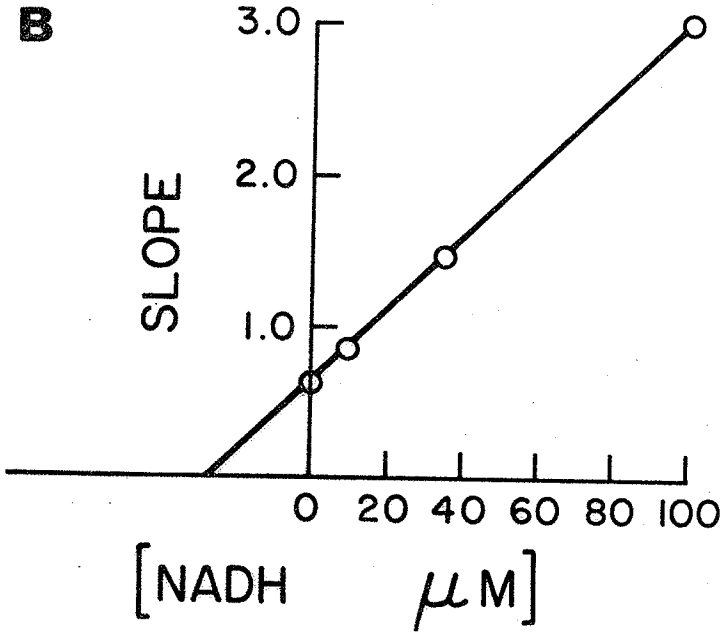
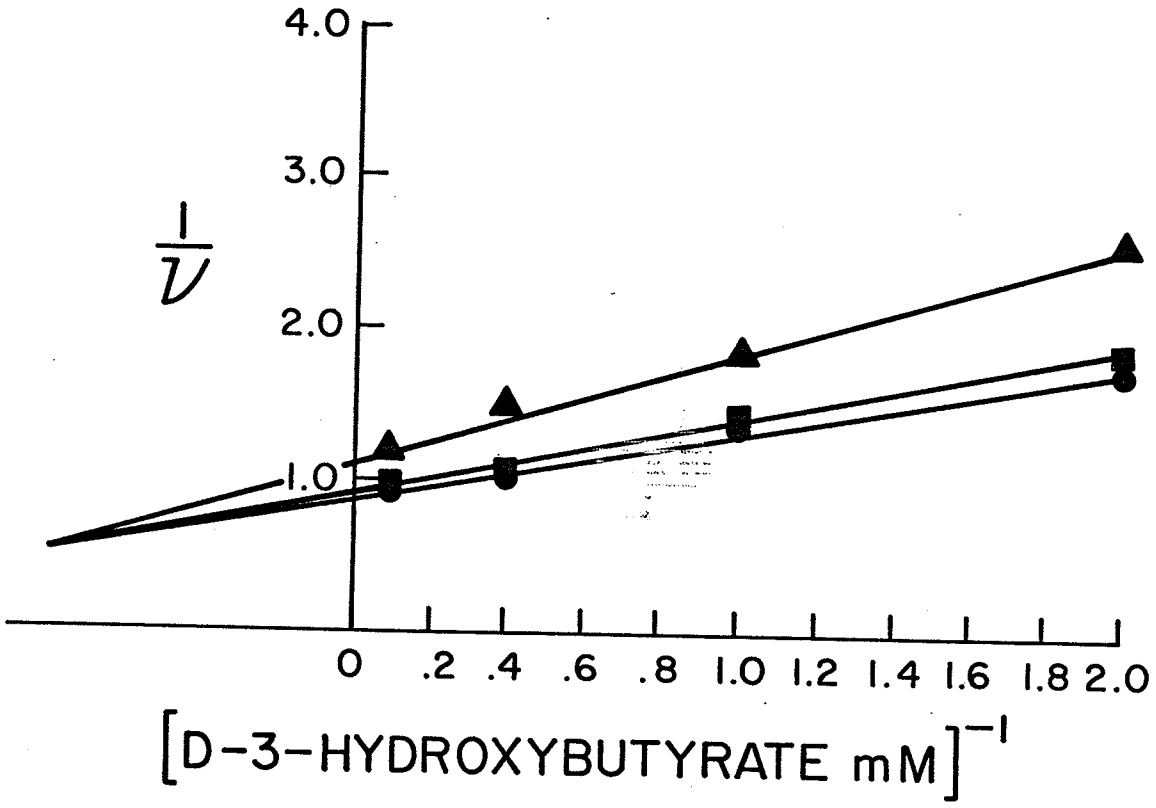

B
SLOPE
3.0
2.0
1.0
0 20 40 60 80 100
[NADH μM]

Figure 29. Product inhibition by NADH with varying concentrations of 3-HB and a fixed saturated concentration of NAD (1.0 mM). Kinetic assays were performed as described in *Materials and Methods*.

● Normal velocity curve (1.0 mM NAD)

■ 35.0 μM NADH

▲ 100.0 μM NADH


4.0
3.0
2.0
1.0
1/v
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
[D-3-HYDROXYBUTYRATE mM]⁻¹

the noncompetitive type. Initially, 100 μM NAD (2 times Km) and 500 μM NAD (11 times Km) were used as possible saturating concentrations of NAD. It was not until 1.0 mM NAD (22 times Km) was used that the inhibition of NADH could be relieved.

DISCUSSION

DISCUSSION

In 1969, Van Caeseele and Lees made the preliminary observation that PHB could be found in heterotrophically (1% glucose) grown *T. novellus*. They reported that *T. novellus* accumulated 4.6% PHB by weight, while the autotrophically (1% sodium thiosulfate) grown cells contained no PHB. These authors prepared electronmicrographs of heterotrophically and autotrophically grown *T. novellus*. In only the heterotroph, large electron transparent inclusions could be seen in the cytoplasm of the cell. After chemical analysis of both the autotrophically and heterotrophically grown cells these inclusions were tentatively identified as polysaccharide as the measurements showed that the polysaccharide content varied appreciably between autotrophically and heterotrophically grown cells and the inclusions also stained with iodine.

Similar electron transparent inclusions have been demonstrated in electronmicrographs of *Hydrogenomonas* H16 (Schlegel *et al.* 1961) and *Ferrobacillus ferrooxidans* (Wang and Lundgren, 1969). These authors have identified the large electron transparent inclusions as PHB grandules. The results obtained in this report would suggest that the inclusions in *T. novellus* observed by Van Caeseele and Lees (1969) might well have been PHB granules.

The physiological conditions of PHB synthesis and degradation (see History) vary from one microorganism to another. As mentioned in the History, numerous microorganisms accumulate PHB during nitrogen

or oxygen depletion. In this study *T. novellus* grown heterotrophically on 1% glucose produced PHB and the degradative enzyme 3-HBDH without limiting aeration or nitrogen concentrations. In this report PHB levels were found to increase during the growth of *T. novellus* on 1% glucose as illustrated in Figure 2, but the optimal physiological conditions for PHB accumulation were not determined. Further growth experiments varying several parameters would be required to optimize conditions for PHB synthesis.

Dawes and Senior (1973) suggest that degradation of PHB does not occur until the exogenous energy or carbon sources are depleted. From Figure 2 it can be seen that the PHB level begins to decrease at 20 h indicating that the exogenous supply of glucose is depleted. An unusual finding (Figure 2) was that 3-HBDH levels increased at the same time as PHB increased. This might indicate a turnover of PHB within the cell. Similar results have been obtained by Thompson and Nakata (1973) with sporulating cells of *Bacillus cereus* T. These investigators found that when the cells were placed in chemically defined sporulation media that after the PHB level started to rise there was only a 2 h lag before the 3-HBDH level started to increase. Shuster and Doudoroff (1962) harvest *R. rubrum* between 18 - 20 h after the stationary phase (18 h) of growth when the polymer has completely been depleted. *T. novellus* on the other hand did not show any significant increase in enzyme levels from a 48 h grown culture as compared to a 22 - 24 h culture.

The enzyme, 3-HBDH, has been purified by numerous investigators from both bacterial and mammalian sources (Table 1). The results of the purification procedure used for 3-HBDH from *T. novellus* were summarized in Table 2. The inital enzyme activity was reported after the ultracentrifugation step as only low levels of activity could be detected in crude extracts. This low activity was presumably due to the presence of NADH oxidase which was removed by the ultracentrifugation step. KCN inhibition of NADH oxidase could not be used because of an increase in absorbance at 340 nm due to formation of a NAD-CN complex. Initially, the method of purification of 3-HBDH from *R. spheroides* (Bergmeyer *et al.* 1967) was used in this investigation. This method involved DEAE-Sephadex batch absorption, $(NH_4)_2SO_4$ fractionation and repeated DEAE-Sephadex chromatography. Using this procedure it was found that 3-HBDH from *T. novellus* had a strong binding affinity to the anion exchanger, DEAE-Sephadex, at pH 8.0. DEAE cellulose has also been used by numerous investigators as an ion exchanger for the purification of 3-HBDH from other bacterial sources (Shuster and Doudoroff, 1962; Delafield *et al.* 1965; Dhariwal and Venkitasubramanian, 1978; Thompson and Nakata, 1973). In the current investigation, after repeated DEAE-Sephadex chromatography (results not indicated) higher specific activities of the enzyme could not be obtained. There are two possible reasons for failure of this method as used to purify 3-HBDH from *T. novellus*. The first reason could be that the contaminating proteins might have the same isoelectric point as the enzyme and would not be separated by charge

differences on DEAE-Sephadex. The second and probably more valid reason could be the loss of 3-HBDH activity during DEAE-Sephadex chromatography as Figure 6 illustrates that the desalted enzyme rapidly loses activity. It was also determined that desalted 3-HBDH activity could be partially recovered by addition of $(NH_4)_2SO_4$ to the reaction cuvette. Unlike 3-HBDH from *T. novellus*, the enzyme from *B. cereus* T (Thompson and Nakata, 1973) requires either Mg^{+2}, Mn^{+2}, Ba^{+2} or Ca^{+2} when stored at $4^{o}C$. The requirement of Mg^{+2}, Mn^{+2} and Ca^{+2} has also been demonstrated for the enzyme from *P. lemoignei* (Delafield *et al.* 1965). There was a greater loss of enzyme activity when desalting with dialysis than by G-25 Sephadex treatement. This loss of enzyme activity through dialysis has also been reported in the enzymes from *R. spheroides* (Preuveneers *et al.* 1973), *R. rubrum* (Shuster and Doudoroff, 1962) and *A. vinelandii* (Jurtshuk *et al.* 1968). The first DEAE-Sephadex column chromatography (Figure 3) eluted 3-HBDH as a single peak with a 6-fold increase in specific activity. Rather than repeated DEAE-Sephadex chromatography the precipitated enzyme from the DEAE-Sephadex column fractions was applied to a G-200 Sephadex superfine column. Initially, the G-200 Sephadex purification procedure also caused inactivation of the enzyme, presumably as a result of the instability of the enzyme in the 0.1 M phosphate pH 8.0 elution buffer. When 1 M $(NH_4)_2SO_4$ was added to the elution buffer it did not alter the running of the column but served to protect the enzyme.

Approximately a three-fold purification occurred using a Blue dextran-Sepharose 4B affinity column (Table 2). Blue dextran, a polyaromatic dye Cibacron blue F3GA linked to dextran, has an affinity for a variety of proteins and enzymes, but shows preferred specificity for enzymes which contain a super-secondary structure known as a dinucleotide fold (Thompson *et al.* 1975). The blue chromophore of blue dextran, mimics the molecular structure of NAD and will bind enzymes which contain the dinucleotide fold (~120 amino acids) which forms the binding site for NAD (Thompson *et al.* 1975). The addition of the nucleotide ligand NAD to the elution buffer serves to remove the enzyme from the blue dextran-Sepharose 4B matrix. Repeating the affinity column procedure or using a NAD linear gradient was unsuccessful in obtaining a further increase in specific activity.

The purified enzyme contained two protein contaminants as determined by polyacrylamide gel electrophoresis (Figure 5a). One of the contaminants was major (dark band) while the other was minor (faint band). By the use of a specific 3-HBDH activity stain the top protein band (furthest from the tracking dye) was determined to be 3-HBDH (Figure 5b). The activity stain involved the use of NBT, a yellow electron acceptor which turns blue when reduced (i.e. with NADH). The use of PMS as an intermediate in the reduction of NBT with NADH, accelerated the appearance of the activity band. The activity stain did not require metal ions (which would be presumably

removed from the enzyme during electrophoresis) thus suggesting the enzyme does not require metal ions for activity. An interesting result (not presented) was that excess $(NH_4)_2SO_4$ when added to the enzyme solution prior to electrophoresis resulted in formation of multiple protein and activity bands on 5% polyacrylamide gels. This presumably was due to aggregation of the enzyme. 3-HBDH from *R. spheroides* (Sigma) after 5% polyacrylamide gel electrophoresis contained numerous (>10) protein bands (results not presented) and indicated a large variation in the location of the enzyme activity band as compared to the enzyme from *T. novellus*.

The molecular weight of 3-HBDH from *T. novellus* was determined to be 110,000 and from *R. spheroides*, 3-HBDH was determined to be 120,000 (Figure 10). Bergmeyer *et al.* (1967) reported that the molecular weight of the enzyme from *R. spheroides* was 85,000. Possible discrepancies in these values could be due to the method used in determination of molecular weight. In this report the gel filtration (G-200 Sephadex superfine) method was used while Bergmeyer *et al.* (1967) used the sedimentation equilibrium (ultra-centrifugation) method. Purifying both enzymes to homogeneity and repeating the molecular weight determination using SDS polyacrylamide gel electrophoresis might resolve the discrepancies in the values obtained by the various methods, but since the purified enzyme was not homogenous, molecular weight determination by SDS polyacrylamide gel electrophoresis could not be done.

The enzyme was determined to have an optimal pH of 8.6 - 9.0 for the forward oxidation reaction (Figure 7). This pH value is similar to other values for 3-HBDH obtained from various bacterial and mammalian sources as illustrated in Table 1.

Unlike the enzymes from *R. rubrum* (Shuster and Doudoroff, 1962) and *P. lemoignei* (Delafield *et al.* 1965) which are inactivated by EDTA and require Mg^{+2} for regaining activity (presumably due to stability) the enzyme from *T. novellus* does not require any metal ions. None of the ions tested in Table 3 had any stimulating effect on enzyme activity. In this manner 3-HBDH from *T. novellus* resembles the enzyme from *R. spheroides* (Bergmeyer *et al.* 1967) and *M. phlei* (Dhariwal and Benkitasubramanian, 1978) which do not require metal ions for activity. Various metal ions, however, did have an inhibitory effect on 3-HBDH activity. The most effective inhibitors were Hg^{+2} and Zn^{+2} while Co^{+2} and Cu^{+2} gave slight inhibition. The mode of action of these inhibitors is by combining with sulfhydryl groups involved at the catalytic site of the enzyme. The effect of Hg^{+2} as an inhibitor of sulfhydryl groups (by forming mercaptides) was confirmed by relief of the inhibition by dithiothreitol, a sulfhydryl reducing agents. The inhibition by Hg^{+2} could also be relieved by other sulfhydryl reducing agents such as glutathione, β-mercaptoethanol and cysteine. The divalent metal ion chelating agent EDTA could not remove Hg^{+2} from the essential sulfhydryl group.

Since the enzyme was thought to contain one or more sulfhydryl groups, various sulfhydryl reducing agents were added to the purified

enzyme but dithiothreitol and cysteine caused only a slight increase in activity while glutathione and βmercaptoethanol were ineffective. Similar results were obtained with the enzymes from *M. phlei* (Dhariwal and Venkitasubramanian, 1978), *P. lemoignei* (Delafield *et al.* 1965), *A. beijerinckii* (Senior and Dawes, 1973) and *R. rubrum* (Shuster and Doudoroff, 1962). However, this does not indicate that the enzyme from *T. novellus* has no sulfhydryl groups but that the enzyme in the purified fraction has most of its sulfhydryl groups already in the reduced form. Various known sulfhydryl inhibitors were tested as inhibitors of 3-HBDH activity. As illustrated in Table 5 both NEM and DTNB had a marked inhibitory effect on 3-HBDH. Inhibition by DTNB could be freely reversed by addition of dithiothreitol, but the inhibition caused by NEM could not be relieved by addition of either dithiothreitol, β-mercaptoethanol, cysteine or glutathione due to the strong bond formation between NEM and the catalytic sulfhydryl group.

The enzymes from *R. rubrum* (Shuster and Doudoroff, 1962) and *A. beijerinckii* (Senior and Dawes, 1973) did not have any sensitive sulfhydryl groups. These variations as to whether or not the enzymes from various sources contain sensitive sulfhydryl groups could be due in part to the assay procedure used by the various investigators. Several agents could protect against sulfhydryl inhibitors and the order ofaddition of inhibitor, substrate, metal ion etc. is crucial in these types of experiments. There was a usual finding regarding

the effect of Zn^{+2} on the enzymes from *M. phlei* (Dhariwal and Venkitasubramanian, 1978) and *R. spheroides* (Bergmeyer *et al.* 1967). Both groups of investigators demonstrated inhibition by sulfhydryl inhibitors but no inhibition by Zn^{+2} could be demonstrated. The enzyme from *M. phlei* was not affected by 33 mM Zn^{+2}, while both $HgCl_2$ and p-chloromercuribenzoate were able to inhibit the enzyme. In contrast, the *T. novellus* enzyme was inhibited by 93% in the presence of 1 mM Zn^{+2}.

Although 3-HBDH is a reversible NAD oxidoreductase and the reverse reaction of acetoacetate going to 3-HB with oxidation of NADH can occur, this reaction is not favoured (results not presented). It was demonstrated that the enzyme, specifically, catalyzes the reduction of NAD and NADP cannot be substituted. NADP did not inhibit or stimulate 3-HBDH activity. Various structural analogues (see Results) were examined for their ability to serve as substrates to reduce NAD or as inhibitors. Only D-lactate was found to be a potent inhibitor of 3-HBDH. This inhibitor is a structural analogue of 3-HB and gave a competitive inhibition plot when 3-HB was varied with a constant amount of NAD as illustrated in Figure 11. Similar competitive inhibition with D-lactate has been demonstrated for 3-HBDH from *A. beijerinckii* (Senior and Dawes, 1973), *P. lemoignei* (Delafield *et al.* 1965) and *R. spheroides* (Bergmeyer *et al.* 1967). A usual finding was that 3-HBDH from *R. rubrum* (Shuster and Doudoroff 1962) was not inhibited by D,L-lactate.

Numerous investigators have demonstrated inhibition with various

analogues which when tested with 3-HBDH from *T. novellus* did not result in any effect on enzyme velocity. For example, pyruvate and α-ketoglutarate are both competitive inhibitors for 3-HBDH from *A. beijerinckii* (Senior and Dawes, 1973). Neither of these compounds had any effect on the enzyme from *T. novellus*. The inhibitory effect of pyruvate (although weak) has also been demonstrated on the enzyme from *P. lemoignei* (Delafield *et al.* 1965). Mammalian 3-HBDH from rat liver mitochondria in some ways resembles the enzyme from *T. novellus* as no inhibition by pyruvate or α-ketoglutarate could be demonstrated for both these enzymes (Berry, 1964). The mammalian 3-HBDH also resembles the enzyme from *R. spheroides* (Bergmeyer *et al.* 1967) in that they are inhibited by succinate and malonate. Neither of these compounds has an effect on the enzyme from *T. novellus*. An interesting observation was that the enzyme from *R. spheroides* (Sigma) was tested with 1.0 mM malonate in our laboratory and we obtained no apparent decrease in enzyme activity. Higher concentrations of malonate (10.0 mM) did cause a slight inhibition with both the enzymes from *R. spheroides* (Sigma) and *T. novellus*. Variations in results on structural analogues and their effects as inhibitors of 3-HBDH from various sources can be due to many parameters. Several investigators (Berry, 1964; Bergmeyer *et al.* 1967) use non-physiological concentrations (10 mM) of the various inhibitors while other (Shuster and Doudoroff, 1962) do not even report the concentrations used.

The amount of enzyme, substrate and most importantly the inhibitor is crucial in this type of experiment and variation in experimental results could be due to any one of these variables. The variations in results obtained by these investigators could be the result of pH changes caused by inadequate buffering of these strongly acidic compounds. The inhibition could be the result of a pH change rather than a physiologically significant type of inhibition.

As mentioned earlier (History), all 3-HBDHs from various sources when tested are competitively product inhibited by NADH. The inhibiton of 3-HBDH by NADH would decrease enzyme activity and supress utilization of PHB until lower levels of reducing power (i.e. exogenous substrate depletion) were in the cell.

Initial velocity data, varying concentrations of one substrate with fixed concentrations of the other (Figure 12 and 13) indicate straight lines on Lineweaver-Burk plots with an intersecting point below the x-axis. Straight line, double reciprocal plots which follow Henri-Michaelis-Menten kinetics are common for enzymes which are not allosteric in nature (Segel, 1975). Both double reciprocal plots were of the intersecting type indicating that a Ping Pong mechanism was not occurring. Since the Kia value was relatively close from one plot to the other, an ordered or sequential mechanism could be proposed. The intercept replots (Figure 14 a and 14b) determine the apparent Km values. The Km values obtained from these plots were 45.5 μM for NAD and 0.77 mM for D-3-hydroxybutyrate. The

slope replots of the initial velocity curves (Figure 15a and 15b) give near identical slope values of 1.4 and 1.5 which would be expected since:

$$\text{Slope} = \frac{Kia\ KmB}{V_{max}} \quad \text{for both slope replots (Segel, 1975).}$$

The use of product inhibition studies allows the determination of the order of substrate addition and product release and proposes a suitable kinetic mechanism for the enzyme (Cleland, 1963a, 1963b, and 1963c). Table 6 is a summary of the various kinetic mechanisms which might occur with a Bi Bi reaction using product inhibition analysis (Cleland, 1963a). The following is a summary of the data obtained from product inhibition analysis of 3-HBDH from *T. novellus:*

Inhibitory Product	Vary NAD		Vary 3-HB	
	Unsat.	Sat. 3-HB	Unsat.	Sat. NAD
Acetoacetate	NC(Fig.16)	UC (Fig.18)[1]	NC (Fig.20)	NC (Fig.22)
NADH	Comp(Fig.24)	Comp (Fig.26)	NC (Fig.27)	- (Fig.29)

[1]Inhibition with 1.0 mM acetoacetate indicated a definite UC double reciprocal plot while 5 and 10 mM concentrations of acetoacetate demonstrated unusual UC double reciprocal plots in that they appear to curve upwards at lower NAD concentrations. The meaning of this result is unknown.

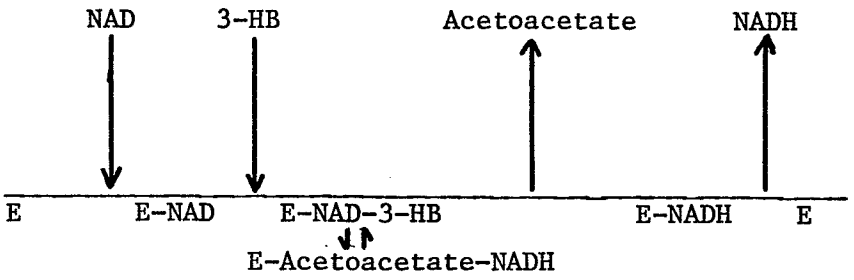
From Table 6 the mechanism of 3-HBDH can be determined to be an ordered Bi Bi where NAD is the first substrate to add to the enzyme and NADH is the last product to leave:

TABLE 6

Product inhibition patterns for Bi Bi Mechanisms.

Mechanism	Inhibitory Product	Variable substrate			
		A		B	
		Unsaturated	Saturated with B	Unsaturated	Saturated with A
Ordered Bi Bi	P	NC*	UC	NC	NC
	Q	Comp	Comp	NC	-
Theorell-Chance	P	NC	-	Comp	Comp
	Q	Comp	Comp	NC	-
Iso Ordered Bi Bi	P	NC	UC	NC	NC
	Q	NC	NC	NC	UC
Iso Theorell-Chance	P	NC	-	Comp	Comp
	Q	NC	NC	NC	UC
Rapid Equilibrium Random Bi Bi	P or Q	Comp	-	Comp	-
Rapid Equilibrium Random Bi Bi and dead end EBQ complex	P	Comp	-	Comp	-
	Q	Comp	Comp	NC	-
Ping Pong Bi Bi	P	NC	-	Comp	Comp
	Q	Comp	Comp	NC	-
Iso Ping Pong Bi Bi (Isomerism of E)	P	NC	-	Comp	Comp
	Q	NC	NC	NC	NC
Di-Iso Ping Pong Bi Bi	P or Q	NC	NC	NC	NC
Random Bi Bi	P or Q	NC	NC	NC	NC

*Abbreviations used: Comp, competitive; UC, uncompetitive, NC, noncompetitive; -, no inhibition.



All replots of both slope and intercept values were linear plots. From the Results, it is evident that higher concentrations of the fixed substrate are required for saturation. Segel (1975) suggests that for overcoming inhibition in an ordered Bi Bi system, saturation does not simply mean ten-times the Machaelis constant. The results obtained in this report (i.e. Figure 29) support this statement.

The kinetic analysis of 3-HBDH from *T. novellus* is consistant with an ordered Bi Bi mechansim. Similar results have been obtained for the enzyme from *R. spheroides* (Preuveneers *et al.* 1973; Hurst *et al.* 1973) and bovine heart mitochondria (Nielsen *et al.* 1972).

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