AN ABSTRACT OF THE THESIS OF

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Title: AN INVESTIGATION OF THE METABOLISM OF POLY-β-HYDROXYBUTYRATE AND ITS POSSIBLE ROLE IN NITROGEN FIXATION IN SOYBEAN ROOT NODULES

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A major objective of this investigation was to determine the possible roles, if any, of poly-β-hydroxybutyrate and products of its metabolic breakdown in symbiotic nitrogen fixation by nodule bactercids. Changes in poly-β-hydroxybutyrate content and in activities of nitrogenase, β-hydroxybutyrate dehydrogenase, and isocitrate lyase were studied under various conditions where the supply of carbohydrate from the soybean plants to the nodule bacteroids was interrupted. Experiments of this type were conducted with detached root nodules, nodules from plants incubated in the dark, and nodules from plants of different ages. The results indicated that nodule bacteroids did not utilize poly-β-hydroxybutyrate until the carbohydrate supply from the host plants was limited by

nodule exicison or incubation in the dark, or by senescence of the host plant. Isocitrate lyase activity was not detected until poly-β-hydroxybutyrate utilization began. The results of these experiments suggest that poly-β-hydroxybutyrate was not capable of fulfilling the requirement for maintenance of high activity of nitrogenase under conditions where the supply of carbohydrate from the host plant was limited.

β-Hydroxybutyrate dehydrogenase from nodule bacteroids was purified and its properties were investigated. Kinetic studies revealed that the mechanism of the β-hydroxybutyrate dehydrogenase reaction was a compulsory order type involving the formation of a ternary complex between the dehydrogenase, NAD^+, and β-hydroxybutyrate.

Three different systems for the transport of electrons to bacteroid nitrogenase were studied. Effective systems included the following: (a) hydrogen in the presence of Clostridium pasteurianum hydrogenase and ferredoxin, (b) NADH generated by the β-hydroxybutyrate dehydrogenase reaction in the presence of an NADH dehydrogenase and either FMN or FAD, (c) NADPH generated by the glucose-6-phosphate dehydrogenase reaction, provided that bacteroid non-heme iron protein, azotoflavin from Azotobacter, and ferredoxin-NADP reductase from spinach were added. It is suggested that the NADPH donor system is physiologically more important than the other systems investigated.

An Investigation of the Metabolism of Poly-β-hydroxybutyrate and Its Possible Role in Nitrogen Fixation in Soybean Root Nodules

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LIST OF ABBREVIATIONS

ATP	Adenosine-5'-triphosphate
CoA	Coenzyme A
DEAE-cellulose	Diethylaminoethyl-cellulose
DCIP	2, 6-Dichlorophenolindophenol
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
HDS	Hydrogen donating system (from C. pasteurianum)
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonate
K_m	Michaelis constant
MES	2-(N-morpholino)-Ethanesulfonate
Na_2EDTA	Disodium ethylenediaminetetraacetate
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADH	Reduced form of nicotinamide adenine dinucleotide
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
PHB	Poly-β-hydroxybutyrate
PPG	Polypropylene glycol
TES	N-tris(hydroxymethyl)methyl-2-aminoethane-sulfonate
Tris	Tris(hydroxymethyl)aminomethane

AN INVESTIGATION OF THE METABOLISM OF POLY-β-HYDROXYBUTYRATE AND ITS POSSIBLE ROLE IN NITROGEN FIXATION IN SOYBEAN ROOT NODULES

INTRODUCTION AND STATEMENT OF PROBLEM

The process of biological nitrogen fixation provides ammonia for the synthesis of protein, which is an essential constituent of all forms of life. In 1960, Donald (32) suggested that of the 10^8 tons of nitrogen fixed biologically each year, the major portion was fixed by legumes. It seems clear that the economic importance of symbiotic nitrogen fixation to the agricultural industry is enormous.

It has been estimated that for each milligram of nitrogen fixed by the legume root nodules, 3 to 19 mg of carbohydrate is consumed (13, 41). Moreover, two or more carbon atoms are required for the export of each fixed nitrogen atom, in the forms of amino acids and amides, from the nodules to the host plant (113). The large carbohydrate requirement for nitrogen fixation was reported to be supplied by the host plant mainly as sucrose (communicated to Dr. Evans by Drs. Kidby and Parker).

It has been established that as much as 50 percent of the dry weight of Rhizobium japonicum bacteroids consists of poly-β-hydroxybutyrate. This storage material may be metabolized to provide energy and carbon skeletons for many bacteria (26).

The primary purpose of this investigation was to study the metabolism of poly-β-hydroxybutyrate in soybean root nodules and to determine the roles, if any, of this polymer and its breakdown products in symbiotic nitrogen fixation. Furthermore, it was the purpose of this investigation to conduct detailed studies on the properties of β-hydroxybutyrate dehydrogenase from nodule bacteroids, and to attempt to couple the reducing power generated by the oxidation of β-hydroxybutyrate to the cell-free nitrogenase system from soybean root nodules.

GENERAL REVIEW OF LITERATURE

Many reviews on the metabolism of poly-β-hydroxybutyrate (PHB) are available. Dawes and Ribbons (26) have presented an extensive review on the role of PHB in bacterial metabolism. Other reviews concerning the metabolism of PHB and other storage materials include those by Dawes and Ribbons (25), Wilkinson (114, 115), and Campbell et al. (18).

The reviews on the subject of nitrogen fixation are even greater in number. The historical aspects of the early work on nitrogen fixation and a consideration of asymbiotic nitrogen fixation have been presented by Wilson (119, 120). A review of symbiotic nitrogen fixation prior to 1958 was made by Allen and Allen (4, Vol. 8, p. 45-118). Reviews on the biochemistry of nitrogen fixation by various organisms include those by Carnahan and Castle (19), Mortenson (82), Burris (16, 17), Hardy and Knight (48), Hardy and Burns (46), and a monograph by Stewart (104).

Occurrence of Poly-β-hydroxybutyrate in Microorganisms

PHB, a polyester composed of the D-(-) stereoisomer of β-hydroxybutyrate, was discovered by Lemoigne (65) in 1923 as a major component of an aerobic bacillus. This polymer subsequently has been found to be an important storage material in a variety of

bacteria. For example, Dawes and Ribbons (26) have listed 25 species of bacteria, including both aerobic and anaerobic organisms, that store this compound. Forsyth et al. (38) reported that Azotobacter vinelandii, Chromobacterium violaceum, free-living Rhizobium, as well as legume root nodules, contain PHB. Furthermore, PHB has been identified in several yeast-like fungi (38) and in certain blue green algae (21). The quantity of PHB in bacteria ranges from a few percent of the dry weight in Pseudomonas solanacearum (38) to as much as 80 percent of the dry weight in Aztobacter chroococcum (95).

Some Chemical and Physical Properties of Poly-β-hydroxybutyrate

PHB samples isolated from many sources have very similar properties. It has an empirical formula $(C_4H_6O_2)_n$ (66). The hydrolytic product of PHB has an optical rotation of -14.4° (64, 90). When hydrolyzed in hot concentrated sulfuric acid, the polymer shows an absorption peak at 235 mμ (63). The polymer is insoluble in many organic solvents but is soluble in hot chloroform (66). Lundgren et al. (70) reported that PHB samples isolated from 16 different species of bacteria showed similar X-ray diffraction patterns. This research established that all samples of the polymer from the various sources possessed the same helical conformation. Also, all

samples exhibited essentially identical infrared spectra with a major absorption peak at 5.7 μ. This peak was reported to correspond to the ester carbonyl stretching mode. PHB samples isolated from different bacteria showed a wide range of melting points and molecular weights. The melting point values ranged from 114° (66, 67) to 188°C (95). Lundgren et al. (70) also reported that molecular weight ranged from 1,000 for the polymer from Bacillus megaterium to 256,000 for the polymer from Pseudomonas saccharophila. PHB isolated from an unidentified Rhizobium has a molecular weight of 128,000 and a melting point of 173°C (6).

The morphology of native PHB granules consists of 100 to 150 Å fibrils making up extended polymeric chains (35). Griebel et al. (44) isolated native PHB granules from Bacillus megaterium and found them to be composed of approximately 98 percent PHB, two percent protein and a small amount of lipid. The lipid components were comprised of an unidentified acetone-soluble compound and phosphatidic acid. The native PHB granules were found to be surrounded by a membrane (44, 71).

Metabolism of Poly-β-hydroxybutyrate

PHB is a storage material that is unique to microorganisms. Its biosynthesis and catabolism have been studied extensively in recent years, mainly by Doudoroff and Stainier, Gibbons, Schlegel,

Wilkinson, Merrick, and their collaborators.

Biosynthesis of Poly-β-hydroxybutyrate

Only a relatively few papers have been published prior to 1968 regarding the metabolic pathways for the biosynthesis of PHB. Merrick and Doudoroff (78) isolated PHB granules from lysozyme-treated Bacillus megaterium KM, which were shown to incorporate ^{14}C-D(-)-β-hydroxybutyryl-coenzyme A (CoA) into PHB. About 40 percent of the total radioactivity added was incorporated into the polymer. The same authors (78) also demonstrated the incorporation of ^{14}C-D(-)-β-hydroxybutyryl-CoA into PHB in the presence of a particulate fraction from Rhodospirillum rubrum. Using a crude extract from B. megaterium, with the addition of coenzyme A, adenosine triphosphate and NADH or NADPH, a small incorporation of ^{14}C-acetate into the polymer was also demonstrated (78).

Recently Merrick and coworkers have published two significant papers concerning the biosynthesis of PHB. Griebel et al. (44), who used a very mild procedure for isolating native PHB granules from Bacillus megaterium, found that the PHB synthetase remained firmly bound to the granules. The pH optimum of PHB synthetase was 7.5. The K_m calculated for D(-)-β-hydroxybutyryl-CoA was 9.25×10^{-5}M. The addition of $MgCl_2$, albumin, or 2-mercaptoethanol stimulated the PHB synthetase activity by about two to four-fold. If all three of

these compounds were added together, the reaction was stimulated by about five-fold. Griebel et al. (44) also reported that PHB synthetase was strongly inhibited by p-mercuribenzoate or N-ethylmaleimide, but only slightly by iodoacetamide. From these results they suggested that PHB synthetase activity was dependent upon a sulfhydryl group.

Although D-(-)-β-hydroxybutyryl-CoA was shown to be the substrate for PHB synthetase (44, 78), the biosynthesis of this thioester remained unclear until Moskowitz and Merrick (86), in 1969, reported results of their studies on L(+)-β-hydroxybutyryl-CoA dehydrogenase from Rhodospirillum rubrum, an enzyme previously described by Stern et al. (103). These workers (86) also identified two enoyl-CoA hydrases. The activities of these enzymes help to explain the formation of D(-)-β-hydroxybutyryl-CoA. The L(+)-β-hydroxybutyryl-CoA dehydrogenase catalyzes the reversible oxidation of L(+)-β-hydroxybutyryl-CoA to acetoacetyl-CoA with NAD^+ as coenzyme. The two enoyl hydrases catalyze the following reactions:

$$\text{L-(+)-β-hydroxybutyryl-CoA} \overset{1}{\rightleftharpoons} \text{crotonyl-CoA} + H_2O$$

$$\text{Crotonyl-CoA} + H_2O \overset{2}{\rightleftharpoons} \text{D-(-)-β-hydroxybutyryl-CoA}$$

Reaction 1 is catalyzed by enoyl-CoA hydrase (L) and reaction 2 by enoyl-CoA hydrase (D). Enoyl-CoA hydrase (D) has a pH optimum

between 8.4 and 8.6. The K_m value for crotonyl-CoA is 9.26×10^{-6} M. Various crotonyl thioesters also were examined as possible substrates for the enoyl-CoA hydrase (D) reaction. Crotonyl pantetheine and crotonyl acyl-carrier protein were hydrated, but only at approximately 50 percent of the rate of crotonyl-CoA. Crotonyl glutathione and acrylyl-CoA did not function as substrates. Trans-2-hexenoyl-CoA was hydrated but at a slower rate than crotonyl-CoA.

Further investigation of PHB synthesis (86) revealed that crotonyl-CoA was incorporated into PHB only if enoyl-CoA hydrase (D) was added, and that L-(+)-β-hydroxybutyryl-CoA was incorporated into PHB only if both enoyl-CoA hydrase (D) and enoyl-CoA hydrase (L) were present. These results confirmed the earlier finding that D (-)-β-hydroxybutyryl-CoA was the substrate for PHB synthetase (44, 78). D (-)-β-hydroxybutyryl acyl-carrier protein was found to be ineffective as a substrate for PHB synthetase, indicating that acyl-carrier protein thioester may not play a significant role in PHB synthesis.

As a result of these findings, Moskowitz and Merrick (86) proposed a pathway for PHB synthesis from acetate. Enzymes for the following reactions have been obtained from R. rubrum:

Acetate $\xrightarrow{1}$ acetyl-CoA $\xrightarrow{2}$ acetoacetyl-CoA $\xrightarrow{3}$ L-(+)-β-hydroxybutyryl-CoA $\xrightarrow{4}$ crotonyl-CoA $\xrightarrow{5}$ D-(-)-β-hydroxybutyryl-CoA $\xrightarrow{6}$ poly-β-hydroxybutyrate

Reactions 1, 2, 3 are catalyzed by acetyl-CoA kinase, thiolase and L-(+)-β-hydroxybutyryl-CoA dehydrogenase, respectively. The acetyl-CoA kinase was reported to be present in R. rubrum by Eisenberg (34) and thiolase and L-(+)-β-hydroxybutyryl-CoA dehydrogenase by Stern et al. (103). Enoyl-CoA hydrase (L) catalyzes reaction 4, and enoyl-CoA hydrase (D) reaction 5. The final reaction is catalyzed by PHB synthetase. All the enzymes appear to be soluble with the exception of PHB synthetase.

Catabolism of Poly-β-hydroxybutyrate

The initial studies on the catabolism of PHB were done by Merrick and Doudoroff (78). They showed that the PHB depolymerase system was membrane bound, and that structural integrity of the PHB granules played an important role in the depolymerase activity (78).

Sierra and Gibbons (99) demonstrated PHB depolymerase activity in Micrococcus halodenitrificans by measuring the anaerobic release of CO_2 from a bicarbonate buffer. The same authors also showed that the depolymerase activity was markedly dependent upon

Na^+ or Li^+ ions (100).

Merrick and Doudoroff (79) showed PHB granules isolated from Bacillus megaterium could be hydrolyzed to D(-)-β-hydroxybutyric acid by a complex enzyme system present in the soluble fraction of PHB-depleted cells of Rhodospirillum rubrum. This system consisted of a thermostable activator, a thermolabile depolymerase, and an esterase. Under certain conditions, trypsin replaced the activator. Various chemical and physical treatments inactivated native PHB granules and made them unsuitable as a substrate for the digestive enzymes. The principal product of depolymerase action was D(-)-β-hydroxybutyric acid, but small amounts of esterified products also were released. These were hydrolyzed by the esterase. PHB granules isolated from Rhodospirillum rubrum also contained a depolymerase system which was membrane bound. Attempts to solubilize the digestive enzyme system from the granules have been unsuccessful.

Merrick et al. (80) investigated the nature of inactivation of native PHB granules by physical and chemical means and found that inactivated granules no longer possessed the distinct morphological features of native PHB. The morphological changes were mainly characterized by membrane fragmentation, loss of coalescence, and surface alteration. Furthermore, substances known to interfere with membrane structural organization also inhibited the depolymerizing

process (76). These data suggest that the membrane of the PHB granules participates, by an unknown mechanism, in the depolymerization of the PHB.

The nature of the esterase from R. rubrum was further studied by Merrick and Yu (81). The esterase attacked the dimeric ester of D(-)-β-hydroxybutyric acid. However, the rate of enzymic hydrolysis of the trimeric ester was much faster than that of the dimeric ester.

Another very interesting PHB depolymerase system from Pseudomonas lemoignei was described by Merrick et al. (77), Delafield et al. (28), and Lusty and Doudoroff (72). This system was reported to be extracellular and to digest purified PHB but not native PHB granules. The principal end product was the dimeric ester of D-(-)-β-hydroxybutyric acid. P. lemoignei also possessed intracellular hydrases that hydrolyzed the dimer to β-hydroxybutyrate. This extracellular depolymerase serves an important function when the organisms utilize PHB as an exogenous source of carbon and energy.

The final product of PHB depolymerase, β-hydroxybutyrate, is further oxidized to acetoacetate, which is then degraded to two molecules of acetyl-CoA. The acetyl-CoA is presumed to be metabolized through the tricarboxylic acid cycle. Evidence for the metabolic pathway of utilization of β-hydroxybutyrate was provided by Sierra and Gibbons (99), who showed that D(-)-β-hydroxybutyrate was

oxidized to acetoacetate by crude extracts from M. halodenitrificans. The acetoacetate was further metabolized when ATP, Mg^{++}, co-enzyme A, and oxaloacetate were added to the extracts, which indicates utilization through the tricarboxylic acid cycle.

β-Hydroxybutyrate dehydrogenase catalyzed the oxidation of D(-)-β-hydroxybutyrate to acetoacetate. This enzyme has been found in every species of bacteria that stores PHB. β-Hydroxybutyrate dehydrogenases from Bacillus megaterium (40), Rhodospirillum rubrum (97), Rhodopseudomonas spheroides (12), Rhodospirillum capsulutus, Rhodopseudomonas palustris (22), Hydrogenomonas strain H16 (94), Pseudomonas lemoignei (27), Azotobacter vinelandii (53) and Rhizobium species (39) have been investigated. β-Hydroxybutyrate dehydrogenase from all the sources studies has proven to have a specific requirement for NAD^{+} as the cofactor and D(-)-β-hydroxybutyrate as the substrate. The bacterial β-hydroxybutyrate dehydrogenase is soluble, and thus is unlike the enzyme from mammalian mitochondria, which is tightly bound to the electron-transport particles (43, 126). Green et al. (43) described a β-hydroxybutyrate dehydrogenase from animal tissue that was specific for L(+)-β-hydroxybutyrate. No such enzyme has been obtained from bacterial sources.

β-Hydroxybutyrate dehydrogenase from many sources requires divalent cations such as Mg^{++}, Mn^{++}, or Ca^{++} for stabilization.

Gavard et al. (40) reported that the addition of Mg^{++} or Mn^{++} stimulated the activity of this enzyme from B. megaterium. On the contrary, Williamson et al. (117) reported that the addition of Mg^{++} or Mn^{++} had no effect on β-hydroxybutyrate dehydrogenase from Rhodopseudomonas spheroids.

According to Shuster and Doudoroff (97) β-hydroxybutyrate dehydrogenase from R. rubrum is cold labile and in this respect is unique. The cold-inactivated enzyme was reactivated by allowing it to return to room temperature. They also reported that the specific activity of the dehydrogenase was dependent upon cultural conditions at the time when cells were harvested. Low specific activities were observed during periods of PHB assimilation, but specific activities were increased at least two-fold in older cells that had been depleted of the polymers. Samples of succinate-grown cells containing no PHB showed low specific activities of β-hydroxybutyrate dehydrogenase.

Fottrell and O'Hara (39, 88) were first to report the existence of isoenzymes of β-hydroxybutyrate dehydrogenase in Rhizobium species. Several Rhizobium strains exhibited two or more isoenzymic forms and one strain of R. leguminosarum exhibited five isoenzymic forms.

Physiological Role of Poly-β-hydroxybutyrate

Macrae and Wilkinson (73, 74) indicated that an increase in the

glucose concentration of the growth medium used for B. megaterium resulted in increased synthesis of PHB. B. megaterium cultured in a medium containing excess carbon, but deficient in nitrogen, synthesized four times as much PHB as was formed in a medium where glucose was limiting. In the PHB-poor cells, nitrogen content decreased by 12 percent in four hours during starvation and by only five percent in PHB-rich cells. The PHB content of the PHB-rich cells, however, decreased to a greater extent than that of the PHB-poor cells. The authors also observed that growth did not occur when PHB-rich cells were held in a medium lacking a carbon source (74). It seems clear that in these bacteria, PHB may serve as an energy source but not as a source of carbon skeletons for the synthesis of cellular components.

The role of PHB in maintaining the survival of bacterial cells under starvation culture conditions was further studied by Stokes and Parson (105). They found that PHB-rich Sphaerotilus discophorus cells survived longer under starvation conditions than cells with little or no PHB. An increase in oxygen supply or the addition of Mg^{++} stimulated the oxidation of PHB (106), and led to a more rapid death of the cells.

Doudoroff and Stanier (33) reported that in Rhodospirillum rubrum a major portion (60 to 90 percent) of the assimilated carbon initially accumulated as PHB. When starved R. rubrum cells were

fed ^{14}C-acetate, 60 percent of the total radioactivity was assimilated into PHB. When ^{14}C-butyrate was supplied to comparable cells, 58 percent of the total radioactivity was assimilated into PHB. The fate of ^{14}C-labeled PHB in R. rubrum was studied for 12 hours in the light under various conditions. In the absence of an exogenous organic substrate more than 90 percent of the polymer was degraded, but much of the ^{14}C of the PHB was redistributed into other cellular components. The rate of PHB degradation was decreased by the addition of butyrate. The addition of succinate had no effect, although succinate was metabolized in R. rubrum. The inability of succinate to protect PHB from degradation became clear when succinate was shown to be photoassimilated principally into a glycogen-like polysaccharide. Similar studies were done with P. saccharophila. Doudoroff and Stanier (33) reported that PHB also served as substrate for endogenous metabolism in the absence of an exogenous carbon source. However, the metabolic rate of P. saccharophila was much slower than that of R. rubrum, and the transfer of PHB carbon to other cell constituents could not be demonstrated.

Stainer and his coworkers (102) also demonstrates that the conversion of stored PHB to other cell materials in R. rubrum required CO_2. R. rubrum cells were incubated in the presence of (a) NH_4Cl and He, (b) He-CO_2 mixture, and (c) NH_4Cl and He-CO_2 mixture. After 16 hours of incubation in the absence of CO_2, the PHB content

decreased slightly with no increase in carbohydrate and nitrogen contents. In the presence of CO_2, but without a nitrogen source, the PHB content decreased 50 percent with a corresponding gain in carbohydrate content. Nitrogen content remained constant. When both a nitrogen source and CO_2 were supplied, the PHB disappeared almost completely from the cells, but they increased in both carbohydrate and nitrogen contents. The authors concluded that PHB may serve as a source of carbon skeletons, energy, and reducing power for further CO_2 assimilation.

Another role of PHB was shown by Slepecky and Law (101). They reported that the breakdown of PHB in Bacillus megaterium provided energy and a carbon source for the sporulation process.

Some Requirements for Nitrogen Fixation

The catalysis of nitrogen reduction by cell-free extracts of nitrogen-fixing microorganisms and nodule bacteroids requires a supply of ATP and an appropriate reductant. Furthermore, in soybean root nodules, two or more carbon atoms are required for the export of each fixed nitrogen atom from the nodules to the host plant (113). Winter and Burris (121), and Mortenson (84) reported that four ATP molecules were required for each pair of electrons transferred in nitrogen fixation by extracts from C. pasteurianum. Hadfield and Bulen (45) reported that five molecules of ATP were

required for each pair of electrons transferred during nitrogen fixation by A. vinelandii extracts. In nitrogen fixation by cell-free extracts from free-living nitrogen-fixing microorganisms and bacteroids, ATP is supplied by an ATP-generating system consisting of creatine phosphate, creatine kinase and ADP. The ATP requirement for in vivo nitrogen fixation is presumed to be provided by the respiratory process.

In the early investigations with extracts of Clostridium pasteurianum (30, 47, 73) high energy-phosphate and reductant for nitrogen fixation were furnished by the phosphoroclastic breakdown of pyruvate. In normal C. pasteurianum cells, ferredoxin functions as an electron carrier between the phosphoroclastic reaction and nitrogenase, but in iron-deficient cells flavodoxin apparently substitutes for ferredoxin as an electron transfer protein (57). In the initial experiment (14) in which nitrogen fixation was demonstrated with cell-free extracts of Azotobacter vinelandii, a heated extract of C. pasteurianum containing ferredoxin and hydrogenase, but lacking nitrogenase, was used to transfer electrons from H_2 to nitrogenase. Subsequently, Bulen et al. (15) discovered that $Na_2S_2O_4$ functioned effectively as an electron donor for nitrogenase, and since then $Na_2S_2O_4$ has been utilized almost exclusively as a reductant for the nitrogenases from Azotobacter and nodule bacteroids because natural electron-donating systems from these organisms have not been discovered. Initial attempts (59) to use pyruvate, α-ketoglutarate or H_2, NADH or NADPH as electron

donors for the nodule bacteroid nitrogenase were unsuccessful. Klucas and Evans (55) reported that NADH supplied by the enzymatic oxidation of β-hydroxybutyrate served as a reducing system for bacteroid nitrogenase-dependent acetylene reduction provided that either benzyl or methyl viologen was supplied. Some evidence (36) was obtained that FMN or FAD and a soluble protein factor from bacteroid extract would substitute for the dye in the NADH-coupled nitrogenase reaction. Yates and Daniel (123) have shown that NADH increased nitrogenase-dependent acetylene reduction by a particulate preparation from Azotobacter chroococcum.

In 1969 Benemann et al. (7) isolated a flavoprotein from Azotobacter vinelandii that functioned in the light-dependent transfer of electrons from photosystem I of spinach chloroplast fragments to Azotobacter nitrogenase. This protein appeared to be identical with the flavoprotein cyrstallized from Azotobacter vinelandii by Hinkson and Bulen (49). Yoch et al. (124) also have characterized a non-heme iron protein (Azotobacter ferredoxin) that functions as a carrier in the photochemical transfer of electrons from photosystem I to nitrogenase. After these discoveries Yoch et al. (125), utilizing a nodule bacteroid extract from our laboratory, presented evidence for the occurrence of an electron transport factor that functioned between photosystem I and Azotobacter nitrogenase. When crude bacteroid nitrogenase was utilized in the photochemical

assay, ethylene production was approximately doubled by the addition of the bacteroid factor. In an abstract Benemann et al. (8) have indicated that acetylene reduction by crude Azotobacter nitrogenase may be coupled to an NADPH-generating system provided that azotoflavin from Azotobacter and ferredoxin-NADP reductase were supplied. Evidence (personal communication with Dr. D. C. Yoch) has been obtained that Azotobacter ferredoxin also is required for NADPH-dependent acetylene reduction by Azotobacter nitrogenase (9).

PART I. INVESTIGATION OF THE RELATIONSHIP BETWEEN POLY-β-HYDROXYBUTYRATE METABOLISM AND NITROGEN FIXATION IN SOYBEAN ROOT NODULES

MATERIALS AND METHODS

Chemicals

Reagent grade chemicals or the highest grade available were obtained from commercial sources. NAD^+, sodium DL-β-hydroxybutyrate, DL-isocitrate lactone (hydrolyzed as recommended by the manufacturer), 2-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA), and tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma Chemical Corporation, St. Louis, Missouri; anhydrous hydrazine from Matheson, Coleman and Bell of Cincinnati, Ohio; chloroform and acetone from J. T. Baker Chemical Company, Phillipsburg, New Jersey. Calcium carbide, used to generate acetylene, and 2, 4-dinitrophenolhydrazine were obtained from Allied Chemical Company, Morristown, New Jersey; levigated alumina from Beckman Spinco Division, Palo Alto, California.

Source of Plant Materials

Soybean plants (Glycine max Merr. var. Chippewa) inoculated with a commercial strain of Rhizobium japonicum (kindly supplied by Dr. Joe Burton of Nitragen Company) were cultured in a greenhouse

in pots of perlite supplied with a nitrogen-free nutrient solution (1). The perlite was sterilized by autoclaving for four hours at 15 pounds per square inch before use. The plants, about ten per pot, were provided with 16-hour day length and were flushed daily with nutrient solution except every fourth day when they were flushed with water to remove any accumulated salts.

Purification and Determination of Poly-β-hydroxybutyrate

Lipid granules suspected to be PHB were isolated from soybean root nodules by the method of Wilkinson and Wilkinson (118). Nodules were macerated in 0.05 Tris-Cl buffer, pH 8.4 using a mortar and pestle. The macerate was squeezed through four layers of cheesecloth and centrifuged at 300 x g for ten minutes. The residue, containing unbroken plant cells and cell debris, was discarded; the supernatant was centrifuged again at 8,000 x g for 15 minutes. The supernatant from this centrifugation was discarded; the residue, containing mainly bacteroids, was washed twice with cold glass-distilled water. The washed bacteroids were dried to a constant weight at 85 °C and were hydrolyzed overnight using 0.2 ml of commercial Clorox (5.25% sodium hypochlorite) per mg of dried bacteroids. PHB granules released from the bacteroids were collected by centrifuging at about 8,000 x g for 20 minutes and washed successively with glass-distilled water and redistilled acetone.

The supernatant from the Clorox hydrolysis step was recentrifuged. The small residue was washed successively with the supernatants from the glass-distilled water washing and from the acetone washing. All the residues were combined and dissolved in boiling chloroform. After cooling to room temperature, the chloroform solution was filtered through a Whatman #1 filter paper. PHB in the filtrate was determined by the method of Law and Slepecky (63), and expressed as percentage of the dry weight of bacteroids.

More highly purified preparations of PHB were used for examination of properties. PHB for this purpose was crystallized from the filtered chloroform solution by adding five volumes of redistilled acetone at -20° C. The crystals were collected by filtration and washed with cold redistilled acetone in a Buchner funnel. For the second crystallization, the crystals were redissolved in boiling chloroform and the acetone precipitation procedure was repeated.

Methods for Study of the Properties of Purified Poly-β-hydroxybutyrate

The melting point of PHB crystals was determined by slowly heating them in a capillary tube immersed in a mineral oil bath.

PHB was hydrolyzed to β-hydroxybutyric acid with anhydrous hydrazine and HCl according to Ottaway (90). Optical rotation of the hydrolytic product at 22° C was measured with a sodium lamp

as a light source. The hydrolytic product was tested as a substrate for β-hydroxybutyrate dehydrogenase, isolated from soybean nodule bacteroids, using the assay conditions discussed subsequently.

The isolated polymers were converted to crotonic acid with hot concentrated sulfuric acid (63). The absorption spectrum of the crotonic acid in concentrated sulfuric acid was recorded with a Cary Model 11 spectrophotometer.

Source and Assay of Enzymes

Nitrogenase

Activity was assayed by reduction of acetylene to ethylene. Soybean root nodules (0.5 g) were placed in 21-ml serum bottles. Two layers of cheesecloth wetted with 0.2 ml of water were placed in each bottle to supply moisture. Bottles were capped with rubber serum stoppers and incubated at 25° C for 30 minutes under 0.25 atm O_2, 0.65 atm argon and 0.1 atm acetylene. The reactions were started by the injection of acetylene and were stopped by adding 0.5 ml of 50% trichloroacetic acid with a hypodermic syringe. Ethylene was assayed by the method of Koch and Evans (58). The quantity of ethylene produced by the complete reaction was corrected for the quantity of ethylene formed in a control reaction which was stopped immediately after the addition of acetylene. Nitrogenase activity was

expressed as nmoles of ethylene formed per hour per g of nodules.

β-Hydroxybutyrate Dehydrogenase

Crude bacteroid extract containing β-hydroxybutyrate dehydrogenase activity was prepared by macerating soybean root nodules in 0.05 M Tris-Cl buffer pH 8.0 (4 ml of buffer for each g of nodules) by use of a mortar and pestle. The macerate was squeezed through four layers of cheesecloth and centrifuged at 300 x g for ten minutes to remove intact host cells and cell debris. The bacteroids were collected by centrifugation at 8,000 x g for 15 minutes, washed twice with 0.05 M Tris-Cl buffer pH 8.0 and resuspended in 0.025 M Tris-Cl buffer, pH 8.0 containing 5 mM $MgCl_2$ (5 ml of buffer for 1 g of bacteroids). The bacteroids were broken in the presence of 2 g levigated alumina per g of bacteroids using a mortar and pestle. A crude cell-free extract was obtained by centrifuging at 35,000 x g for 30 minutes. All steps were carried out at 0-4° C.

β-Hydroxybutyrate dehydrogenase activity was assayed in a mixture that contained, in μmoles: Tris-Cl (pH 8.0), 100; $MgCl_2$, 3; NAD^+, 1.2; sodium DL β-hydroxybutyrate, 20; and crude bacteroid extract containing about 0.2 mg of protein. The final volume of the reaction mixture was 3 ml. The reactions were started by adding enzyme, and the activity at 25° C was followed spectrophotometrically by measuring the increase in the absorbancy of NADH at 340 mμ with

a Cary Model 11 spectrophotometer.

Isocitrate Lyase

The first steps for preparation of bacteroid crude extract for assay of isocitrate lyase activity were the same as those for the preparation of β-hydroxybutyrate dehydrogenase. The entire method was based on that of Johnson et al. (52) with some modifications. After washing twice with 0.05 M Tris-Cl buffer, pH 8.0, the bacteroids were suspended in 0.025 M Tris-Cl buffer, pH 8.0 containing 0.01 M 2-mercaptoethanol and 0.1 mM Na_2 EDTA (5 ml of buffer for 1 g of bacteroids). After adding 0.2 g of cold levigated alumina per g of cells, the cell suspension was disrupted in a MSE sonicator. In order to minimize heating during sonication, the cell suspension was sonicated for two minutes in an ice bath, cooled to 1 °C and sonicated for another two minutes. Cell-free extracts were obtained by centrifugation at 35,000 x g for 30 minutes. Endogenous pyridine nucleotides were removed by stirring in 5 mg of activated charcoal per ml of crude extract for five minutes (20). Charcoal and adsorbed pyridine nucleotides were removed by centrifugation.

Isocitrate lyase activity was determined by the procedure of Daron and Gunsalus (23). The complete reaction mixture contained, in μmoles: Tris-Cl (pH 7.9), 200; $MgCl_2$, 6; cysteine-HCl, 4; sodium isocitrate, 15; and charcoal treated crude extract containing about

4 mg of protein. The final volume of the reaction mixture was 3.0 ml. Reactions were conducted under N_2 at 25° C in 21-ml serum bottles capped with rubber stoppers. Reactions were started by adding isocitrate, and were stopped after ten minutes by injecting 0.2 ml of 80% trichloroacetic acid with a hypodermic syringe. The precipitated protein was removed by centrifugation, and glyoxylate in the supernatant was determined colorimetrically as the 2, 4-dinitrophenylhydrazone. The absorbancy of the complete reaction mixture was corrected for the absorbancy of the reaction mixture lacking substrate.

Poly-β-hydroxybutyrate Depolymerase

The bacteroid residues used for assaying PHB depolymerase activity were prepared anaerobically according to Klucas et al. (56). After the bacteroids were broken in the French press, the ruptured cells were centrifuged at 48,000 x g for 50 minutes. The supernatant was discarded. The residue containing PHB granules and other debris was suspended in 0.05 M Tris-Cl buffer, pH 8.5 (1 ml of buffer for 1 g of residue) in air. This suspension exhibited PHB depolymerase activity. The pH of the suspension was maintained at 8.5 during incubation at 30° C by adding 0.5 M KOH. Samples (5 ml) were taken for β-hydroxybutyric acid assay. The first two samples were taken at 15-minute intervals and the subsequent samples were

taken at 30-minute intervals. Samples were placed immediately in a boiling water bath. The precipitated protein and PHB granules were removed by centrifugation. β-hydroxybutyric acid in the supernatant was determined by an enzymatic method in the presence of hydrazine and excess NAD^+ according to Williamson and Mellancy (116, p. 459-461).

Protein was determined by the biuret method (42). The micro-Kjeldahl method (110, p. 208) was used for nitrogen determination.

General Procedure for Study of the Relationship Between the Metabolism of Poly-β-hydroxybutyrate and Nitrogen Fixation in Soybean Root Nodules

For studies of changes in PHB content and changes in activities of nitrogenase, β-hydroxybutyrate dehydrogenase and isocitrate lyase in detached nodules, 28-day-old plants grown under the conditions described under "Source of Plant Materials" were used. The nodules were detached from the plants and washed twice in cold glass-distilled water to remove perlite and other debris. The excised nodules (100 g) were surface-sterilized by gently shaking for five minutes in one liter of 2% sodium hypochlorite solution. Subsequent manipulations were carried out under sterile conditions. The surface-sterilized nodules were washed with five liters of cold glass-distilled water, blotted dry with cheesecloth and placed in two 2-liter flasks. Moist cheesecloth was placed int the flasks to supply moisture. The flasks were

maintained at room temperature and flushed continuously with filtered air. Nodules were withdrawn at various intervals for assay of nitrogenase, β-hydroxybutyrate dehydrogenase and isocitrate lyase activities and for determination of PHB content.

For studies of changes in PHB content and in activities of the same three enzymes in nodules from soybean plants that had been incubated in the dark, 28-day old plants were again utilized. The plants were placed in a darkened growth chamber maintained at 27° C. The root medium in each pot was flushed daily with nitrogen-free nutrient solution (1) except on every fourth day when it was flushed with water. Nodules were removed from these plants at various intervals for the determination of PHB content and for assay of enzyme activities.

To study the changes in PHB content and in enzyme activities during the growth period of the soybean plants, nodules from plants of different ages grown under the conditions described in "Source of Plant Materials" were used.

RESULTS AND DISCUSSION

Some Chemical and Physical Properties of Poly-β-hydroxybutyrate

PHB was reported to occur in free-living Rhizobium species, in acetone-dried nodules from Mimoso pudica, and nodules of Pueraria species by Forsyth et al. (38). Alper et al. (6) have reported the molecular weight and melting point for PHB from an unidentified species of Rhizobium. However, no one has published conclusive evidence of the identity of the lipid granules isolated from soybean nodule bacteroids. The data in Table 1 indicate that the lipid granules isolated from Rhizobium japonicum bacteroids contained PHB. The melting point of the purified granules (Table 1) was within the range of values for authentic PHB (70). Law and Slepecky (63) found that PHB was hydrolyzed in hot concentrated sulfuric acid and that the product, β-hydroxybutyric acid, may be dehydrated to yield crotonic acid. They (63) also reported that crotonic acid in concentrated sulfuric acid has a characteristic absorption peak at 235 mμ. Bacteroid lipid granules, after heating in concentrated sulfuric acid, showed an absorption peak at 235 mμ. The lipid granules from soybean nodules were resistant to mild acid and base hydrolysis. Consequently, hydrazinolysis was used to break down the granules (90). Optical rotation of the hydrolytic

Table 1. A Summary of Some Properties of Purified Poly-β-hydroxybutyrate from Soybean Nodule Bacteroids.

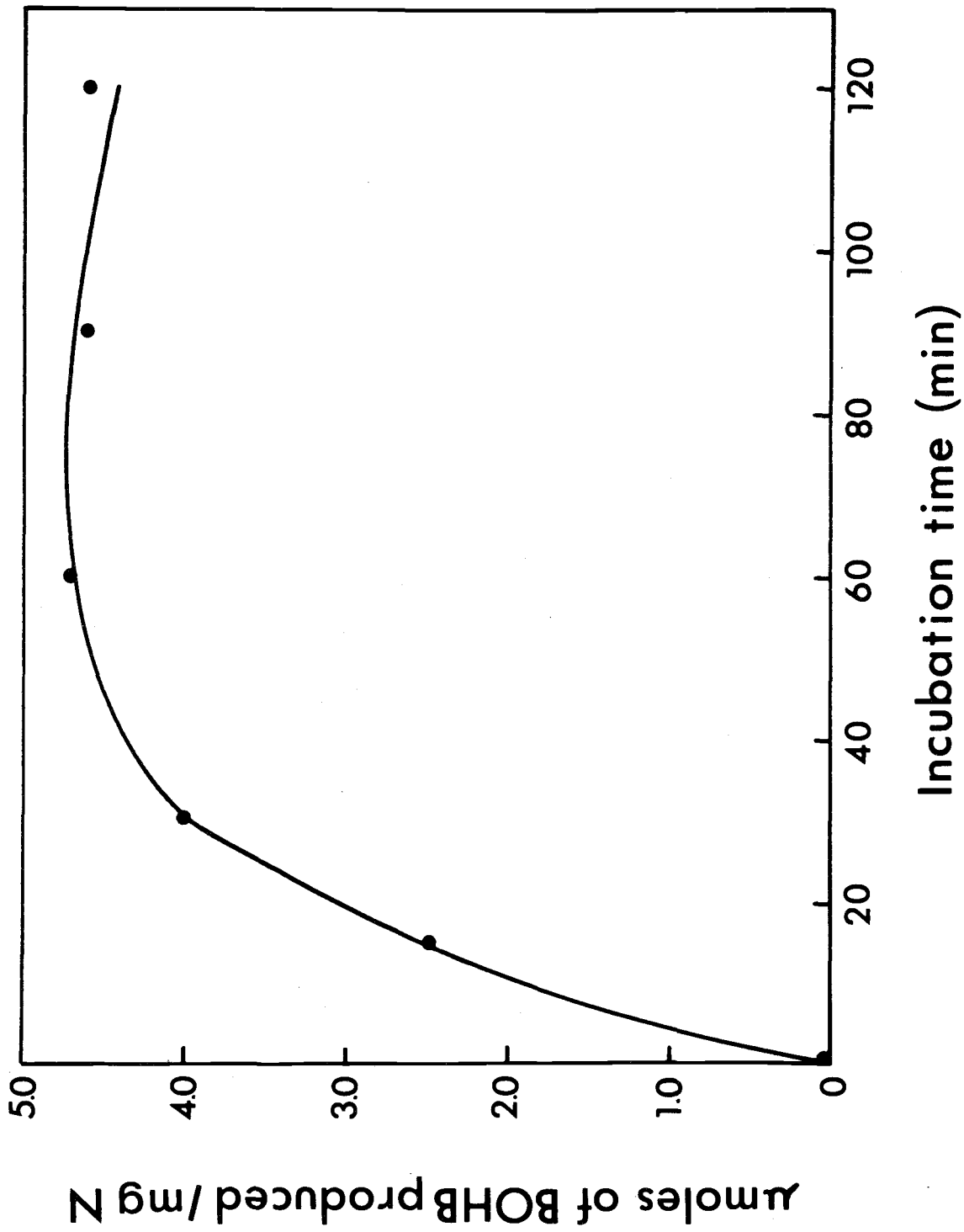
Properties	Observation
Solubility	Boiling chloroform
Melting point	169-170 ° C
$[\alpha]_{22}^{D}$ of hydrolytic product	-14.8 °
Absorption peak in conc. H_2SO_4 after heating	235 mμ
Hydrolytic product as a substrate for β-hydroxy-butyrate dehydrogenase	Active

product of the bacteroid lipid granules agreed closely with the values reported by Ottaway (90) and Lehninger (64) for β-hydroxybutyric acid. The hydrolytic product also was found to function as a substrate for β-hydroxybutyrate dehydrogenase from nodule bacteroids. These results provide conclusive evidence that the lipid granules isolated from the bacteroids were high in PHB content.

Poly-β-hydroxybutyrate Depolymerase Activity in Soybean Root Nodules

Cell-free extracts of Rhodospirillum rubrum contain a soluble as well as a particulate PHB depolymerase (79). The particulate system is responsible for the self-digestion of the native PHB granules. It was observed in our laboratory by Dr. Klucas and Dr. Koch that buffers of high ionic strength and pH were required for the preparation of nitrogenase extracts from bacteroids. If buffer systems of this type were not used, ruptured bacteroids produced acid (pH 4-5) and as a result enzymes were inactivated. Figure 1 provides evidence that at least part of the acidity was due to β-hydroxybutyric acid liberated from PHB by depolymerase. Samples taken after 30 minutes of incubation, for example, containing 6.5 mg of nitrogen per ml, showed a concentration of 0.03 M β-hydroxybutyric acid. The PHB depolymerase activity appeared to cease after 60 minutes of incubation (Figure 1). Although no systematic efforts were made to

Figure 1. Time course of poly-β-hydroxybutyrate depolymerase activity in nodule bacteroids. The experimental conditions were described under "Materials and Methods." "BOHB" is the abbreviation for β-hydroxybutyrate.


μmoles of BOHB produced /mg N
0
1.0
2.0
3.0
4.0
5.0
20
40
60
80
100
120
Incubation time (min)

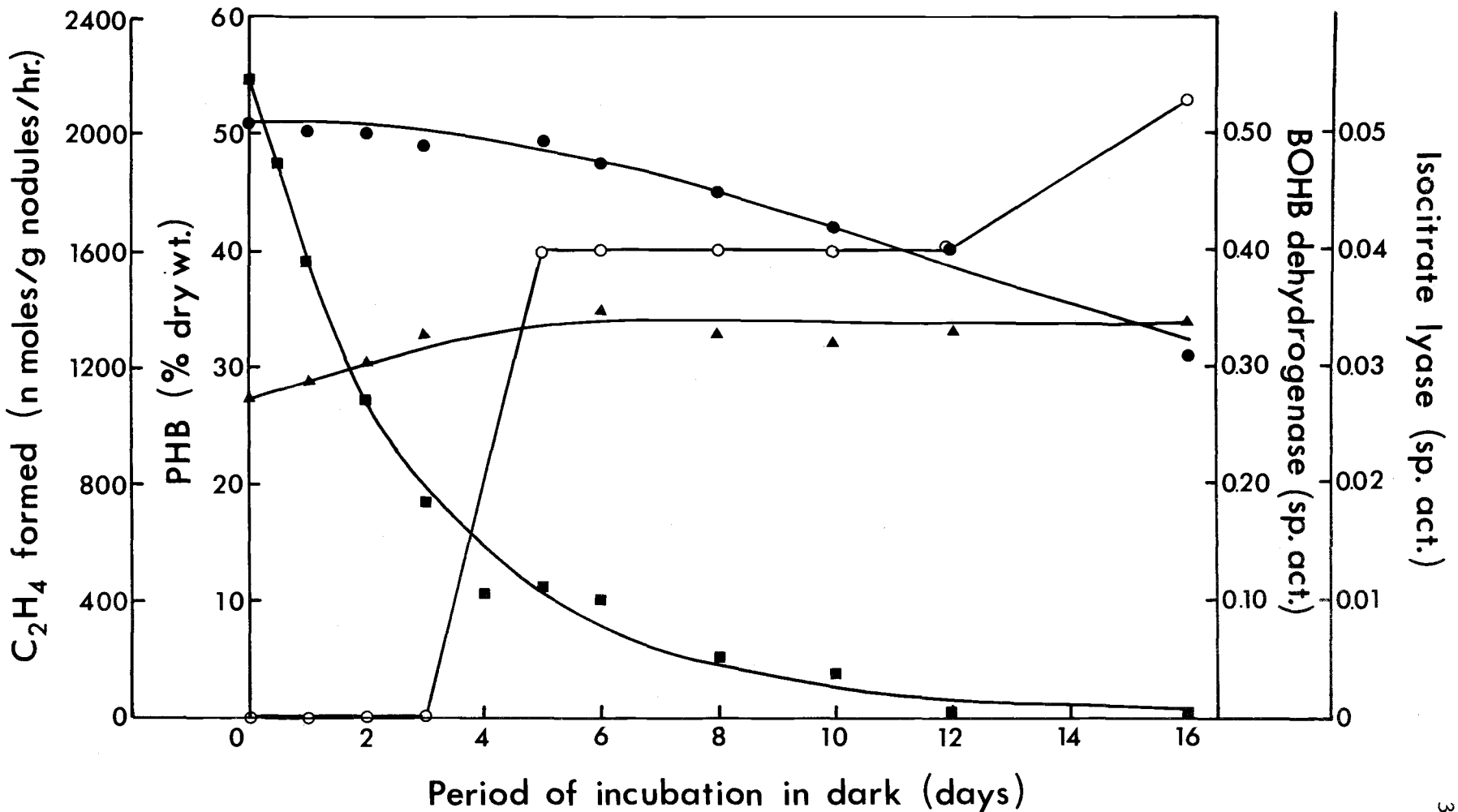
characterize the PHB depolymerase, the results (Figure 1) indicated that the depolymerase from bacteroids is associated with the particulate fraction of the ruptured cells.

The Relationship of Poly-β-hydroxybutyrate Metabolism to Nitrogen Fixation in Root Nodules from Soybean Plants Incubated in the Dark

The process of symbiotic nitrogen fixation requires a source of energy (ATP), a supply of reducing power and sufficient carbon skeletons for the incorporation of fixed ammonia into amino acids and amides. These requirements must be supplied directly or indirectly by the host plants. It is generally believed that the host plants supply the bacteroids with sucrose, which provides the substrates for generation of ATP, reducing power, and carbon skeletons. Since it has been found that as much as 50 percent of the dry weight of soybean nodules bacteroids is PHB, it was of interest to investigate the possibilities that this polymer may serve as a source of energy, reducing power, and carbon skeletons for nitrogen fixation particularly under conditions where the supply of carbohydrate from the host plants was no longer available.

When intact soybean plants were placed in a growth chamber in the dark, the nitrogenase activity of the nodules decreased very rapidly (Figure 2). After three days in the dark, the nitrogenase activity had decreased to about 37 percent of the activity observed

Figure 2. Changes in the activities of nitrogenase (■), β-hydroxybutyrate (BOHB) dehydrogenase (▲), isocitrate lyase (o), and in the poly-β-hydroxybutyrate content (●) of root nodules during maintenance of the intact soybean plants in the dark. The specific activities (sp. act.) of β-hydroxybutyrate dehydrogenase and isocitrate lyase are expressed as μmoles of NAD reduced per minute per mg of protein and μmoles of glyoxylate formed per ten minutes per mg of protein, respectively.


C_2H_4 formed (n moles/g nodules/hr.)
PHB (% dry wt.)
BOHB dehydrogenase (sp. act.)
Isocitrate lyase (sp. act.)
Period of incubation in dark (days)
2400
2000
1600
1200
800
400
0
60
50
40
30
20
10
0.50
0.40
0.30
0.20
0.10
0.05
0.04
0.03
0.02
0.01
0
0
2
4
6
8
10
12
14
16

originally. In contrast (Figure 2), the PHB content changed little, if any, after three days incubation. After five days incubation, the PHB content began to decrease more rapidly; however, at this time, the nitrogenase activity had decreased to 20 percent of the original activity. The appearance of isocitrate lyase activity after five days of incubation suggested that PHB may be converted to C-4 dicarboxylytic acids through the glyoxylate cycle. After 12 days of incubation, isocitrate lyase activity increased further. β-hydroxybutyrate dehydrogenase activity increased slowly up to the sixth day, but remained fairly constant thereafter.

From the results of this experiment, it appears that PHB alone cannot fulfill all the requirements for nitrogen fixation when the supply of carbohydrate ordinarily provided by the host plant has been interrupted.

Although there is insufficient experimental evidence to determine why nitrogenase activity decreases rapidly when the carbohydrate supply from the host plant is interfered with, the following rational seems worthy of discussion.

PHB metabolism may supply the bacteroids with sufficient energy and reducing power for nitrogen fixation, but the breakdown of this storage material may not supply the needed carbon skeletons at an appropriate time. From the high activity of PHB depolymerase (Figure 1), it seems clear that PHB in bacteroid may be rapidly

hydrolyzed to β-hydroxybutyric acid. Furthermore (Figure 2), the unusually high activity of β-hydroxybutyrate dehydrogenase, a key enzyme participating in the catabolism of PHB (26), shows that an active system is available for oxidation of β-hydroxybutyrate to acetoacetate with the concomitant reduction of NAD^+. The oxidation of NADH through oxidative phosphorylation could provide the ATP, and NADH *per se* may provide the reducing power for nitrogen fixation. Furthermore, acetoacetate may be broken down to acetyl-CoA, a product that may be oxidized through the tricarboxylic acid cycle to yield additional reducing power and ATP (99). Klucas and Evans (55) have demonstrated that NADH generated from the oxidation of β-hydroxybutyrate supported cell-free nitrogenase-dependent acetylene reduction provided that either benzyl viologen or methyl viologen, a diaphorase, and ATP generating system were present.

Pate *et al.* (91) in 1969 have reported that detached root nodules from broad bean (*Vicia faba* L.) could be induced to exude bleeding sap from the transport system. The excreted sap was collected, and its composition determined by an amino acid analyzer. The results revealed high concentrations of asparagine, aspartic acid, glutamine and glutamic acid and that these unusually high concentrations were restricted to conditions where the fixed nitrogen was being exported from the areas of the nodule where nitrogen fixation occurred. An experiment conducted by the author has revealed that bleeding sap

from soybean nodules contained asparagine, aspartic acid, glutamine and glutamic acid at concentration of 125, 29, 20, and 5 μmoles per ml of nodule bleeding sap, respectively. These data support the contention of Pate et al. (91) that carbon skeletons are essential for the transport of fixed nitrogen out of nodules.

If the carbon skeletons were unavailable, the fixed ammonia would be expected to accumulate sufficiently to repress the synthesis of nitrogenase. The evidence available does not indicate that ammonia per se is particularly inhibitory to nitrogenase activity. Since the addition of sucrose, fructose, glucose, pyruvate, succinate or malate to nodules, that exhibited low nitrogenase activity as a result of dark incubation, failed to stimulate nitrogenase activity, it is suggested that accumulated ammonia repressed the synthesis of nitrogenase rather than inhibited its activity. This conclusion is supported by the work of Oppenheim and Marcus (89) who observed that ammonia repressed nitrogenase formation in Azotobacter vinelandii within one hour after the addition of ammonia to culture medium.

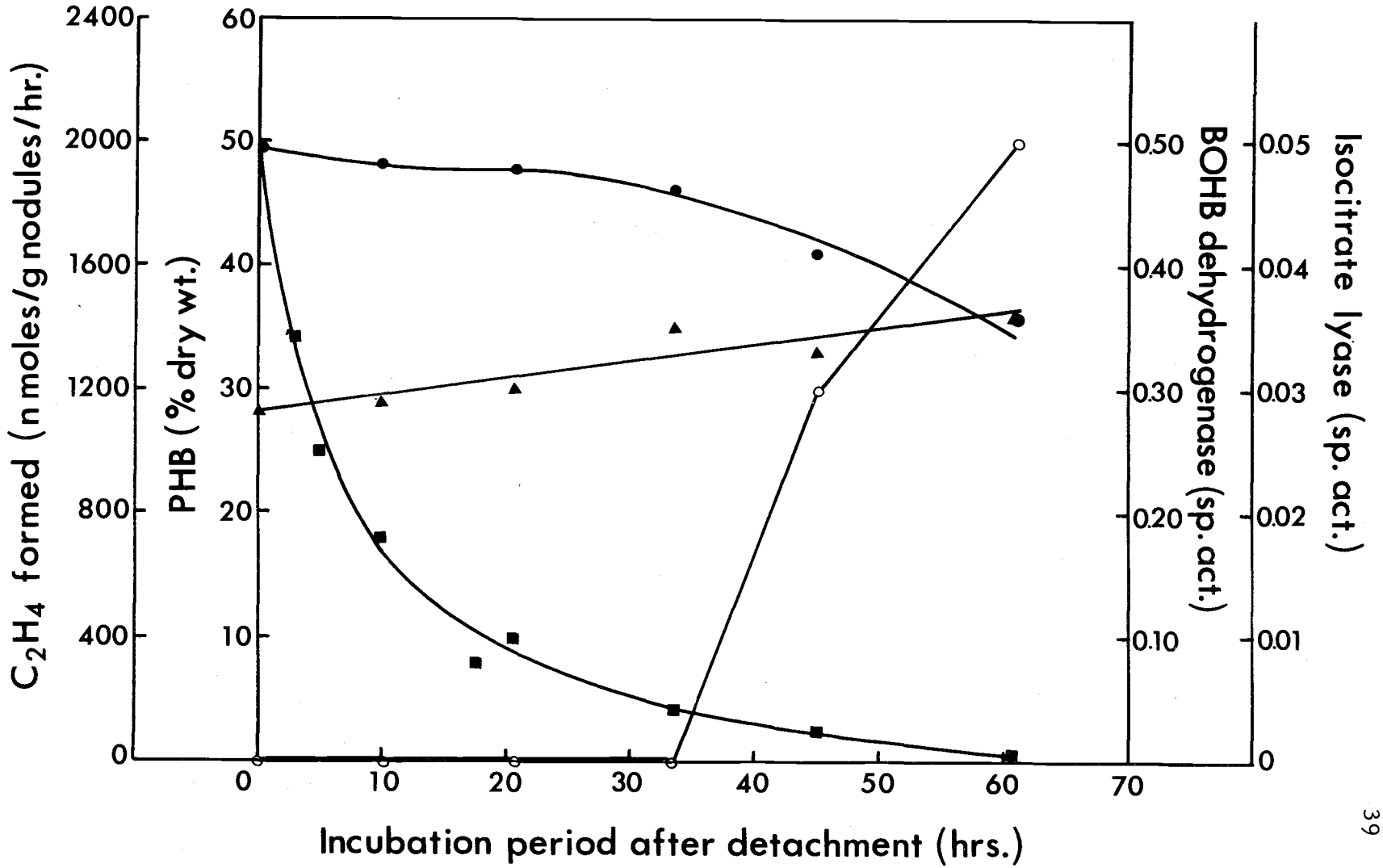
Johnson et al. (52) found no significant isocitrate lyase activity in soybean nodule bacteroids. Isocitrate lyase is one of the key enzymes of the glyoxylate cycle which provides a mechanism for synthesis C-4 intermediates of the tricarboxylic acid cycle from acetyl-CoA derived from acetate or fatty acit metabolism. C-4

intermediates also are essential carbon skeletons needed for the synthesis of amino acids and amides. Under conditions where bacteroids were sustained by PHB alone, no obvious mechanism for the maintenance of C-4 intermediates would be available unless isocitrate lyase were present and the glyoxylate cycle functional. Lack of C-4 carboxylic acids undoubtedly would lead to the accumulation of fixed ammonia, a product known to repress the nitrogenase synthesis. As indicated in Figure 2, isocitrate activity was not detected in bacteroids until the fifth day of incubation of plants in the dark. The isocitrate lyase activity apparently was induced by some unidentified control mechanisms related to the depletion of the carbohydrate supply from the host plants.

The Relationship of Poly-β-hydroxybutyrate Metabolism to Nitrogen Fixation in Detached Root Nodules

The supply of carbohydrate from the host plants to the bacteroids is severed when nodules are detached from the host plants. As shown in Figure 3, removal of nodules from the host plant resulted in changes in PHB content, nitrogenase, β-hydroxybutyrate dehydrogenase, and isocitrate lyase activities. The trends of these results were similar to those presented in Figure 2. As shown in Figure 3, PHB content of detached nodules did not decrease rapidly until 35 hours after detachment of nodules. The slight increase in the

Figure 3. Changes in the activities of nitrogenase (■), β-hydroxybutyrate (BOHB) dehydrogenase (▲), isocitrate lyase (o), and in the poly-β-hydroxybutyrate (PHB) content (●) of the detached nodules during incubation. The definitions of specific activities (sp. act.) of β-hydroxybutyrate dehydrogenase and isocitrate lyase are included in the legend of Figure 2.

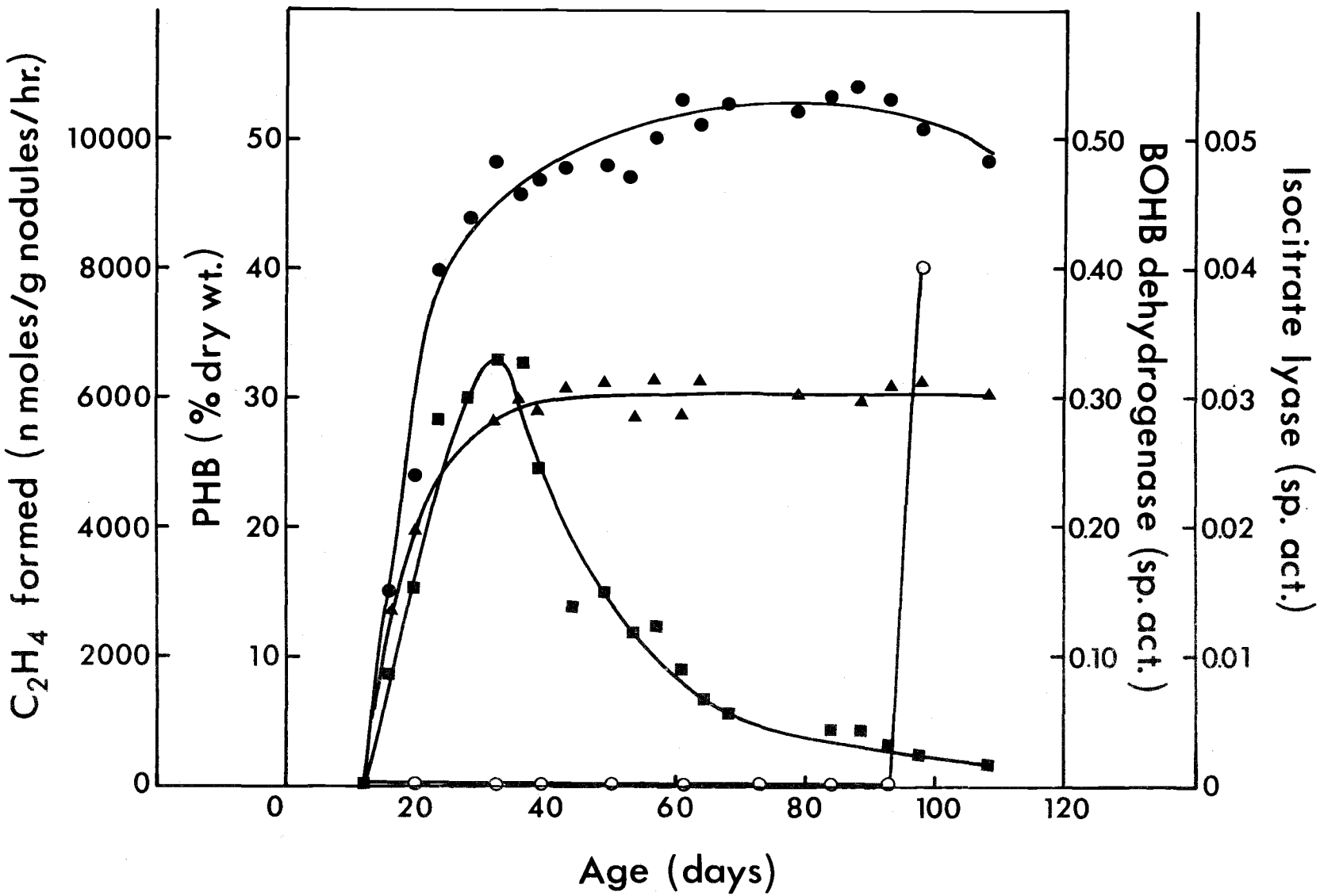

C_2H_4 formed (n moles/g nodules/hr.)
PHB (% dry wt.)
BOHB dehydrogenase (sp. act.)
Isocitrate lyase (sp. act.)
Incubation period after detachment (hrs.)
2400
2000
1600
1200
800
400
0
60
50
40
30
20
10
0.50
0.40
0.30
0.20
0.10
0.05
0.04
0.03
0.02
0.01
0
0
10
20
30
40
50
60
70

specific activity of β-hydroxybutyrate dehydrogenase suggests an increased rate of oxidation of β-hydroxybutyrate during the period when PHB was utilized. The sudden appearance of isocitrate lyase activity after 34 hours of incubation and the concomitant disappearance of PHB suggested the conversion of PHB to C-4 dicarboxylic acids through the glyoxylate cycle.

As indicated in Figure 3, again PHB utilization appeared not to be able to maintain the nitrogenase activity. Although nitrogenase activity deteriorated rapidly during the first ten hours after nodule excision, PHB content decreased only about one percent in this period. These results like those mentioned previously (Figure 2) provide no support for a postulated role of PHB as a substrate capable of supporting nitrogen fixation.

The Relationship of Poly-β-hydroxybutyrate Metabolism to Nitrogen Fixation Through the Growth Period of the Nodules

Parallel studies on the metabolism of PHB and nitrogen fixation of soybean nodules throughout the growth period were conducted in hopes of providing evidence regarding any possible interaction between the two processes. As shown in Figure 4, nitrogenase activity increased rapidly, reaching a maximum at 33 days after planting but fell equally rapidly after the maximum rate was attained. In contrast the PHB content of nodules increased strikingly up to 25 days.


C_2H_4 formed (n moles/g nodules/hr.)
PHB (% dry wt.)
BOHB dehydrogenase (sp. act.)
Isocitrate lyase (sp. act.)
Age (days)
0
2000
4000
6000
8000
10000
10
20
30
40
50
0.10
0.20
0.30
0.40
0.50
0.01
0.02
0.03
0.04
0.05
0
20
40
60
80
100
120

After this PHB increased more gradually until plants reached an age of 80 days. After plants were 85 days old, the PHB content decreased to some extent. Again, isocitrate lyase activity was detected at about the time when PHB began to appear. These data suggest that the bacteroids were sustained by PHB after carbohydrate was limited by plant senescence. These data (Figure 4) again indicate no direct interaction between PHB metabolism and nitrogen fixation.

A more direct role of PHB does emerge from the data shown in Figures 2, 3 and 4. It appears that the bacteroids are not sustained on PHB as a source of energy, reducing power, and carbon skeletons until the carbohydrate supplies are exhausted. Almon (5) reported that the bacteroids were unable to divide and revert to free-living forms. These findings, however, have been challenged (personal communication with Dr. Parker and Dr. Evans). Perhaps, PHB serves as an energy source for free-living Rhizobium when they are released from nodules and when they are independent of their host. If further experiments prove that bacteroids also can revert to viable Rhizobium cells, then PHB also may serve as a storage form of energy for these cells.

PART II. PURIFICATION AND SOME PROPERTIES OF β-HYDROXYBUTYRATE DEHYDROGENASE FROM *RHIZOBIUM JAPONICUM* BACTEROIDS

MATERIALS AND METHODS

Chemicals

The sources of the chemicals used were as follows: sodium DL-β-hydroxybutyrate, NAD^+, NADH, $NADP^+$, Tris buffer, p-chloromercuribenzoate, p-hydroxymercuribenzoate, p-chloromercuribenzene-p-sulfonate, and streptomycin sulfate (Sigma Chemical Corporation, St. Louis, Missouri); polypropylene glycol (P-400), acetoacetate and glycine (Matheson, Coleman and Bell, Cincinnati, Ohio); enzyme grade ammonium sulfate (Mann Research Laboratories, New York, N. Y.); Bio-gel P-100 (Bio-Rad Laboratories, Richmond, Calif.); DEAE-cellulose (DE-32, Reeve Angel-Scientific Division, Clifton, N. J.); and MES, TES, and HEPES (Calbiochem, Los Angeles, Calif.) All other chemicals used were reagent grade or the highest grade available and obtained through usual commercial sources.

Source of *Rhizobium japonicum* Bacteroids

Rhizobium japonicum bacteroids were isolated from soybean (*Glycine max* Merr. Var. Chippewa) root nodules. Plants inoculated

with a commercial strain of Rhizobium japonicum (kindly supplied by Dr. Joe Burton of the Nitragen Co.) were cultured in a greenhouse or in a growth chamber in pots of perlite supplied with a nitrogen-free nutrient solution. The perlite was sterilized in an autoclave at 15 pounds per square inch for four hours before being utilized. Pots were flushed daily with nitrogen-free nutrient solution (1) and every fourth day with water to remove any accumulated salts. The plants were supplied with supplemental light (about 600 ft candles at three feet) for 16 hours each day. When plants were 30-35 days old, they were removed from the pots, and nodules were harvested and washed in cold tap water. Within an hour after excision, the nodules were used for the preparation of extracts or were frozen and stored at -70° C.

Determinations

Sodium D(-)-β-hydroxybutyrate was determined by use of β-hydroxybutyrate dehydrogenase in presence of excess NAD^+ as described by Williamson and Mellanby (116, p. 459-461). Freshly redistilled acetoacetate was standardized manometrically (110, p. 198-199) and stored at -70° C. NADH was assayed by use of the extinction coefficient of 6.22×10^3 M^{-1} cm^{-1} at 340 mμ (50). NAD^+ was determined by use of the extinction coefficient of 17.8×10^3 M^{-1} cm^{-1} at 259 mμ (98). Protein concentration was determined by the

method of Lowry et al. (69) and by absorbancy measurements at 260 and 280 mμ (112).

Standard Assay Conditions

Enzyme activity at 25° C was assayed by following the reduction of NAD^+ spectrophotometrically at 340 mμ using a Cary Model 11 spectrophotometer. The standard assay mixture in a final volume of 3 ml, contained in μmoles: Tris-Cl buffer (pH 8.0) 100; $MgCl_2$, 3; NAD^+, 1.2; sodium DL-β-hydroxybutyrate, 20. Reactions were initiated by adding enzyme and the rates, which remained linear, were followed for two minutes. One unit of enzyme was defined as the amount that catalyzed the reduction of 1 μmole of NAD^+ per minute under the conditions of the standard assay.

Purification of the Enzyme

Freshly harvested or frozen soybean root nodules were macerated in 0.05 M Tris-Cl buffer pH 8.4 (2 ml buffer for each 1 g of nodules) by use of an Omnimixer operated at top speed for two minutes. This and the subsequent steps were conducted at 0-4° C. The macerate was squeezed through four layers of cheesecloth and centrifuged at 300 x g for ten minutes to remove cell debris. The bacteroids were collected by centrifugation at 8,000 x g for 15 minutes and were washed twice with 0.05 M Tris-Cl buffer at pH 8.4. The

washed bacteroids were resuspended in 0.05 M Tris-Cl buffer, pH 8.0 containing 0.01 M $MgCl_2$ (one volume of buffer per g of bacteroids) and were ruptured with an Aminco French press at a pressure of 16,000 pounds per square inch. After breaking the cells, another volume of buffer was added and then after stirring for ten minutes, the crude extract was collected by centrifugation at 48,000 x g for 50 minutes. To 1 ml of crude extract, 4 mg of streptomycin sulfate (in a 5% solution at pH 8.2) were added slowly with stirring to partially remove nucleic acids. The precipitate was collected by centrifugation at 40,000 x g for 15 minutes and discarded. Polypropylene glycol was added slowly with stirring to the supernatant fluid at a concentration of 75 ml for each 100 ml of extract. The precipitate was collected by centrifugation at 40,000 x g for 15 minutes and was dissolved in 0.05 M Tris-Cl buffer, pH 8.0 containing 0.01 M $MgCl_2$ in a volume equivalent to half the volume of crude extract. Undissolved proteins were removed by centrifugation. To 100 ml of this solution, 35 ml of polypropylene glycol was added, centrifuged to remove precipitated protein, and another 42 ml of polypropylene glycol per 100 ml of solution was added. The pelleted protein was dissolved in 0.05 M Tris-Cl buffer, pH 8.0 with 0.01 M $MgCl_2$ in a volume equivalent to half the volume of crude extract, and was further purified by collecting a fraction of protein precipitated between 40 and 60 percent ammonium sulfate saturation. The precipitated protein

was dissolved in 0.025 M Tris-Cl buffer, pH 7.0 containing 5 mM $MgCl_2$ (a volume equivalent to 10% of the volume of crude extract). The solution was applied directly to a Bio-Gel P-100 column (2.5 x 45 cm) which previously had been equilibrated with 1.1 of 0.025 M Tris-Cl buffer, pH 7.0 containing 5 mM $MgCl_2$. The column was eluted with same buffer at rate of 0.5 ml per minute. Fractions of 3.2 ml were collected, and the most active fractions (18 through 23) were combined. The pooled eluate was further purified by a DEAE-cellulose column (2 x 20 cm) which also had been equilibrated with 0.025 M Tris-Cl buffer, pH 7.0 containing 5 mM $MgCl_2$. The column was eluted with the same buffer at 0.5 ml per minute, and fractions of 2 ml were collected. The most active fractions (70 through 77) were pooled. The purified enzyme was frozen and stored in liquid nitrogen until used.

RESULTS AND DISCUSSION

Enzyme Purification

The result of a typical purification is shown in Table 2. The final step indicates a 108-fold purification and a 21% yield. Rhizobium japonicum bacteroids are an excellent source of this enzyme. The specific activities of crude extracts from Rhodopseudomonas spheroides (12) and Rhodospirillum rubrum (97) are no more than 0.11, whereas the specific activity of crude extracts from bacteroids routinely is around 0.20. The specific activity of 21.7 of the most purified bacteroid enzyme (Table 2) may be compared with a specific activity of 17.2, which is the highest value reported (12) in the literature. The most purified preparation (step 7, Table 2) was examined by polyacrylamide gel electrophoresis by the method of Davis (24). Five protein bands were detected, three of which exhibited β-hydroxybutyrate dehydrogenase activity in the nitro blue tetrazolium assay (37). From the intensities of the bands showing activity, it was estimated that the preparation was no more than 70 percent pure.

Throughout the enzyme purification procedure NAD^+ was used as the cofactor in the assay. (See Materials and Methods.) A series of experiments in which $NADP^+$ was utilized in place of NAD^+ consistently gave negative results.

Table 2. Summary of the Purification* of β-Hydroxybutyrate Dehydrogenase

	Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Enzyme yield (%)	Purification
1.	Crude extract	290	1433	0.20	100	--
2.	Streptomycin sulfate ppt.	269	1016	0.27	93	1.3
3.	First polypropylene glycol ppt.	249	513	0.48	86	2.4
4.	Second polypropylene glycol ppt.	218	121	1.80	75	8.9
5.	Ammonium sulfate ppt., 40-60%	172	71	2.44	59	12.1
6.	Bio Gel P-100 column eluate	130	35	3.76	45	18.6
7.	DEAE-cellulose column	61	3	21.70	21	108.0

*Details of the purification are discussed in the text.

Properties of the Purified Enzyme

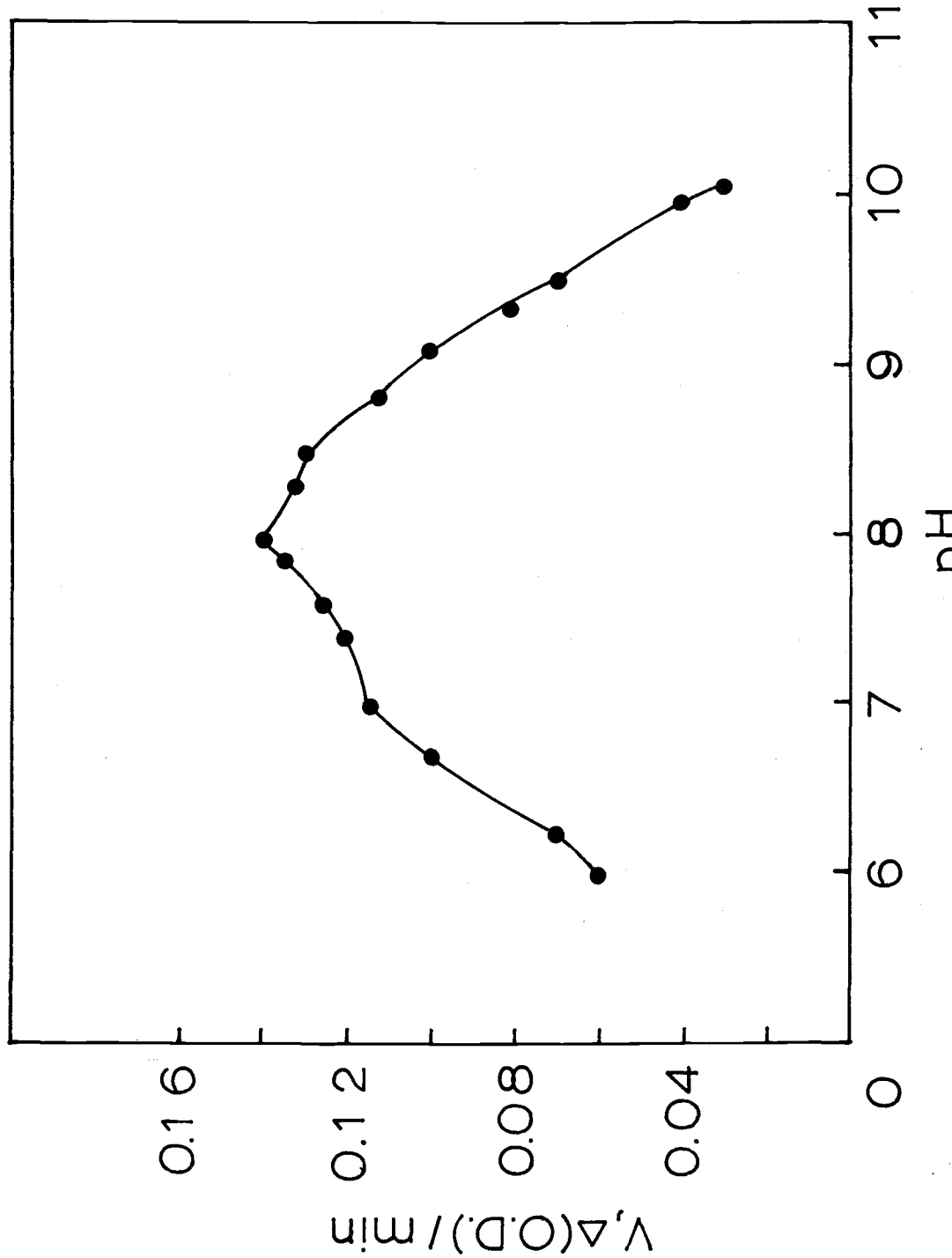
Optimum pH

Tris, TES, HEPES, MES and histidine were used as buffers for the determination of pH optimum of the β-hydroxybutyrate dehydrogenase. None of these buffers appeared to inhibit the enzyme appreciably as indicated by the observation that essentially the same activities were obtained with different buffers at the same pH. Optimum activity (Figure 5) was observed between pH 7.0 and 8.5. Enzyme activity decreased rapidly above pH 8.5 and below pH 7.0.

Stability

The purified enzyme (0.18 mg protein per ml) in 0.025 M Tris-Cl buffer at pH 7.0 containing 5 mM $MgCl_2$ was stable for at least four days when in an ice bath. When frozen and stored in liquid nitrogen, no loss of activity was observed after one month. The bacteroid enzyme like β-hydroxybutyrate dehydrogenase from several other sources, (12, 53, 94, 97) requires Mg^{++} ions for stability. In an experiment in which ions were removed from the purified enzyme by use of a Bio-Gel column, the specific activity of the enzyme decreased 50 percent within five minutes and was completely inactivated after three hours in an ice bath.

Figure 5. Effect of pH on β-hydroxybutyrate dehydrogenase activity. The composition of the reaction mixtures and the procedure for enzyme assay were described under "Standard Assay Conditions" with the exceptions that the pH of the reaction mixtures varied as indicated and the buffer system was modified as follows: in reactions at pH values ranging from 6.0 to 6.7, 100 μmoles of MES were added; in reactions from 6.7 to 8.3, 100 μmoles of TES or HEPES were added; in reactions ranging between 7.4 to 9.3, 100 μmoles of Tris were added, and in reactions between 9.1 to 10, 100 μmoles of histidine were utilized.


V, Δ(O.D.) / min
0.16
0.12
0.08
0.04
0
6
7
8
9
10
11
pH

Inhibition by Glycine

As shown by the results in Table 3, the enzyme is inhibited by glycine. The concentration of glycine required to exhibit appreciable inhibition was rather high; however, the effect was more pronounced as the pH of the assay mixture increased. As indicated in Figure 6, 0.05 M concentration of glycine inhibited the enzyme activity 29 percent at pH 8.0, and 71 percent at pH 9.1. No inhibition was observed at pH 7.5 or below. These data suggest that the anionic form of glycine was responsible for the inhibition. In an investigation of other inhibitors it was found that glutamic acid, glutamine, histidine, alanine, glycinamide, arginine and NH_4^+ had no effect on the enzyme, whereas glycylglycine inhibited about the same magnitude as glycine.

Inhibition by Thiol Reagents

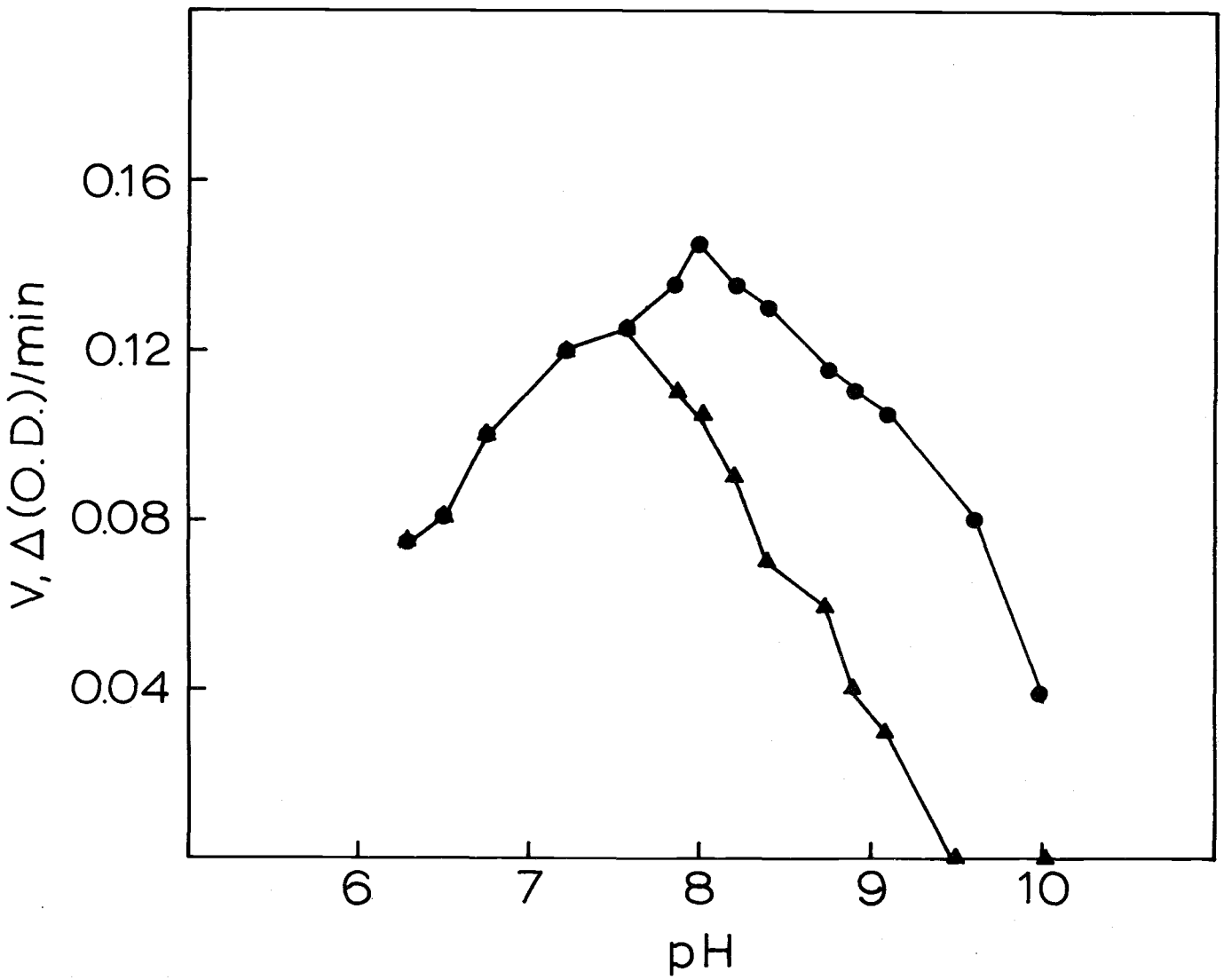
Bergmeyer and coworkers (12) reported that β-hydroxybutyrate dehydrogenase purified from Rhodopseudomonas spheroides was extremely sensitive to a number of thiol inhibitors, especially those of the mercurial type. In contrast, Shuster et al. (97) reported that the same enzyme from Rhodospirillum rubrum exhibited no sensitivity toward thiol inhibitors. β-Hydroxybutyrate dehydrogenase from R. japonicum bacteroids was (Table 4) highly sensitive toward

Table 3. Effect of Glycine on the Activity of β-Hydroxybutyrate Dehydrogenase

Concentration of glycine in reaction mixtures (mM)	Inhibition (%)
0	0
2.5	3
5.0	9
25.0	24
50.0	46

The composition of reaction mixtures and the experimental procedures for the enzyme assay were the same as described under "Standard Assay Conditions" with the exceptions that $MgCl_2$ was omitted, and glycine was added. The concentrations of glycine were varied as indicated. The reactions were initiated by the addition of enzyme (4 μg of protein in 0.1 ml of 0.025 M Tris-Cl buffer, at pH 7.0 containing 1 mM $MgCl_2$).

Figure 6. Effect of pH (●) and effect of glycine at various pH values (▲) on β-hydroxybutyrate dehydrogenase activity. The composition of the reaction mixtures and the procedure for enzyme assay were described under "Standard Assay Conditions" but with some modifications. In addition to the modification described in Figure 5, the following changes were made: $MgCl_2$ was omitted from the reaction mixtures. For study of the effect of glycine, 150 μmoles of twice crystallized glycine were added. The reactions were initiated by the addition of enzyme (4 μg of protein in 0.1 ml of 0.025 M Tris-Cl buffer, at pH 7.0 containing 1 mM $MgCl_2$).


0.16
0.12
0.08
0.04
V, Δ (O.D.)/min
6
7
8
9
10
pH

mercuric acetate but not toward other thiol inhibitors. It was observed that the inhibition of the enzyme by mercuric acetate was prevented by preincubating the enzyme with divalent cations, NAD^+ or NADH but not with β-hydroxybutyrate. The effectiveness of protective agents listed in decreasing order was as follows: NAD^+, Mn^{++}, NADH, Mg^{++}, and Ca^{++} (Table 5). Co^{++} and Zn^{++} inhibited the enzyme activity.

Kinetics and Mechanisms of Reaction

The kinetics of enzyme reactions involving two substrates has been developed by Alberty (2). For those two-substrate enzymic systems having the general reaction $A + B \rightleftharpoons C + D$ in which both substrates interact with the enzyme before dissociation of either product, the following rate equations developed by Alberty should be obeyed (2).

$$V_f/v = 1 + K_A/[A] + K_B/[B] + K_{AB}/[A][B] \quad (1)$$

$$V_r/v = 1 + K_C/[C] + K_D/[D] + K_{CD}/[C][D] \quad (2)$$

A and B are substrates involved in the forward reaction and C and D are substrates involved in the reverse reaction. NAD^+, β-hydroxybutyrate, NADH and acetoacetate are the corresponding substrates for β-hydroxybutyrate dehydrogenase reaction. K_A, K_B, K_C, and K_D are the corresponding apparent Michaelis constants.

Table 4. Inhibition of β-Hydroxybutyrate Dehydrogenase by Thiol Inhibitors

Thiol inhibitor	Final conc. (μmoles/3 ml)	Inhibition (%)
Mercuric acetate	0.01	20
	0.06	68
	0.10	100
Cadmium chloride	0.10	0
	0.50	27
	1.00	27
Sodium arsenite	0.10	0
	1.00	0
	5.00	0
p-Chloromercuribenzoate	0.10	0
	1.00	0
	10.00	60
p-Hydroxymercuribenzoate	0.50	0
	1.00	40
	10.00	90
p-Chloromercuribenzene-sulfonate	0.50	0
	1.00	30
	5.00	90
Potassium acetate*	0.10	0

The composition of reaction mixtures and the experimental procedures for the enzyme assay were the same as described under "Standard Assay Conditions" with the exceptions that $MgCl_2$ was omitted, and thiol inhibitors were added. The concentrations of thiol inhibitors in the reaction mixtures were varied as indicated. The reactions were initiated as described in Table 3.

*Potassium acetate was tested in order to confirm the observation that the mercuric ions and not acetate ions were responsible for the inhibition.

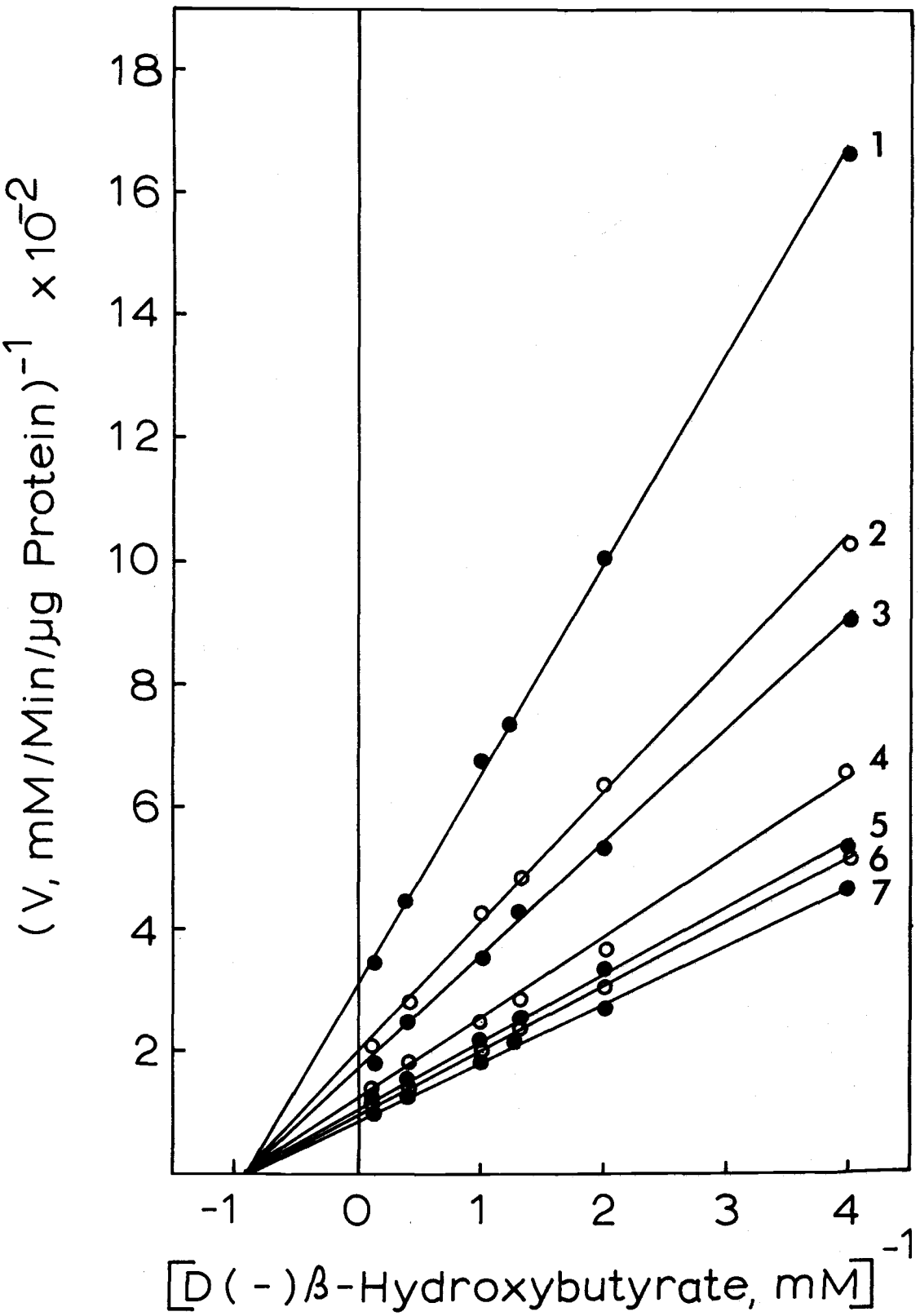
Table 5. Protection of β-Hydroxybutyrate Dehydrogenase Against Inactivation by Mercuric Acetate

Protective agents	Final concentration (mM)	Inhibition (%)
Calcium chloride	3	50
	2	64
	0.50	86
	0.25	100
	0	100
Magnesium chloride	1	0
	0.50	25
	0.25	54
	0.10	75
	0	100
Manganese chloride	1	0
	0.50	15
	0.25	15
	0.10	40
	0	100
NAD^+	1	0
	0.50	0
	0.25	17
	0.10	42
	0.05	100
	0	100
NADH	0.25	32
	0.05	56
	0	100

The composition of the reaction mixtures and the experimental procedures for the enzyme assay were the same as described under "Standard Assay Conditions" with the following modifications: $MgCl_2$ was omitted from the standard assay mixtures, and the protective agents were added. The concentrations of the protective agents were varied as indicated. Enzyme (4 μg of protein in 0.1 ml of 0.025 M Tris-Cl buffer, at pH 7.0 containing 1 mM $MgCl_2$) was added to the reaction mixtures. After 30 seconds of incubation at 25° C, 0.3 μ moles of mercuric acetate was added. The reactions were initiated by the addition of NAD. When NAD was used as the protective agent, the reactions were initiated by the addition of β-hydroxybutyrate.

K_{AB} is defined as the product of the dissociation constant of A and Michaelis constant K_B; similarly, K_{CD} is product of the dissociation constant of C and the Michaelis constant K_D. V_f and V_r are the maximal reaction velocities for the forward and reverse reactions, respectively.

These constants can be calculated from double reciprocal plots (68) using kinetic data obtained through studies in which one substrate is varied while the second is held constant at various levels. This graphical analysis method was developed by Vestling (111, vol. 5, p. 137-173). Experiments were performed to determine the various kinetic constants for the β-hydroxybutyrate dehydrogenase reaction. The double reciprocal plots for a series of experiments in the forward direction where D(-)-β-hydroxybutyrate was varied at several concentrations of NAD^+ are shown in Figure 7. Similar data obtained from experiments in which the NAD^+ concentration was varied at different levels of β-hydroxybutyrate are presented in Figure 8. Figures 9 and 10 depict the double-reciprocal plots for the reverse reaction. In order to calculate the kinetic constants, secondary graphs were made which involve plots of reciprocal apparent maximal velocities (Figures 7 through 10) versus reciprocals of concentrations of substrates (i.e., NAD^+, β-hydroxybutyrate, NADH and acetoacetate) which were held constant at a series of different concentrations. Figure 11 shows a secondary plot where the reciprocals


(V, mM/Min/µg Protein)$^{-1}$ x 10^{-2}
18
16
14
12
10
8
6
4
2
-1
0
1
2
3
4
[D(-)ß-Hydroxybutyrate, mM]$^{-1}$
1
2
3
4
5
6
7

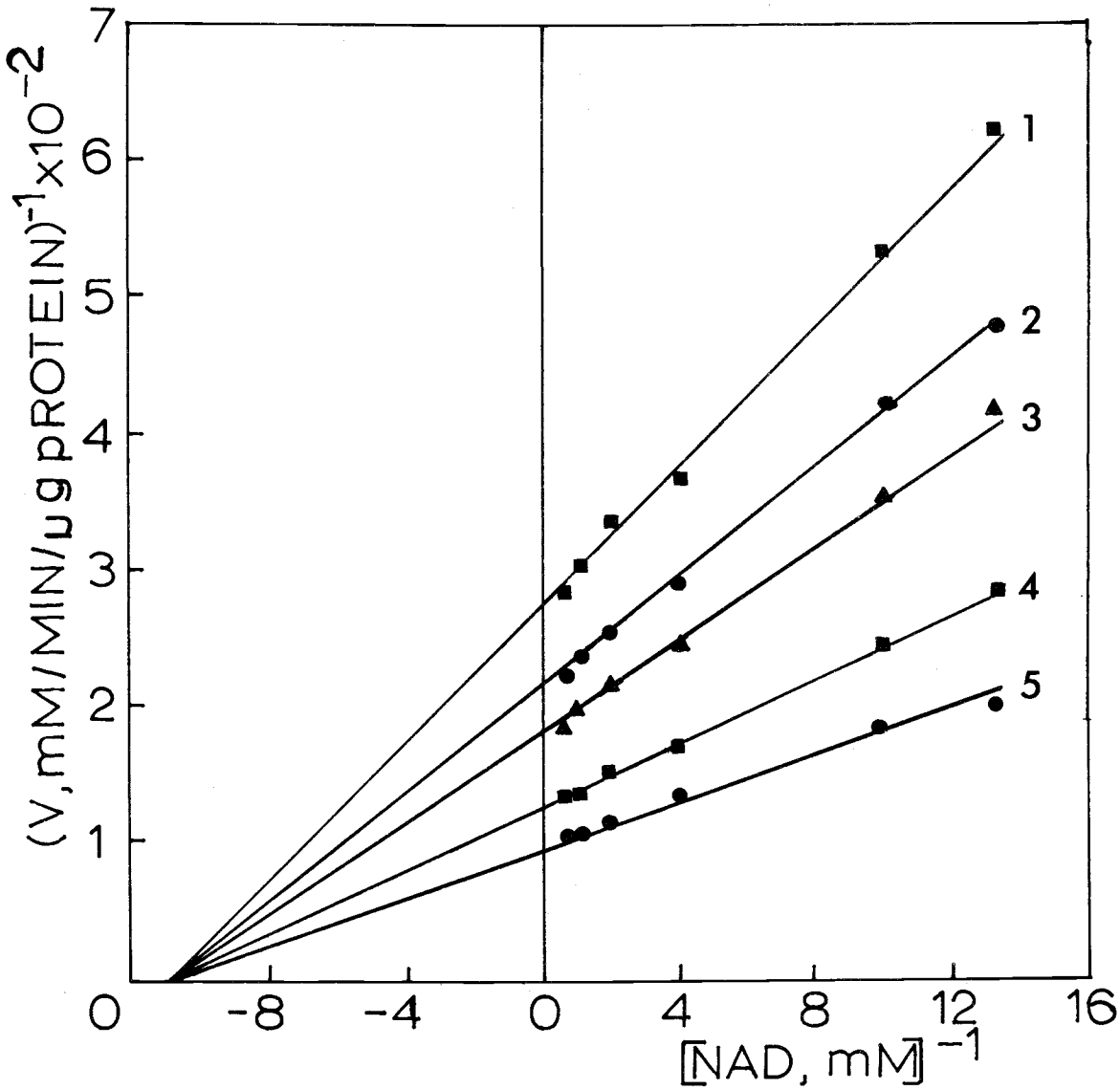


Figure 8. Plot of reciprocals of initial reaction velocities (1/v) versus reciprocals of millimolar concentrations of NAD^+. D(-)-β-hydroxybutyrate concentrations were maintained at 0.50 mM (curve 1), 0.75 mM (curve 2), 1.00 mM (curve 3) 2.50 mM (curve 4), and 7.50 mM (curve 5). Velocity (v) was determined as a function of NAD concentrations which was varied in the range from 7.50×10^{-2} mM to 150.00×10^{-2} mM. Velocity (v) is expressed as in Figure 7. The composition of reaction mixtures and other experimental procedures were the same as described under "Standard Assay Conditions" with the exceptions of Tris-Cl buffer at pH 8.4 was used; NAD and D(-)-β-hydroxybutyrate concentrations were varied as indicated.

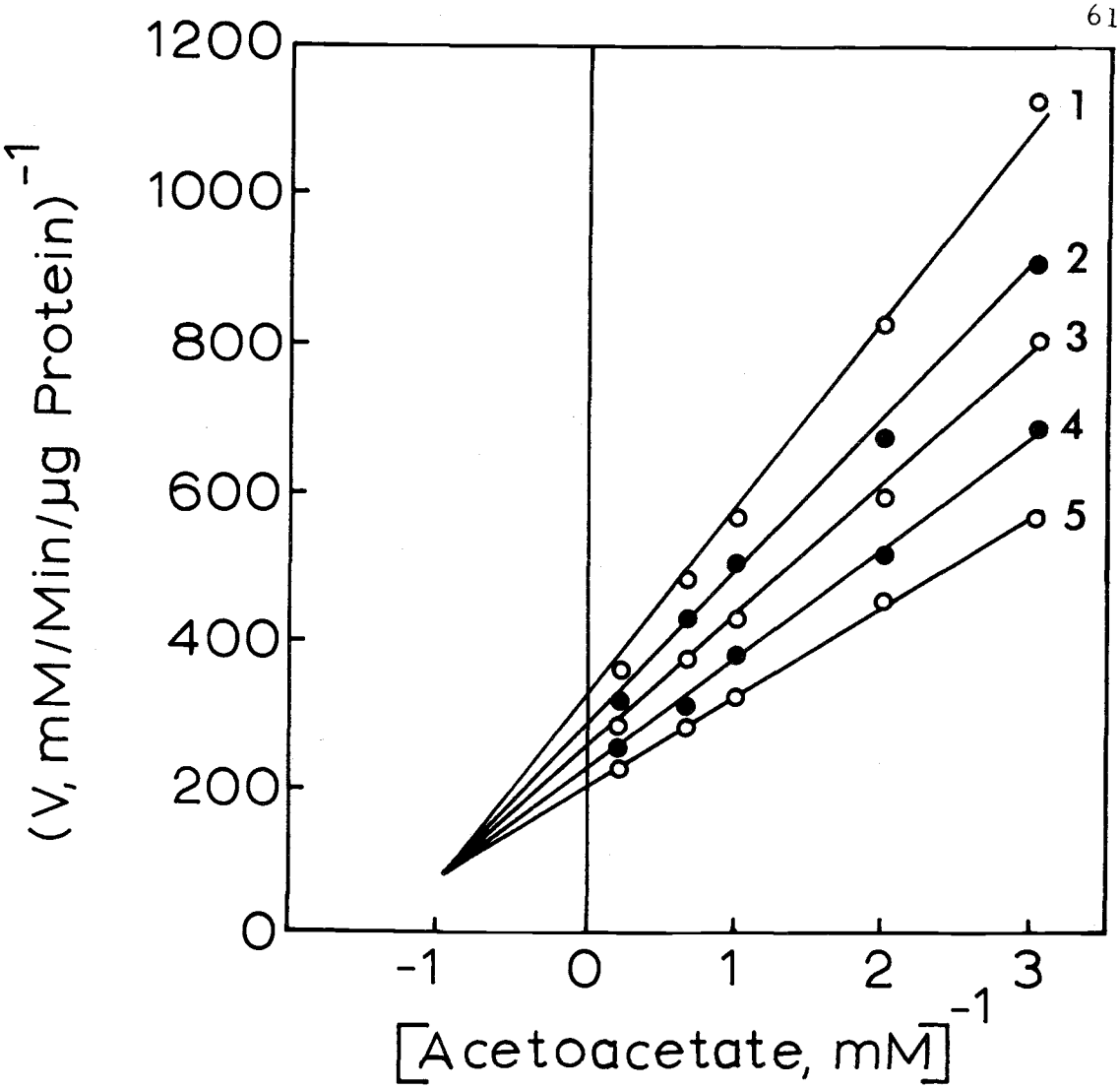
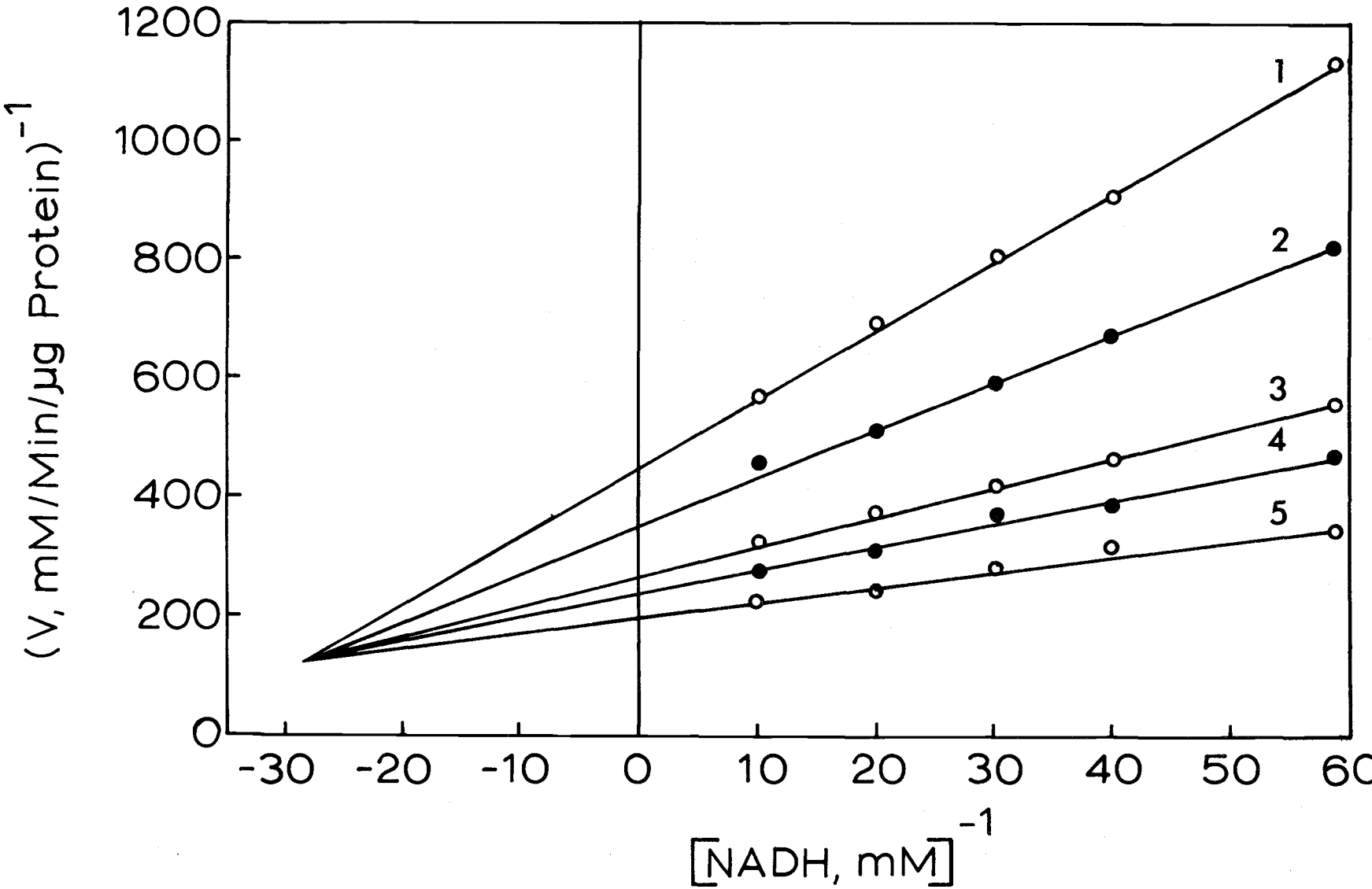


Figure 9. Plot of reciprocals of initial reaction velocities (1/v) versus reciprocals of millimolar concentrations of acetoacetate. NADH concentrations were held constant at 1.70×10^{-2} mM (curve 1), 2.50×10^{-2} mM (curve 2), 3.30×10^{-2} mM (curve 3), 5.00×10^{-2} mM (curve 4), and 10.00×10^{-2} mM (curve 5). Velocity (v) was determined as a function of acetoacetate concentration, which was varied in the range from 0.33 mM to 4.50 mM. Velocity (v) is expressed as the millimolar concentration of NADH oxidized in the reaction mixture per minute per µg of protein. The composition of reaction mixtures and other experimental procedures were the same as described under "Standard Assay Conditions" with the exceptions that Tris-Cl buffer at pH 8.4 was used, and D(-)-β-hydroxybutyrate and NAD were replaced by acetoacetate and NADH, respectively. The concentrations of acetoacetate and NADH were varied as indicated.

Figure 10. Plot of reciprocals of initial reaction velocities (1/v) versus reciprocals of millimolar concentrations of NADH. Acetoacetate concentrations were held constant at 0.33 mM (curve 1), 0.50 mM (curve 2), 1.00 mM (curve 3), 1.50 mM (curve 4), and 4.50 mM (curve 5). Velocity (v), expressed as in Figure 9, was determined as a function of NADH concentration, which was varied in the range from 1.70×10^{-2} mM to 10.00^{-2} mM. The composition of reaction mixtures and other experimental procedures were the same as described under "Standard Assay Conditions with the exceptions of Tris-Cl buffer at pH 8.4 was used; D(-)-β-hydroxybutyrate and NAD were replaced by acetoacetate and NADH, respectively. The concentrations of acetoacetate and NADH were varied as indicated.


1200
1000
800
600
400
200
0
(V, mM/Min/µg Protein)$^{-1}$
-30
-20
-10
0
10
20
30
40
50
60
[NADH, mM]$^{-1}$
1
2
3
4
5

of apparent maximal velocities (y-axis intercepts of Figure 7) are plotted versus the reciprocals of NAD^+ concentrations in Figure 7. Similarly, Figures 12, 13, and 14 represent analogous secondary plots of data from Figures 8, 9, and 10, respectively. These secondary plots were used to evaluate the kinetic constants which are summarized in Table 6.

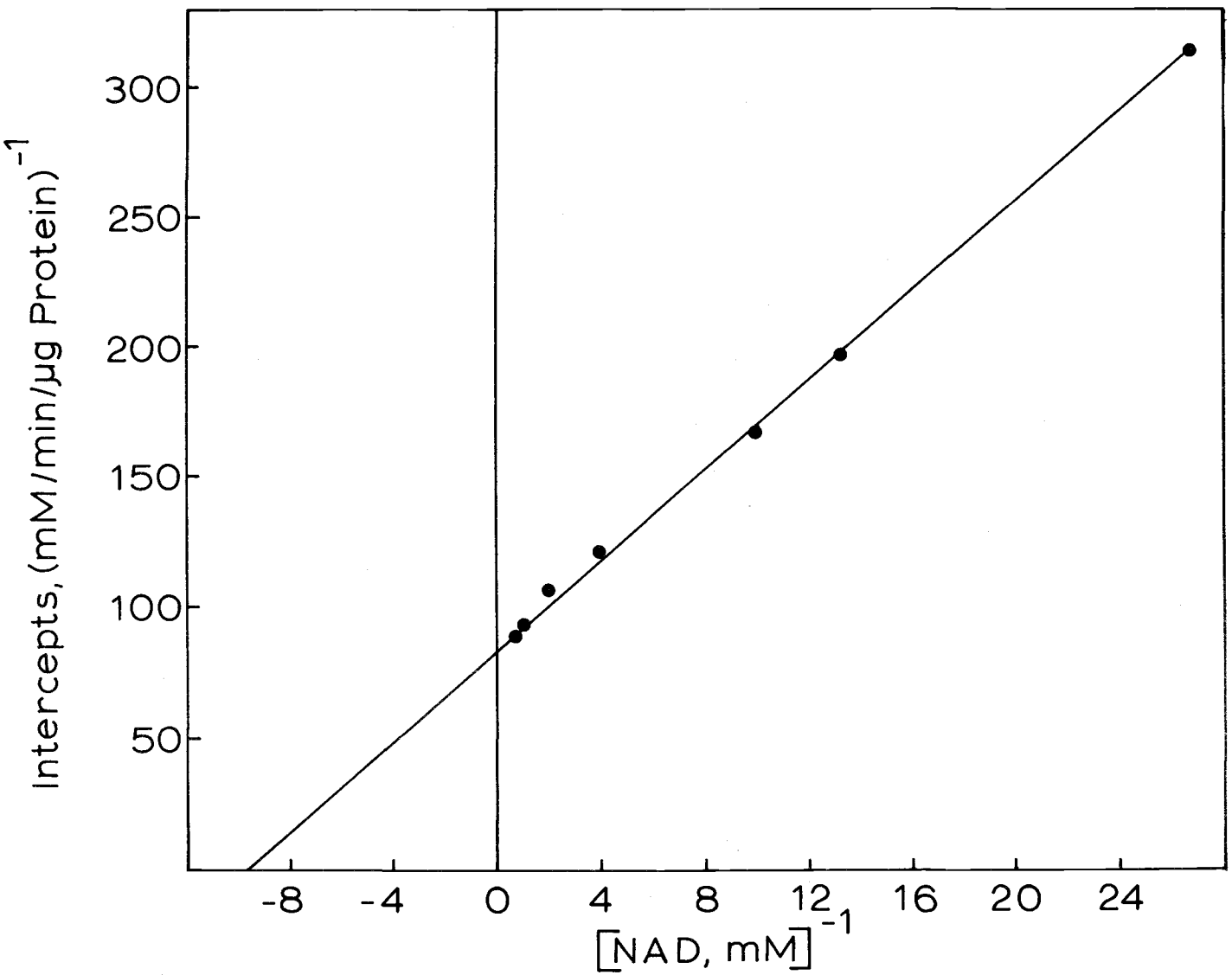
Dehydrogenases generally exhibit one of the three types of reaction mechanisms: (1) compulsory order mechanism without a ternary complex or the Theorell-Chance mechanism (107); (2) compulsory order mechanism in which a ternary complex is formed; (3) random mechanism. Alberty (2) has described in detail the value of correlating the apparent equilibrium constant determined experimentally with kinetic constants obtained for bisubstrate enzymes and reported that for the Theorell-Chance mechanism the following relationship should be obeyed:

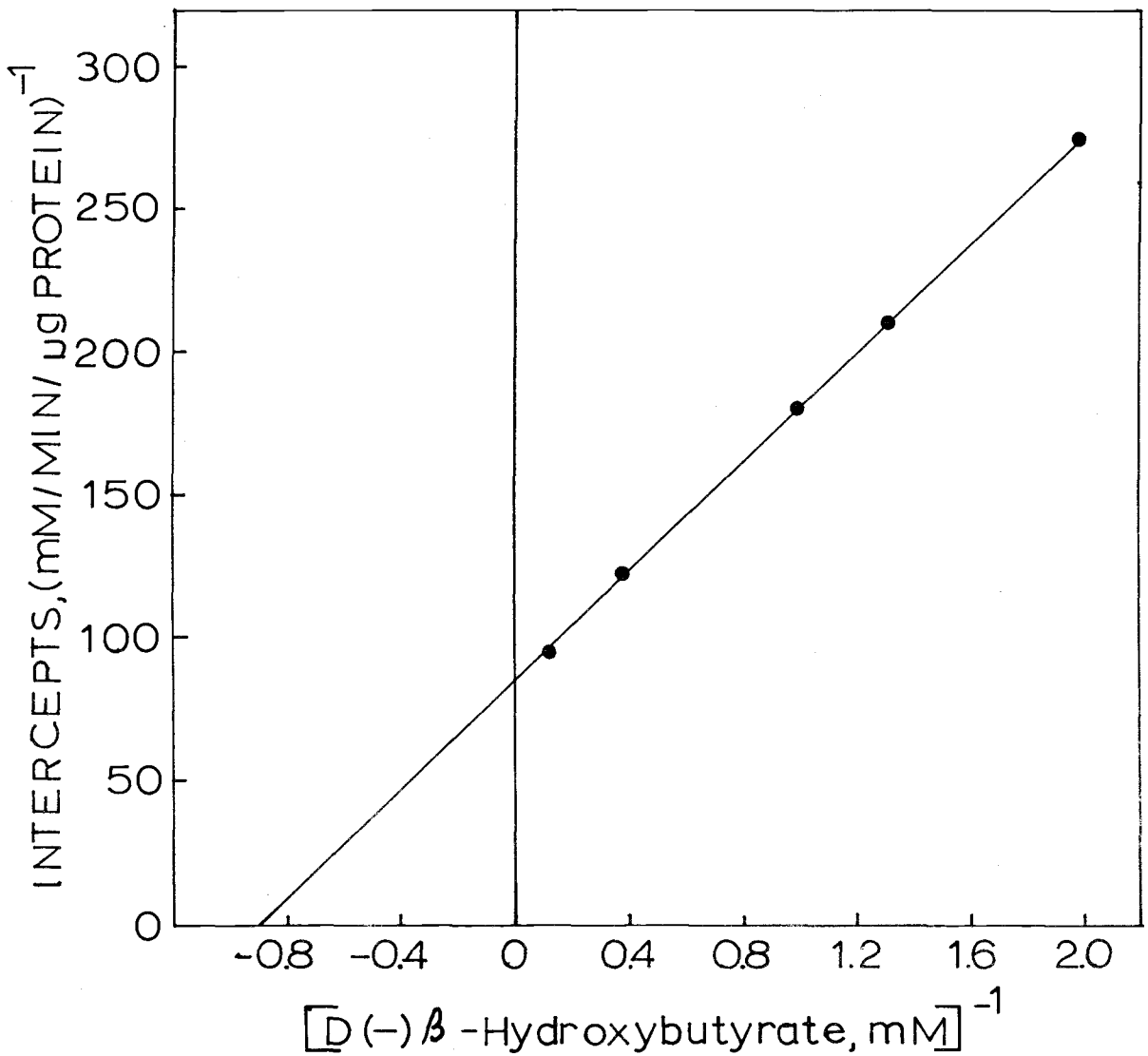
$$K_{app} = V_f^3 K_C K_D / V_r^3 K_A K_B \qquad (3)$$

For the reactions in which the compulsory order with the ternary complex and random mechanism are applicable, the following relationship should prevail:

$$K_{app} = V_f K_{CD} / V_r K_{AB} \qquad (4)$$

Figure 11. Plot of reciprocals of maximal reaction velocities ($1V_m$), obtained from y axis intercepts in Figure 7 versus reciprocals of millimolar concentrations of NAD^+. The intersections of this plot with the y axis evaluates $1/V_f$, and that with the x axis, $-1/K_A$.


Intercepts, (mM/min/µg Protein)$^{-1}$
300
250
200
150
100
50
-8
-4
0
4
8
12
16
20
24
[NAD, mM]$^{-1}$


INTERCEPTS, (mM/MIN/ ug PROTEIN)$^{-1}$
300
250
200
150
100
50
0
-0.8
-0.4
0
0.4
0.8
1.2
1.6
2.0
[D (−) β -Hydroxybutyrate, mM]$^{-1}$

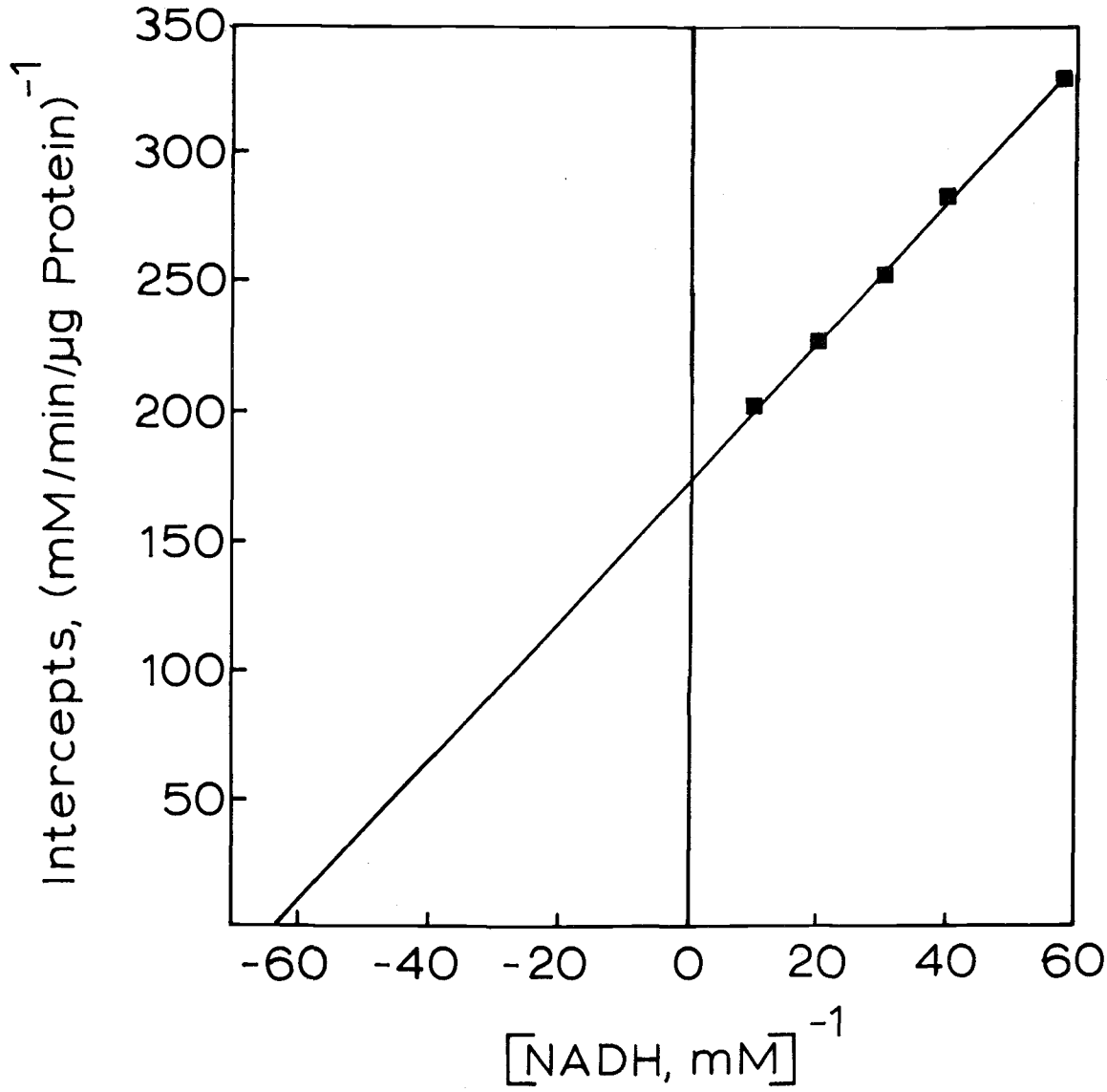


Figure 13. Plot of reciprocals of maximal reaction velocities ($1/V_m$), obtained from y axis intercepts in Figure 9 versus reciprocals of millimolar concentrations of NADH. The intersections of this plot with the y axis evaluates $1/V_r$, and that with the x axis, $-1/K_C$.

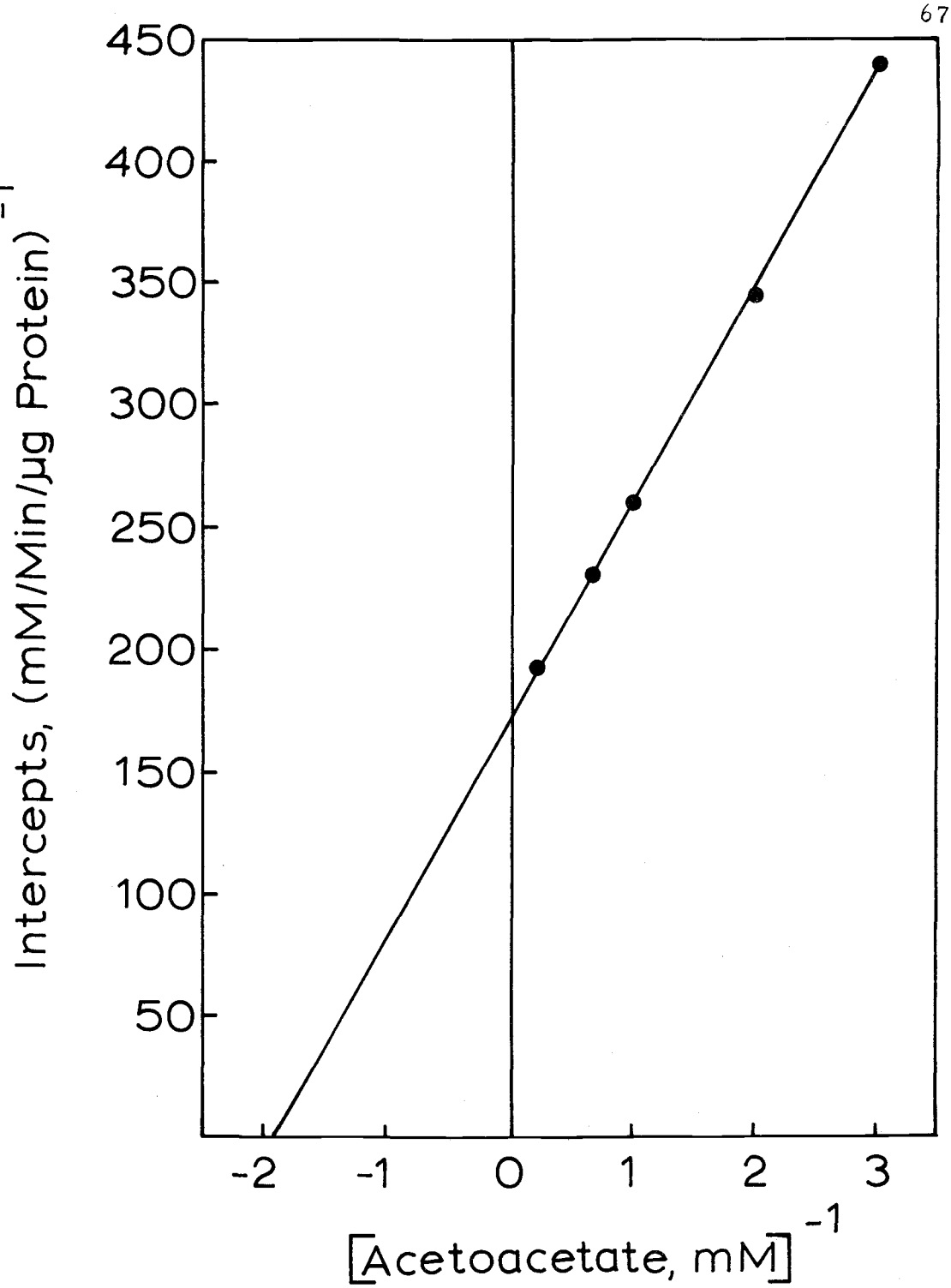


Figure 14. Plot of reciprocals of maximal reaction velocities ($1/V_m$), obtained from y axis intercepts in Figure 10 versus reciprocals of millimolar concentrations of acetoacetate. The intersections of this plot with the y axis evaluates $1/V_r$, and that with the x axis, $-1/K_D$.

Table 6. Kinetic Constants of the β-Hydroxybutyrate Dehydrogenase System

K_A	9.70×10^{-2} mM
K_B	1.14 mM
K_{AB}	1.03×10^{-1} mM^2
V_f	1.19×10^{-2} mM/min./μg of protein
K_C	1.28×10^{-2} mM
K_D	5.31×10^{-1} mM
K_{CD}	1.85×10^{-2} mM^2
V_r	5.72×10^{-3} mM/min./μg of protein

The kinetic constants are defined under "Results and Discussion."

With the data presented in Table 6, K_{app} values of 5.49×10^{-1} and 3.75×10^{-1} were calculated with the equations 3 and 4, respectively. Krebs et al. (62) reported that the K_{app} value for the β-hydroxybutyrate dehydrogenase system from Rhodopseudomonas spheroides was 4.18×10^{-1} in experiments conducted at pH 8.55.

The difference between the calculated values of 5.49×10^{-1} (by equation 3) and 3.75×10^{-1} (by equation 4) and the reported value of 4.18×10^{-1}, makes it difficult to exclude or substantiate the Theorell-Chance mechanism. Recently, Alberty (3) has proposed that the use of the product inhibition method as a device for obtaining useful information in distinguishing the three possible mechanisms of dehydrogenases. If the product is present initially with substrates for the forward reaction or the reverse reaction, significant kinetic effects are expected that would modify the general kinetic equations (equations 1 and 2). The mode of modification would depend on the type of mechanism of reaction. The modified equations are actually the kinetic equations for inhibition. Determination of the type of inhibition by the double reciprocal plots of $1/v$ versus $1/[s]$ in the presence of varying concentrations of products leads to the elucidation of the type of reaction mechanism. The relationships between the mechanism of reaction and the mode of product inhibition are summarized in Table 7.

Experiments were conducted to determine the types of product

Table 7. The Relationships Between Mechnaism of Reaction and the Mode of Product Inhibition.

Reaction mechanism	Added product	Type of inhibition	
		Substrate A varied	Substrate B varied
Theorell-Chance	C	competitive	noncompetitive
	D	noncompetitive	competitive
Compulsory order with formation of ternary complex	C	competitive	noncompetitive
	D	noncompetitive	noncompetitive
Random	C	competitive	competitive
	D	competitive	competitive

For the oxidation of β-hydroxybutyrate (the forward reaction), the substrates varied, A and B were NAD and β-hydroxybutyrate, respectively. The substrate varied determined the initial velocity of the reaction. When NAD concentrations were varied while holding β-hydroxybutyrate at a constant level, the addition of product C (NADH) or D (acetoacetate) revealed a mode of inhibition that is characteristic of reaction mechanism. When β-hydroxybutyrate concentrations were varied, and NAD maintained at a constant level, the addition of C or D revealed another mode of inhibition. For the reduction of acetoacetate (the reverse reaction) C (NADH) and D (acetoacetate) were the substrates varied, and A (NAD) and B (β-hydroxybutyrate) were the products.

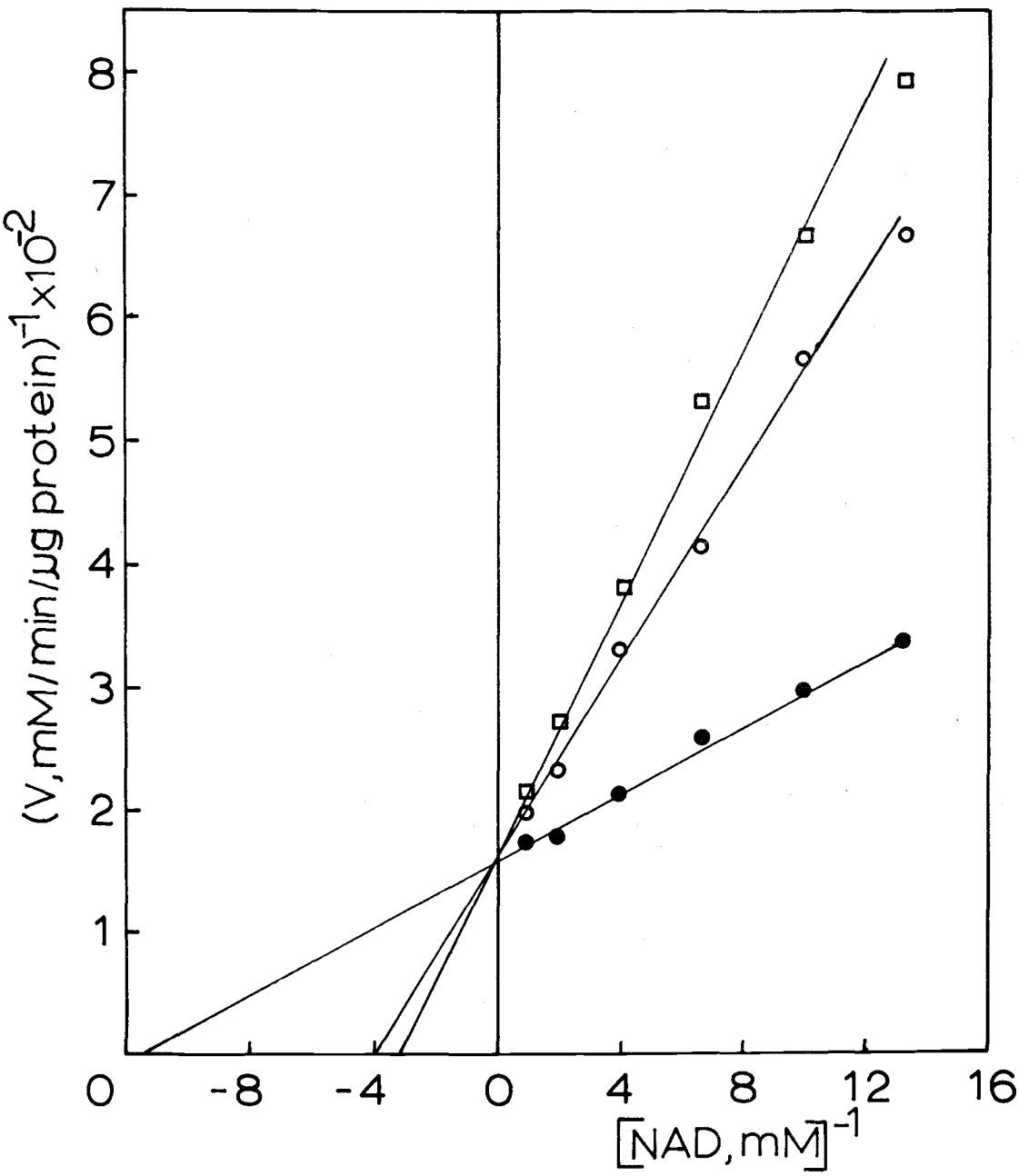


Figure 15. Plot of reciprocals of initial reaction velocities (1/v) versus reciprocals of millimolar concentrations of NAD^+. The concentrations of NADH were: 0.00 (•), 0.07 mM (o), and 0.10 mM (□). D(-)-β-hydroxybutyrate concentration was maintained at 1.25 mM, and the NAD^+ concentration varied in the range from 7.50×10^{-2} mM to 100.00×10^{-2} mM. Velocity (v) is expressed as in Figure 7. The composition of reaction mixtures and the experimental procedures were the same as described under "Standard Assay Conditions" with the exceptions that the concentrations of D(-)-β-hydroxybutyrate and NAD were varied as indicated.

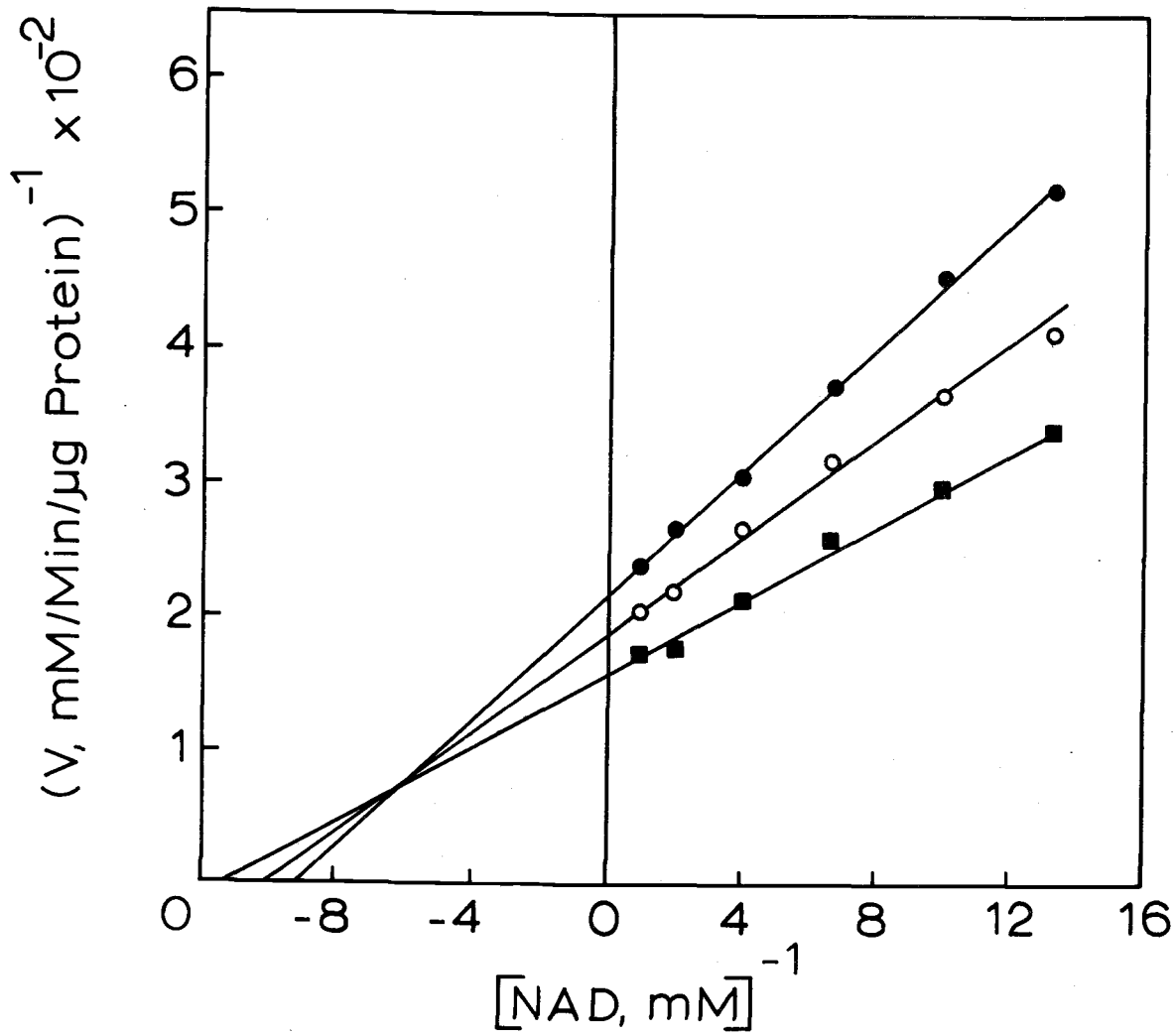


Figure 16. Plot of reciprocals of initial reaction velocities (1/v) versus reciprocals of millimolar concentrations of NAD^+. The concentrations of acetoacetate were: 0.00 (■), 0.30 mM (o), and 0.80 mM (●). Other details of the plot are the same as described in Figure 15.

Figure 17. Plot of reciprocals of initial reaction velocities (1/v) versus reciprocals of millimolar concentrations of D(-)-β-hydroxybutyrate. The concentrations of NADH were: 0.00 (o), 0.07 mM (●), and 10.10 mM (▲). The NAD concentration was maintained at 0.15 mM, and D(-)-β-hydroxybutyrate concentration was varied in the range from0.25 mM to 5.00 mM. Velocity (v) is expressed as in Figure 7. The composition of reaction mixtures and the experimental procedures were the same as described under "Standard Assay Conditions" with the exceptions that NAD and D(-)-β-hydroxybutyrate concentrations were varied as indicated.

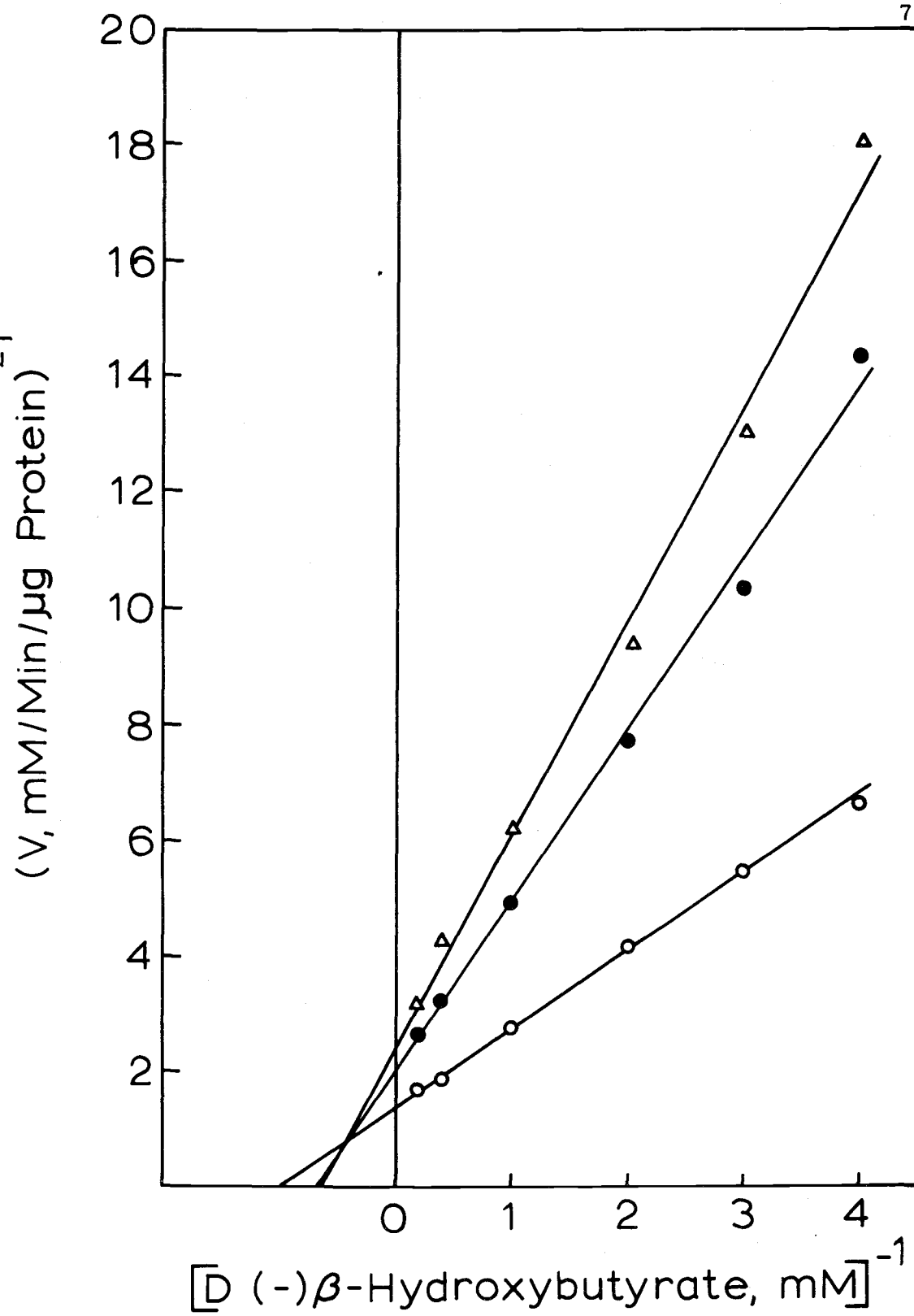
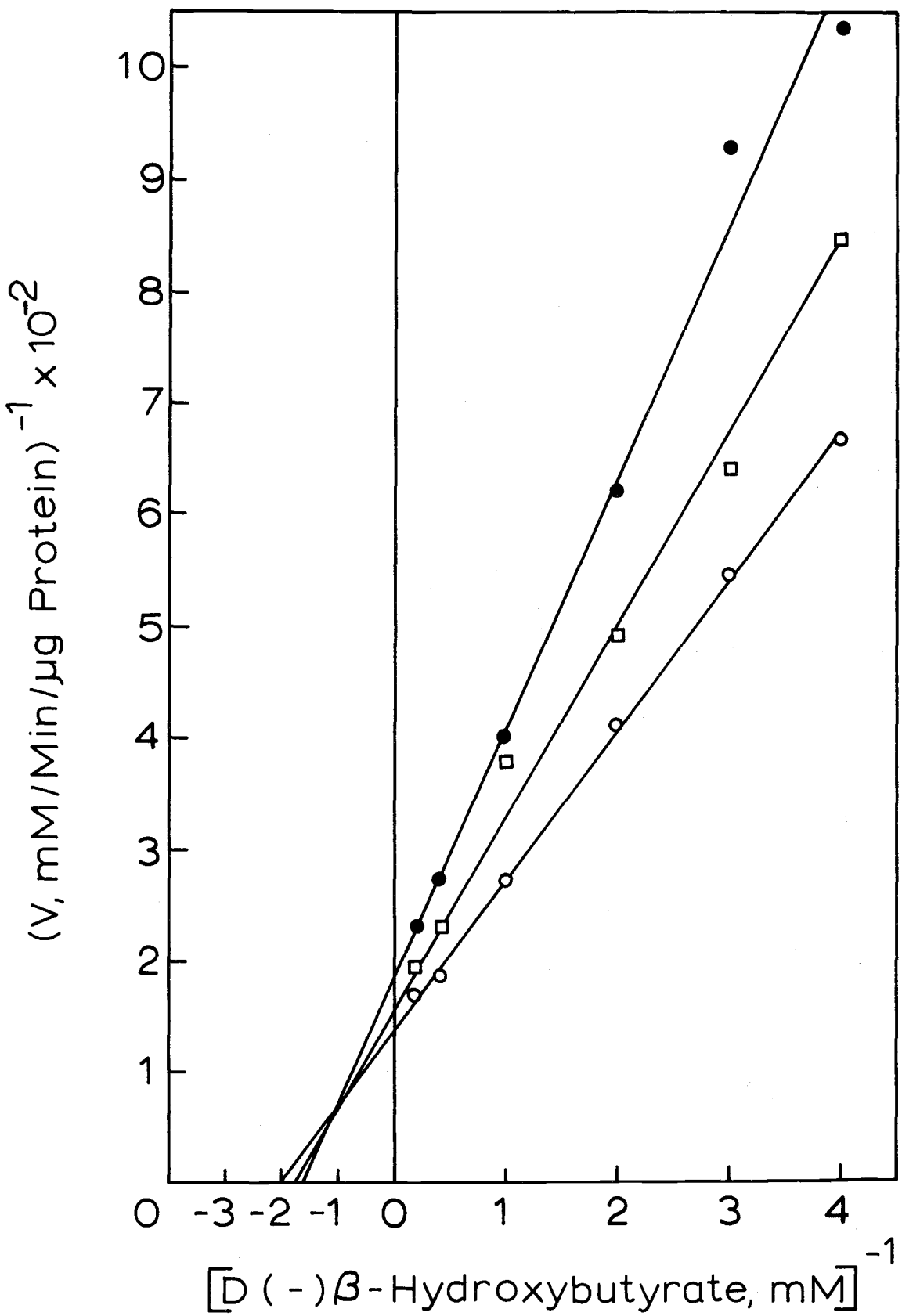

20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
(V, mM/Min/µg Protein)$^{-1}$
[D (-)β-Hydroxybutyrate, mM]$^{-1}$

Figure 18. Plot of reciprocals of initial reaction velocities (1/v) versus reciprocals of millimolar concentrations of D(-)-β-hydroxybutyrate. The concentrations of acetoacetate were: 0.00 (o), 0.30 mM (□), and 0.60 mM (●) Other details of the plot are the same as described in Figure 17.


(V, mM/Min/µg Protein)$^{-1}$ x 10^{-2}
10
9
8
7
6
5
4
3
2
1
0
-3
-2
-1
0
1
2
3
4
[D (-) β-Hydroxybutyrate, mM]$^{-1}$

inhibition. In Figure 15 are presented double reciprocal plots for 1/v versus 1/[NAD] with NADH present at different levels. As indicated in Figure 15, the inhibition is competitive. Similar plots in which acetoacetate was varied are shown on Figure 16. The inhibition in this graph is noncompetitive. Double reciprocal plots of 1/v versus 1/[β-hydroxybutyrate] in the presence of differenct concentrations of NADH and acetoacetate are presented in Figures 17 and 18, respectively. The inhibition exhibited in both figures is noncompetitive. Analogous experiments were conducted for the reverse reaction (reduction of acetoacetate). The detail data were plotted but are not presented in this report. The types of inhibition observed were the same as those for the forward reaction. From these data and the information summarized in Table 7, it appears that β-hydroxybutyrate dehydrogenase reaction proceeds by a compulsory order mechanism with the formation of a ternary complex between the dehydrogenase, NAD^+, and β-hydroxybutyrate.

PART III. ELECTRON TRANSPORT SYSTEMS FOR NITROGENASE FROM *RHIZOBIUM JAPONICUM* BACTEROIDS[1]

MATERIALS AND METHODS

Chemicals

Reagent chemicals of the highest purities available were purchased from commercial sources. NAD^+, $NADP^+$, FMN, FAD, sodium DL-β-hydroxybutyrate, disodium glucose-6-phosphate, DL-glyceraldehyde-3-phosphate and DL-isocitric acid-lactone (hydrolyzed as recommended by the manufacturers) and D-glyceraldehyde were purchased from Sigma Chemical Corporation, St. Louis, Missouri. Acetaldehyde was obtained from Matheson, Coleman and Bell of Cincinnati, Ohio, and redistilled before use. The sources of buffers, gases, and other chemicals utilized have been listed by Koch *et al.* (60). Solutions of reagents used in enzyme assays were adjusted to the desired pH values before they were added to reaction mixtures.

Source and Assay of Enzymes

Crude soybean bacteroid nitrogenase and a polypropylene glycol precipitate of bacteroid nitrogenase were prepared and assayed as described by Klucas *et al.* (56).

[1]Some of the experiments reported in this section were conducted in conjunction with Dr. Robert V. Klucas.

Bacteroid non-heme iron protein, estimated to be 90 percent pure was prepared by a procedure involving precipitation by protamine sulfate and a heat treatment then chromatography on DEAE cellulose (60). Activity of the protein was assayed by its capacity to function as an electron carrier between photosystem I of spinach chloroplast fragments and a bacteroid nitrogenase prepared by polypropylene glycol precipitation.

Azotoflavin and Azotobacter ferredoxin prepared by methods described by Beneman et al. (7) and Yoch et al. (124) were kindly supplied by Dr. D. C. Yoch and were assayed for activity by the procedure utilized for the assay of bacteroid non-heme iron protein (60).

Crude C. pasteurianum extract containing active nitrogenase and a heated C. pasteurianum extract lacking nitrogenase but containing hydrogenase and ferredoxin were prepared from air-dried C. pasteurianum cells by the procedure utilized by Bulen et al. (14).

The cell walls and other components in the pellet resulting from the preparation of a crude bacteroid nitrogenase (56) were utilized for the preparation of an acetone powder (120, vol. 1, p. 15-26). An extract of the powder was prepared by mixing 1 g of the powder with 4 ml 0.05 M TES buffer (pH 7.1), shaking for 20 minutes at 25° C then centrifuging at 2° C for 20 minutes at 30,000 x g. The supernatant extract was assayed for NADH-dehydrogenase (benzyl

viologen as the receptor) by the method utilized by Naik and Nicholas (87) and for NADH dehydrogenase by use of 2,6-dichlorophenolindophenol (DCIP) as described by Mahler (75, vol. 2, p. 701-711).

An electrophoretically homogenous preparation of ferredoxin-NADP reductase from Scendesmus obliquus, purified initially by the method of Powls, Wong and Bishop (93) and further by preparative gel electrophoresis was kindly supplied by Dr. Norman I. Bishop. Activity was assayed by the method of Jagendorf (51, vol. 6, p. 430-434). This preparation catalyzed the reduction of 64 μmoles of DCIP per minute per mg protein.

Dehydrogenase activities were assayed spectrophotometrically by measurement of the increase in the absorbancy of NADH or NADPH at 340 mμ. β-Hydroxybutyrate dehydrogenase was assayed by a modified method of Shuster and Doudoroff (97), glucose-6-phosphate dehydrogenase by the method of DeMoss (29, vol. 1, p. 328-334), isocitric dehydrogenase by the method of Plaut (92, vol. 5, p. 645-651), glyceraldehyde-3-phosphate dehydrogenase by the method of Krebs (61, vol. 1, p. 407-411) and aldehyde dehydrogenase and glyceraldehyde dehydrogenase by the method of Seegmiller (96, vol. 1, p. 511-514). In these assays either NAD^+ or $NADP^+$ were used as indicated in Table 1.

Table 8. Some Dehydrogenase Activities of a Crude Extract of Soybean Nodule Bacteroids.

Enzyme	Substrate	Specific Activities*	
		With NAD	With NADP
β-hydroxybutyric acid dehydrogenase	β-hydroxybutyrate	0.20	0.00
Glucose-6-phosphate dehydrogenase	Glucose-6-phosphate	0.01	0.02
Isocitrate dehydrogenase	Isocitrate	0.00	0.04
Glyceraldehyde dehydrogenase	Glyceraldehyde	0.01	0.00
Glyceraldehyde-3-phosphate dehydrogenase	Glyceraldehyde-3-phosphate	0.06	0.00
Acetaldehyde dehydrogenase	Acetaldehyde	0.02	0.01

*Specific activity is defined as μmoles of NAD or NADP reduced per minute per mg of protein.

General Procedure for NADH- or NADPH-coupled Acetylene Reduction Assays

Reactions were carried out in serum bottles of 21 ml capacity. All reagents except nitrogenase and the NADH or NADPH-generating system were added to the bottles (maintained in ice), then each was closed with a rubber serum stopper and evacuated and flushed three times with purified argon. The components of the NADH or NADPH-generating system were added by use of a hypodermic syringe and the bottles again were evacuated and flushed three times with argon. In the final gassing 0.9 atm of argon and 0.1 atm of C_2H_2 were added and nitrogenase was injected into the bottles with a hypodermic syringe. Reactions were incubated with shaking at 25 °C for either 30 minutes or one hour, and terminated by the addition of 0.5 ml of 15% trichloroacetic acid. Ethylene which is in the gas phase over the reaction mixture, was assayed by the method described by Kelly, Klucas, and Burris (54). Other details are indicated in legends of tables and figures.

Other Assays

The protein content in extracts was assayed by a modification of the Folin method (69) using crystalline bovine serum as the standard.

RESULTS AND DISCUSSION

Some Oxidative Capacities of Bacteroid Extracts

From the investigations of Tuzimura and Meguro (109) and Thorne and Burris (108), it is apparent that soybean nodule bacteroids oxidize fructose-1-6-diphosphate and a whole series of citric acid cycle acids. Conclusions regarding the utilization of carbohydrates have not been consistent. According to Kidby and Parker (personal communication with Dr. Evans), sucrose is the major substrate supporting nitrogen fixation by bacteroids of Lupinous species. Bergersen and Turner (11) found that succinate, fumarate and to some extent pyruvate stimulated nitrogen fixation by isolated soybean nodule bacteroids suspended in a sucrose-free medium, and Koch and Evans (unpublished results) observed that nitrogen fixation by washed bacteroids from soybeans was enhanced by succinate, β-hydroxybutyrate and to a lesser extent by pyruvate.

Since the reducing capacity and the energy for the ATP supply required for nitrogen fixation must be derived from the oxidation of substrates, it was of interest to survey bacteroid extracts for some dehydrogenase activities. As indicated in Table 8 a crude bacteroid extract prepared by the method that retained nitrogenase activity exhibited relatively high β-hydroxybutyrate and glyceraldehyde-3-phosphate dehydrogenase activities when NAD^+ was supplied as the

acceptor. Low but measurable activities of glucose-6-phosphate, acetaldehyde and glyceraldehyde dehydrogenases were detected in presence of NAD^+ and moderate activities of isocitrate and glucose-6-phosphate dehydrogenase activities were observed in reactions containing $NADP^+$. Although no systematic effort was made to extract maximum dehydrogenase activities, the present results indicate that crude extracts of bacteroids exhibit relatively high activities of β-hydroxybutyrate and glyceraldehyde-3-phosphate dehydrogenases.

H_2 as a Source of Electrons

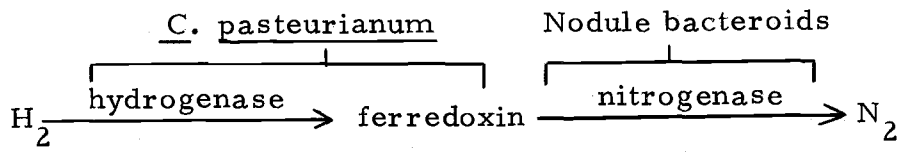
Dixon (31) has observed that bacteroids from the nodules of Pisum sativum possess hydrogenase activity and Bergerson (10) briefly mentioned that cell-free extracts of soybean nodule bacteroids evolve H_2 from NADH. These observations together with demonstrated capacity of H_2 to serve as an electron donor for nitrogenase in crude extracts of Clostridium pasteurianum (14) encouraged tests of H_2 as a possible electron donor for bacteroid nitrogenase. It is clear (Table 9) that bacteroid nitrogenase supplied with an ATP-generating system and H_2 failed to reduce acetylene to ethylene. In contrast a reaction containing crude C. pasteurianum extract, H_2 and an ATP-generating system catalyzed the reduction of acetylene to ethylene at a rate comparable to that obtained with $Na_2S_2O_4$. When a heated C. pasteurianum extract (HDS) (containing hydrogenase and

Table 9. Donation of Electrons to Nodule Bacteroid Nitrogenase by H_2 in Presence of a Heated Extract (HDS) of Clostridium pasteurianum.

Type of crude nitrogenase added	Electron donor system	C_2H_4 produced (nmoles/min/mg protein)
Clostridium pasteurianum	H_2	40
Clostridium pasteurianum	H_2 and HDS	37
Clostridium pasteurianum	$Na_2S_2O_4$	34
None	HDS and $Na_2S_2O_4$	0
Nodule bacteroid	H_2	0
Nodule bacteroid	H_2 and HDS	22
Nodule bacteroid	$Na_2S_2O_4$	20
Nodule bacteroid	$Na_2S_2O_4$ and HDS	12

Each reaction in a final volume of 1.5 ml contained an ATP generating system consisting of (7.5 μmoles ATP, 50 μmoles of creatine phosphate, 0.2 mg creatine phosphokinase, 10 μmoles of $MgCl_2$) 100 μmoles of TES buffer at pH 7.2 and 20 μmoles of $Na_2S_2O_4$ as indicated. Protein (mg) added to reactions as indicated were as follows: crude Clostridium pasteurianum extract, 5.6; nodule bacteroid extract, 8; and heated Clostridium pasteurianum extract (HDS) 2.9. Flasks were evacuated and filled with 0.2 atm of C_2H_2, 0.5 atm of H_2 where indicated and all reactions were made to 1 atm with argon. Reactions were incubated for 15 minutes at 30 degrees C.

ferredoxin but lacking nitrogenase) was incubated with nodule bacteroid extract, an ATP-generating system and H_2, acetylene was reduced at a rate approximately equivalent to that observed when $Na_2S_2O_4$ was added. Since H_2 and HDS from C. pasteurianum exhibited no nitrogen-fixing capacity alone it is clear that bacteroid nitrogenase may be coupled to H_2 provided that a hydrogen donating system from C. pasteurianum is supplied. These reactions are summarized in scheme A.

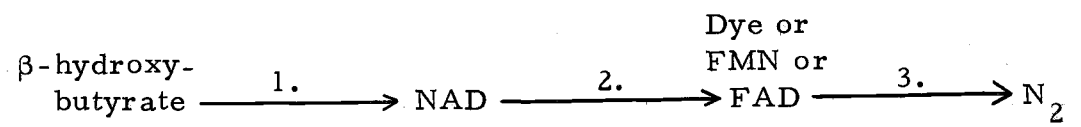


Scheme A

Coupling to NADH

It had been established by Forsyth et al. (38) that legume nodule bacteroids accumulate large amounts of poly-β-hydroxybutyrate and that the β-hydroxybutyrate dehydrogenase in bacteroid extracts is unusually active. Klucas and Evans (55) have reported that NADH generated by the β-hydroxybutyrate dehydrogenase system served effectively as a reducing system for bacteroid nitrogenase provided that a low potential dye such as benzyl or methyl viologen was added. Further investigations (36) revealed that FAD or FMN substituted for

dyes in the NADH-coupled bacteroid-nitrogenase reaction. From the data (Table 10) it is clear that acetylene reduction proceeded at a relatively rapid rate in a complete reaction containing an NADH-generating system, an ATP-generating system, crude bacteroid nitrogenase, FAD and an extract from an acetone powder of bacteroids. Less than one nmole of acetylene was reduced when the ATP-generating system, or nitrogenase, or FAD was omitted. Other experiments (55) have shown that the reaction proceeded at less than ten percent of the normal rate when β-hydroxybutyrate was omitted. From the results (Table 10) it also is obvious that benzyl viologen substituted for FAD in the NADH-coupled reaction. The sequence of electron transport in these coupled reactions is illustrated by scheme B.



1. β-hydroxy-butyrate dehydrogenase
2. NADH dehydrogenase
3. Nitrogenase

Scheme B

Further investigations revealed that either FMN or FAD was effective as cofactors in the NADH-coupled nitrogenase system. Maximum rates (Figure 19) were observed with either FMN or FAD at a concentration of about 0.5 mM.

Table 10. Components Required for Coupling an NADH-generating System to Bacteroid Nitrogenase-dependent Acetylene Reduction.

Reaction Mixture	C_2H_4 formed (nmoles/30 min)
Complete*	516
Without NAD	10
Without ATP-generating system	1
Without crude nitrogenase	1
Without FAD	1
Without bacteroid acetone-powder extract	225
Without FAD, with benzyl viologen	960

*The complete reaction in a final volume of 2.5 ml included: an NADH-generating system (consisting of 1 μmole of NAD, 200 μmoles of β-hydroxybutyrate, and endogenous β-hydroxybutyrate dehydrogenase); an ATP-generating system (see legend of Table 9); 100 μmoles TES buffer at pH 7.5; bacteroid acetone-powder extract (8 mg protein); crude bacteroid nitrogenase (8.9 mg protein), and 1 μmole of FAD.

Where indicated 1 μmole of benzyl viologen was added instead of FAD. Reactions were evacuated and flushed six times with argon then filled with 0.1 atm C_2H_2 and 0.9 atm argon and then incubated for 30 minutes at 25 degrees C. Ethylene was measured as described in Materials and Methods.

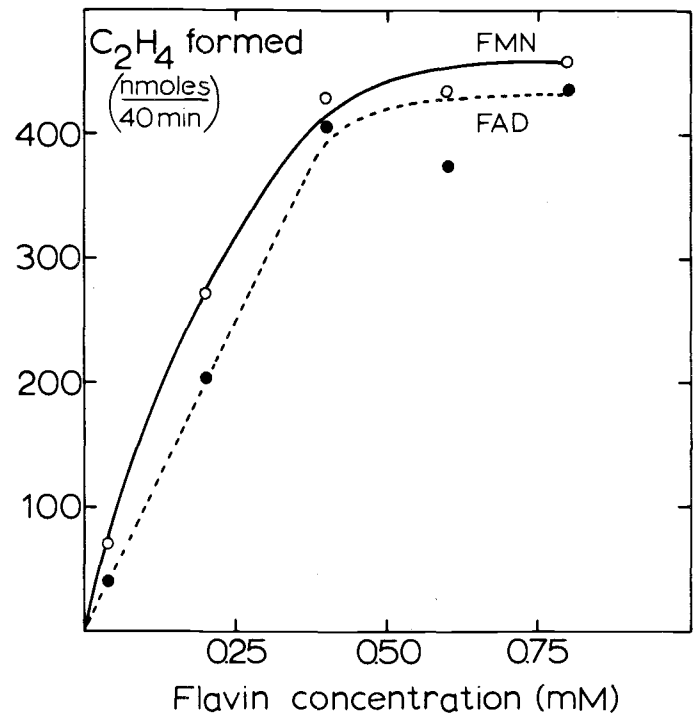


Figure 19. Effects of FAD and FMN on the rate of nitrogenase-dependent acetylene reduction. The complete reaction mixture in a final volume of 2.5 ml contained the components listed for the complete reaction in the legend of Table 10 with the exception that the concentrations of FAD or FMN were varied as indicated. Reactions were gassed, flushed, incubated and assayed as indicated in Table 10.

When FAD (or FMN) was utilized in the coupled reaction (scheme B) a marked stimulation in acetylene reduction resulted from the addition of acetone-powder extract of bacteroids (Table 10). The active factor in this extract was heat-labile and therefore was assumed to be a protein. The NADH-coupled reaction (Figure 20) responded strikingly to increasing concentrations of acetone-powder extract. The lag in the initial rates of the reactions presumably was caused by incomplete removal of O_2 from the vessels. In an effort to identify the active factor in the acetone-powder extract an experiment (analogous to that described in Figure 20) was conducted with the exception that certain reactions received additional highly purified β-hydroxybutyrate dehydrogenase or purified NADH dehydrogenase from bacteroids. The total units of both β-hydroxybutyrate dehydrogenase and NADH dehydrogenase in the reactions were determined. The results (unpublished) showed that the bacteroid nitrogenase contained sufficient β-hydroxybutyrate dehydrogenase for maximum reaction rate and that additional purified β-hydroxybutyrate dehydrogenase failed to increase the rate of acetylene reduction. In contrast, the addition of the acetone-powder extract consistently increased the rate of acetylene reduction, and furthermore the increase was correlated positively with the total units of NADH dehydrogenase in the reactions. From these results it was concluded that NADH dehydrogenase was an essential component of the

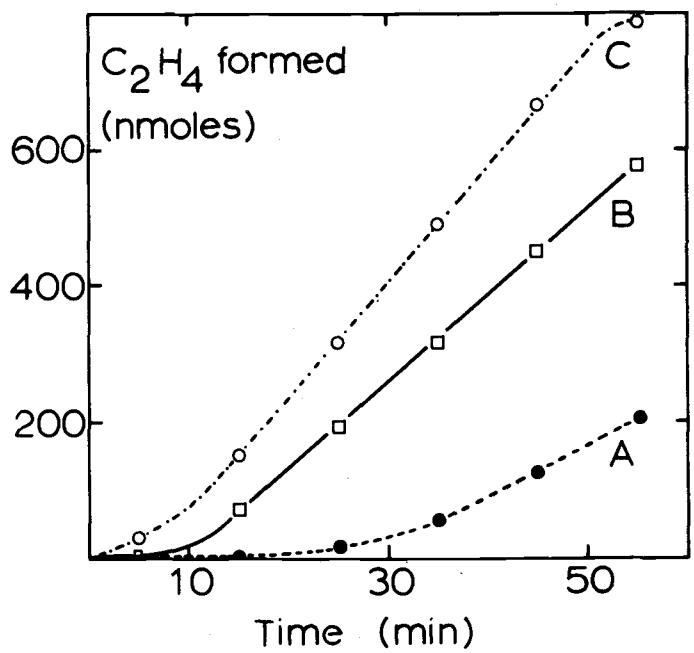


Figure 20. The effect of different concentrations of bacteroid acetone-powder extract on the rate of nitrogenase-dependent acetylene reduction. Reactions contained the components listed for the complete reaction (Table 10) with the exception that acetone-powder extract was varied as follows: without acetone-powder extract, curve (A); 4.0 mg of protein from acetone-powder extract, curve (B); and 8 mg of protein from acetone-powder extract, curve (C). Reactions were gassed, flushed and incubated as described in Table 10.

NADH-coupled nitrogenase reaction (scheme B).

In other experiments in which benzyl viologen was used in place of either FAD or FMN in the coupled reactions, the addition of the NADH dehydrogenase had no consistent stimulatory effect on acetylene reduction. One possible explanation for this observation is that the nitrogenase preparation contained sufficient NADH dehydrogenase for a maximum rate.

Coupling to NADPH

Recently, Koch et al. (60) have isolated and characterized a non-heme iron protein from the bacteroids of soybean nodule bacteroids that is similar to but not identical with ferredoxin from Azotobacter. This protein functions as a carrier in the light-dependent transfer of electrons from photosystem I to nitrogenase. When the non-heme iron protein became available, experiments were designed in which bacteroid non-heme iron protein and other factors were utilized in attempts to couple NADH and NADPH-generating systems to bacteroid nitrogenase. The addition of the non-heme iron protein alone or along with azotoflavin to a reaction containing an NADH-generating system (analogous to the complete reaction of Table 10) but without FAD or benzyl viologen resulted in no consistent acetylene reduction. Experiments were conducted in which NADPH was continuously generated by a glucose-6-phosphate dehydrogenase system. Complete

reactions also contained a PPG precipitate of bacteroid nitrogenase, an ATP-generating system, bacteroid non-heme iron protein, ferredoxin-NADP-reductase from spinach and azotoflavin from Azotobacter. The complete reaction (Table 11) catalyzed the reduction of acetylene to ethylene at a reproducible rate. Activity was essentially dependent upon the ATP-generating system, the NADPH-generating system, bacteroid non-heme iron protein, ferredoxin-NADP reductase and azotoflavin. A time course of the complete reaction and a control reaction lacking the NADPH-generating system is presented in Figure 21. Although the azotoflavin in this experiment (Table 11) was derived from Azotobacter, evidence has been obtained by Koch et al. (60) that soybean bacteroids contain a flavoprotein that functions in the light-dependent transfer of electrons from photosystem I to bacteroid nitrogenase. From preliminary experiments it appears to be analogous to the azotovlavin from Azotobacter. From these results we conclude that it is possible to reconstitute an electron transport system in which electrons are transferred from an NADPH-generating system to bacteroid nitrogenase. It is also clear that NADH dehydrogenase from bacteroids does not substitute for the ferredoxin-NADP reductase in this system.

An experiment similar to that described in Table 11 was conducted with the exception that both azotoflavin and Azotobacter ferredoxin were added and bacteroid non-heme iron protein was omitted.

Table 11. Requirements for Coupling a NADPH-generating System to Acetylene Reduction by Bacteroid Nitrogenase.

Reaction Mixture	C_2H_4 formed (nmoles/hr)
Complete*	94.0
Without ATP-generating system	1.0
Without NADPH-generating system	0.8
Without bacteroid NHIP	0.8
Without azotoflavin	2.0
Without azotoflavin and bacteroid NHIP	0.5
Without ferredoxin-NADP reductase	0.5
Without ferredoxin-NADP reductase, with NADH dehydrogenase	0.4
Without nitrogenase	0.5

*Each complete reaction in a final volume of 1.5 ml contained an ATP-generating system (see legend of Table 9) an NADPH-generating system (consisting of 1 μmole of NADP, 50 μmoles of glucose-6-phosphate and 0.15 mg of crystalline glucose-6-phosphate dehydrogenase) 0.25 mg of bacteroid non-heme iron protein (NHIP), 0.9 mg of azotoflavin, 0.48 mg of NADP-ferredoxin reductase and 4 mg of bacteroid nitrogenase (specific activity of 40 nmoles C_2H_4/min/mg of protein) from a PPG precipitate.

The NADH dehydrogenase (0.8 mg of protein) was added where indicated as a crude extract of an acetone powder of nodule bacteroids. Reactions were evacuated, flushed, gassed, incubated and assayed as indicated in Table 10 with the exceptions that the period of incubation was 1 hour rather than 30 minutes.

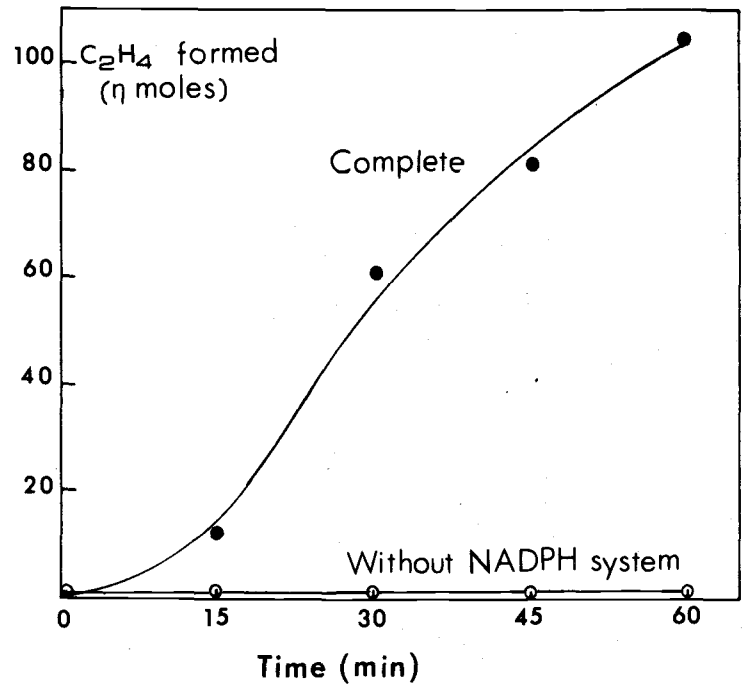
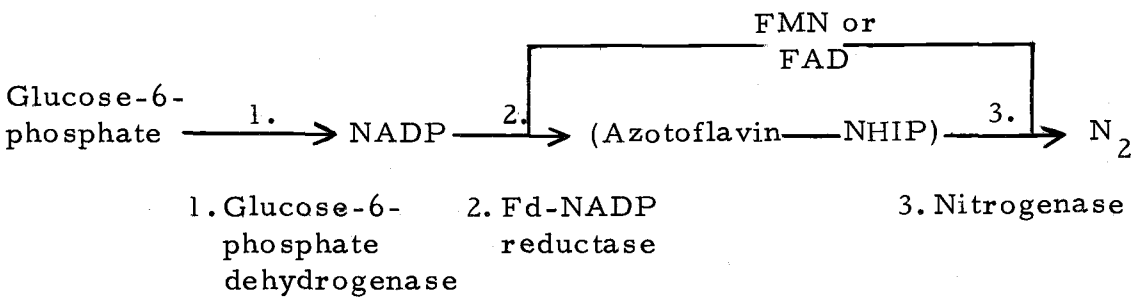


Figure 21. Time course for the reduction of acetylene in the presence and absence of an NADPH-generating system. The experimental conditions for the complete reaction were the same as described for the complete reaction in Table 11 with the exceptions that the period of incubation were varied and the NADPH-generating system was omitted as indicated.

Again, a relatively rapid rate of the complete reaction was observed that was essentially dependent upon an ATP-generating system, an NADPH generating system, azotoflavin and Azotobacter ferredoxin, and ferredoxin-NADP reductase. A strikingly increased rate of activity on the acetylene reduction was obtained when 0.5 μmole of FMN was added to the complete system.

Another experiment was conducted similar to that described in Table 11 with the exception that 0.5 μmole of FMN was added to each reaction in addition to other cofactors (Table 12). In this case activity in the complete reaction was not dependent upon bacteroid non-heme iron protein or azotoflavin, however, ferredoxin-NADP reductase was essential. The relatively low activity observed when a bacteroid acetone-powder extract was added instead of ferredoxin-NADP reductase suggests the presence of a ferredoxin-NADP reductase in the acetone-powder extract used in this experiment.

The results in Tables 11 and 12 may be interpreted with the aid of the electron transport sequence outlined in scheme C.



Scheme C

Table 12. Requirements for Coupling an NADPH Generating System to Acetylene Reduction by Bacteroid Nitrogenase in Presence of FMN.

Reaction Mixture	C_2H_4 formed (nmoles/hr)
Complete*	237
Without bacteroid NHIP	394
Without azotoflavin	340
Without azotoflavin and bacteroid NHIP	258
Without Fd-NADP reductase	3
Without Fd-NADP reductase, with bacteroid acetone powder extract	36
Without Fd-NADP reductase, bacteroid NHIP and azotoflavin	1

*Each complete reaction mixture in a final volume of 1.5 ml contained the components indicated for the complete reaction in the legend of Table 11 and in addition 0.5 μmole of FMN.

The acetone powder extract added as indicated contained 0.8 mg of protein. Reactions were flushed, gassed and incubated as described in the legend of Table 10.

Electrons may be transported from an NADPH-generating system such as glucose-6-phosphate dehydrogenase through ferredoxin-NADP reductase to azotoflavin and bacteroid non-heme iron protein to N_2 *via* bacteroid nitrogenase. Ferredoxin from *Azotobacter* obviously will substitute effectively for the bacteroid non-heme iron protein. There is no evidence indicating whether azotoflavin or bacteroid non-heme iron protein accepts electrons first from the previous donor in the sequence (seheme C). When either FMN or FAD was added to reactions along with azotoflavin and bacteroid non-heme iron protein (or Azotobacter ferredoxin) electrons were transferred from the NADPH-generating system *via* ferredoxin-NADP reductase directly to FMN and then to N_2 *via* nitrogenase. Under these conditions, azotoflavin and bacteroid non-heme iron protein apparently were by-passed. In the experiments conducted to date, it has not been possible to effectively substitute an NADH-generating system for the NADPH-generating system in reactions where azotoflavin and bacteroid non-heme iron protein were added. Bacteroids may contain a transhydrogenase that transfers electrons from NADH to NADP, but this possibility so far has not been adequately investigated. Further research obviously is essential before the components of this system are completely resolved and all the necessary constituents for the coupled reaction can be obtained from bacteroids. The results reported, however, are convincingly reproducible and are

considered as an excellent guide toward designing additional experiments.

Stimulation of $Na_2S_2O_4$-supported Nitrogenase Activity by Cofactors

Recently Yates (122) has reported that cytochrome C_4, NADH dehydrogenase, Azotobacter ferredoxin, azotoflavin, and a dialyzable factor from Azotobacter stimulated nitrogenase-dependent acetylene reduction, in reactions in which $Na_2S_2O_4$ was used as the reductant. The response to these compounds ranged from one to about three-fold in reactions where low concentrations of Azotobacter nitrogenase was added. Furthermore, Yates (122) observed that the addition of NADH dehydrogenase to partially purified nitrogenase from Azotobacter protected the nitrogenase from O_2 damage. He concluded that the stimulation of nitrogenase by the factors investigated very likely was caused by an effect on dissociation or disaggregation of the nigrogenase. The possibility that the added factors were involved in electron transport to nitrogenase was discounted. The inhibition of O_2 damage to nitrogenase by NADH dehydrogenase also was interpreted on the basis of an interaction of the NADH dehydrogenase with nitrogenase in a unique way that prevented the deleterious effects of O_2.

We considered it necessary, therefore, to examine the influence of azotoflavin, Azotobacter ferredoxin, and bacteroid non-heme iron

protein on the activity of $Na_2S_2O_4$-dependent bacteroid nitrogenase. From the results (Table 13) the addition of azotoflavin, a combination of azotoflavin and Azotobacter ferredoxin, bacteroid non-heme iron protein all at concentrations equivalent to those used in previous experiments resulted in increased nitrogenase activity ranging from 5 to 47 percent. None of the added components alone exhibited nitrogenase activity. Although our results are consistent with those reported by Yates (122) convincing evidence is available indicating a role of azotoflavin and bacteroid non-heme iron protein in electron transport to nitrogenase. In experiments in which an NADPH-generating system was used as the reductant, the addition of azotoflavin or bacteroid non-heme iron protein to the coupled assay resulted in a 45-fold or greater increase in nitrogenase activity. This response may be contrasted to a one to three-fold increase from added cofactors in assays where $Na_2S_2O_4$ was used (122). Furthermore, in those assays in which azotoflavin was included, the addition of the NADPH-generating system caused an obvious reduction in azotoflavin converting the protein from its normal yellow to a blue color that is characteristic of the reduced semiquinone form of azotoflavin (49). The reduction of azotoflavin was dependent upon ferredoxin-NADP reductase and the NADPH-generating system. Furthermore, Beneman et al. (7) reported that the incubation of azotoflavin in the light with chloroplast fragments, ascorbate and DCIP resulted in the reduction

Table 13. Effect of Azotoflavin, Azotobacter Ferredoxin and Bacteroid Non-heme Iron Protein (NHIP) on Nitrogenase-dependent Acetylene Reduction With $Na_2S_2O_4$ as the Electron Donor

No.	Reaction Mixture	Ethylene formed (nmoles/20 min)	Stimulation (%)
1	Complete	1292	--
2	Complete + 0.9 mg azotoflavin	1904	47
3	Complete + 1.5 mg azotoflavin and Azotobacter ferredoxin	1564	21
4	Complete + 0.2 mg of bacteroid NHIP	1360	5
5	Complete + 0.9 mg of azotoflavin + 0.2 mg bacteroid NHIP	1904	47
6	Same as reaction 2 but without nitrogenase	1	--
7	Same as reaction 3 but without nitrogenase	1	--
8	Same as reaction 4 but without nitrogenase	1	--
9	Same as reaction 5 but without nitrogenase	1	--

The complete reaction mixture in a final volume of 1.5 ml contained an ATP generating system and $Na_2S_2O_4$ (see Table 9) a 20-35% PPG preparation of nitrogenase (4 mg of protein).

The procedure for evacuation, gassing, and incubation was the same as that described in the legend of Table 10.

of azotoflaven.

Experiments were carried out in our laboratory in which about 60 nmoles of bacteroid non-heme iron protein was incubated in the light in presence of ascorbate, DCIP, photosystem I and an ATP-generating system. Reactions were then placed in the dark and bacteroid nitrogenase and acetylene added. In this reaction acetylene was reduced in the dark but no acetylene was reduced by a control reaction conducted entirely in the dark or another control reaction (conducted in the light) which was complete with the exception that non-heme iron protein was omitted. We conclude, therefore, that the evidence available strongly indicates that azotoflavin and bacteroid non-heme iron protein participate in electron flow from NADPH to nitrogenase. This role may be in addition to Yates' (122) postulated role of this and other compounds as substances influencing the conformational status of nitrogenase.

SUMMARY

An investigation was conducted to determine the roles of poly-β-hydroxybutyrate and its metabolic products in symbiotic nitrogen fixation by nodule bacteroids. Detailed investigations were carried out on β-hydroxybutyrate dehydrogenase and on systems for the transport of electrons to nodule bacteroid nitrogenase. The results of these experiments are summarized as follows:

1. As much as 50 percent of the dry weight of soybean nodule bacteroids was found to be poly-β-hydroxybutyrate.
2. Nodule bacteroids possessed an active poly-β-hydroxybutyrate depolymerase system.
3. Poly-β-hydroxybutyrate content of the bacteroids failed to decrease rapidly until the supply of carbohydrate from the soybean host plants to the nodule bacteroids was limited by (a) excision of the nodules from the plants, (b) incubation of the plants in the dark, or (c) the onset of senescence in the host plant.
4. Poly-β-hydroxybutyrate alone failed to support a high rate of nitrogenase activity in nodules after the carbohydrate supply from the host was interrupted.
5. Isocitrate lyase activity in bacteroids was not detected until poly-β-hydroxybutyrate utilization became apparent.

6. Nodule bacteroids exhibited an unusually active β-hydroxybutyrate dehydrogenase but the activity of this enzyme did not change significantly as a result in changes in the apparent rate of utilization of poly-β-hydroxybutyrate.

7. A procedure for purifying the bacteroid β-hydroxybutyrate dehydrogenase was developed which involved two polypropylene glycol fractionations an ammonium sulfate fractionation, gel filtration, and ion exchange chromatography. A 108-fold purification was obtained.

8. β-Hydroxybutyrate dehydrogenase exhibited optimum activity at pH values between 7.0 and 8.5, required Mg^{++} ions for stability, and was inhibited by glycine and mercuric acetate. This latter inhibition was protected by the addition of divalent cations, NAD, or NADH.

9. Kinetic studies revealed that the mechanism of the β-hydroxybutyrate dehydrogenase reaction involved a compulsory order type with the formation of a ternary complex between the dehydrogenase, NAD, and β-hydroxybutyrate.

10. A K_{app} value of 3.75×10^{-1} was calculated using data obtained from kinetic studies.

11. Hydrogen functioned as an effective electron donor for nitrogenase from bacteroid provided that hydrogenase and ferredoxin from Clostridium pasteurianum were present.

12. Electrons were effectively transferred from β-hydroxybutyrate to bacteroid nitrogenase in a reaction containing β-hydroxybutyrate dehydrogenase, NADH dehydrogenase, NAD, and either FMN or FAD.

13. Glucose-6-phosphate donated electrons to bacteroid nitrogenase in a system that including glucose-6-phosphate dehydrogenase, NADP, ferredoxin-NADP reductase from spinach, azotoflavin from Azotobacter, and a bacteroid non-heme iron protein. It is suggested that the NADPH donor system is physiologically more important than the other systems investigated.

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