COMPARISON OF SOLUBLE REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE OXIDASES FROM VEGETATIVE CELLS AND SPORES OF *CLOSTRIDIUM BOTULINUM* 62-A

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THESIS

This is to certify that the

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ABSTRACT

COMPARISON OF SOLUBLE REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE OXIDASES FROM VEGETATIVE CELLS AND SPORES OF *CLOSTRIDIUM BOTULINUM* 62-A

by John Henry Green

A comparison has been made of the properties of a vegetative cell and a heat stable spore reduced nicotinamide adenine dinucleotide (NADH) oxidase from a strain of *Clostridium botulinum* 62-A. Both NADH oxidases were soluble and could be separated from their respective particulate diaphorases by centrifugation at 32,000 g for 6-8 hours. Forty and eighty-fold purifications respectively were obtained for vegetative cell and spore NADH oxidases.

Comparisons were based on heat inactivations, solubility in ammonium sulfate solutions, substrate (NADH) and inhibitor (atabrine) kinetics, relative activity in buffers between pH 5.2 to 9.4, cofactor requirements, elution from an anion exchange column, estimations of molecular weights derived from gel filtrations, immunological responses, and serological cross reactions between heterologous antigens and antibodies. Differences were found in all of the above properties and are given below.

The spore NADH oxidase was extremely heat stable and survived 65 C for 2 hours with little or no loss while the vegetative oxidase lost 90% activity in 3 minutes at 65 C. The spore oxidase precipitated in a narrow band at 55% ammonium sulfate saturation while the vegetative oxidase precipitated over a wide band showing a peak at 70% saturation.

The spore oxidase exhibited less affinity for the substrate than the corresponding vegetative cell enzyme. Its Michaelis constant (K_m) was 8 times greater than the vegetative oxidase K_m of 7.9×10^{-6} M The kinetics also showed that atabrine is a non-competitive inhibitor for both enzymes and was less inhibitory to the spore oxidase. The pH optimum of both enzymes was approximately 7.5 but the spore oxidase displayed more activity in the lower pHs while the vegetative oxidase was more active in the pH range above optimum.

A difference was shown in their ionic charge when both NADH oxidases were mixed together and eluted from a DEAE cellulose column by a linear NaCl gradient. They appeared separately in the effluent, the spore enzyme being eluted first.

Several attempts to remove the flavin by acid-ammonium sulfate treatment revealed that this cofactor was

tightly bound to the spore oxidase. The flavin was readily removed from the vegetative oxidase and flavin adenine dinucleotide (FAD), not flavin mononucleotide (FMN), reactivated the enzyme.

The spore oxidase was excluded from the imbibed volumes of both Sephadex G-100 and G-200 gel particles and was estimated to have a molecular weight of 200,000 or greater. The vegetative oxidase was included in both and was therefore 100,000 molecular weight or less. The spore oxidase elicited higher levels of antibodies in rabbits than did the vegetative cell oxidase. Neither NADH oxidase would cross react with its heterologous antibody in a precipitation reaction.

The above differences in characteristics studied showed a distinct difference between the vegetative cell and spore NADH oxidases.

The evidence excluded the possibility of the conversion of a single vegetative cell enzyme molecule into a heat stable spore NADH oxidase. Stable spore proteins may originate by the polymerization of vegetative proteins or de novo protein synthesis during sporulation.

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By

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INTRODUCTION

In the family Bacillaceae, both aerobic and anaerobic species are capable of forming endospores which are metabolically dormant and usually heat resistant. The thermal stability of endospores is of economic interest in the food and drug industry. Certain of the sporeforming bacteria are pathogenic and represent a hazard to livestock and human lives.

Sporogenesis in itself is of academic interest, for here can be observed an unusual case of morphogenesis in which a normally labile vegetative cell differentiates into a biological entity able to withstand extremely adverse conditions. This transformation entails morphological, biochemical and physical changes, and it has received attention from many scientific disciplines.

The attempts at resolving the phenomenon of spore durability have been multifold but no clear explanation for the resistance of spores exists today. Studies on the nature of spores have been concerned with the factors influencing sporulation and germination, fine structural changes, mineral and water content, formation and function of dipicolonic acid (DPA), the development of heat

resistant proteins, and interrelationships of other constituents associated with bacterial spores. One of the above topics, heat resistant proteins, is the area with which this present research is concerned.

The elucidation of bacterial spore heat resistance depends in part upon resolving the mode of transformation from labile proteins to stable proteins. When biological systems are subjected to elevated temperatures, their proteins are among their most vulnerable entities. By comparison, nucleic acids are relatively heat stable and consequently have received little attention when factors involved in thermal destruction are being considered. Spores have a mechanism or mechanisms by which they are able to preserve proteins and other constituents necessary for germination and outgrowth. Of the many spore enzymes which have been identified in extracts, all are heat stable in the intact spore and some are heat stable in the spore extracts. These extractable, heat resistant proteins provide reasonable systems for studying mechanisms of spore stability. Of the many enzymes which have been identified in spore extracts, some are heat stable and have been shown to develop during sporogenesis.

Simmons (1961) reported a reduced nicotinamide adenine dinucleotide oxidase (NADH oxidase, formerly called

DPNH oxidase) found in both vegetative cells and spores of Clostridium botulinum. The spore NADH oxidase is heat stable in extracts while the vegetative enzyme is a normally labile flavoprotein.

The discovery of analogous enzymes in both vegetative cells and spores suggested a possible means of studying the formation and properties of a heat stable protein. A wide variety of possible mechanisms may exist for the transformation from the vegetative protein state to the spore protein state. In general, these could be stated briefly as consisting of four alternatives. First, the spore protein would essentially have the same primary, secondary, and tertiary structure as the analogous vegetative enzyme, heat resistance being accomplished by the addition or the modification by non-protein factors such as DPA, metal ions or pH. The second alternative might involve a structural modification of the original vegetative protein to form a heat resistant spore enzyme, the primary protein structure of the two analogous enzymes remaining essentially the same. The third possibility is polymerization or depolymerization of vegetative proteins to form stable spore structures. Fourthly, the spore enzyme may be synthesized de novo during sporulation and has little or no primary structural relationship to its analogous vegetative enzyme. Elimination of one or more of

these possible alternatives would help to elucidate the origin of one known stable protein.

The research presented in this thesis is a study of the characteristics of the spore and vegetative NADH oxidases of C. botulinum. Differences in heat stability, molecular size, enzyme kinetics, ionic charges, solubility, serological reactions and other characteristics will be presented and evaluated. The possible significance of the difference between these two analogous enzymes will be discussed in relationship to the formation of resistant spore protein.

LITERATURE REVIEW

Sporogenesis: Biochemical and Morphological Considerations

Ever since Alfred Koch (1888) first reported his observations on the sporogenesis of several sporeforming bacteria, scientists have been interested in this unique process of development. In the sixty years following this report, most studies were confined to routine microscopic observations, requirements for growth and sporulation, some biochemical studies, and antigenic characteristics. Good reviews of these early works have been written by Cook (1932) and Knaysi (1948).

In recent years, new biochemical and morphological findings have put a different emphasis on the study of bacterial spores. Several of the important initial findings that stimulated the new biochemical and morphological approaches to sporogenesis will be mentioned.

The first major biochemical approach to studying spores was made by Hills (1949, 1950) who observed that specific factors from yeast extract were necessary for the germination of *Bacillus anthracis*. He determined from fractionations of yeast extract that several different

amino acids, glucose, and certain nucleotides were stimulative for rapid germination of spores of the genus Bacillus. The amino acids, L-alanine, and tyrosine and the nucleoside adenosine were found to be the only nutrients necessary for the germination of a few species of these Bacillus spores. The stereoisomer, D-alanine, was occasionally found to inhibit germination, especially in spores of Bacillus subtilis. Hills concluded that a biochemical action on the part of the spores was probably responsible. This research stimulated subsequent biochemical studies of bacterial spores and thus led to further important findings.

Stewart and Halvorson (1953) were studying the effects of L- and D-alanine on the germination of Bacillus cereus spores and found an active alanine racemase in the intact spores. Later (1954) they studied this enzyme in spore extracts and showed that it was heat stable when associated with particulate matter. Since this first report of alanine racemase, a variety of oxidative and non-oxidative enzymes, all heat resistant in the intact spore, have been reported (Lawrence, 1957; Murty, 1957; and Levinson, 1957). Church and Halvorson (1955, 1956) reported that the oxidative enzymes of aerobic spores were activated by heat shocking the spores. Most spore enzymes appear to be dormant until activated by heat shocking, mechanical rupture or germination.

Powell and Strange (1953) observed that a number of organic compounds were secreted during the initial stages of germination and that excretion was one of the first changes associated with the germination process. An unusual organic acid with an absorption band between 250 and 300 mμ was detected in these exudates. Powell (1953) later identified this compound as pyridine 2:4 dicarboxylic acid, dipicolinic acid, by spectrophotometric comparison with known organic compounds. This compound is found uniquely in spores of Bacillaceae in the approximate range from 5 to 15% of their dry weight. It is released during germination (Powell, 1957 and Rode and Foster, 1960). DPA has been shown to develop during sporogenesis, before the formation of heat resistant Clostridium roseum spores (Halvorson, 1957 and Collier, 1957). A similar relationship was found for B. cereus (Hashimoto, Black and Gerhardt, 1960). Church and Halvorson (1959) were able to vary the amount of DPA in B. cereus spores by changing the phenylalanine content of the media. They found that spores containing less DPA per spore were less heat resistant. The above and similar findings show that DPA is associated with spore heat resistance. As yet, no direct evidence or mechanism by which DPA functions in spore heat resistance has been shown.

Our initial knowledge of spore enzymes and, to a limited extent, spore physiology came from studies of germination. Hardwick and Foster (1952) developed a useful technique for studying spore formation, which they termed endotrophic sporulation. Actively growing vegetative cells are removed from their nutrient medium and replaced in distilled water. They were able to show that at a certain stage of growth, the vegetative cells became irreversibly committed to sporulation. This technique has since been used by many authors to study sporulation, primarily in the aerobic sporeforming bacilli. Lund (1957) and Day (1960) reported unsuccessful attempts at endotrophic sporulation in anaerobic sporeforming bacilli (PA 3679 and C. botulinum, respectively); however, Collier (1957) was successful with Clostridium roseum.

Hardwick and Foster (1953), using endotrophically produced spores, studied seventeen different enzyme systems in vegetative cell and spore extracts of several Bacillus species. Their spore extracts were devoid of enzyme activity. The conclusion they drew from these experiments was that sporogenesis occurs at the expense of proteins pre-existing in the vegetative cell. Foster and Perry (1954) further substantiated this claim in their studies of the free amino acid, purine and pyrimidine pool found in

Bacillus mycoides cells. Isotopic tracer techniques revealed an appreciable reduction in this pool of free materials as the cells underwent endotrophic sporulation. They concluded that spore synthesis during sporulation drew from the vegetative cell's pool of free materials, perhaps at the expense of degraded vegetative constituents.

The higher levels of divalent metal ions in spores than in cells, especially calcium, was observed by Curran, Brunsteller and Myers (1943). Their spectrochemical analyses showed a definite increase in calcium and a slight increase in copper and manganese. Spores were lower in potassium and phosphorous content. Grelet's studies (1952a, b, c) focused attention on the fact that specific chemical species such as calcium, magnesium and manganese were necessary for sporulation. In his studies, Grelet (1952c) indicated an association between the amount of calcium in the medium and the amount and heat resistance of spores produced Curran (1957) showed that magnesium ions could replace calcium ions in synthetic media with the result that the same number of spores were produced, but they were less heat resistant. Knaysi (1961a, b), using a spodogram technique, found most of the mineral content of spores was concentrated in or near the spore cortex. In vegetative cells, the mineral content was evenly distributed.

The development of plastic embedding and ultrathin sectioning, coupled with electron microscopy, has provided useful techniques for studying bacteria and spores. Robinow (1953) was among the first to use these techniques to study spore structures. His observations revealed the complexity of the spore coat and membrane layers in resting spores of B. cereus and B. megaterium.

Fine structural changes occurring during sporogenesis have been observed and reported by other workers. Hashimoto and Naylor (1958) were among the first to apply these above techniques to studies of synchronized cells of Clostridium sporogenes that were undergoing sporulation. Hashimoto, Black and Gerhardt (1960) made similar observations on sporulating cells of B. cereus and they coupled their studies with other observable spore characteristics such as the synthesis of DPA, radioactive phosphorus uptake, and the appearance of heat stable spores. Young and Fitz-James (1959a, b, c) studied the synchronized sporulating cells of B. cereus and B. cereus var. Alesti using an electron microscope and various chemical techniques Their observations reveal the development of a spore septum which forms and encloses future spore desoxynucleic acid (DNA) They also observed an active ribonucleic acid (RNA) turnover during sporulation. Fitz-James (1960, 1962) has described

the possible role that mesosomes and cytoplasmic membranes may play in the early development and formation of the spore septum in the genus Bacillus and in Clostridium patinovorum. These and similar observations show that sporulation is an ordered process with definite spore structures being formed in conjunction with or followed by the appearance of other spore characteristics.

Recent interest has been directed to observations on the formation and properties of the spore cortex. Young and Fitz-James (1962) have described the cortex formation in B. cereus. Warth, Ohye and Murrell (1963a, b) have studied the mucopeptide, containing α, ϵ -diaminopimelic acid (DAP), that is closely associated with spore cortex. This recent interest in spore cortex is due to its close association with heat resistance and will be discussed later.

Spore Protein Synthesis

Some enzymes found in spores are heat stable analogues of vegetative enzymes while others have no counterpart in the vegetative cell. Many enzymes show a decrease or increase in activity during sporulation or undergo conversion from a soluble system to a particulate system, or from particulate to soluble systems. In many studies, periodic sampling of synchronized cultures

have shown that some spore enzymes are synthesized during sporogenesis.

A system for this synthesis would require an active turnover of RNA. Studies of sporulating cultures show that an active turnover of RNA does occur and that there is little or no synthesis of DNA (Young and Fitz-James, 1959b, c; Rosas de Valle and Aronson, 1962; and Day, 1963).

Working with asporogenic strains (Sp^-) of Bacillus subtilis, Schaeffer and Ionesco (1960) observed that the addition of purified DNA from sporogenic strains (Sp^+) to Sp^- strains caused the latter to sporulate. It seems evident that the vegetative cells of sporeforming bacteria have DNA containing the information for sporogenesis. These genes for synthesis of spore matter are apparently inactive until some mechanism, as yet unknown, "instructs" the cells to initiate sporulation whereby they form the RNA necessary for spore synthesis.

The synthesis of spore protein during sporogenesis has been demonstrated by several workers. Heat stable glucose dehydrogenase from B. cereus has been shown to develop after exponential growth of vegetative cells, but prior to the appearance of forespores (Bach and Sadoff, 1962). A similar finding was made for heat resistant spore catalase of B. cereus (Sadoff, 1961). Szulmajster and Schaeffer

(1961a, b) studied several NADH oxidizing systems (NADH oxidase, -dehydrogenase, -cytochrome c reductase, and diaphorase) in Sp^- and Sp^+ strains of B. subtilis They observed a definite increase in the various NADH oxidizing enzymes during the sporulation of Sp^+ strains For the corresponding interval, there was not a similar increase in the Sp^- strains. In the same study, the authors found a decrease in succinic cytochrome c reductase, cytochrome c oxidase and cytochrome c peroxidase during sporulation of Sp^+ strains; no corresponding decrease in Sp^- strains

Hanson, Srinivasan and Halvorson (1963) have studied changes in enzymatic activities, during sporulation of B. cereus, by periodically sampling synchronized cultures between early vegetative stages through maximum spore production. Vegetative cells, apparently lacking a functional tricarboxylic acid (TCA) cycle enzyme system, rapidly synthesize aconitase, malic dehydrogenase and other enzymes during the cell's transition into a spore. Anti-sporogenic agents, such as α-Picolinic acid, not only prevented sporulation when added to the medium but inhibited formation of these active TCA cycle enzymes. The authors feel that the formation of certain TCA cycle enzymes may be necessary prior to the beginning of sporulation They suggest that the antisporogenic agents may function by merely inhibiting this enzyme formation

Doi and Halvorson (1961) have made comparisons between vegetative and spore electron transport systems in B. cereus and they observed a variety of differences. The primary difference was the change from a predominantly cytochrome-rich, particulate system of vegetative cells to a soluble, flavoprotein oxidase system found in spores. They also showed that the soluble oxidases of spores function in electron transport during dormancy and germination.

Aubert and Millet (1963), using Sp^+ and Sp^- strains of Bacillus megaterium, were able to induce β-galactosidase during sporulation of the Sp^+ strains. Just prior to sporulation, the cells were washed and resuspended in fresh media. At any time, up to the appearance of 100% refractile spores (about six hours), they were able to induce β-galactosidase in the Sp^+ strains. After this time, the enzyme could no longer be induced in the Sp^+ strains. There was no time limit for the induction of this enzyme in the Sp^- strains.

Monro (1961) has studied the formation of a parasporal inclusion body ("crystal") during the sporulation of Bacillus thuringiensis, an insect larva pathogen. The "crystal" can be readily isolated in pure form and it is composed entirely of protein. Tracer studies using carbon^{14} labeled

amino acids, added at various times prior to and during sporogenesis, show that the "crystals" are synthesized during sporulation. This same study with labeled amino acids showed that many of the spore proteins were also synthesized during sporulation.

The various reports cited reveal that protein synthesis is active during sporogenesis and ceases abruptly when the mature, refractile spore is formed In some cases, a loss or gain of a certain enzyme activity has been noted during sporogenesis Increased RNA synthesis has been detected and this also indicates an active protein synthesizing mechanism.

Antigenic Studies of Spores

Kabat (1958) points out the following limitation in the use of serological techniques to study and compare protein structures. An antigenic structure is a site or series of sites on the protein molecule and not the whole molecule. Alterations of part of the protein structure can occur without changes in the antigenic structure and more than one antibody can react with a given protein.

For nearly 60 years microbiologists have attempted serological comparisons between spores and vegetative cells of Bacillaceae. They have encountered some limitations

which in some ways are peculiar to sporeforming bacteria. The question of possible vegetative contamination of spore preparations can always be asked. Another consideration is the effect on spore antigens of heat or chemicals used to destroy vegetative material. Thirdly, when viable spores are injected into animals, there is the possibility of spore germination, *in vivo*, producing vegetative antigens.

In his classical serological studies on spores, Defalle (1902), was aware of these problems. He used vegetative cells, viable and heat treated spores as antigens. Observations were made by agglutination or sensitizing reactions with whole cells or spores and he was able to distinguish distinct vegetative and spore antigens. When cross reactions between vegetative cells and spore antibodies were observed, they were generally attributed to either vegetative impurities in the antigenic preparation or possible spore germination in the animal. In many experiments, vegetative antibodies did not react with spores.

Howie and Cruickshank (1940), using several *Bacillus* species were able to produce crops containing 100% spores. In their experiments, they did not observe a cross reaction between vegetative cells and spore antibodies. They concluded that vegetative cells were distinctly different

from spores in their antigenic structure. The same authors did observe some common H (flagelar) and O (somatic) antigen between vegetative cells and old cultures of Clostridium sporogenes. They noted, however, that the old cultures of C. sporogenes they used as antigens were not pure spores. Norris and Wolf (1961) did find one common H antigen between cells and spores of Bacillus alvei, but found no other common antigens between spores and cells of eleven other Bacillus species.

Bekker (1944) heated spores of various Bacillus species for 30 minutes at 120°C and injected these into rabbits. When the resulting serum was tested with viable spores, it was noted that the autoclaving had little or no effect on changing the antigenic properties of spores. Control sera were produced by injecting viable spores. Antisera from autoclaved spores did not react with vegetative cells and antigens from various Bacillus species were mutually distinct. Similar observations had been made by Defalle and others.

Vennes and Gerhardt (1957, 1959) have produced specific antibodies against isolated vegetative cell structures of B. megaterium. The isolated structures were found to be, for the most part, antigenically distinct Antibodies produced from isolated spore wall did not show

any cross reaction with cell walls or any other cell component.

Norris (1961) in his review of spore antigens, sums up the six decades of research since Defalle. He concludes that spore antigens are distinct from vegetative cell antigens. When apparent cross reactions have been shown between cells and spore antibodies, there is always the question of germination or vegetative contamination.

MATERIALS AND METHODS

Production of Cells and Spores

Cells and spores were produced from Clostridium botulinum 62-A, American Type Culture Collection (ATCC 7948). The strain that was used by Simmons (1961) was employed for most of these experiments. The stock cultures were stored at -20 C as 0.5 ml suspensions of spores.

The medium used for spore cultures was a modification of the 4% Trypticase (Baltimore Biological Laboratories, Baltimore, Md.) medium developed by Day (1960). It contained 1 ppm thiamine and following mineral salts: 200 ppm each of $MgCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$ and $CaCl_2$; 20 ppm each of $FeSO_4$, $ZnSO_4$ and $CuSO_4$; and 50 ppm of K_2HPO_4.

Vegetative cultures were grown in a medium containing 2% Trypticase, 1 ppm thiamine and 0.2% dextrose. In both vegetative and spore cultures, 0.2% sodium thioglycoloate was used only in the inoculating series to be described below.

The procedure for growing these cultures was modified from Simmons (1961) and was adapted to 18 liters of media in carboys incubated at 37 C in water baths. To establish actively growing cultures, the following

inoculating sequence was employed. A tube containing 0 5 ml of frozen spore suspension was thawed, poured into 10 ml of media, heat-shocked at 100 C for 1-2 minutes and incubated at 37 C for 12 to 18 hours until turbid growth was apparent. This initial inoculation culture was then transferred to a flask, containing 120 ml of medium and incubated at 37 C for 4 hours. The entire contents of this flask was added to 480 ml of medium and this was incubated for 4 hours. Finally, this culture was transferred into 2400 ml of medium and incubated 4 hours yielding a 3 liter inoculum of actively growing cells. This last flask was poured into the carboy containing 15 liters of medium. In spore cultures, sterilized mineral salts were also added at this time The age of the culture (in hours) was measured from the time of this final inoculation until the culture was removed from the 37 C water bath and cooled.

The cultures were routinely sampled and examined microscopically. In spore cultures, swelling appeared at approximately 14 to 16 hours and the maximum percentage of refractile spores in sporangia were observed at about 24 hours These cultures were then allowed to incubate another 24 to 36 hours followed by 5 to 10 days storage at 4 C. Harvesting of spore cultures was done when microscopic examination showed that the spores were free of their sporangia.

Vegetative cell cultures were harvested after 7 to 11 hours of growth at which time they were rapidly chilled in ice baths to arrest growth and then harvested immediately.

Harvesting of the spores and cells was accomplished either batchwise in Servall centrifuges (model G or SS-4, Ivan Sorvall Inc., Norwalk, Conn.), or in a closed model Sharples Super Centrifuge (type 313D-49A-24BY, Sharples Corp., Philadelphia, Penn.). The collected cells and spores were washed 3 to 5 times in cold 0.85% NaCl solutions and were then stored in the freezer at -20 C.

Assay Procedures

The NADH oxidase activity was routinely assayed by following the reduction in absorption at 340 mμ in a Beckman DU spectrophotometer (Beckman Instruments Inc., Fullerton, Calif.). To a diluted portion of enzyme in a 3 ml cuvette (1 cm light path), the following final concentrations of materials were added: 10 μM flavin adenine dinucleotide (FAD); 0.067 M phosphate buffer, pH 7 7; approximately 125 μM NADH; and distilled water to a total volume of 3.0 ml. It was found necessary to mix the enzyme and FAD and incubate them at room temperature for several minutes in order to measure maximal activity. A unit of enzyme activity is defined as a 0.01 optical

absorption change at 340 mμ using an initial concentration of 125 μM NADH. Units are reported as 0.01 ΔOD_{340}/minute/ml of sample. Specific activity is the number of units per ml of sample divided by the mg/ml of protein for that sample.

Protein levels were determined by turbidimetric procedures (Stadtmann, Novelli and Lipmann, 1951), the Lowry technique (1951), or the spectrophotometric determination of Warburg and Christian (1942). Choice of the method employed depended upon the experimental situation. Protein concentrations are reported as mg of protein per ml of extract or suspension.

The assay procedure for diaphorase was identical to NADH oxidase assay except 10 to 100 ppm of methylene blue was added to the mixture. The total activity was measured by recording the decrease in absorption at 340 mμ and diaphorase was estimated as the activity above the known NADH oxidase activity for the same sample. Units are reported as 0.01 ΔOD_{340}/minute/ml of sample.

An assay for the spore enzyme was necessary to distinguish between vegetative and spore NADH oxidase activity. This was accomplished by testing the enzymatic activity before and after heating a portion of the sample to 70 C for 10 minutes. Spore enzyme was little affected by this heat treatment while vegetative enzyme was completely

denatured. Spore enzyme can also be identified serologically by reacting rabbit antispore serum with the sample, removing the precipitate by centrifugation, and assaying for remaining NADH oxidase activity. Only spore NADH oxidase is removed by antibodies against the spore enzyme.

The toxin of C. botulinum was qualitatively assayed by the following procedure. A half milliliter of the material in question was injected intraperitonealily into a mouse weighing approximately 25 g. Death of the mouse within 3 to 4 days was interpreted as indicating the presence of toxin. Preparations used for rabbit immunization were negative by this toxin assay. All preparations, following either vegetative or spore enzyme purification heating procedure, had little or no detectable toxin.

Purification Procedures

Purification of the spore and vegetative cell NADH oxidases was accomplished by the following procedures. All extracts, unless otherwise specified, were suspended in 0.067 M 2-amino-2-(hydroxy methyl)-1,3-propanediol (tris) buffer at pH 7.7 and maintained at 0 to 4 C. Routine assays for activity and protein concentration were performed at the various procedures to determine specific activity.

Homogenates were prepared in a high speed Servall Omnimixer at full speed, using a medium sized cup (50 ml

capacity) which was cooled by immersion in an ice bath. Cells or spores, 10 to 12 grams, were mixed with 45 grams of Superbrite #110 beads (Minnesota Mining and Manufacturing Co., St. Paul, Minn.) and tris buffer. The duration of the run was usually 10 minutes. Cell debris and glass beads were removed by centrifugation, washed and often rehomogenized for recovery of more enzyme. The supernatant fluids were then pooled and referred to as initial extracts.

The extracts were subjected to centrifugation for 6-8 hours at 32,000 g. This procedure removed the diaphorase and other particulate matter which interfered with enzyme assay and other subsequent procedures.

The nucleic acids which would interfere with protein fractionation procedures (below) were removed by the following sequence of steps. The extracts were treated with ribonuclease and desoxyribonuclease (Calibiochem, Los Angeles, Calif.), 1 mg each per 100 ml of extract, and incubated 37 C for two hours in the presence of 0.005 M Mg^{++} (Hatch, et al., 1961). The nucleic acid degradation products formed were separated from the enzyme during subsequent ammonium sulfate and gel filtration procedures.

Extracts containing a heat stable enzyme can be subjected to controlled heating procedures to remove other

proteins which are less stable. Both vegetative and spore NADH oxidases exhibit some degree of thermal stability. The extracts were placed in a boiling water bath and when the treatment temperature was approached, transferred to a water bath of the proper temperature. Vegetative extracts were treated for 3 minutes at 50 C. Spore extracts were treated for 30 minutes at 75 C. Denatured material was removed by centrifugation.

Proteins can be precipitated from an aqueous solution by a procedure known as "salting out" in which ammonium sulfate is often used as the salt. The increased concentrations of highly ionic salts force out of solution the organic solutes (proteins) which have low affinities for the solvent. A protein with a specific affinity for water can usually be "salted out" by a definite concentration of salt and thus separated from other proteins with different affinities. Variation in protein behavior can be achieved by altering the type of salt used, the solution temperature and the pH (Dixon and Webb, 1958, 1961).

Initially the proteins were precipitated by slowly adding ammonium sulfate until 90% saturation was achieved The precipitated proteins were separated from their supernatant solutions by centrifugation. The proteins were resuspended in a small volume of tris buffer and either

of two routine procedures was followed. In the first method, solid ammonium sulfate was slowly stirred into the protein suspension maintained at 0 C. The pH varied with the addition of ammonium sulfate and was approximately 5.2 to 5.4 as 90% saturation was approached. The second method employed a saturated solution of ammonium sulfate adjusted to a pH of 6.5 with NH_4OH. The fractionation was carried out at room temperature by slowly adding the calculated amount of ammonium sulfate solution to give the desired concentration.

Active enzyme fractions were further purified by gel filtration on Sephadex G-100 or G-200 columns (Pharmacia, Uppsala, Sweden). Fractions were collected and assayed for activity and protein concentration. Most of the purified vegetative and spore enzymes used for rabbit immunization, serological reactions, and kinetic studies were from these columns.

Gel Filtration

Sephadex gel filtration columns were prepared according to the manufacturer's instructions and consisted of two sizes: 2 Sephadex G-100 of approximately 3.5 cm x 100 cm each and several Sephadex G-100 or G-200 of approximately 2.5 cm x 60 cm. The total volume (V_t) was determined from

the geometry of the column and the void volume (V_o), by measuring the volume between start until the first protein material appeared in the effluent. The imbibed volume (V_i) was determined from the formula: $V_i = a\ W_r$, where a equals dry weight of Sephadex used and W_r = water regain for a given gel.

The solute (protein) distribution coefficient (K_D) can be determined from experimental results using the formula

$$\frac{V_e - V_o}{V_i} = K_D \qquad \text{(Flodin, 1962)}$$

where V_e is the effluent volume in which the solute (NADH oxidase) appears. In the event that $V_e = V_o$, the solute has been completely excluded from the imbibed volume and K_D is considered to be 0. The molecular size of a solute having a K_D of 0 would be equal to or greater than the exclusion size which is approximately 100,000 and 200,000 mol. wt. for Sephadex G-100 and G-200 respectively.

The eluant used in gel filtration columns was 0.01 M tris buffer at pH 7.7. A constant flow rate was achieved by use of a Sigmamotor Pump (Sigmamotor, Inc., Middleport, N.Y.) and 2 to 5 ml fractions were collected.

Procedures for Enzyme Characteristics

The heat inactivation studies were performed on NADH oxidases derived from either vegetative cells or spores of C. botulinum. The source of the vegetative NADH oxidase was active Sephadex G-100 fractions from routine purification procedures. For this particular investigation the spore NADH oxidase preparation was modified. The spore extracts were heated at 60 C for 5 minutes instead of 75 C for 30 minutes. Aliquots of enzyme were heated at 55 to 100 C in individual tubes (13 x 100 mm) placed in a regulated water bath. At various times, tubes were removed, cooled in an ice bath, supernatant fluid collected by centrifugation and assayed for enzyme activity. Loss of activity was determined by comparison to unheated controls.

Enzyme kinetics were studied by noting the effect of substrate concentration on the oxidase activity. The NADH concentration was varied over the range of 20 μM to 240 μM while maintaining a constant concentration of enzyme. The effect of atabrine as a noncompetitive inhibitor of flavin enzymes was ascertained by repeating the above experiments in the presence of 0.12, 0.6 and 1.2 mM atabrine. Michaelis constants for substrate (K_m) and inhibitor (K_i) were determined from the slopes of Lineweaver and Burk (1934) plots of data and the maximum velocity,

V_{max}, determined from the line intercept on the ordinate axis.

The effect of pH on enzyme activity was determined in 0.2 M buffers over the pH range of 5.2 to 9.4 using a constant amount of enzyme and NADH. The activity was reported in terms of percent of maximum activity.

The flavin cofactor requirements for vegetative cell and spore NADH oxidase were studied by attempts at removal of the cofactor component and then activation of the resulting enzyme with the addition of either FAD or flavin mononucleotide (FMN). A vegetative cell NADH oxidase suitable for these studies was obtained from ammonium sulfate fractionation or gel filtration procedures. The acid-ammonium sulfate technique for flavin removal (Warburg and Christian, 1938) was attempted several times in an endeavor to free the spore NADH oxidase of its cofactor.

Immunization and Serological Procedures

The vegetative cell and spore NADH oxidases (antigens) employed in either immunization or serological reaction procedures were obtained from respective Sephadex G-100 columns as described. Two different immunization methods were tried with different sets of Dutch Belt rabbits. All suspensions used in immunization procedures described below

were maintained from 0 to 4 C until just prior to rabbit injection. All glassware used for serological reactions was thoroughly cleaned with non-ionic detergent.

The first method employed either vegetative cell or spore antigens suspended in Freund's adjuvants, as directed by the manufacturer (Difco, Detroit, Michigan), and injected by subcutaneous and intramuscular routes. The first injection consisted of 0.5 to 1 ml of antigenic material containing 1 to 2 mg of protein and suspended in an equal volume of Freund's complete adjuvant. In subsequent injections, similar suspensions were prepared with Freund's incomplete adjuvant and administered once a week for 4 to 6 weeks. Over a period of a year a total of ten rabbits were injected with vegetative protein and four with spore protein by this above method. An alternative immunization method using only vegetative suspensions was also attempted. Foot pads (toe pads) of a rabbit were injected with 0.2 to 0.5 ml of vegetative protein suspension (no adjuvants) followed 3 to 4 weeks later by one subcutaneous injection of 0.5 ml protein suspension (Leskowitz and Waksman, 1960).

Blood was collected by cardiac puncture withdrawing 15 to 20 cc from each rabbit. The fresh blood was placed in clean 25 x 150 mm tubes and slanted, incubated at room temperature for 2-4 hours, then stored overnight at 4 C.

Serum was decanted from these tubes and clarified by centrifugation. Further treatment of the serum was necessary in order to remove serum enzymes that oxidized NADH. An ammonium sulfate fraction of 0-40% saturation was prepared from the serum proteins. This fraction was resuspended in 0.85% NaCl solution and used in the serological reactions described below. A control serum was prepared from blood drawn from non-immunized rabbits and treated in a similar manner.

The method of Cohn and Torriani (1952) was used to determine cross reactions between spore or vegetative cell NADH oxidases. Vegetative and spore antigens, obtained from Sephadex G-100 columns, were serially diluted in 0.85% NaCl solution. The dilutions were then distributed equally into several sets of 13 x 100 mm clean test tubes. To each identical set of dilutions, an equal volume of antiserum, control serum, or 0.85% NaCl solution was added. All tubes were incubated at room temperature 1 to 2 hours, stored at 4 C overnight and any precipitate was removed by centrifugation. The supernatant fluid from each tube was collected and assayed for NADH oxidase activity. The dilution sets receiving the various serums were compared to the controls. Spore antisera usually had to be diluted 10 to 100 fold prior to reaction with spore antigen.

EXPERIMENTAL RESULTS

Purification of Vegetative and Spore NADH Oxidase

Several procedures in sequence were utilized to purify the spore and vegetative NADH oxidases. The highest purification ratios obtained varied from 25 to 40 fold for vegetative enzyme and from 60 to 80 fold for spore enzyme.

The initial extracts were subjected to centrifugation for 6 to 8 hours at 32,000 g to remove particulate matter. There was an initial loss of 10 to 15% of total activity following this centrifugation

Table 1 shows a distribution of enzymatic activities found in initial extracts, supernatant fluid and resuspended pellets. It is apparent that methylene blue (MB) stimulates the oxidation of NADH by the particles and has little or no effect on the activity of the supernatant fluid. This result indicates that the initial loss was due to removal of a diaphorase.

The centrifugation procedure also removes the particulate protein from the initial extracts which amounts to 50% or more of the total protein. Both the spore and vegetative cell NADH oxidase remain in the supernatant fluid

and thus are considered soluble enzymes.

Table 1 Diaphorase activity in spore and vegetative cell extracts before and after centrifugation.

Source (fraction) in 10 μM FAD	Vegetative (units)	Spore (units)
Initial Extract		
no MB	96	75
100 ppm MB	180	126
Supernatant Fluid		
no MB	92	76
100 ppm MB	99	81
Resuspended Pellets		
no MB	9	6
10 ppm MB	30	15
100 ppm MB	66	36

Results of heat inactivation studies are presented in Figure 1. The information obtained from these heat inactivation studies was applied to the heating step of the purification procedure. The selection of 75 C treatment for the spore extract was based on the convenience of adjusting and controlling the temperature of a laboratory water bath.

The purified spore and vegetative cell NADH oxidases exhibit notable differences in their heat stability. Simmons (1961) also noted similar differences based on experiments with crude preparations. He reported a heat activation of the spore enzyme at 70 C but the present

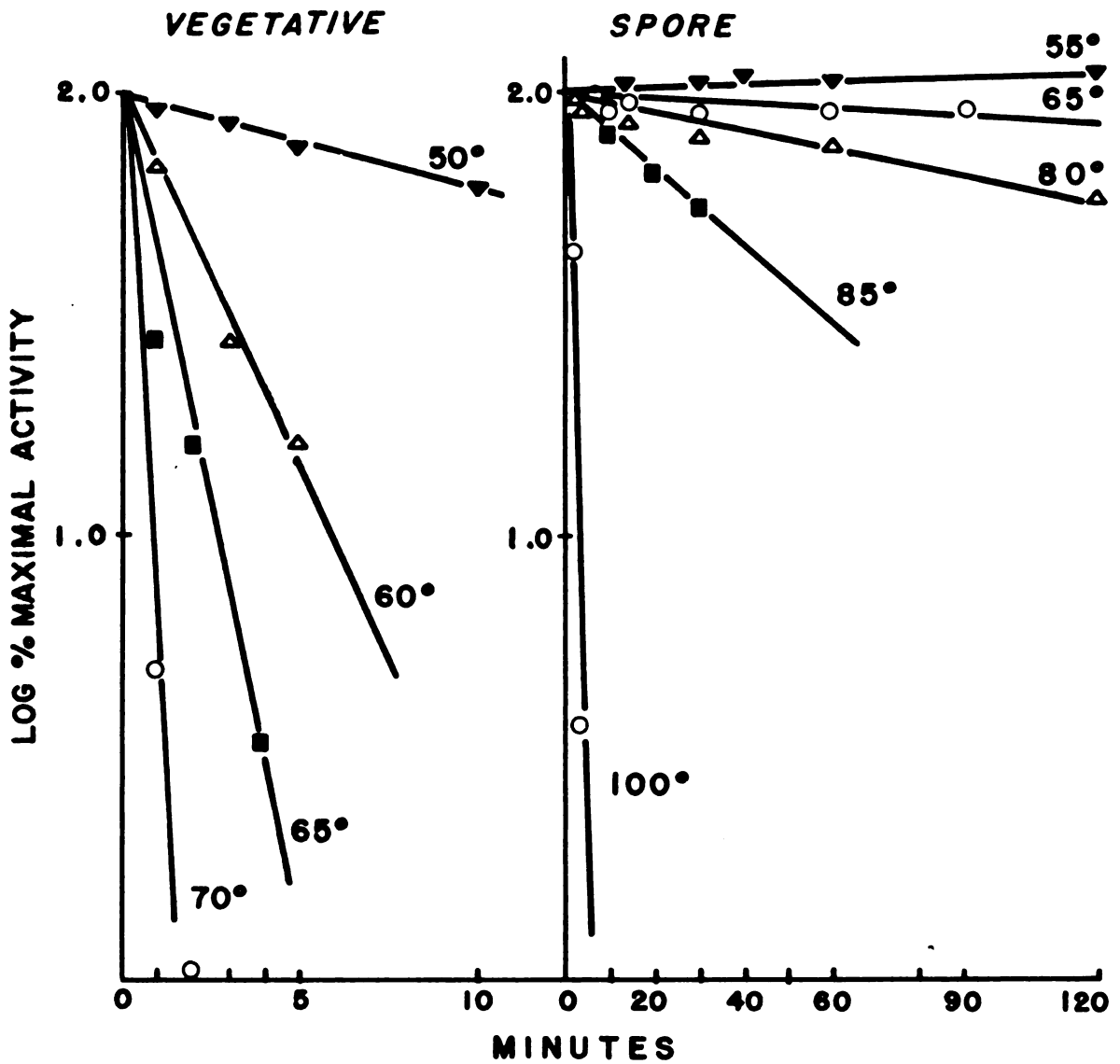

Figure 1. Heat inactivation determinations of vegetative cell and spore NADH oxidases.

data show heat activation at 55 C with a slight loss at 65 C. The activation of the spore enzyme could be explained by the presence of a NAD reducing system that was labile at 55 C. Data in Table 2 indicate that the same spore extract used in thermal inactivation studies does not reduce NAD under the conditions of the NADH oxidase assay. This apparent activation may be a property of the spore NADH oxidase.

Table 2. Attempt to reduce NAD with spore enzyme.

Spore Enzyme in 10 μM FAD	Activity (units)
Control	
125 μM NADH	195
250 μM NAD	0
500 μM NAD	0

A detailed study was performed to determine the range of ammonium sulfate concentration necessary to precipitate the vegetative or spore NADH oxidases. Vegetative or spore proteins which had been initially precipitated by 90% saturated ammonium sulfate were resuspended in tris buffer and dialyzed 4 to 6 hours to remove excess salts. Calculated amounts of ammonium sulfate were added to these suspensions to give 5 or 10% increases in saturation in

the range of 33 to 90% saturation. Assays were performed to determine NADH oxidase activity and protein concentration of precipitates which formed within these increments.

The results of NADH oxidase precipitation by either method described in Methods and Materials were very similar. Figures 2 and 3 represent the enzyme and protein profiles resulting from increments of 5 to 10% over the ammonium sulfate saturation range studies. The spore enzyme precipitates within a narrow range at an ammonium sulfate concentration of 55% saturation. The vegetative preparations precipitated over a wide band of ammonium sulfate concentrations with a peak at 70% saturation.

The vegetative cell NADH oxidase profiles reveal a small initial peak coinciding with the spore enzyme profiles. In Figure 3, this was labeled peak A, and is heat resistant. This spore-like enzyme appearing in vegetative extracts will be referred to again in this thesis.

The vegetative NADH oxidase that was obtained from the ammonium sulfate fractionation of these procedures was partially inactive and FAD was needed for maximal activity. This vegetative enzyme was used for two flavin cofactor studies that will be described in detail later.

The nucleic acids constituted more than 20% dry weight in crude extracts as detected by the Warburg and

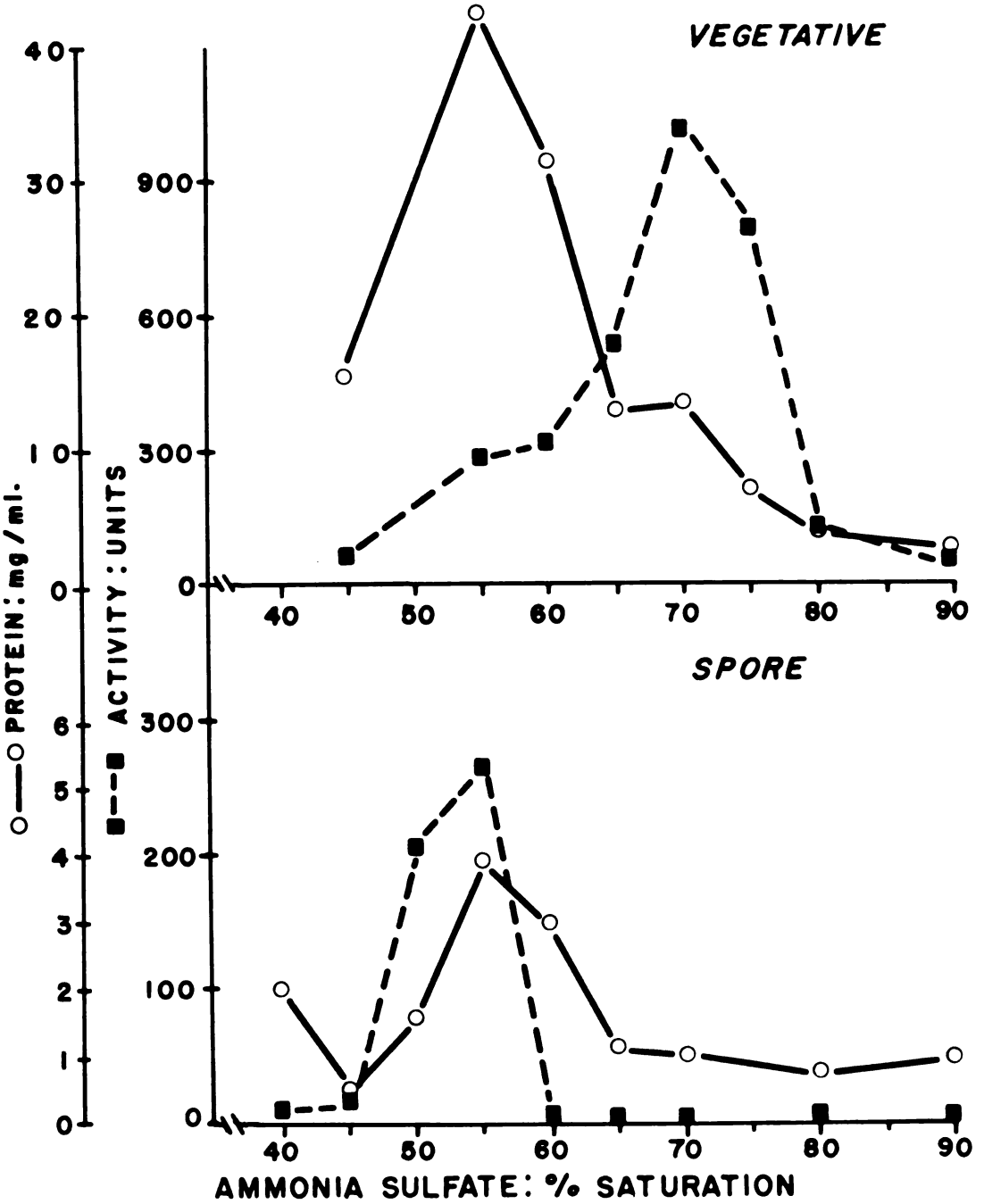

Figure 2. Vegetative and spore precipitation profiles of NADH oxidase activity and protein in various saturations of ammonium sulfate at 0 C.

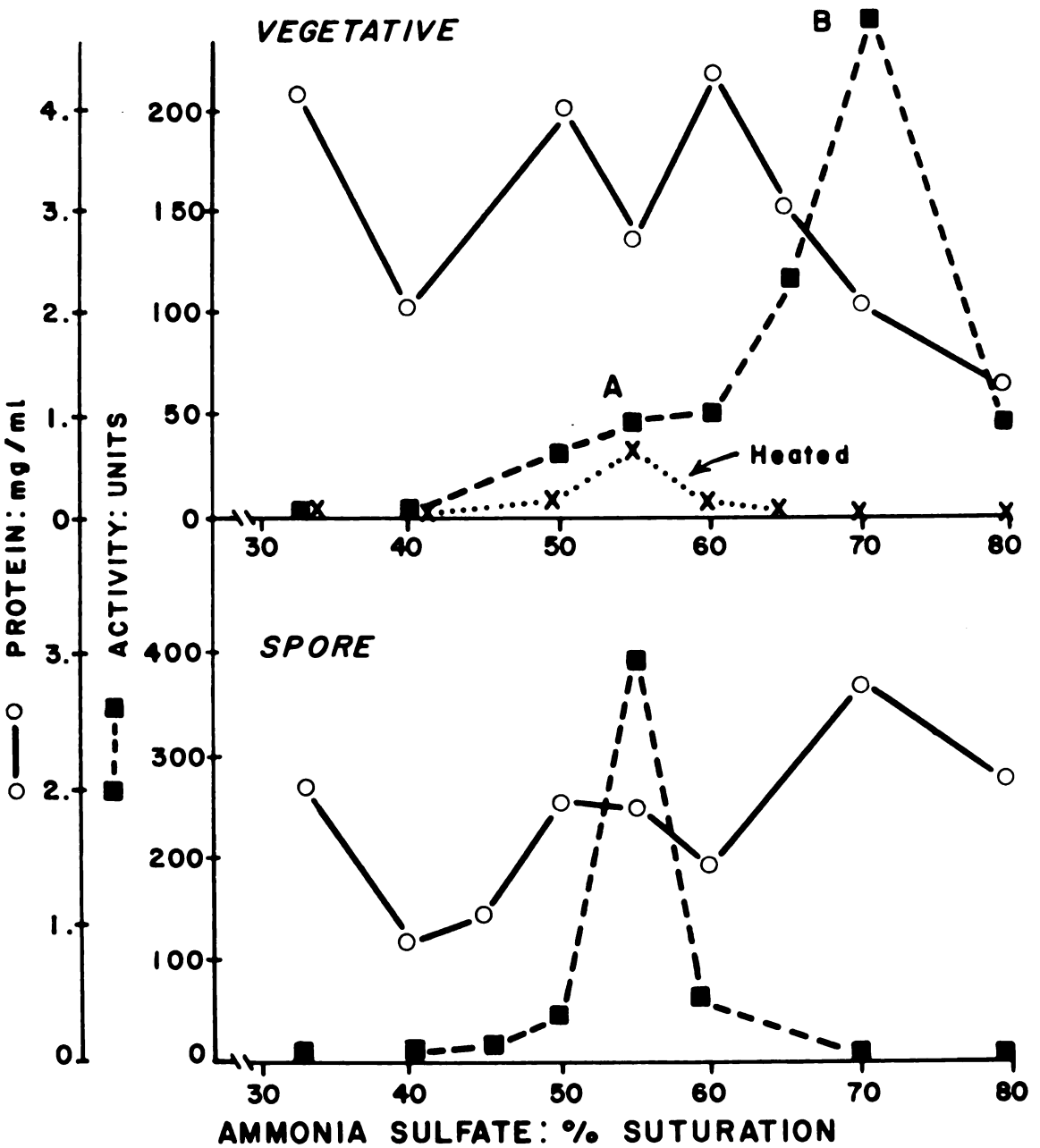

Figure 3. Vegetative and spore precipitation profiles of NADH oxidase activity and protein in various saturations of ammonium sulfate at room temperature and a constant pH of 6.5. In the vegetative profiles, a spore-like enzyme, peak A, is identified by the heating method (X ..... X). Peak B is vegetative enzyme.

Christian (1942) spectrophotometric method. After the initial 0-90% ammonium sulfate treatment, they were reduced to about 3% of the resuspended precipitate and after further ammonium sulfate fractionation were less than 1% by dry weight of all succeeding suspensions.

Further purification of vegetative cell and spore NADH oxidases was accomplished by gel filtration procedures Two large Sephadex G-100 columns of approximately 700 cc each, were used for these enzyme purifications.

Tables 3 and 4 show results from purification of vegetative cell and spore NADH oxidases. These procedures, previously described in detail, are briefly indicated in the first vertical column. The gel filtrations reported represent single fractions showing the highest purification ratios. There is no loss of either NADH oxidase from these gel filtrations.

Gel Filtrations

Gel filtration elution profiles were determined by protein and NADH oxidase assays. The vegetative cell and spore NADH oxidase profiles from both Sephadex G-100 and G-200 are shown in Figures 4 and 5 respectively.

Table 5 lists the K_D values calculated from the Flodin (1962) equation The effluent volume (V_e) was

Table 3. Purification procedures and typical results for vegetative cell NAD oxidase from C. botulinum.

Procedure	Volume (ml)	Activity (units/ml)	Total units (x 10^3)	Protein (mg/ml)	Specific Act. (units/mg)	Purification	Yield (%)
Initial extract	246	396	97.	30.7	12.9	1.0	100
Centrifugation 7-1/2 hr, 32,000g = g	238	360	85	18.5	19.5	1.53	87.5
RNAse-DNAse 37 C + 50 C 3 min treatments	222	267	59.5	7.8	34.0	2.65	61.5
0-90% $(NH_4)_2SO_4$	100	550	55.0	17.0	32.3	2.52	56.5
55-70% $(NH_4)_2SO_4$	10	4500	45.0	49.5	91.0	7.1	46.3
Sephadex G-100	3	510	1.53	1.33	380.0	29 5	1.57

Table 4. Purification procedures and typical results for spore NADH oxidase from *C. botulinum*.

Procedure	Volume (ml)	Activity (units/ml)	Total units ($\times 10^3$)	Protein (mg/ml)	Specific Act. (units/mg)	Purification	Yield (%)
Initial extract	310	96	29.8	20.5	4.7	1.0	100
Centrifugation 6 hr, 32,000g = g	300	87	26.2	5.0	17.3	3.6	88
RNAse-DNAse 37 C + 75 C 30 min treatments	280	60	16.7	0.9	66.5	14.0	56
0-90% $(NH_4)_2SO_4$	100	138	13.8	2.0	69.0	14.7	46.5
45-55% $(NH_4)_2SO_4$	7	1740	13.1	11.2	155.0	33	44
Sephadex G-100	3	330	0.990	0.95	345	73	3.3

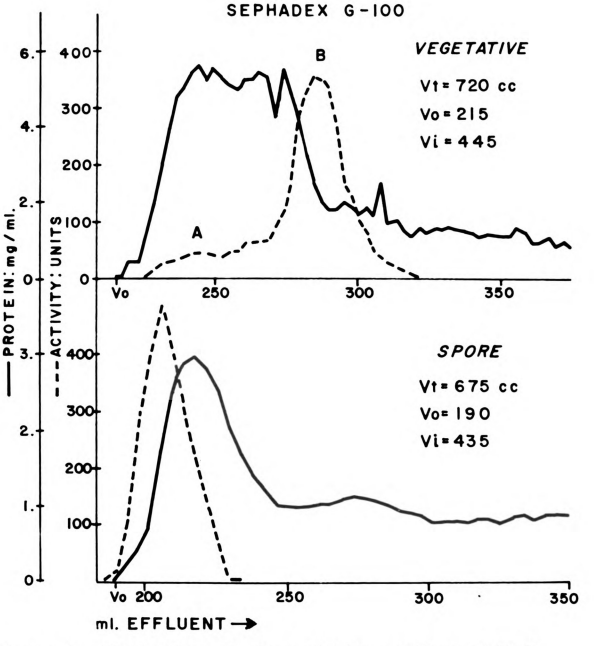

Figure 4. Vegetative and spore elution profiles of NADH oxidase activity and protein from respective Sephadex G-100 columns. In the vegetative NADH oxidase profile, purified enzyme was routinely collected from peak B fractions. Profiles in region of peak A were studied in detail (to be described, Figure 6).

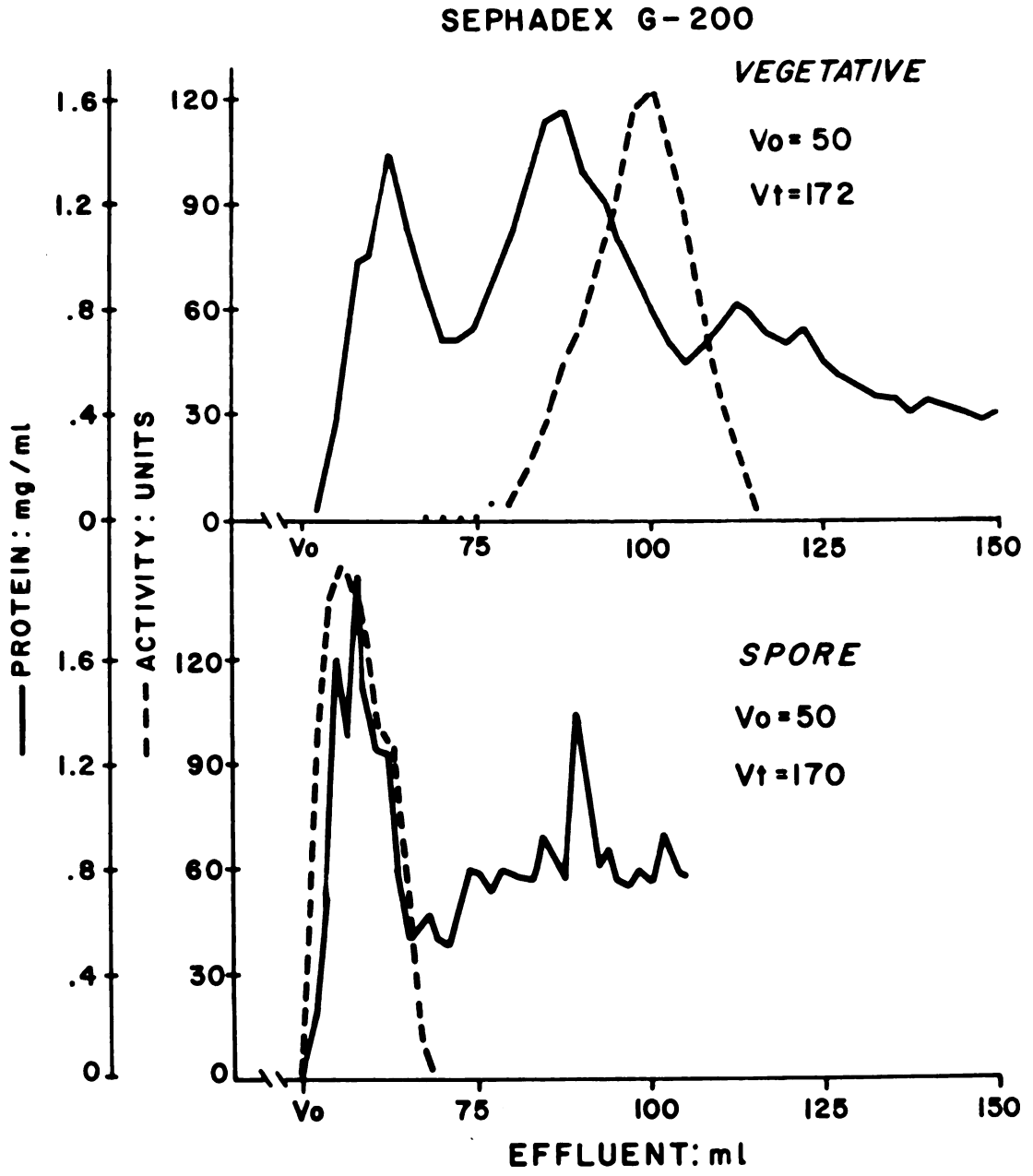

Figure 5. Vegetative and spore elution profiles of NADH oxidase activity and protein from respective Sephadex G-200 columns.

Table 5. K_D values determined from gel filtrations for vegetative cell and spore NADH oxidase from C. botulinum.

NADH oxidase activity peak	Column	
	Sephadex G-100	Sephadex G-200
Spore	0	0.01
Vegetative	0.110	0.280
peak A	0.035	----

determined from the various elution profiles. It is evident that the spore NADH oxidase is excluded, or nearly excluded, from the imbibed volumes of the gel particles of Sephadex G-100 and G-200. The vegetative NADH oxidase "enters" the imbibed volume of the gel particles of either Sephadex G-100 and G-200. This implies that the spore enzyme is equal to or greater than the greatest exclusion size of the columns, in this case approximately 200,000 molecular weight for Sephadex G-200. The vegetative NADH oxidase is included in the imbibed volumes of both Sephadex G-100 and G-200. This implies that it is equal to or less than the smallest exclusion size, approximately 100,000 molecular weight for Sephadex G-100.

The vegetative cell NADH oxidase elution profiles from Sephadex G-100 columns reveal an initial peak, labeled A

in Figure 4, whose position nearly coincides with that of the spore enzyme. A detailed study of this region was performed as follows. Pooled fractions were routinely assayed for NADH oxidase activity and protein concentration. They were then retested for spore enzyme by heating and serological methods. Protein concentrations were also redetermined on the heated samples. The results, seen in Figure 6, show that peak A contains a heat resistant NADH oxidase that precipitates with antispore serum. The majority of the other proteins in this region are also heat stable. Similar evidence from ammonium sulfate fractionation studies, previously mentioned (Figure 3), also show that a heat resistant, spore-like NADH appears early in cultural development.

Separation of Mixed Vegetative and Spore Enzymes

Equal units of vegetative cell and spore NADH oxidase were mixed and placed on Sephadex G-100 and G-200 columns and a diethyl-amino ethyl (DEAE) cellulose column.

A 1 cm x 20 cm ion exchange (DEAE) column was prepared according to Peterson and Sober (1962) and adjusted to a pH of 7.7 with 0.01 M tris buffer. Proteins were eluted from this column by using a linear NaCl gradient between 0.07 to 0.2 M (Bock and Ling, 1954). A Sigmamotor Pump was used to control the flow rate at 50 ml per hour and 5 ml

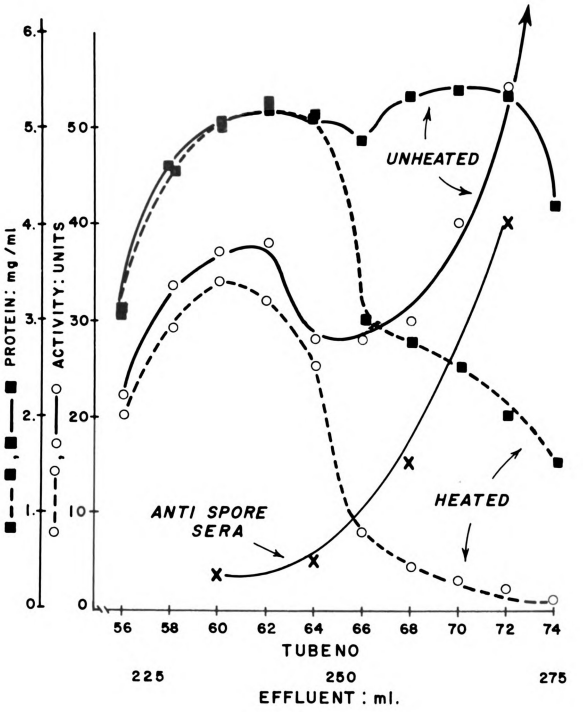

Figure 6. Normal and heat resistant elution profiles of NADH oxidase activity and protein, also serological reaction profile, from region of peak A, Figure 4. Spore-like enzyme is identified by both heating and serological (X ----- X) methods of assay.

fractions were collected. The effluent fractions from these columns were assayed for proteins, total NADH oxidase activity, and for heat resistant NADH oxidase activity. These elution profiles are shown in Figures 7, 8, and 9.

The elution profiles show that the vegetative cell and spore NADH oxidases have been separated on Sephadex G-100 and G-200. A complete separation is obtained on Sephadex G-200 and this establishes a useful means to distinguish or separate spore from vegetative NADH oxidases. These results could have been predicted from the K_D values However, the actual mixing of these two NADH oxidases, eluting them from gel filtration columns and being able to demonstrate separate peaks definitely establishes that the vegetative and spore NADH oxidases differ in molecular size.

The elution profile for spore enzyme on DEAE cellulose is quite narrow compared to the vegetative enzyme which has a tendency to trail. These results indicate probable differences in ionic charge between the spore and vegetative NADH oxidase.

Determination of Enzyme Characteristics

Studies of enzyme kinetics, pH optima, and cofactor requirements were performed on the vegetative cell and spore NADH oxidases The purpose was to show the differences that

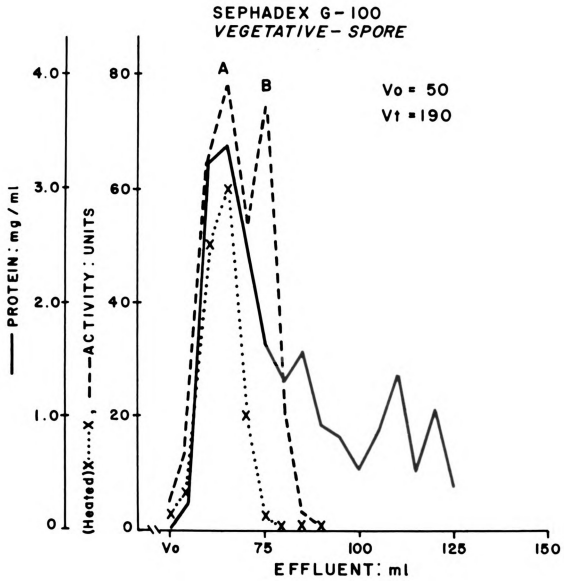

Figure 7. Profiles for NADH oxidase activity and protein eluted from a Sephadex G-100 column loaded with a mixture of vegetative and spore enzyme extracts. Spore enzyme, peak A, is identified by the heating method (X ..... X) which also identifies peak B as vegetative enzyme.

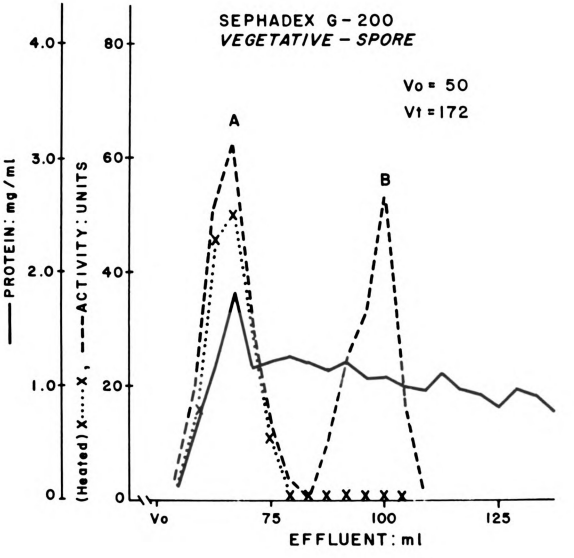

Figure 8. Profiles for NADH oxidase activity and protein eluted from a Sephadex G-200 column loaded with a mixture of vegetative and spore enzyme extracts. Spore enzyme, peak A, is identified by the heating method (X ..... X) which also identifies peak B as vegetative enzyme.

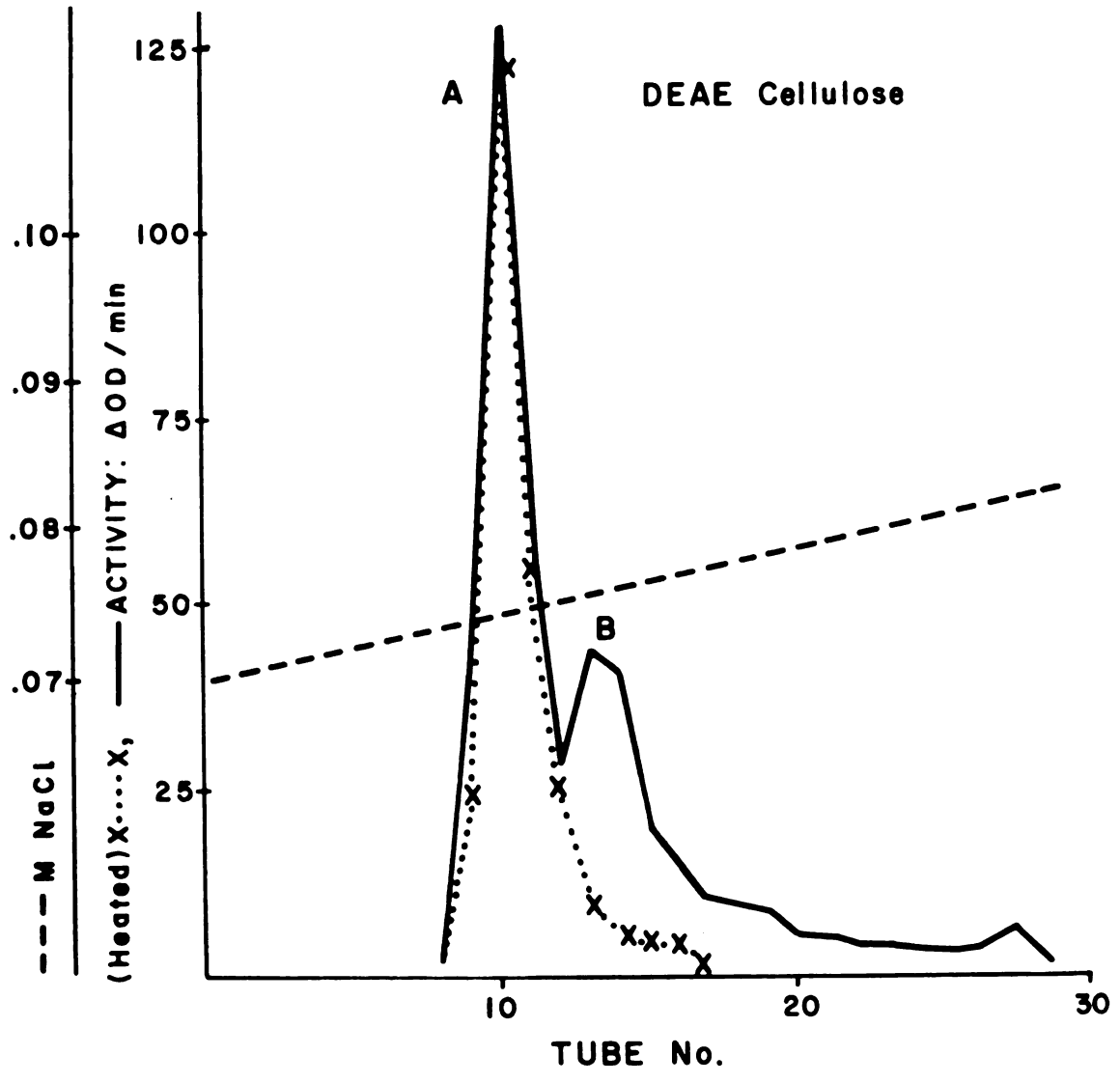

Figure 9. Profiles for NADH oxidase activity eluted from a DEAE cellulose column loaded with a mixture of vegetative and spore enzyme extracts. Spore enzyme, peak A, is identified by the heating method (X ..... X) which also identifies peak B as vegetative enzyme.

might exist between these two enzymes. Figure 10 represents typical Lineweaver and Burk plots of the relationship between substrate concentration and the reaction rates. From these plots the kinetic values were determined. Table 6 summarizes the Michaelis constants, K_m and V_{max}, for spore and vegetative enzymes and the K_i values for various concentrations of atabrine, a non-competitive inhibitor. The K_m of the spore enzyme is approximately 8 times that of the vegetative enzyme implying a greater substrate binding by the vegetative enzyme. Inhibitor kinetic values indicate that the spore enzyme is less inhibited by atabrine.

The optimum pH for activity was determined for vegetative and spore NADH oxidases and the resulting curves are seen in Figure 11. The pH optimum for the two enzymes appears to lie between 7.3 to 7.6. It is apparent the spore enzyme is more active in the lower pH regions than the vegetative enzyme. Conversely, the vegetative enzyme shows more activity in the pH regions above the optimum pH.

Differences in affinity for FAD were noted between vegetative and spore NADH oxidases. The vegetative enzyme readily loses half or more of its activity after ammonium sulfate fractionation but the activity can be restored by the addition of FAD. Following elution from Sephadex columns,

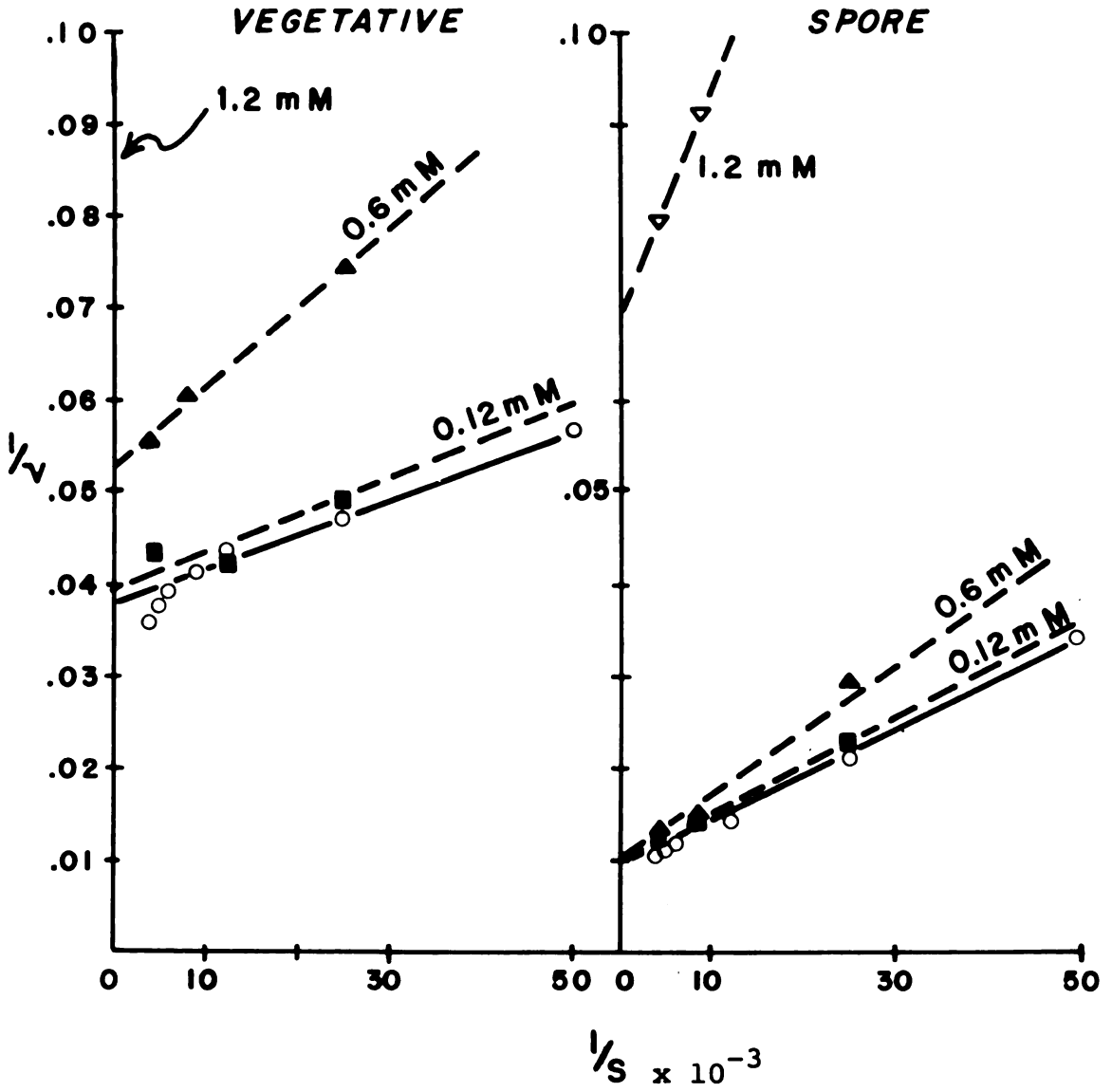

Figure 10. Effect of various concentrations of substrate and inhibitor on reaction velocities of both vegetative cell and spore NADH oxidases. Results of substrate (solid lines) and substrate in different atabrine concentrations (dashed lines) are shown above.

Table 6. Enzyme kinetic constants determined for vegetative cell and spore NADH oxidase from C. botulinum.

Constant 20 to 240 μM NADH	Inhibitor concentration (atabrine)	Vegetative NADH oxidase	Spore NADH oxidase
K_m	----	7.9×10^{-6} M	5.9×10^{-5} M
V_{max}	----	130	280
K_i	0.12 mM	1.2×10^{-5}	$5. \times 10^{-5}$
	0.60 mM	2.3×10^{-5}	9×10^{-5}
	1.2	*	4.5×10^{-4}

*Completely inhibited.

the vegetative NADH oxidase is nearly free of FAD. Several unsuccessful attempts were made to resolve the partially purified spore NADH oxidase by the Warburg and Christian acid-ammonium sulfate treatment. The cofactor remained strongly bound to the spore NADH oxidase and the enzyme was rapidly destroyed below pH of 3. The best experimental results for removal of flavin from spore enzyme are shown in Figure 12. Comparison is made to a vegetative enzyme from an ammonium sulfate fractionation. In neither case does FMN restore activity. Simmons (1961) also treated his cell-free preparations to remove flavin groups and reported about 55% reactivation with FMN.

The effect of incubation time on activity was noted when FAD and enzyme were mixed prior to assaying. Figure 13

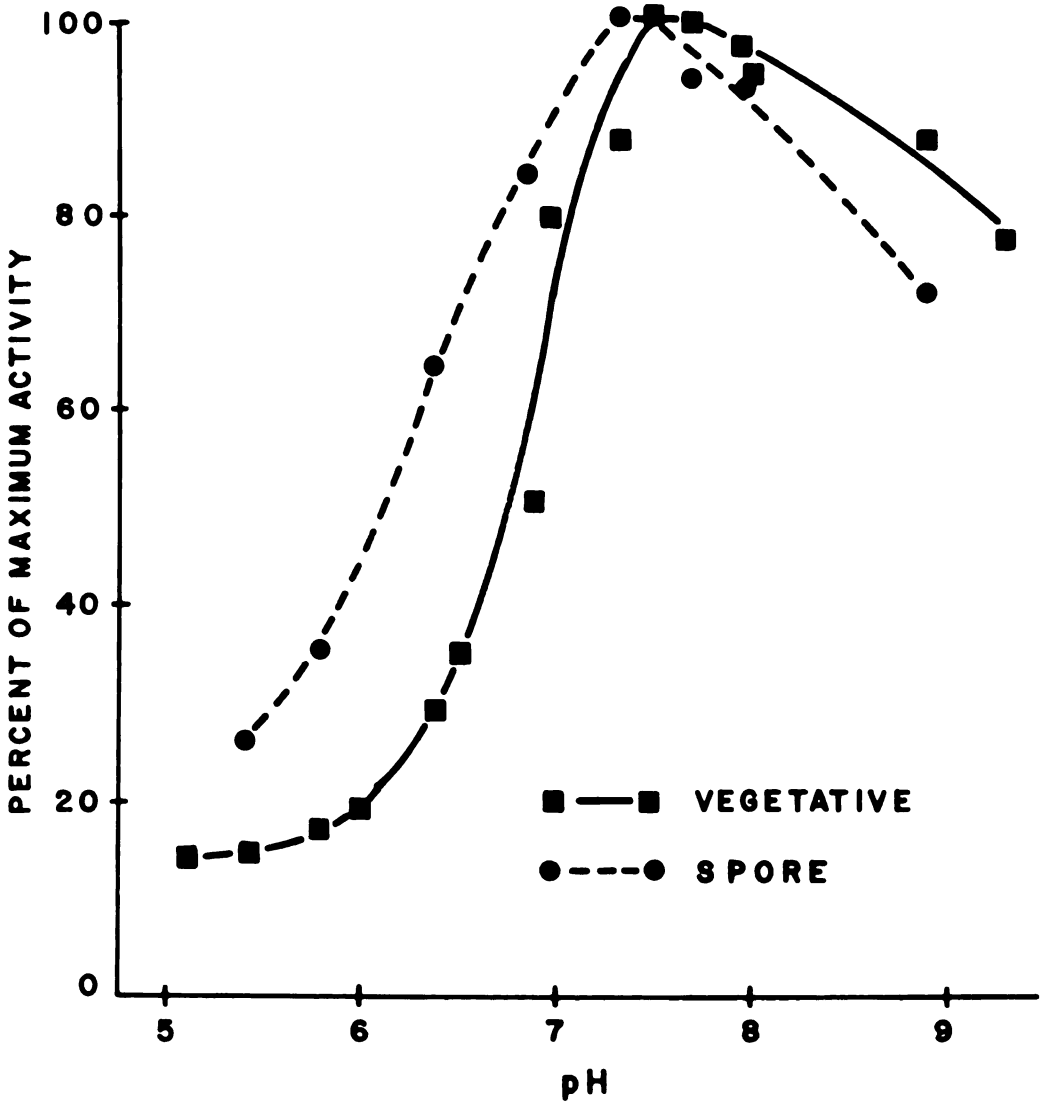

Figure 11. Plot of relative velocity vs. pH of medium for both vegetative cell and spore NADH oxidase.

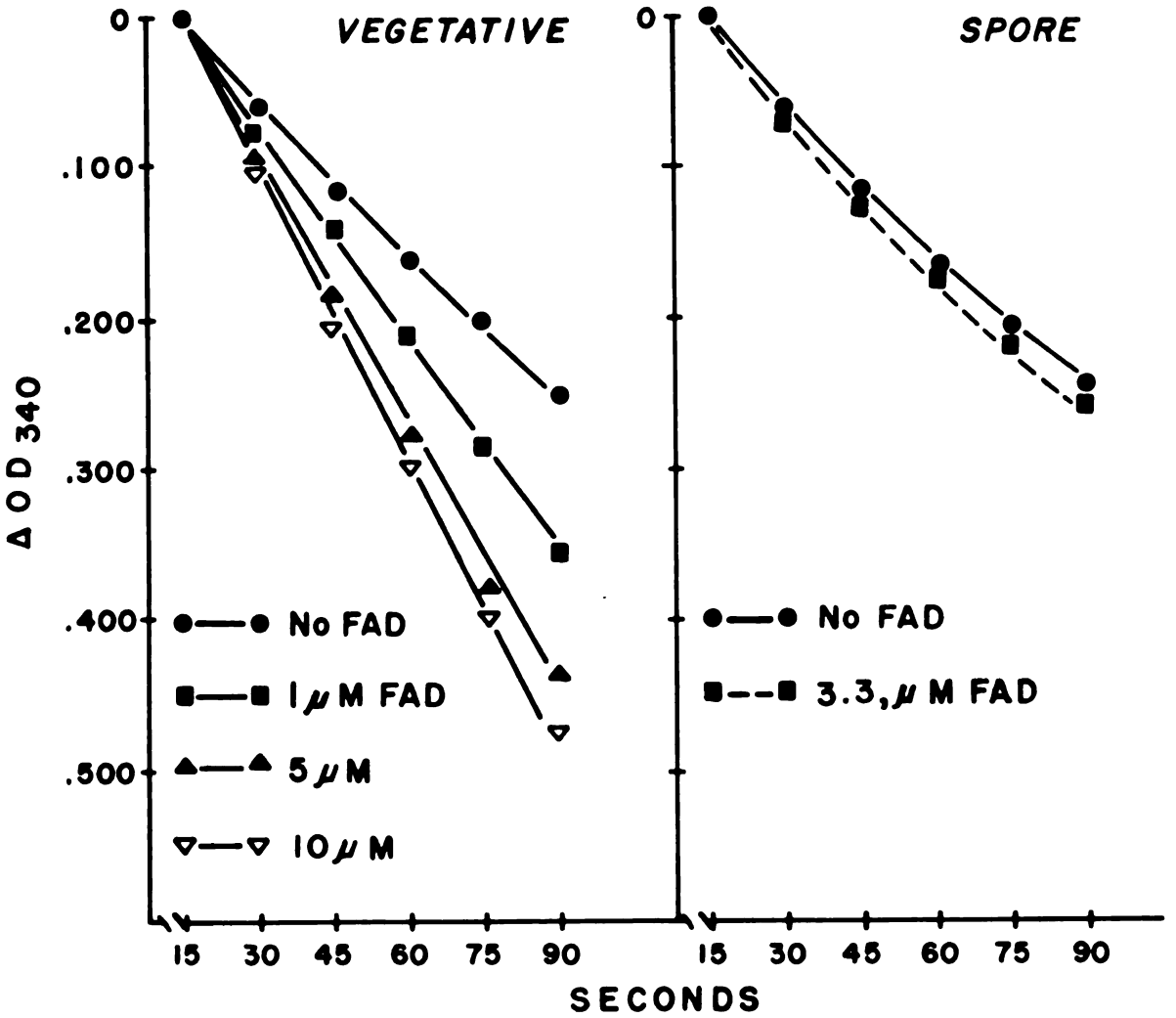

Figure 12. Flavin reactivation of both vegetative cell and spore NADH oxidases. In either enzyme assay, the presence of 10 μM FMN has no effect and simulates the no FAD curve.

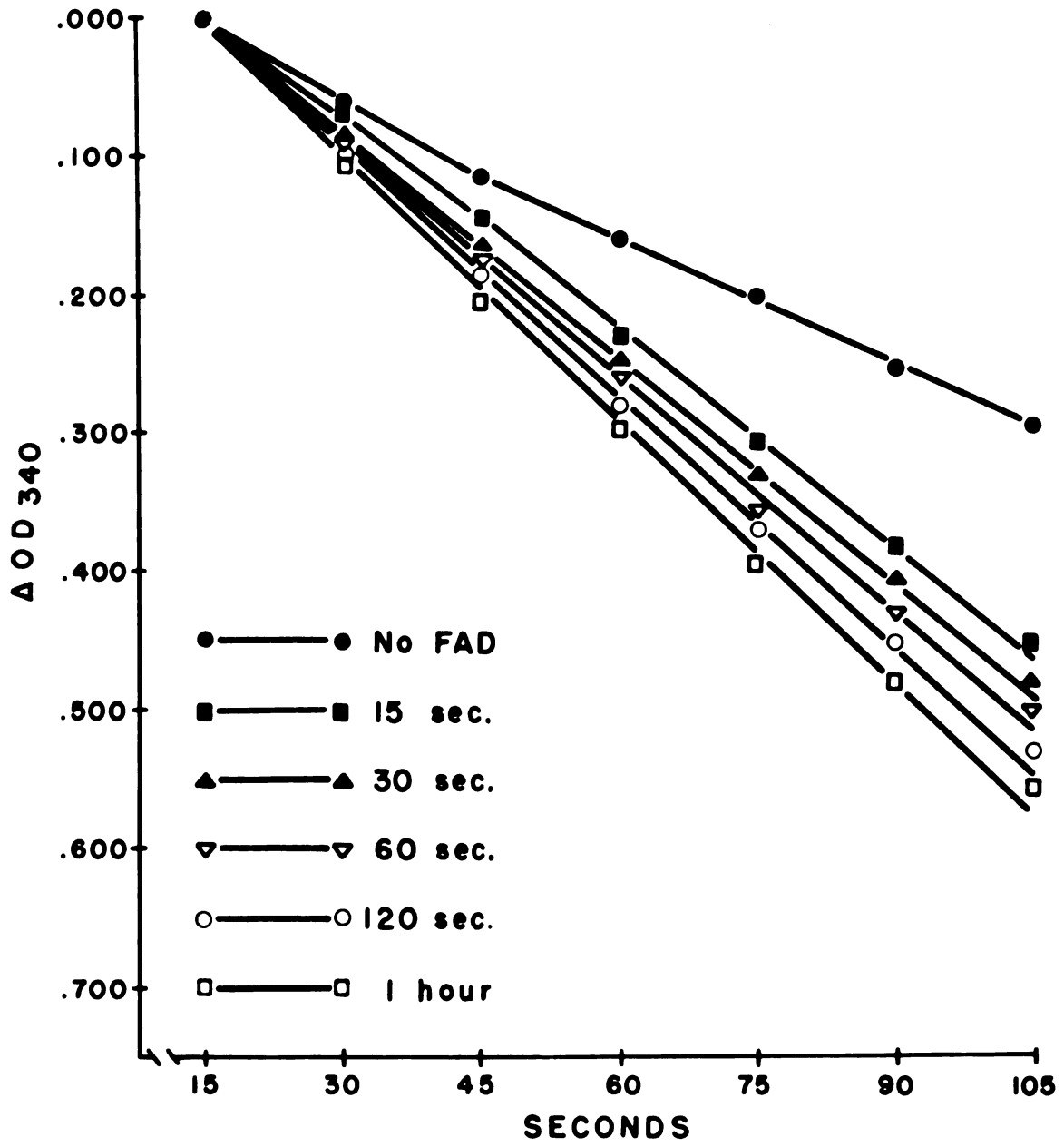

Figure 13. Effect of vegetative cell NADH oxidase and FAD incubation on maximal activity. The above enzyme-FAD incubation times include the 15 seconds between initiating the assay reaction and the first observation of reduction in absorption at 340 mμ.

represents the data from experiments using a constant amount of enzyme and FAD (10 μM) and varying only the time of incubation. In assaying procedures, the FAD was always pre-incubated with the enzyme for 3 minutes.

A vegetative cell NADH oxidase obtained from gel filtration column was used in another study and results are seen in Table 7. Various concentrations of FAD, ranging from 1.67 μM to 30 μM and 10 μM FMN, were added to this inactive vegetative cell NADH oxidase. FMN reactivated the enzyme about 10% of the maximal activity obtained by FAD. For the enzyme concentration in this experiment, the amount of FAD used in routine assays (10 μM) achieved maximal activity.

Table 7. Flavin activation of vegetative cell NADH oxidase.

μM Flavin (concentration)	Activity: units
0.0	6
10 FMN	21
1.67 FAD	84
3.30	114
10	132
20	135
30	135

Serological Studies

Serological studies were performed in an endeavor to find antigenic relationships between vegetative cell and spore NADH oxidases. If these two antigens had one or more common antigenic sites, then a cross reaction would be expected between either protein and antibodies formed against the other protein. If they were identical or very closely related, either protein would react fully with its heterologous antibody.

The Cohn and Torriani technique used is described in Methods and Materials. It was selected for the following reason. It is an ideal method for showing a specific serological reaction when a wide variety of antigens and their homologous antibodies are present in the same mixture. If the antigen is an enzyme, then the specific antibody-antigen reaction is detected by removal of the enzyme from the mixture.

The results of serological studies are presented in Figures 14 and 15 and are typical for vegetative and spore antigen-antibody reactions. The striking result of these investigations is that there is no cross reaction between vegetative cell or spore NADH oxidase and heterologous antisera.

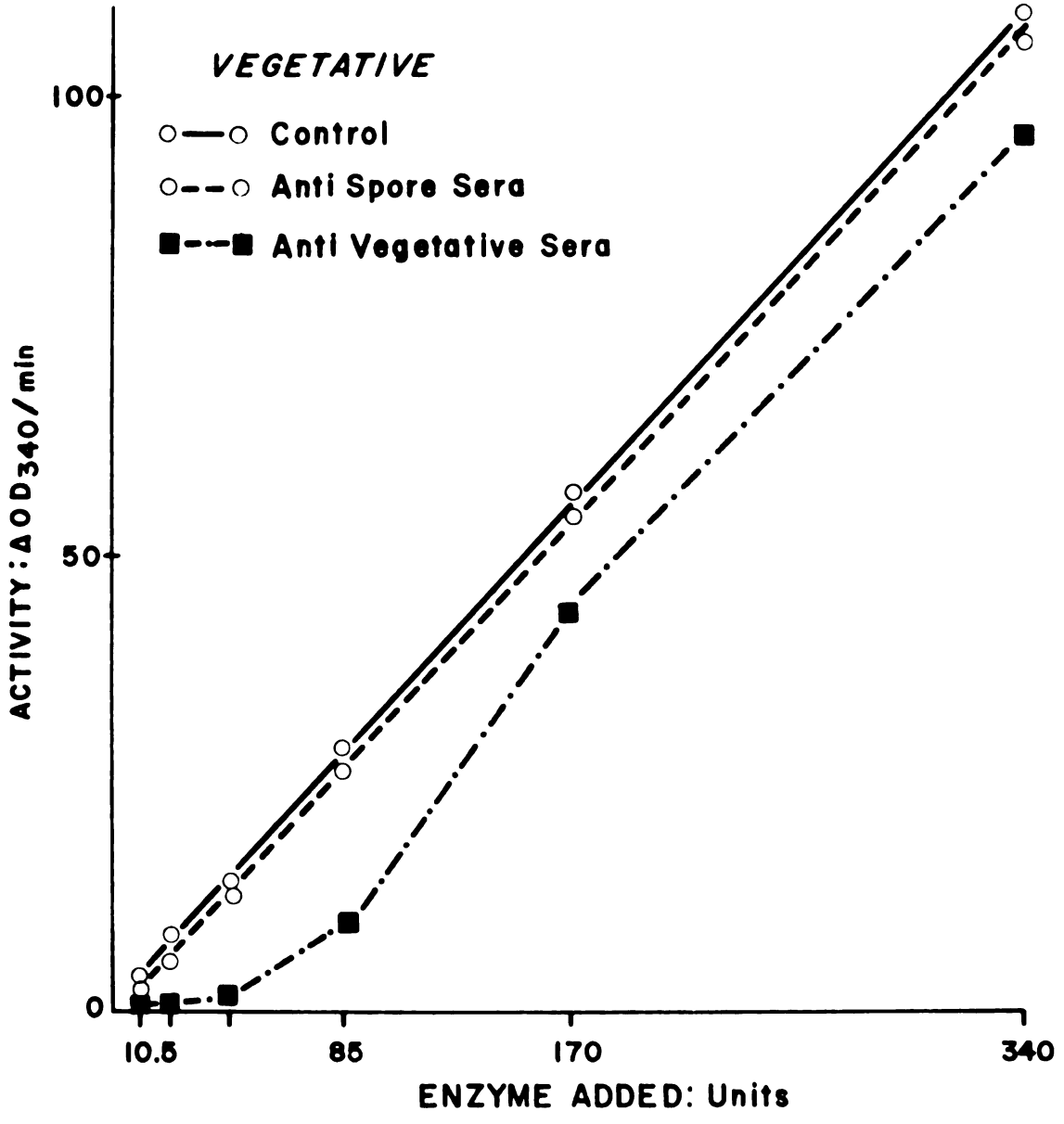

Figure 14. Serological reactions with vegetative cell NADH oxidase as antigen. Both saline and serum controls (0 ——— 0) and the antispore sera curve are identical and indicate no cross reactions. The anti vegetative sera curve is typical for all anti vegetative rabbit sera tested.

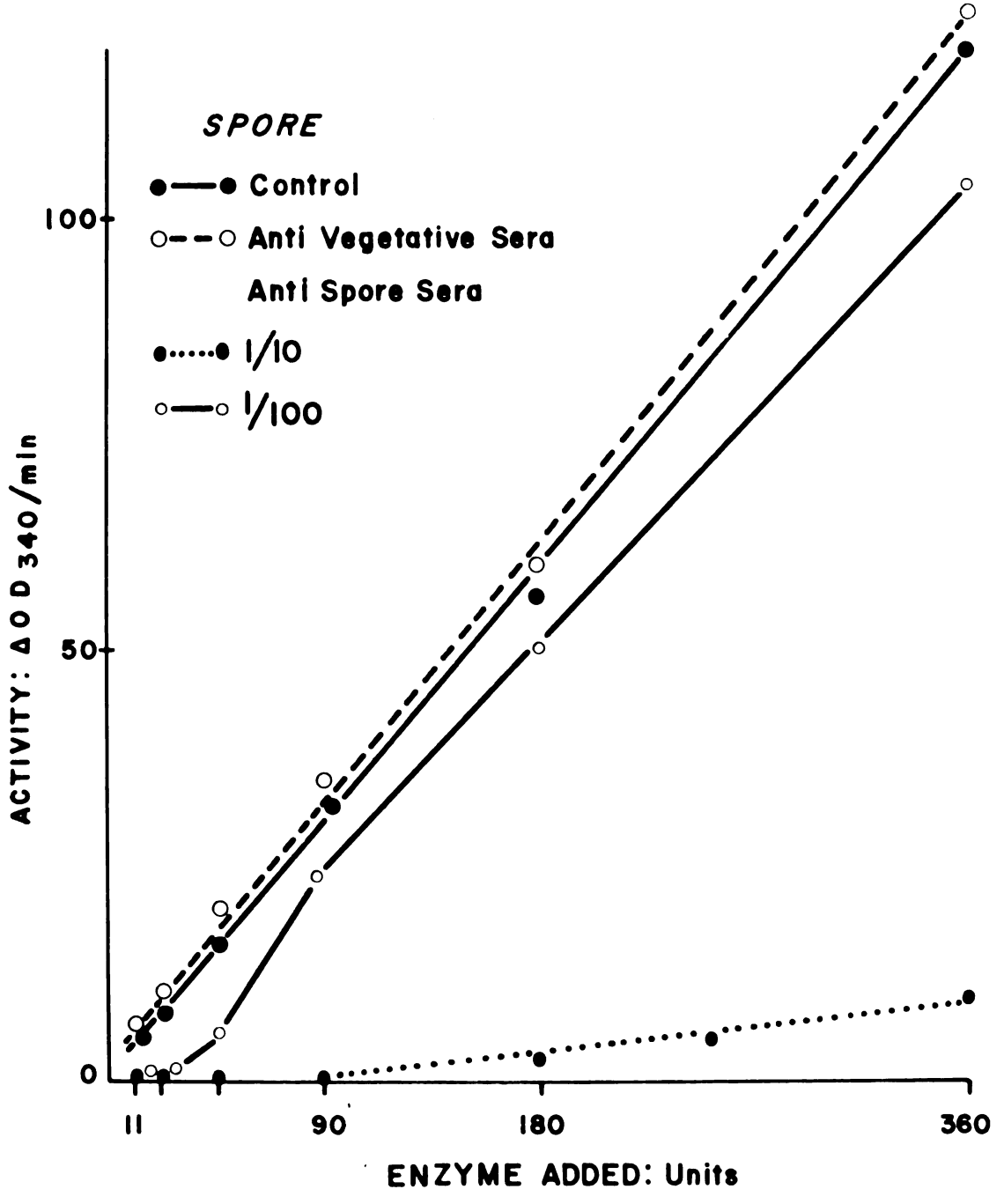

Figure 15. Serological reactions with spore NADH oxidase as antigen. Both saline and serum controls (0 —— 0) and the anti vegetative sera are identical and indicate no cross reactions. The diluted (1/10 and 1/100) anti spore sera curves are typical for all anti spore rabbit sera tested. Undiluted anti spore sera completely remove the spore NADH oxidase (antigen) in these concentrations.

Rabbits receiving spore antigen produced high levels of antibody. Undiluted antispore sera completely removes spore NADH oxidase for the enzyme levels shown in Figure 15. All rabbits receiving antigen derived from vegetative cells by either Freund's method or by the foot pad technique, had low titers of antibodies against the vegetative NADH oxidase. These observed differences in titers indicate an apparent distinction in antigenic properties of these enzymes.

DISCUSSION

The purpose of this research, as outlined in the Introduction, was to compare the properties of vegetative cell and heat resistant spore NADH oxidases of C. botulinum. It was hoped that the comparison of these analogous enzymes would elucidate some possible mode by which a heat resistant spore protein could be derived from vegetative cells.

A comparison of the properties of these two enzymes shows the following differences. Besides the extreme differences in their heat stability, there appear to be differences in their solubility at various levels of ammonium sulfate saturations. There are differences in their ionic surface charge as observed by characteristic differences in their elution from DEAE cellulose anion exchange chromatographic columns. The optimum pH activities of the two enzymes show a slight but consistent difference. The important kinetic differences are their reaction with substrate and inhibitor and their binding capacities for the flavin cofactor.

The above cited differences could be interpreted as resulting from some modification of the vegetative NADH oxidase molecule. However, there are two characteristics which show distinct protein structural and molecular size

differences between these analogous enzymes.

In the first case, they do not cross react serologically. Current interpretation of this information is that the two antigens (enzymes) are different. Kabat (1958) points out that antigenic sites on a protein molecule are not necessarily an expression of the whole molecule. Therefore, differences in one or all expressed antigenic sites cannot be absolutely interpreted as complete differences in protein structure. The possibility that the basic vegetative protein structure, has been changed to form a spore protein should not be completely disregarded.

The information obtained from gel filtration studies shows a definite molecular size difference between the two proteins The spore enzyme has an estimated molecular weight of 200,000 or more and is therefore at least twice the size of the vegetative enzyme which is estimated at 100,000 or less The fact that gel filtration separates solutes by molecular size is well documented (Flodin, 1962; Granath and Flodin, 1961; Killander and Flodin, 1962). Molecular size differences quite definitely exclude the possibility that a single vegetative enzyme molecule has been modified into a single spore enzyme molecule. However, the interpretation that a vegetative NADH oxidase molecule has polymerized with one or more other proteins molecules, including other

vegetative NADH oxidases, is not excluded. Such a polymerization could lead to the formation of a protein which need not react serologically with its monomer.

In the Introduction, four general alternatives were given to embrace all conceivable ways a spore protein might be derived from vegetative cells. The first two alternatives were concerned with possible modification of a single vegetative protein, either by extrinsic protein factors such as DPA and calcium or intrinsic protein structural changes. Possible non-protein modifications and protein structural modifications of vegetative cell NADH oxidase molecules can be eliminated as the sole means of forming heat resistant spore NADH oxidase.

The second two alternatives were briefly stated as either polymerization or depolymerization of vegetative proteins to form stable structures, or the de novo synthesis of a spore protein structurally unrelated to pre-existing vegetative proteins. As mentioned, our current findings cannot completely exclude either of these two as operational for spore NADH oxidase from C. botulinum.

In reviewing the literature, some authors have indicated evidence for de novo synthesis of spore proteins. Isotopic tracer studies show the incorporation of labeled amino acids into the spore protein (Hardwick and Foster, 1952

and 1953; Foster and Perry, 1954, and Monro, 1961). The existence of spore enzymes not found in the vegetative cell certainly support this view (Bach and Sadoff, 1962). Other authors, working with spores as antigens, generally indicate spores and their vegetative cells are antigenically distinct (Norris, 1962).

There is no a priori reason for all spore proteins to arise de novo and the possibility does exist that cells utilize some protein conserving mechanism. Although the enzymes observed in the present work are distinctly different, it is not possible to exclude polymerization as a means of formation. If this is truly the case, the flavin cofactor may be involved in some way in the mechanism of heat resistance.

Some current theories explaining spore heat resistance can also be considered in the light of the results and the above interpretation. The main stress has been to find a direct connection between calcium ions, DPA and a mechanism for protecting spore constituents, especially proteins. Young (1959) demonstrated that DPA could form a DPA-amino acid complex which was calcium dependent and she speculated that a similar complex might exist with spore proteins. Doi and Halvorson (in Halvorson and Howitt, 1961) demonstrated that DPA stabilized a soluble NADH oxidase from B. cereus, but Powell (1957) was unable to

show any increase in thermal stability of her soluble adenosine deaminase. Halvorson and Howitt suggest that if DPA protects proteins directly, such stabilization might be selective for those enzymes requiring divalent ions for tertiary structure.

If a DPA-Ca-NADH oxidase complex was responsible for the extreme heat resistance of the spore enzyme, it was unique to the particular strain of C. botulinum 62A used. There exists in our laboratory a culture of C. botulinum 62A which does not possess the stable spore NADH oxidase (Day, 1963).

Lewis, Snell and Burr (1960) have proposed a mechano-chemical hypothesis for spore heat resistance and dormancy. Their concept involves an elastic layer, such as the spore cortex, that would swell and exert pressure upon the vital core and thus force water out. The water content within the interior would be greatly reduced and this semi-dehydrated state could explain both dormancy and heat resistance. Support for their hypothesis comes from several sources: concentration of metal ions in the periphery of spores, Knaysi (1961a, b); apparent location of DPA in spore periphery, Hashimoto and Gerhardt (1960); the effect of Ca:DPA ratio in heat resistance, Levinson, Hyatt and Moore (1961); and base (ion) exchange studies of spores, Alderton and Snell (1963).

The Lewis hypothesis may explain a possible mechanism for the intact spore, but it does not explain the development of certain heat stable proteins. Bach and Sadoff (1962) have shown the appearance of a heat stable protein prior to the development of definite spore structures such as spore cortex. This research performed on NADH oxidases of C. botulinum shows the early development of a heat stable, spore-like protein in vegetative cells as shown in Figures 3 and 6. At this stage, the heat stability of spore NADH oxidase cannot be explained by a contractible spore cortex.

Any theory which attempts to explain the unique heat resistance of Bacillaceae spores must incorporate into it a mechanism whereby the configuration of the spore proteins is maintained. A viable spore must have enzyme systems capable of initiating germination, carrying on the synthesis of outgrowth and finally capable of producing a viable vegetative cell. Failure of any proteins in this reaction sequence result in what the microbiologist would normally classify as a non-viable organism.

CONCLUSIONS

A study of the enzymatic characteristics of vegetative cell and spore NADH oxidases from a strain of Clostridium botulinum 62-A has been presented. The heat stability of the isolated spore enzyme was tested and compared to that of the vegetative enzyme. At 60 C, the protein derived from vegetative cells was readily denatured while the spore enzyme was unaffected by this heat treatment. These results would tend to validate this as a test system for studies of mechanisms of heat resistance.

Certain similarities were found for vegetative cell and spore NADH oxidases. Both are soluble in their extracted state and can be separated from their respective particulate diaphorases by centrifugation. The pH optima (approximately 7.5) are not significantly different. However, a slight difference is observed in the spore enzyme's higher relative activity in pHs below optimum and the vegetative enzyme is conversely more active above optimum pH.

This study has revealed the following significant differences between the two analogous NADH oxidases. There are the above mentioned differences in the thermal stability of the purified enzymes as well as their respective

solubilities in solutions of ammonium sulfate. The spore NADH oxidase precipitates at 55% saturation while the vegetative enzyme precipitates at 70% saturation.

The enzymes differ in their reactions with substrate and the inhibitor atabrine. The K_m value for tne spore enzyme is about 8 times greater than that for the vegetative NADH oxidase. This can be interpreted as showing that the spore enzyme has a lesser affinity for substrate. On the other hand, spore enzyme is less inhibited by atabrine than the vegetative enzyme. This may be related to the relative abilities of the two proteins to bind cofactor.

Important differences in their ability to bind flavin were observed. The flavin can be completely removed from the vegetative cell NADH oxidase by relatively mild treatments and FAD, not FMN, restores activity. Stronger acid-ammonium sulfate treatments fail to remove an appreciable amount of flavin from the spore enzyme. Thus, there are only indications that FAD may be the primary cofactor.

The results of gel filtration studies show that there are at least two fold differences in the molecular size of the two oxidases. Estimations of 100,000 or less molecular weight for the vegetative NADH oxidase and 200,000 or more for the spore enzyme have been made. Differences in

the charge on the two proteins is indicated by their elution characteristics on DEAE cellulose anion exchange columns.

Neither oxidase interacts with the heterologous antibodies elicited by its analogous enzyme. This can be interpreted as indicating that there exist no structural similarities in their antigenic sites. Apparent differences in their antigenic properties are observed by quantitative differences in antibody formation in rabbits receiving similar immunization treatments.

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