THE GLUCOSE DEHYDROGENASE OF *BACILLUS CEREUS*, A MODEL FOR THE STUDY OF SPORE HEAT RESISTANCE

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ABSTRACT

THE GLUCOSE DEHYDROGENASE OF BACILLUS CEREUS; A MODEL FOR THE STUDY OF SPORE HEAT RESISTANCE

by John A. Bach

This research was undertaken in an effort to simplify the study of spore heat resistance by separating heat resistance from viability. The assumption was made that the heat resistance of spore enzymes contributes to the heat resistance of whole spores. From this it followed that a heat stable spore enzyme and its homologous heat labile counterpart from germinated spores or vegetative cells could be purified and used as a model system in the study of spore heat resistance. For this reason, various extracts of Bacillus cereus were examined for a satisfactory enzyme system.

Glucose dehydrogenases were found in extracts of vegetative cells, spores and germinated spores, which had different levels of heat resistance but appeared to be very similar by other criteria. The vegetative cell enzyme, which subsequently was shown to be spore protein, had a half-life of 15 minutes at 69 C and retained this level of heat resistance throughout purification. The enzyme from spores was slightly less heat resistant than the vegetative cell enzyme. The

glucose dehydrogenase from germinated spores was heat labile, having a half-life of about one minute at 50 C.

Various procedures were employed to test the similarity of the enzymes in an attempt to show that they are homologous proteins. A study of the time course of biosynthesis of glucose dehydrogenase in a growing culture of Bacillus cereus revealed that neither a heat labile nor heat stable enzyme is present in the culture during the exponential growth phase. A heat stable glucose dehydrogenase appears after the exponential growth phase has ended, and prior to the detection of dipicolinic acid and cellular heat resistance. This enzyme is present in high concentration in spores and germinated spores, but, as stated before, the enzyme from germinated spores is markedly heat labile.

The glucose dehydrogenases from vegetative cells and spores were purified extensively and characterized according to their catalytic and serological properties. Purification was accomplished by the following steps: extraction by mechanical disruption of vegetative cells or spores, heating to 65 C, protamine precipitation of nucleic acids, ammonium sulfate fractionation, and DEAE cellulose column chromatography. A purification as high as 360 fold was obtained by this procedure. The Michaelis

constants of the vegetative cell enzyme were determined and compared to the Michaelis constants of the spore enzyme reported in the literature. The constants for both enzymes were very similar. Purified enzymes from vegetative cells and spores were injected into rabbits to produce specific antisera. The two enzymes were compared by a modified immunodiffusion technique and found to be serologically identical.

On the basis of the results obtained by serological and enzymatic comparisons, the coincidence of the production of this enzyme with the production of spores, and the coincidence of the change in heat resistance of this enzyme with the change in heat resistance of whole spores, it was concluded that the glucose dehydrogenase from vegetative cells, spores and germinated spores is essentially the same enzyme with different levels of heat resistance. The resistant glucose dehydrogenase is easily purified, is stable during storage, and is readily assayed. All of these considerations favor the use of this enzyme system as a model for studying spore heat resistance at the molecular level.

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by

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INTRODUCTION

Bacterial endospores are produced, by members of the genera Bacillus and Clostridium, as a resistant, dormant stage in the life cycle of these organisms. Endospores are formed within vegetative cells during the stationary growth phase of the culture. After endospore formation is completed, the surrounding vegetative cell material (sporangium) is usually digested, and the spores are released. In qualitative chemical composition, these spores differ from vegetative cells only in their content of dipicolinic acid (pyridine 2,6 dicarboxylic acid). Dipicolinic acid has not been found in exponentially growing vegetative cells. In biological characteristics, however, spores differ grossly from vegetative cells, being almost inactive metabolically, and resistant to many of the physical and chemical agents capable of killing vegetative cells. During a process called germination, the typical resistant properties of spores are lost, and certain latent metabolic enzymes once again become active. At the same time, large amounts of soluble material are released from the spores, including peptides and the calcium salt of dipicolinic acid. If environmental conditions are favorable, the germinated spores grow into true vegetative cells which repeat the life cycle by reproducing

and sporulating exactly as their predecessors did.

One of the most unique properties of bacterial spores is their extremely high resistance to heat. The spores of some bacterial species are capable of withstanding autoclaving at 115 C for as long as three hours, while, in contrast, the vegetative cells of this species, and most other sporeformers, are killed in a few minutes at 55 C to 65 C (Curran, 1952). Although this is an extreme example, it is, nevertheless, true that most bacterial spores are much more resistant to heat than the vegetative cells from which they were formed.

To study the effect of any factor on the heat resistance of bacterial spores, a suitable means of measuring that heat resistance is needed. Up to the present time, this has been done by enumerating the survivors of a given heat treatment. However, the detection of these survivors depends on the proper functioning of the multitude of reactions involved in germination, outgrowth, and reproduction. Although these reactions have nothing to do with spore heat resistance, they may be affected by the factor under study. Thus, the study of spore heat resistance with such a complex, variable system can not be expected to yield reliable information. A more suitable method of studying heat resistance would be one which does not depend on spore viability, or perhaps one

which does not even depend on whole spores. In fact, the simplest, most direct method of studying heat resistance would be at the molecular level, providing of course that spore heat resistance is expressed at the molecular level.

Because many of the essential components of cells, such as proteins and nucleoproteins, are extremely labile to heat, it is reasonable to suspect that these compounds are protected from heat denaturation in spores, and, conversely, unprotected in vegetative cells and germinated spores. If this protective mechanism involves molecular structure, rather than just a change in cell environment, it should be possible to isolate and purify heat resistant spore material and homologous heat sensitive vegetative cell or germinated spore material. Together, these two types of cellular material would constitute a simple, cell-free model system for studying spore heat resistance without the problems inherent in spore viability methods. In fact, if the two types of material were actually homologous and could be sufficiently purified, a simple physical-chemical comparison might indicate at least one mechanism of spore heat resistance. The most reasonable approach to this problem would be to select, for examination, one cellular component which is heat stable in spores and heat labile in vegetative cells and germinated

spores. In addition, this component should be easily purified and must possess some measurable index of denaturation. The obvious choice for such a component is some type of enzyme, since enzyme denaturation is easily detected by loss of enzymatic activity.

The enzyme catalase, from vegetative cells and spores of Bacillus cereus, appeared to fulfill the requirements for such a model system, but extensive attempts by Sadoff, Kools, and Ragheb (1959) to crystallize the heat stable enzyme failed because of the low cellular concentration of this enzyme, the poor yields obtained during purification, and the instability of the enzyme during storage at low temperatures. It became apparent that, to obtain the amount of pure protein needed for a physical-chemical comparison, a new model was needed. For this reason, the glucose dehydrogenase from Bacillus cereus was examined as a possible model for the study of spore heat resistance. The isolation, partial purification, and partial characterization of this enzyme system is the topic of this thesis.

HISTORICAL REVIEW

Many heat resistant enzymes have been found in resting spores of Bacillus cereus and in cell free extracts of these spores. Most of these enzymes differ greatly in their catalytic specificities and almost as greatly in their level of heat resistance. Although the heat resistance of many of these enzymes has been reported, to the author's knowledge, only catalase has been purified and characterized expressly for the purpose of formulating a mechanism of spore heat resistance.

In connection with their studies on the germination requirements of Bacillus cereus spores, Stewart and Halvorson (1953) described an alanine racemase which converted L-alanine to D-alanine. The enzyme was completely resistant to heating at 80 C for two hours while it was in the intact spore.

Lawrence and Halvorson (1954) found a heat resistant catalase in spores of Bacillus cereus which was completely resistant to heating at 80 C. When this enzyme was extracted from the spore, its heat resistance was somewhat diminished. In addition to the stable catalase, a heat labile catalase was also present in spore extracts, but the labile enzyme was

not active in intact spores.

A remarkably heat stable adenosine ribosidase which cleaved adenosine into adenine and ribose was found in spores of Bacillus cereus by Lawrence (1955). This enzyme retained 50 per cent of its activity after heating at 100 C for four hours. Powell and Hunter (1956) reported the same adenosine ribosidase to be stable both in intact and disintegrated spores. These authors also described an adenosine deaminase in Bacillus cereus spores. The deaminase in the intact spores was stable to heating at 60 C for several hours. However, in spore homogenates or germinated spores, the enzyme was destroyed in 15 minutes at 60 C.

Sadoff, Kools and Ragheb (1959) found, in vegetative cells of Bacillus cereus, a heat stable catalase which had heat resistance similar to that of spore catalase. Recent immunochemical data (unpublished) indicate that the heat stable vegetative catalase is identical to the heat stable catalase found in spores by Lawrence and Halvorson (1954).

Church and Halvorson (1955) discovered a DPN-specific glucose dehydrogenase in extracts of Bacillus cereus spores. This enzyme was partially purified and characterized by Doi, Church and Halvorson (1959) in connection with their study of the intermediate metabolism

of aerobic spores. The effect of heat on this enzyme was not reported.

MATERIALS AND METHODS

A. Organism and Medium

Bacillus cereus T. was the organism used throughout this study. Stock cultures were maintained by allowing the organism to sporulate on nutrient agar slants, and refrigerating the slants at 4 C until needed.

All vegetative cell crops, and most of the spore crops, were grown at 30 C, in G medium, as described by Stewart and Halvorson (1953). This medium contains the following components per liter:

$ZnSO_4$	0.00001 g.
$CuSO_4$	0.00001 g.
$CaCl_2$	0.00001 g.
$FeSO_4.7H_2O$	0.000001 g.
K_2HPO_4	1.0 g.
$(NH_4)_2SO_4$	4.0 g.
Yeast Extract	2.0 g.
$MnSO_4.H_2O$	0.1 g.
$MgSO_4$	0.8 g.
Dextrose	4.0 g.
Antifoam B	0.7 ml.

Some of the spore crops were grown at 28 C with the dextrose concentration reduced to three grams per liter.

This modification prevented premature lysis of the sporulating cells by lowering their oxygen requirement, thereby increasing spore yield.

B. Growth of Vegetative Cell and Spore Crops

Large amounts of vegetative cells and spores were needed to obtain enough glucose dehydrogenase for purification and characterization of the enzyme. All of the vegetative cell crops and some of the spore crops were produced in three liter fermenters (New Brunswick Scientific Company). The medium in these fermenters was oxygenated by forced aeration and mechanical agitation. Some of the spore crops were produced in 18 liter pyrex carboys in which the medium was oxygenated by forced aeration through six, radially arranged sparger tubes. The force of the air flowing through the spargers rotated and agitated the medium, thereby increasing the efficiency of oxygenation. The temperature of both the New Brunswick fermenter and the carboy was maintained by a thermostatically controlled water bath.

The large volume of medium used in either fermenter required a large, actively growing inoculum. This was prepared in dimpled flasks, each containing 50 milliliters of G medium. The dimpled flasks are 500 milliliter Erlenmeyer flasks with four depressions, equally spaced

around the lower side of the flask. These depressions cause turbulence when the flasks are shaken, thereby increasing the oxygenation of the medium. Serial transfers through an increasing number of dimpled flasks synchronizes the physiological age of the cells and increases the volume of the inoculum.

The following specific procedure was used to grow vegetative cell and spore crops. A nutrient agar slant was inoculated from a stock culture and incubated at 30 C approximately nine hours. A dimpled flask of medium was inoculated from the nine hour slant and shaken for four hours. Ten milliliters of culture from this flask were put into each of two more flasks, and these flasks were shaken for two hours. Ten milliliters of culture from the two flasks were put into each of seven more flasks, and these flasks were shaken for two hours. The entire contents of the seven flasks were transferred into a New Brunswick fermenter tank containing 2,500 milliliters of medium. The tank contents were agitated at 450 revolutions per minute, and aerated at a rate of five liters per minute. If the 18 liter carboy fermenter was used, about 1.5 liters of a two hour culture from the New Brunswick fermenter were used for inoculation.

Vegetative cell crops were harvested at about seven

and one quarter hours after inoculating the fermentor. For spore crops, the cultures were allowed to grow until microscopic examination of stained smears indicated a large proportion of free spores. The vegetative cell and spore crops were harvested in a Sharples Super Centrifuge, type T-41-23 1-1-H (The Sharples Specialty Company). Spores were cleaned by differential centrifugation in water or dilute saline until practically free of vegetative debris and germinated spores. Vegetative cells were washed once or twice in 0.1 molar, pH 7.6, tris (trishydroxymethylaminomethane) buffer. The cells and spores were cleaned in the cold in a Servall centrifuge, model SS-1 (Ivan Sorvall, Incorporated). Unless specifically stated otherwise, all further mention of centrifugation implies the use of this centrifuge. The cells and spores were stored at -20 C.

C. Preparation of Cell-free Extracts

All cell-free extracts were obtained by mechanical disruption of cells in a Servall Omnimixer (Ivan Sorvall, Incorporated). In most cases, three parts, by weight, of number 110 superbrite beads (Minnesota Mining and Manufacturing Company), one part, wet weight, of cell or spore paste, and enough 0.1 molar, pH 7.6, tris buffer to fill the 80 milliliter Omnimixer cup were mixed

at top speed for 10 to 30 minutes. The cup was cooled in crushed ice. After breakage, the brei was centrifuged at 32,700 times gravity for 15 to 60 minutes, and the supernatant liquid was retained for enzyme studies.

D. Glucose Dehydrogenase Assay

Glucose dehydrogenase was assayed spectrophotometrically at 340 millimicrons using DPN and glucose as substrates. This assay is based on the difference in light absorption of DPN and DPNH. Both compounds absorb at 260 millimicrons, but only DPNH absorbs at 340 millimicrons. Since glucose dehydrogenase catalyses the oxidation of glucose and reduction of DPN, the rate of increase in absorbance at 340 millimicrons can be used to express enzyme activity. One unit of glucose dehydrogenase activity is defined as an increase of 0.001 optical density units per one hundred seconds at 340 millimicrons.

The assay procedure was the same for almost all experiments. In addition to enzyme, the assay cuvettes contained the following reagents: 300 micromoles of pH 7.6, tris buffer; 100 micromoles of glucose; 2 micromoles of DPN; and water up to three milliliters. Endogenous cuvettes contained no glucose but otherwise were the same. One exception to this procedure was that used

for assaying glucose dehydrogenase in the heat inactivation of vegetative cell extract. The assay cuvettes in this experiment contained the following reagents: 50 micromoles of pH 7.3, tris buffer; 100 micromoles of glucose; 1 micromole of DPN; and water up to three milliliters. All assays were done at approximately 32 C in a Beckman spectrophotometer, model DU (Beckman Instruments, Incorporated). The temperature was maintained by heat from the hydrogen lamp.

E. DPNH Oxidase Assay

DPNH oxidase was also assayed spectrophotometrically, using DPNH as the only substrate. The oxidation of DPNH decreased the absorption at 340 millimicrons. One unit of DPNH oxidase activity is defined as a decrease of 0.001 optical density units per 100 seconds at 340 millimicrons. In addition to enzyme, the assay cuvettes contained the following reagents: 300 micromoles of pH 7.6, tris buffer; 0.5 micromole of DPNH; and water up to three milliliters. Endogenous cuvettes contained no DPNH.

F. Assay for Heat Resistance

Heat inactivation rates were used to screen cell extracts for heat resistant enzymes and to determine the inactivation kinetics of purified enzymes. In general, these rates were obtained by heating samples at a fixed temperature for various times and determining the enzyme

activity of each heated sample. Since the heating times vary widely among samples, the time required for temperature equilibration of the samples constitutes a different percentage of the heating time for each sample. This introduces an error in the time-temperature value reported for each sample. When screening for heat resistant enzymes, this error is unimportant but in kinetic studies it can not be ignored. For this reason, two methods of heating samples were used, one for crude extracts and another for purified enzyme solutions.

Crude extracts were heated in rubber stoppered, 15 millimeter, pyrex test tubes. Before adding extract, each tube was pre-heated to the inactivation temperature in an unstirred water bath. After adding the extract, the tubes were stoppered and agitated for one minute to increase the rate of heat transfer. The tubes were withdrawn and iced at various intervals after the addition of the extract and the heated extracts were centrifuged to remove precipitated protein before activity measurements.

Purified enzyme solutions were heated in thin-walled capillary tubing. The tubes were made by drawing 23 millimeter pyrex test tubes to a diameter of two millimeters in a gas-oxygen flame. These capillary tubes had extremely thin walls to facilitate heat transfer and re-

duce the temperature lag time. In addition, they had sufficient volume to hold the amount of enzyme solution needed for an accurate assay. Highly purified enzyme was drawn into the tubes and both ends were sealed by melting them in a flame. At zero time, all of the tubes were placed simultaneously in a stirred water bath. Each tube was withdrawn after the specified time interval and cooled in a 10 C water bath. The enzyme activity of each sample was measured without prior centrifugation, since no visible precipitate had formed.

G. Purification of Glucose Dehydrogenase

To determine the properties of the glucose dehydrogenases from vegetative cells and spores, both crude extracts had to be purified. The purification procedure for both extracts was similar to that used by Doi, Church and Halvorson (1959) in their purification of spore glucose dehydrogenase. The crude extract was heated just up to 65 C, cooled immediately and rapidly in ice, and centrifuged to remove precipitated material. The heated extract was then concentrated by adding solid ammonium sulfate slowly, with stirring, until the solution was saturated. The precipitated protein was collected by centrifugation, separated from the supernatant liquid, and resuspended in one tenth the original volume of 0.1 molar, pH 7.6, tris buffer. The pH of the extract was not controlled during

the ammonium sulfate saturation.

Nucleic acids were removed from the concentrated extract because they interfere with ammonium sulfate fractionation. They were precipitated by adjusting the pH of the concentrated extract to 5.1 with acetic acid and adding small amounts of 2 per cent protamine sulfate in a fine stream while stirring the extract. The precipitate was removed by centrifugation, and the 280/260 ratio (see section H. of MATERIALS AND METHODS) of the supernatant liquid was determined. This procedure was repeated until a ratio of 0.7 or greater was obtained. The supernatant liquid was then dialysed against 0.1 molar, pH 7.6, tris buffer for about 12 hours to remove excess protamine sulfate. Additional precipitation occurred during dialysis, but, since the precipitate contained no glucose dehydrogenase, it was removed by centrifugation and discarded.

The protein remaining in the dialysed extract was fractionated with ammonium sulfate. The desired saturation percentage was obtained by measuring the volume of the extract before each addition of ammonium sulfate, and adding a calculated amount of solid salt, slowly and with constant stirring. No attempt was made to control the pH of the solution during addition of the ammonium sulfate. Each precipitated fraction was collected by centrifugation and resuspended in 0.1 molar, pH 7.6,

tris buffer.

The ammonium sulfate fractions which contained glucose dehydrogenase were further fractionated on an anion exchange column. The material used in the column was N,N-diethylamino ethyl cellulose (DEAE cellulose). The column was prepared by pouring a suspension of DEAE cellulose in 0.05 molar, pH 7.6, tris buffer into a 10 millimeter chromatographic tube. The packed volume of the column was approximately 20 milliliters.

The ammonium sulfate fractions containing glucose dehydrogenase were combined and dialysed against 0.05 molar, pH 7.6, tris buffer to prepare them for adsorption on the column. Dialysis at this buffer concentration precipitated a large amount of material, but, since this material was enzymatically inactive, it was discarded. The supernatant solution was added to the column and washed through with buffer. The effluent from washing contained no glucose dehydrogenase, indicating that the enzyme was adsorbed on the DEAE cellulose.

Adsorbed protein was removed from the column by gradient elution. The gradient was obtained by using two reservoirs in a series arrangement. The lower reservoir was an aspirator bottle containing 180 milliliters of buffer, and the upper reservoir was a separatory

funnel containing 1.5 molar sodium chloride solution. Since the system was open only at the bottom of the column and the top of the separatory funnel, every drop of buffer-sodium chloride mixture leaving the aspirator bottle was replaced by a drop of 1.5 molar sodium chloride. The result was a non-linear increase in the sodium chloride concentration of the buffer between the limits, zero and 1.5 molar. Five milliliter fractions of the eluate were collected automatically by a linco fraction collector (Research Instruments Company). Each fraction was assayed for protein, glucose dehydrogenase, and chloride ion.

H. Protein and Nucleic Acid Assay

It was necessary to know the protein content of solutions obtained during enzyme purification in order to be able to calculate specific activities. Two methods were used for these determinations.

In samples containing more than 10 per cent nucleic acid, protein was determined colorimetrically with the Folin-Ciocalteu phosphomolybdotungstic acid reagent. (Lowry et al 1951). This reagent reacts with tyrosine residues and the peptide chains in proteins to give a blue color. A Bausch and Lomb "Spectronic 20" spectrophotometer (Bausch and Lomb Optical Company) was used for the readings. The wave length was 660 millimicrons.

In samples containing less than 10 per cent nucleic acid, protein was determined spectrophotometrically at 280 and 260 millimicrons, according to the method of Warburg and Christian (1942). This method depends on light absorption at 280 and 260 millimicrons by protein and nucleic acid. Both protein and nucleic acid absorb at these wavelengths, but, since their absorption coefficients differ, the ratio of absorbance at 280 millimicrons to absorbance at 260 millimicrons (280/260 ratio) can be used to determine the relative amounts of each in a given sample. Information on the relative amounts of protein and nucleic acid, plus the optical density at 280 millimicrons, is used to calculate the actual concentration of each in the samples. A Beckman DU spectrophotometer was used for these readings.

I. Chloride Ion Assay

The sodium chloride concentration required to elute each fraction from the cellulose column was estimated from the chloride ion concentration of the fractions. This information was used to compare the exchange characteristics of both glucose dehydrogenases. The chloride ion concentration in each fraction was determined by titration with silver nitrate using potassium chromate as an adsorption indicator. Since protein affects the adsorption endpoint, a correction had to be made for the

protein in each fraction. The correction factor was obtained empirically by titrating known concentrations of sodium chloride and protein, with silver nitrate.

J. Acetone Extraction of Whole Cells

A quantitative method for assaying glucose dehydrogenase in small cell samples was needed to study the rate of formation of this enzyme in growing cultures. Several assay procedures were tried, but most of these were not successful. Whole cells could not be assayed directly because they were impermeable to substrates used in the assay. Heating the cells to 69 C for two minutes did not increase their permeability. Breaking the cells in the Omnimixer diluted the activity excessively because of the large cup volume and the large volume of buffer required to thoroughly wash the glass beads.

Acetone extraction of the cell samples increased their permeability to assay substrates and also permitted the assay of small cell samples. Glucose dehydrogenase assay by acetone extraction was done in the following manner. Cell samples were taken from a New Brunswick fermentor at various times during growth, washed by alternate centrifugation and resuspension in 0.5 per cent saline, and suspended in just enough saline to disperse the cells. One part of each suspension was pipetted into

13 parts of acetone, at -15 C, and centrifuged immediately in an International clinical centrifuge, model CL (International Equipment Company). The centrifuge was operated in a deep freeze at -15 C. After centrifugation, the acetone was decanted, and the extracted cells were allowed to dry for about ten hours in the freezer. The dried cells were resuspended in 0.5 per cent sodium chloride, heated for two minutes at 69 C, and assayed for glucose dehydrogenase by the same procedure used for cell extracts.

K. Dipicolinic Acid Assay

Dipicolinic acid (DPA) was determined colorimetrically by the method of Janssen, Lund and Anderson. (1958) In this method, DPA is extracted from the cells by autoclaving, and complexed with divalent iron to produce an orange color. The DPA concentration was determined in a Bausch and Lomb "Spectronic 20" spectrophotometer, using chemically prepared DPA as a standard. The wave length was 440 millimicrons.

L. Production of Immune Serums

Immune serums, containing antibodies against enzyme proteins, were needed for serological comparisons. These serums were obtained in the following manner. Samples of highly purified enzyme were concentrated by dialysing against 20 per cent polyvinylpyrrolidone. One part of con-

centrated enzyme and one part of Freund's complete adjuvant (1944) were emulsified by forcing in and out of a one cubic centimeter tuberculin syringe and injected subdermally in the necks of rabbits. These rabbits had been heart bled prior to injection to obtain normal serum for serological controls. Twenty-four days after injection, the rabbits were heart bled to obtain immune serums. The blood was incubated at room temperature for three hours to start clot retraction and refrigerated for about three days to finish the retraction. The serum was poured off, centrifuged to remove blood cells, and stored at -20 C. Forty-six days after the first injection, a second, smaller injection of enzyme plus Freund's incomplete adjuvant (1944) was given to keep the antibody level high. At fifty-six days, the rabbits were bled again to obtain more immune serum.

M. Serological Comparison of Enzymes

The serological comparison of spore and vegetative cell glucose dehydrogenase was accomplished by the Ouchterlony agar gel diffusion method (1949a, 1949b) and by Consden and Kohn's (1959) modification of this method. In the agar gel diffusion method, several wells are cut in a layer of solid agar, sealed with melted agar, and filled with various antigens and antisera. The antigens

and antisera diffuse through the agar, until, in a region of optimal proportions, they combine and precipitate, forming a visible line. Since various antigens and antisera differ in diffusion rates and optimal proportion values, this method is capable of separating the components of a compound antigen-antibody reaction. Consden and Kohn's modification of the Ouchterlony technique is the substitution of cellulose acetate film for the agar gel. In this method, moist cellulose acetate film is spotted with very small amounts of antigens and antisera, and incubated under mineral oil to isolate the aqueous phase. During incubation, the antigens and antisera diffuse through the aqueous phase, and precipitate in the cellulose acetate matrix. After incubation, the film is washed and stained with a suitable dye to reveal the precipitin lines.

A slight modification of the cellulose acetate technique, especially useful in this study, is the enzymatic identification of precipitin lines. This method depends on the enzymatic reduction of DPN (in the presence of glucose) by serologically precipitated glucose dehydrogenase, and the fluorescence of DPNH under a Black-ray ultraviolet lamp (Ultraviolet Products, Inc.). The procedure consists of spreading a solution of glucose and DPN on the completed cellulose acetate film, and examining

the film for fluorescent precipitin lines.

N. Spore germination

The heat labile glucose dehydrogenase was present only in germinated spores. Germination was accomplished by the following procedure. Five grams, wet weight, of partially germinated spores, which had been stored at -20 C for about five months, were suspended in 50 milliliters of germinating solution, and heat shocked at about 70 C for ten minutes. The germinating solution contained 0.2 per cent alanine and 0.02 per cent adenosine in 0.07 molar, pH 7.0 potassium phosphate buffer. After heat shock, the suspension was incubated at 30 C for five hours, and then refrigerated at 4 C for 12 hours. At the end of this time, almost all of the spores were germinated, as indicated by their permeability to crystal violet stain. In addition, a small number of germinated spores had grown into vegetative cells.

RESULTS

A. Screening for Heat Resistant Enzymes

The purpose of this study was to identify and purify an enzyme system which could be used as a model in the study of spore heat resistance. Any enzyme would be useful, as long as it had a heat stable and heat labile form. Results of experiments on the catalase of Bacillus cereus suggested that heat labile enzymes are present during all stages of vegetative growth, and that heat stable enzymes appear near the beginning of the stationary phase. For this reason, stationary phase cell extracts were screened for heat resistant enzymes by the method given in Section F. of MATERIALS AND METHODS.

The heat inactivation curve for glucose dehydrogenase, the first enzyme examined, is given in figure 1. This graph of enzyme activity versus time of heating indicates that the glucose dehydrogenase loses half of its activity in about 15 minutes at 69 C. A half-life of this magnitude means that the enzyme has a greater than average resistance to heat.

B. "Activation" of Glucose Dehydrogenase

An interesting feature of the curve in figure 1 is the large increase in activity during the first minute of heating. This increase was reminiscent of the activation of glucose oxidation in spores of Bacillus cereus,

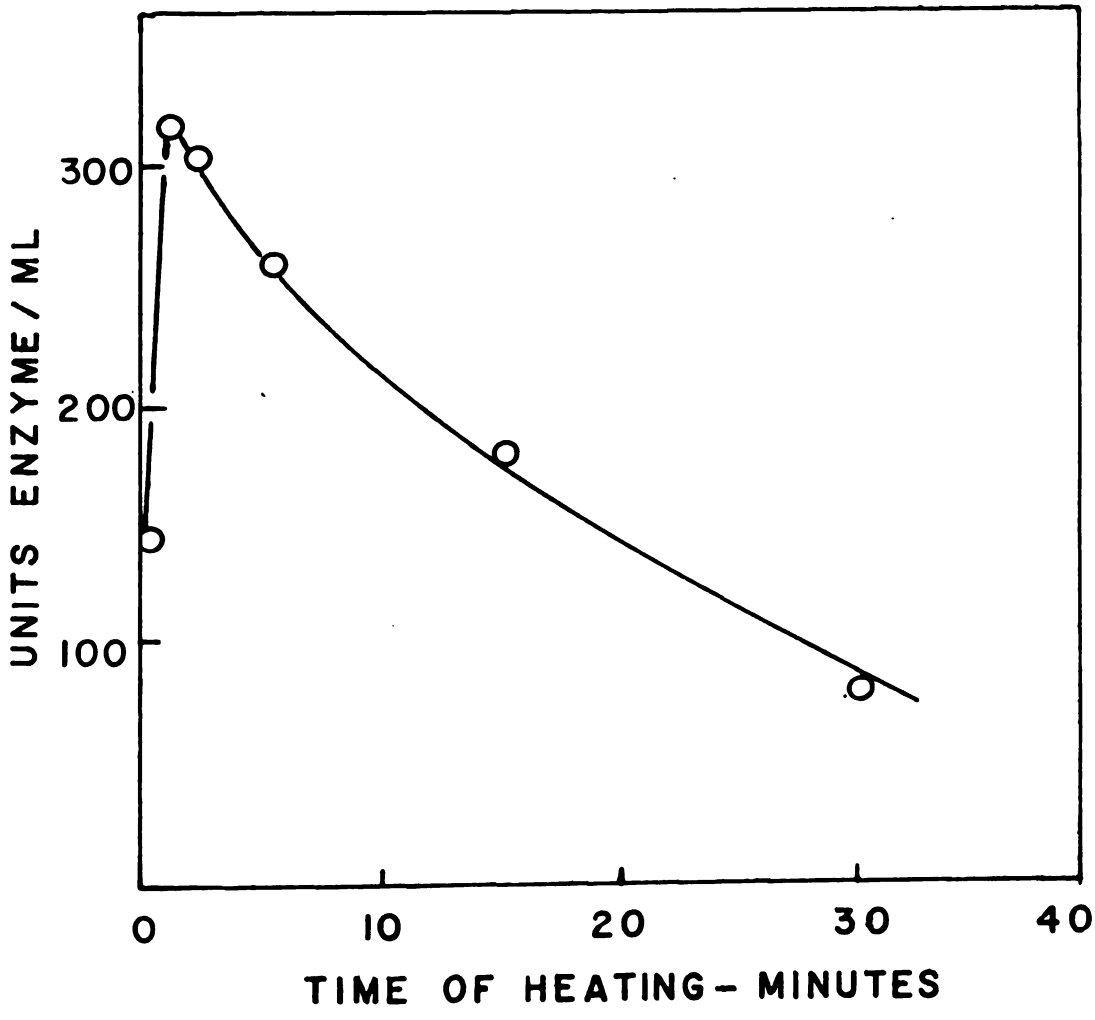


Figure 1. The heat inactivation at 69 C of the glucose dehydrogenase in an extract of vegetative cells of Bacillus cereus. This curve also shows the apparent heat activation of the enzyme.

reported by Church and Halvorson (1957). This increase could be the result of true activation or merely an expression of the difference in heat stability of two coupled enzymes - in this case, glucose dehydrogenase and DPNH oxidase.

To test the latter possibility, samples of extracts were heated to 60 C for various times, and assayed for both glucose dehydrogenase and DPNH oxidase. The lower temperature was used to slow the apparent activation to a measurable rate. The results are plotted in figure 2, which shows the activity for each enzyme versus heating time at 60 C. As DPNH oxidase activity decreases due to thermal inactivation, the activity of glucose dehydrogenase increases. Apparently the loss of the oxidase permits DPNH to accumulate at a faster rate.

C. Time Course of Glucose Dehydrogenase Biosynthesis

Although glucose dehydrogenase had been found in spores of Bacillus cereus (Church and Halvorson 1955) and in stationary phase cells or forespores of the same organism, neither a heat labile nor heat stable glucose dehydrogenase could be found in exponentially growing cells. Therefore, biosynthesis had to be initiated at a time between the exponential and stationary growth phase of this organism.

In order to observe the appearance of the enzyme

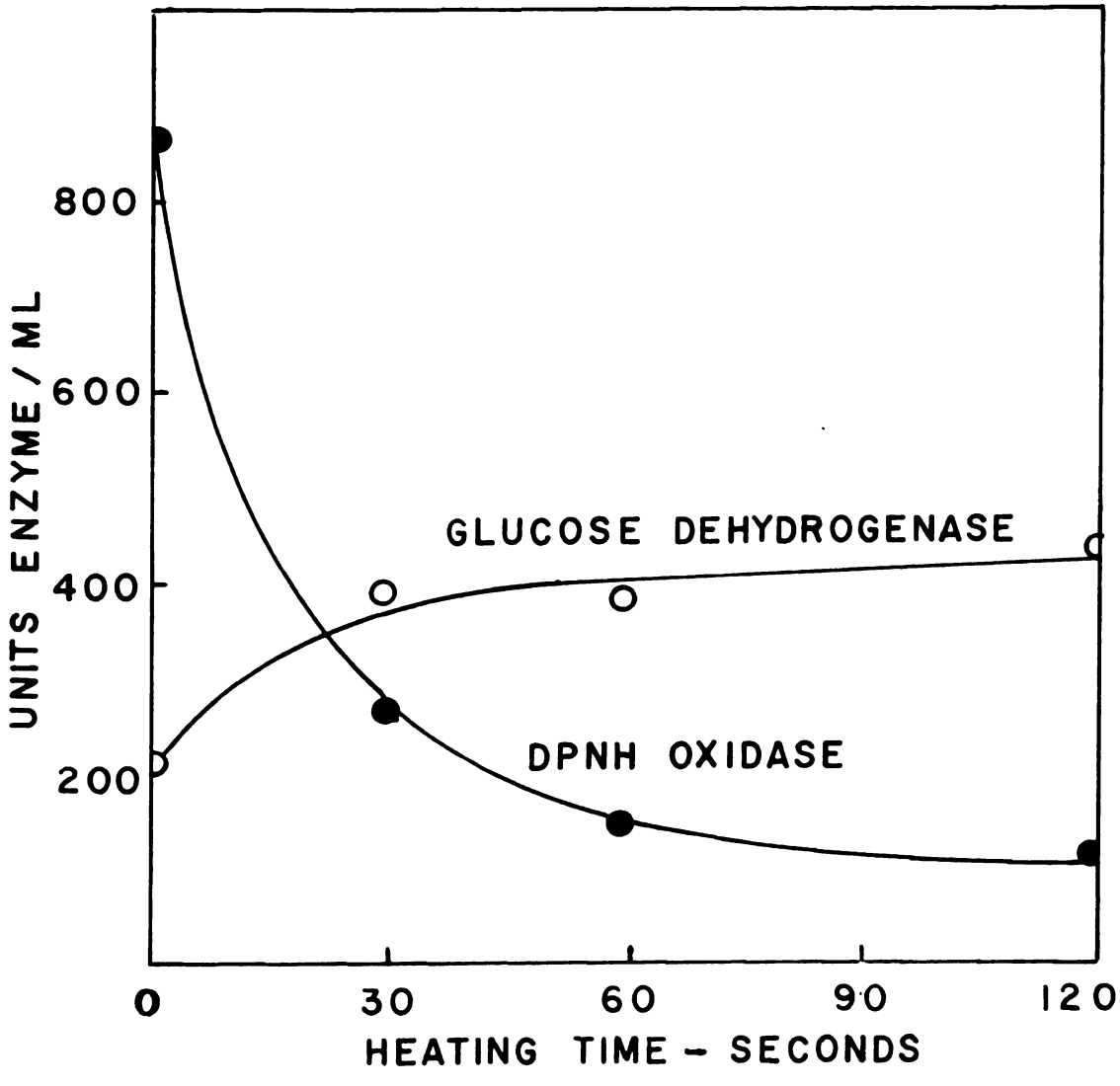


Figure 2. A comparison of the effect of heating at 60 C on the glucose dehydrogenase and the DPNH oxidase in an extract of vegetative cells of Bacillus cereus.

and to follow its concentration throughout the sporulation process, cell samples were harvested from the New Brunswick fermentor at various times during growth. The cells were acetone dried, heated, and assayed for glucose dehydrogenase activity. The results are shown in figure 3 as a graph of glucose dehydrogenase activity and dipicolinic acid concentration versus culture time. The curve for dipicolinic acid is included as a parameter of sporulation. Glucose dehydrogenase increased rapidly after six hours, but then appeared to decline after eight hours. However, the decline in glucose dehydrogenase corresponds to an increase in the number of spores, as indicated by increased dipicolinic acid concentration. This suggested that spores are more refractory to acetone extraction than vegetative cells and thus remain impermeable to the assay substrates in spite of the acetone treatment.

To check the validity of the acetone extraction procedure, an alternate extraction method was employed. Two large samples were taken from a fermentor at nine and thirteen hours after inoculation, ruptured in the omnimixer, heated at 69 C for two minutes and assayed for glucose dehydrogenase. The enzyme concentration of the culture, obtained by this procedure, was considerably higher than the concentration obtained by acetone

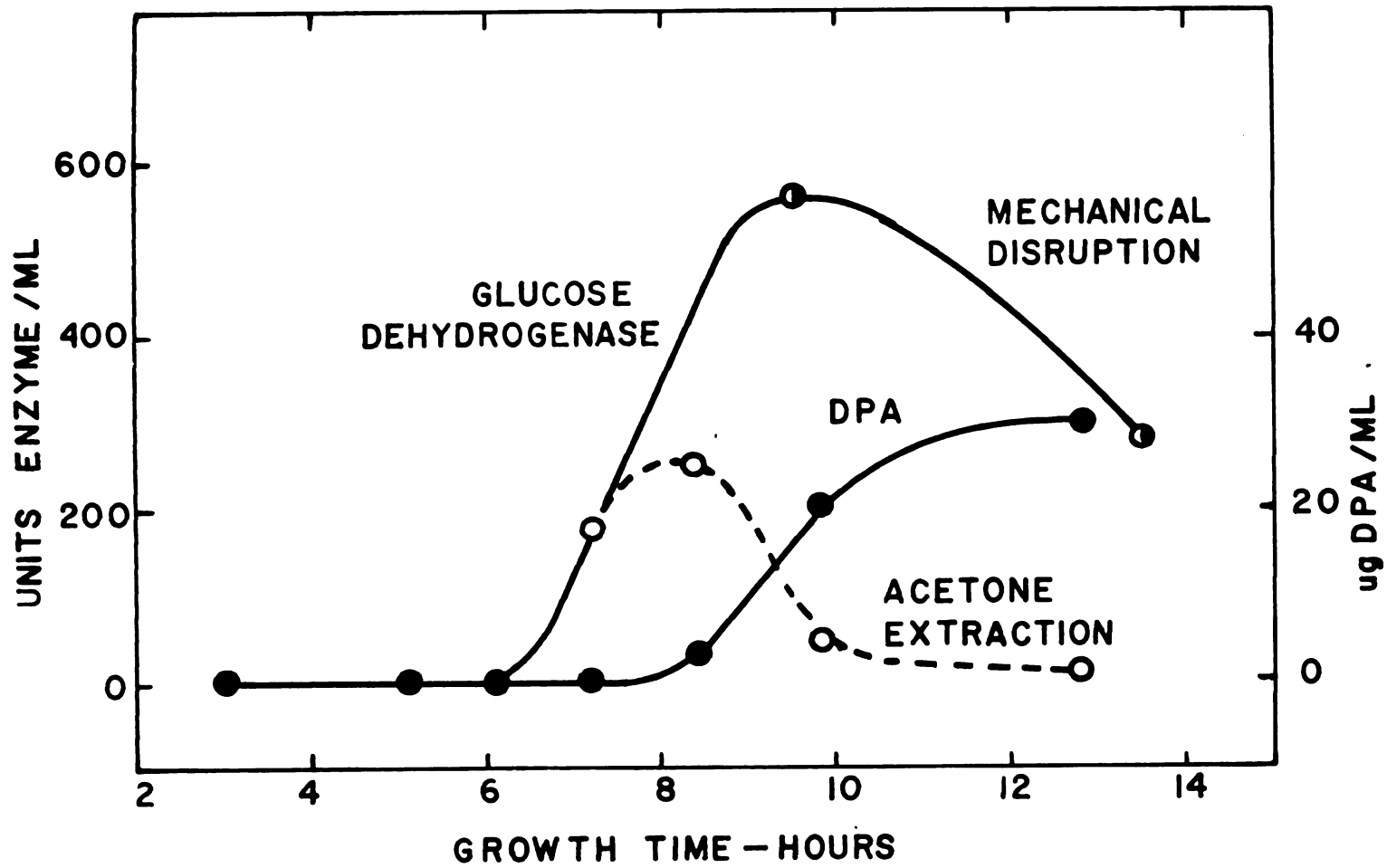


Figure 3. A time course of the biosynthesis of heat resistant glucose dehydrogenase and dipicolinic acid by a growing culture of Bacillus cereus.

extraction. These data point out the fact that the apparent enzyme concentration can be grossly altered by changing the extraction method.

D. Purification of Vegetative Cell and Spore Glucose Dehydrogenase

The results for the purification of vegetative cell and spore glucose dehydrogenase are summarized in tables 1 and 2. The meaning of the various columns is as follows: Specific activity is defined as units of enzyme per milligram of protein; purification number is the ratio of specific activity in a particular step to the specific activity in the heated extract; and yield is the percentage of total enzyme activity remaining at a particular step, relative to the total activity in the cell extract.

The total activity of the crude extracts cannot be determined directly, because they contain DPNH oxidase. Loss of the oxidase during purification results in an apparent increase in the total amount of glucose dehydrogenase, while, at the same time, glucose dehydrogenase is lost by inactivation. To obtain an estimate of the total amount of glucose dehydrogenase in the extracts, all increases in activity, throughout the purification, were added to the apparent total activity of the crude extracts.

TABLE 1

Purification of the glucose dehydrogenase from vegetative cells of Bacillus cereus

Procedure		Specific Activity	Purification	Yield %
Heated Extract		44	1	62
Protamine and Dialysis (0.1 M. tris)		195	4	72
Ammonium Sulphate Fractionation	54-75%	155	4	44
	75-86%	1,720	39	
	86-96%	334	8	
Combined, Reheated, Dialysed (0.05 M. tris)		3,420	75	46
DEAE Cellulose Chromatography	Fract. 9	29,400	660	17
	Fract. 10	12,600	290	13
	Fract. 11	2,240	51	7

TABLE 2

Purification of the glucose dehydrogenase from spores of Bacillus subtilis

Procedure		Specific Activity	Purification	Yield %
Heated Extract				82
Protamine and Dialysis (0.1 M. tris)		75	1	84
Ammonium Sulphate Fractionation	0-54%	88	1	4
	54-100%	972	13	76
DEAE Cellulose Chromatography	Fract. 4	10,800	144	27
	Fract. 5	1,230	18	7

The specific activity and purification number of the heated spore extract are not presented because the protein concentration of the extract was not determined. Instead, the specific activity of the protamine and dialysis step is considered a purification of one. The heated spore extract probably did not have a specific activity much lower than that of the dialysed extract because very little precipitation occurred during the protamine and dialysis step.

Although the purification obtained in the 75-86 per cent saturated ammonium sulphate fraction was far greater than that in both the 54-75 and 86-96 per cent saturated fractions, all three fractions were combined before cellulose chromatography in order to conserve total activity.

The similarity between vegetative cell and spore glucose dehydrogenase was indicated by their solubility and charge properties. Both enzymes were precipitated at an ammonium sulfate concentration of approximately 80 per cent of saturation (see Doi, Church and Halvorson, 1959, for data on spore enzyme), and both enzymes were eluted from the cellulose column at a sodium chloride concentration of about 0.1 molar.

E. Characterization of Vegetative Cell and Spore Glucose Dehydrogenase

1. Heat resistance

The stability of some proteins is increased

when they are conjugated with nucleic acids. Since the crude vegetative cell and spore extracts contained a large amount of nucleic acid, the heat resistance of the glucose dehydrogenase in these extracts might have been due to binding with this material. To eliminate this possibility and to compare the heat resistance of the two enzymes, purified vegetative cell and spore enzymes were tested for heat resistance by the capillary tube method. The vegetative cell enzyme used in this experiment had been purified 290 times and contained less than 1 per cent nucleic acid. The spore enzyme had been purified 20 fold and contained about 6 per cent nucleic acid.

Inactivation data for both enzymes are presented in figure 4 as the logarithm of activity versus time of heating at 69 C. The purified vegetative cell enzyme has a half-life of 17 minutes compared to a half-life of 15 minutes for the crude vegetative cell extract. The inactivation of the purified vegetative cell enzyme follows first order reaction kinetics very closely. On the other hand, the inactivation of purified spore glucose dehydrogenase does not appear to follow first order kinetics and this enzyme has a half-life of only one minute at 69 C.

Since the removal of practically all nucleic acid

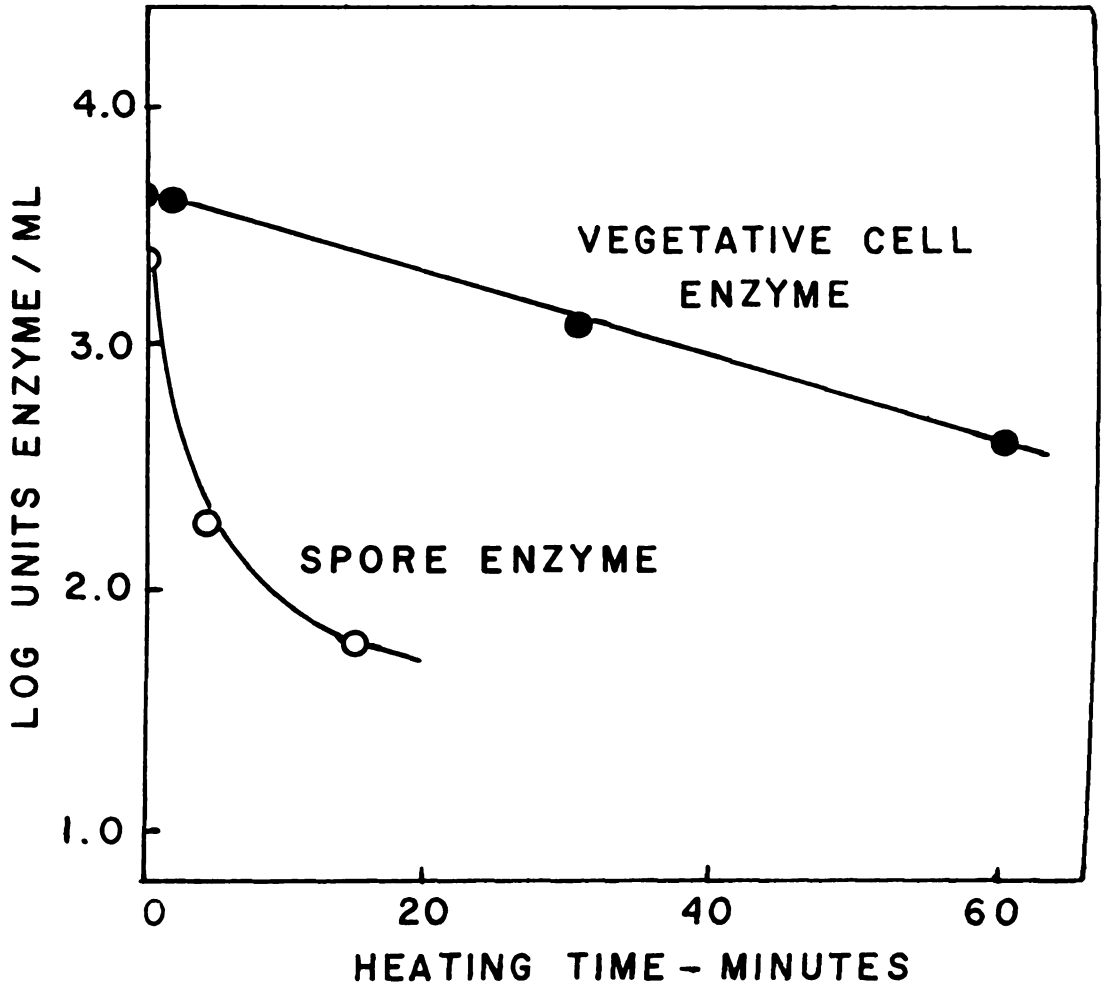


Figure 4. The heat inactivation at 69 C of the purified glucose dehydrogenases from vegetative cells and spores of Bacillus cereus.

from the vegetative cell extract does not lower the heat stability of glucose dehydrogenase from this source, it is unlikely that nucleic acids contribute to the heat stability of this enzyme.

2. Michaelis constants

To prove the enzymatic similarity of vegetative cell and spore glucose dehydrogenase, the Michaelis constants of the two enzymes were compared. The Michaelis constants of the vegetative cell glucose dehydrogenase were determined from the data in figure 5. These are Lineweaver-Burke plots (1934) of enzyme activity versus substrate concentration. The enzyme preparation used for this experiment was an 86 per cent saturated ammonium sulfate fraction, having a specific activity of 11,700. The spore glucose dehydrogenase Michaelis constants were reported by Doi, Church, and Halvorson (1959). Table 3 is a compilation of results obtained for both enzymes which shows that the enzymes have similar substrate affinities.

3. Serology

The vegetative cell and spore glucose dehydrogenases were compared serologically to test the structural similarity of the two enzymes. The results obtained by the agar gel diffusion technique are shown in the photographs in figure 6. These plates demonstrate clearly that

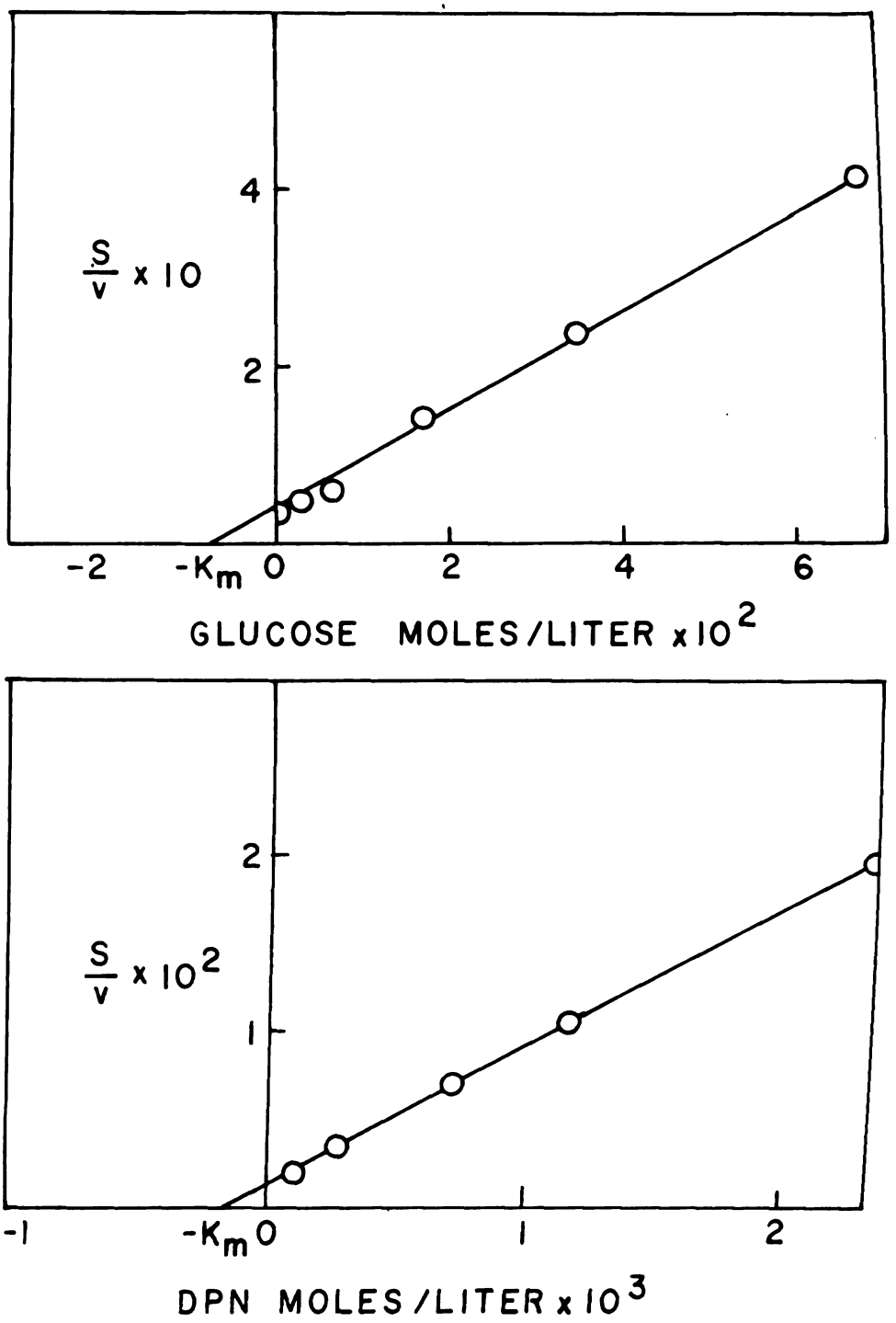


Figure 5. The effect of substrate concentration on the activity of the glucose dehydrogenase from vegetative cells of B. stearothermophilus. In this figure, S is the substrate concentration and v is the change in optical density per minute at 340 millimicrons.

TABLE 3

Michaelis constants for the glucose dehydrogenases from vegetative cells and spores of *Bacillus megaterium*

Enzyme	Substrate	K_m
Vegetative Cell Enzyme	Glucose	7.0×10^{-3}
Spore Enzyme	Glucose	6.7×10^{-3}
Vegetative Cell Enzyme	DPN	2.0×10^{-4}
Spore Enzyme	DPN	9.1×10^{-4}

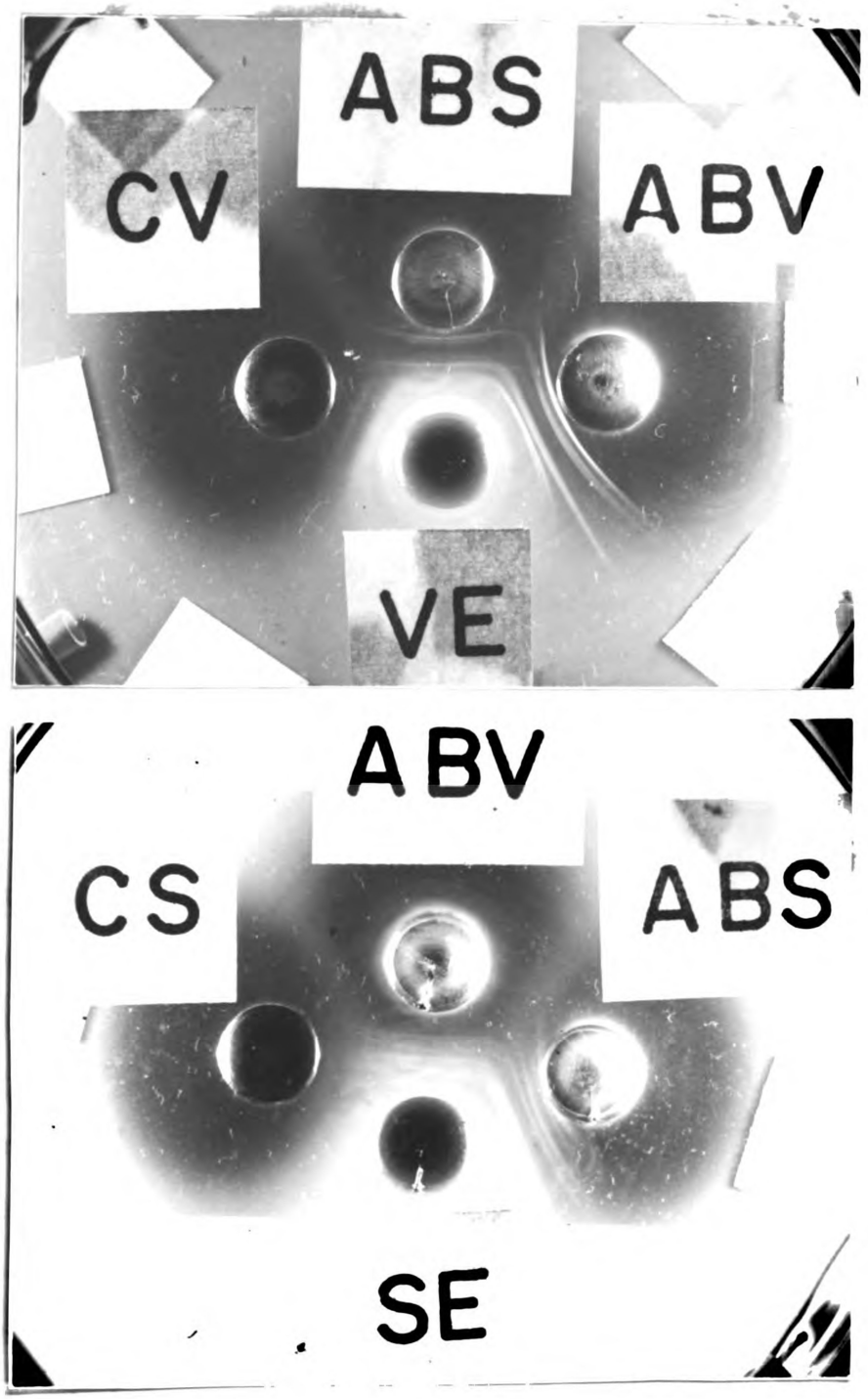
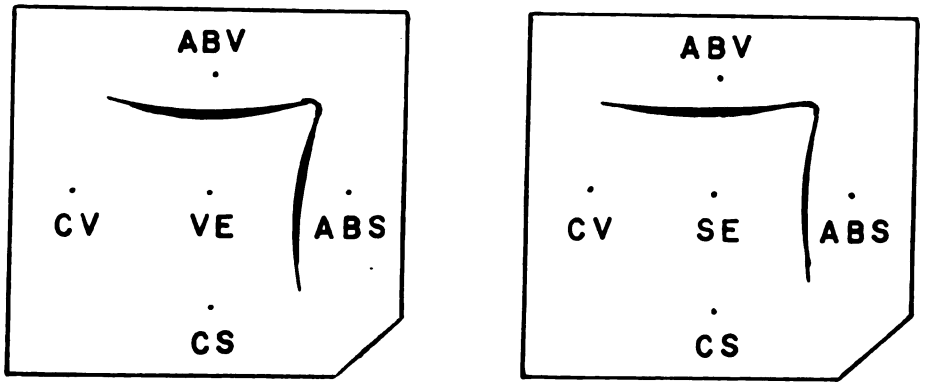


Figure 6. A serological comparison of the glucose dehydrogenases from vegetative cells and spores of Bacillus cereus by the agar gel method. See page 42 for key to well contents.

at least two heat resistant proteins are present both in vegetative cells and spores. To determine if one of the proteins was glucose dehydrogenase, a mixture of DPN and glucose was spread over the agar, and the precipitin lines were examined for fluorescence under an ultraviolet lamp. Although fluorescence was observed, it could not definitely be attributed to the precipitin lines, because of the large amount of unprecipitated glucose dehydrogenase in the agar.

The serological identity of the two enzymes was proved by using cellulose acetate film in place of agar and developing the precipitin lines with a DPN-glucose mixture. The arrangement of antigens and antisera on the film was essentially the same as the arrangement used for the agar diffusion experiment, but, when a DPN-glucose mixture was used to develop the <u>washed</u> film, the only visible precipitin lines were those produced by the reaction of glucose dehydrogenase with its homologous antibody. After spreading the glucose-DPN mixture on the washed films, distinct fluorescent lines appeared between the antigens and each antiserum, but not between the antigens and the normal serum controls. These results are presented diagrammatically in figure 7, where the inked lines represent fluorescence.



KEY

ABV - Serum containing antibodies against vegetative cell enzyme
ABS - Serum containing antibodies against spore enzyme
VE - Vegetative cell enzyme
SE - Spore enzyme
CV - Control serum from rabbits receiving vegetative cell enzyme
CS - Control serum from rabbits receiving spore enzyme

Figure 7. A serological comparison of the glucose dehydrogenases from vegetative cells and spores of Bacillus cereus by the cellulose acetate film method.

F. Heat Labile Glucose Dehydrogenase

Because vegetative cells contained no heat labile glucose dehydrogenase, a germinated spore extract was examined for this enzyme. A glucose dehydrogenase was found, in this extract, which was completely inactivated in less than one minute at both 70 and 60 C. At 50 C, this enzyme had a half-life of less than one minute, compared to a half-life of seventeen minutes at 69 C for the vegetative cell enzyme.

DISCUSSION

Following the exponential growth of Bacillus cereus cultures, a complex series of events occurs resulting in the production of heat resistant spores. Heat resistant glucose dehydrogenase is synthesized by these cells early in the sporulation process and is eventually incorporated into spores. After germination, when the spores have completely lost their heat resistance, the glucose dehydrogenase is still present in high concentration but it too has lost its heat resistance. The glucose dehydrogenase can not be detected after vegetative growth commences because it is diluted by the multiplication process.

The results of serological and enzymatic comparisons, the coincidence of the production of this enzyme with the production of spores, and the coincidence of the change in heat resistance of this enzyme with the change in heat resistance of spores, all indicate that the glucose dehydrogenase in vegetative cells, spores, and germinated spores is the same enzyme with different levels of heat resistance. For this reason, if both the heat labile and heat stable forms can be completely purified, this enzyme system should provide an excellent model for studying spore heat resistance. However, as

stated in the introduction, the purpose of this study was not merely to obtain any model system, but one in which the enzymes are plentiful, easily purified, and reasonably stable during storage. Enzymes with these characteristics can be accumulated in sufficient quantity to permit physical-chemical characterization.

The vegetative cell glucose dehydrogenase appears to meet these requirements since it is very stable, and can be purified easily and in high yields. Although its cellular concentration is not especially high, its stability during storage will permit the accumulation of the large amount of pure enzyme needed for its complete characterization. In fact, a partially purified preparation of this enzyme has been stored for eight months, at -20 C, without losing appreciable activity.

On the other hand, the accumulation of germinated spore glucose dehydrogenase may not be as easy because this enzyme is heat labile. However, since the spore enzyme is much more stable, large amounts of ungerminated spores, rather than germinated spores, could be accumulated. Germination of the spores and purification of the heat labile enzyme could then be accomplished in a short time, with minimum loss of enzyme activity.

The aberrant inactivation kinetics of the spore

glucose dehydrogenase, shown in figure 4 might be explained as the combined kinetics of a mixture of heat stable and heat labile enzymes. A similar observation was made by Lawrence and Halvorson (1954) who studied the catalase from spore extracts of Bacillus cereus. Such a mixture of enzymes might be caused by contamination of the spore crop with germinated spores or by a decrease in heat resistance of some of the stable enzyme during purification and storage of the extract. Contamination of the spore crop with germinated spores can be discounted as a cause of the aberrant kinetics because the spore extract was heated to 65 C during purification. If the spore extract contained any germinated spore enzyme at that time, it would have been completely inactivated and the kinetics obtained for the purified spore extract would have been of first order. Hence, a decrease in heat resistance of the spore enzyme during purification and storage seems the only satisfactory explanation for the observed results.

The loss of heat resistance by purified spore glucose dehydrogenase might take place by the same mechanism as the loss in heat resistance of this enzyme during germination of whole spores. If this is true, perhaps this "cell-free germination" could be initiated

at will by conditions similar to those required for germination of whole spores. Such a system would permit one to study loss of heat resistance by a "dynamic" method rather than by a "static" comparison of heat stable and heat labile enzymes. Much more information about the mechanism of heat resistance could be obtained from a dynamic system than from a static one because loss in heat resistance may involve more than one simple step. In fact, the loss of heat resistance by a series of events is suggested by the spore enzyme inactivation curve shown in figure 4. If only two levels of heat resistance were possible for each glucose dehydrogenase molecule - those of the vegetative cell and germinated spore enzymes - the bend in this curve would be extremely sharp at 69 C, because of the large difference in inactivation rates of the two enzymes. Instead, the rather broad bend observed indicates the possibility of glucose dehydrogenase with heat resistance intermediate to that of the vegetative cell and germinated spore enzymes. Thus, the loss of heat resistance by this enzyme may be a sequential phenomenon, requiring more than one unit event for the change from maximum to minimum heat resistance. This statement, however, should be considered as no more than an hypothesis, since the meager inactivation data

in figure 4 can not support any real conclusions. The heat inactivation results in figure 4 do, nevertheless, contain important implications for the purification of this model system. If physical-chemical comparisons of the heat stable and heat labile enzymes are to be made, the stable enzyme must be obtained from vegetative cells because the enzyme at this stage of spore development retains its heat stability during storage. On the other hand, since loss of heat resistance by purified enzyme appears to depend on the maturity of the spores from which the enzyme is isolated, the heat stable enzyme from mature spores would be useful in "cell-free germination" studies.

Compared to other heat resistant spore enzymes and to the whole spores themselves, the stability of the glucose dehydrogenase from vegetative cells and spores of Bacillus cereus is rather low. For example, the adenosine ribosidase found in Bacillus cereus spore extracts by Powell and Hunter (1956) had a half-life of four hours at 100 C, compared to a half-life of fifteen minutes at 69 C for the glucose dehydrogenase. However, the glucose dehydrogenase has both a heat stable and heat labile form, and the difference in heat stability of the two forms is quite large. Thus, the glucose dehydrogenase appears to be a reasonable model for the

study of heat resistance. The fact that the change in heat resistance of the glucose dehydrogenase parallels the change in heat resistance of whole spores during germination is additional proof of its usefulness as a model system. Correlation with the heat resistance of whole spores is, in fact, a requirement for any enzyme system to be used for the study of spore heat resistance, since the mere presence of a heat resistant enzyme in spores does not necessarily mean it contributes to spore heat resistance. For example, a very heat stable inorganic pyrophosphatase was found by Johnson and Johnson (1959) in Azotobacter agilis, a nonsporeforming mesophile. This enzyme obviously bears no relation to cellular heat resistance because the cell from which it is derived is not heat resistant.

In spite of the foregoing considerations, a certain amount of caution must be exercised in predictions of the usefulness of this model system for determining the mechanism of spore heat resistance. Since the purified heat stable glucose dehydrogenase is rapidly inactivated at temperatures used to heat shock spores prior to germination, and the heat labile glucose dehydrogenase is abundantly present in these germinated spores, it is obvious that differences in structure of these two purified enzymes cannot completely account for the heat

resistance of the enzyme in intact spores. It is possible that the structural differences between these enzymes are diminished when they are extracted, or that intraspore environment also plays a role in the protection of this enzyme while it is in the spore. In any event, the author feels that the application of the model system, described in this thesis, to the study of spore heat resistance is almost certain to yield information of a fundamental nature, and to provide at least the beginning of a theory for the mechanism of spore heat resistance.

SUMMARY

A model system for the study of the heat resistance of spores of Bacillus cereus has been described. This system consists of a heat stable glucose dehydrogenase from vegetative cells and spores, and a heat labile glucose dehydrogenase from germinated spores. In crude extracts, the vegetative cell enzyme has a half-life of about fifteen minutes at 69 C, while the germinated spore enzyme has a half-life of about one minute at 50 C.

An increase in the glucose dehydrogenase activity of vegetative cell extracts was observed during the first minute of heating at 69 C. This apparent heat activation was investigated because of its possible connection with the heat activation of glucose oxidation in whole spores. It was concluded that the heat inactivation of DPNH oxidase, without grossly affecting the glucose dehydrogenase activity, was responsible for the apparent heat activation of the extracts.

Biosynthesis of the heat stable glucose dehydrogenase is initiated shortly after the exponential growth of cultures ceases and before any dipicolinic acid can be detected in the cells. The heat stable enzyme is present in the cells during most of the sporulation process but a true measure of its concentration during this

period was impossible with the extraction procedures available.

The enzyme from vegetative cells was purified about 600 times, and the enzyme from spores was purified about 175 times by the following steps: extraction by mechanical disruption of cells or spores, heating to 65 C, protamine precipitation of nucleic acids, ammonium sulphate fractionation, and DEAE cellulose column chromatography.

The purified enzymes from vegetative cells and spores had almost identical substrate affinity and serological characteristics, but differed in heat stability and kinetics of heat inactivation. The difference in heat stability and heat inactivation kinetics was attributed to a partial conversion of the heat stable enzyme to the heat labile enzyme.

The possibility of completely purifying the glucose dehydrogenase system and using it as a model for studying spore heat resistance was discussed.

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