SEDIMENTATION BEHAVIOR OF THE GLUCOSE DEHYDROGENASE FROM SPORES OF *BACILLUS CEREUS*

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ABSTRACT

SEDIMENTATION BEHAVIOR OF THE GLUCOSE DEHYDROGENASE FROM SPORES OF *BACILLUS CEREUS*

by John W. Kools

Glucose dehydrogenase from germinated spores of *Bacillus cereus* was used as a model for the study of the heat resistance of bacterial endospores. It had previously been found that the heat resistance of this enzyme, *in vitro*, was a function of the NaCl and hydrogen ion concentrations of its environment. In solutions of 5 M NaCl, the enzyme was as heat resistant as it was in the intact spores and appeared to have dissociated into two or more molecules. A study was therefore made of the relationship between the state of aggregation of the protein and its heat resistance.

Gel filtration was used in an attempt to separate the aggregated from the disaggregated enzyme. This was not a successful approach although an excellent separation of glucose dehydrogenase from contaminating proteins was obtained. A combination of $(NH_4)_2SO_4$ fractionation, heat denaturation of contaminating proteins, and gel filtration with G-100 Sephadex through a 900 ml column resulted in a purification of 90-100% and a yield of enzyme that approached 100%.

The G-100 Sephadex was suspended in and eluted with a buffer system composed of 0.05 M imidazole chloride buffer pH 6.5 and containing 0.5 M NaCl. This concentration of NaCl gave sufficient heat resistance to the enzyme so that the column could be run at room temperature.

The two sedimentation techniques used in this study employed linear sucrose gradients for the stabilization of the boundary of the sedimenting protein. These gradients were 0-3 per cent and 5-20 per cent respectively. The location of the boundary after the centrifugation runs was determined by sampling the tube and assaying the enzyme activity of each sample.

No change in the sedimentation properties of the glucose dehydrogenase was observed over a pH range of 6.5-8.0 in 0.05 M imidazole chloride buffer pH 6.5 at 20 C. The sedimentation constant was 8.4 S.

The effect of the NaCl concentration was determined in 0.05 M imidazole chloride buffer pH 6.5. Without any NaCl present, the molecular weight of the enzyme was 37,000, but it was partially dissociated into 22,000 molecular weight subunits in 3 M NaCl at 20 C. From 3-5 M NaCl the sedimentation constant decreased with a corresponding decrease in the diffusion constant, indicating a shape change with no change in the molecular weight.

The sedimentation studies were conducted at 20 C while thermal inactivation studies had been conducted at

elevated temperatures. Although the temperature of the preparative centrifuge could be regulated only over a limited range, sedimentation studies in 3 M NaCl at temperatures of 5, 20, and 27 C indicated an undissociated, partially dissociated, and completely dissociated enzyme respectively. On the other hand, sedimentation studies in 0.05 M imidazole chloride buffer, indicated only an undissociated enzyme at 5 and 20 C. It would therefore appear that heating can cause disaggregation of the enzyme and that this phenomenon occurs readily as the concentration of NaCl increases. As a consequence of these observations it would appear that the property of heat resistance resides in the subunit of the enzyme.

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By

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INTRODUCTION

Members of the family Bacillaceae are characterized by their ability to form dormant cells called endospores. The endospores are extremely heat resistant in comparison to vegetative cells of the same family and to other microorganisms in general. The mechanism by which these spores are made stable has been studied with the primary approach dealing with the cytological and chemical differences between the labile vegetative cells and the resistant spores. Many theories have been derived from these studies to explain heat resistance, such as a dipicolinic acid-calcium-protein complex (Young, 1959) and a gelled core (Black and Gerhardt, 1962); but there is no direct experimental evidence to confirm or refute these hypotheses.

In an attempt to by-pass the difficulties that arose when the mechanism(s) of heat resistance was studied in intact spores, an enzyme extracted from the spores was used as a model. The justification for this approach lies in the fact that all of the enzymes within the spore which are needed for its germination (and possibly outgrowth) must necessarily be heat resistant (Kools, 1960).

Recently an enzyme, glucose dehydrogenase, was isolated from the spores of Bacillus cereus, and its heat resistance was found to be a function of the pH and NaCl concentration of the suspending medium. The enzyme could be rendered as stable in vitro as it was in the intact spore. There was also dissociation of the enzyme into smaller molecular weight fragments in solutions of high NaCl concentration (Bach, 1963).

The purpose of the study reported in this thesis was an investigation of the relationship between the heat resistance and the state of aggregation of glucose dehydrogenase in solutions of various salt and hydrogen ion concentrations.

HISTORICAL REVIEW

The heat resistance of bacterial endospores has played an important role in the history of microbiology. Much of the furor over the theory of spontaneous generation might never have occurred if early investigators had appreciated the extent of the resistance of spores. For example, John Tyndall, in trying to disprove the theory of spontaneous generation, found in some cases that more than five and one-half hours at 100 C was necessary to sterilize his media. From these experiments, he concluded that heat resistant organisms had contaminated his media. Ferdinand Cohn, in 1876, demonstrated that certain bacteria were able to produce heat resistant endospores and that these could be microscopically differentiated from vegetative cells (Stanier, Doudoroff, and Adelberg, 1963).

A spore has a unique structure. The external wall of the spore consists of an outer coat which is often seen to be split into two layers. Inside this coat lies a thick electron-transparent layer called the cortex. The inner spore coat seems to develop from the interior portion of the cortex and becomes the cell wall of the new vegetative cell when germination occurs. Inside the inner spore wall

is a mass of cytoplasm called the core. The spores of B. cereus are characteristically covered, in addition, by a loose-fitting envelope called the exosporium (Hashimoto, Black and Gerhardt, 1960).

Over a period of 70 years, many studies have been made in an attempt to elucidate the mechanism of heat resistance in bacterial endospores. Subsequently, a number of theories have been proposed to explain this unusual attribute. The majority of these theories were based on the chemical and structural differences that existed, or were thought to exist, between the spores and the vegetative cells. Among these were included:

1. A non-permeable spore coat (Lewith, 1890).
2. A relatively low water content in the spores (Davenport and Castle, 1895).
3. A high concentration of dipicolinic acid (DPA) and calcium in the spores (Powell, 1953).
4. A relatively high thiol content in spores, i.e., cystine and cysteine (Vinter, 1961).

By virtue of the discovery of the extensive permeability of spores to water and solutes, the concept of heat resistance due to a non-permeable spore coat could be discounted (Murrell, 1961; Gerhardt and Black, 1961; and Black and Gerhardt, 1962).

On the other hand, Black and Gerhardt (1962) pointed out that the spore interior is relatively dry compared to the

cytoplasm of a vegetative cell but not to the extent envisioned by Davenport and Castle (1895). They were able to determine the weight of the interstitial water surrounding the spores thereby allowing them to make a valid dry weight determination. The dormant spores of B. cereus contained 65% water, germinated spores 73% water, and the vegetative cells 77% water by weight. Murrell (1961), using density determinations of dry spore material and an estimate of water in spore pellets found that any anhydrous portion of the spore would account for less than 10% of the total spore volume. This value was confirmed by Black and Gerhardt (1962).

Another hypothesis concerning the water content of spores had been set forth which predicted the existence of a dry, central core (Lewith, 1890). The experiments of Black and Gerhardt (1962) showed that about 90% of the volume of this central core was permeable to water. Thus, it would seem that water is distributed throughout the spore but that the core has a relatively lower concentration of water. The authors have proposed that:

> the core of the dormant spore exists as an insoluble and heat stable gel, in which cross-linking between macro-molecules occurs through stable but reversible bonds so as to form a high-polymer matrix with entrapped free water.

They further suggested that this cross-linking was due to the presence of disulfide bonds, and, in support of this hypothesis, they mentioned the high concentration of cystine-cysteine groups in spores (Vinter, 1961).

Dipicolonic acid comprises 4-15% of the dry weight of spores, whereas none is found in vegetative cells. It was shown that this compound must be present in order for spores to be heat resistant (Halvorson and Howitt, 1961; Church and Halvorson, 1959; Black, Hashimoto and Gerhardt, 1960). During spore germination the DPA is released into the medium in the form of a calcium chelate with the subsequent loss of heat resistance of the spores. Calcium was also implicated in the heat resistance of spores (Curran, Brunstetter and Myers, 1943; and Black et al., 1960). Young (1959) found that peptides from spores form a complex with the calcium-DPA chelate and has, therefore, suggested that a DPA-calcium-protein complex could exist. However, no meaningful experiments leading to an understanding of the mechanism of the heat resistance due to DPA have been performed.

Spores are heat resistant and it follows that their essential proteins are also heat resistant under conditions prevailing in the spore interior. This forms the basis for a different approach to the problem, that of studying individual enzymes from the spore. Enzymes extracted from spores have been found to be either heat stable or heat labile, suggesting that some proteins are intrinsically stable, while others are stabilized by factors in their environment. Some spore enzymes are particulate and heat stable when first extracted but tend to lose this stability if they

become soluble. Powell and Hunter (1956) extracted a stable adenosine ribosidase from spores of B. cereus which was capable of withstanding a temperature of 100 C for 1 hour. They did not state whether the enzyme was particulate or soluble. The adenosine deaminase from the same organism lost its heat resistance upon disintegration of the spore. Another enzyme from spores of B. cereus, alanine racemase, has been found to be particulate and relatively stable when extracted by means of brief sonic oscillation. The enzyme loses heat resistance and becomes soluble when subjected to extensive sonic oscillation (Stewart and Halvorson, 1953; Stewart and Halvorson, 1954).

Bach (1963) has recently described an enzyme, glucose dehydrogenase, from the spores of B. cereus which was labile when extracted from the spore but which could be made stable by the addition of sodium chloride to the enzyme solution. His experiments indicated that this protein was stabilized by salts normally present in spores. When the sodium chloride was added to glucose dehydrogenase, the enzyme disaggregated into two or more units.

In contrast to the above examples, two intrinsically heat resistant, non-spore proteins have been studied. The first of these, the flagella from thermophilic bacteria, were much more stable than those from mesophilic bacteria (Adye, Koffler, and Mallett, 1957). The heat resistance of these proteins was maintained through extensive purification

and no stabilizing material was detected (Koffler, Mallett, and Adye, 1957; and Adye et al., 1957).

The alpha-amylase from Bacillus coagulans has been crystallized without any evidence for the existence of a stabilizing factor. When the organism was grown at 55 C, the crystalline enzyme retained 88 to 90% of its activity after being heated for 60 minutes at 90 C. However, when the same organism was cultured at 35 C, only 6-10% of its activity remained when it was subjected to the preceding conditions (Campbell, 1954).

Many protein molecules exist as aggregates of peptides which are referred to as subunites. Pedersen (1940) has reviewed the research concerning the dissociation of proteins in varying hydrogen ion concentrations and concluded that "for a given protein, the molecular weight as a rule has its maximum value within a given pH range around its isoelectric point." This dissociation of a protein at a given pH may also be influenced by the salt concentration of the surrounding medium. For example, haemoglobin in moderately concentrated salt solutions (0.5-1.0 M) was dissociated into molecules having half the normal molecular weight.

McFarlane (1935) found that the dissociation of a protein can further be influenced by the presence of another, different protein. He discerned that globulin from horse serum dissociated in the presence of albumin.

It has, in fact, been observed that the presence of even an amino acid can cause disaggregation of a protein molecule. For example, 0.001% thyroxine produces appreciable dissociation of thyroglobulin (Lundgren, 1936). In contrast to the effect of the addition of a protein or amino acids to the solution, the dilution of the protein suspension resulted in the appearance of several disaggregation products. The same author also noted that urea favored the dissociation of thyroglobulin.

Temperature also seems to have some role in protein dissociation. Pedersen (1950) reported that gamma globulin disaggregated when the temperature of the substance in the ultracentrifuge was raised from 10 to 30 C.

Svedberg and Brohult (1938) showed that haemocyanin at pH 6.5 was dissociated into half molecules by irradiation with ultraviolet light of wave lengths of 280 millimicrons. Brohult (1937) reported that the haemocyanin molecule could be partially split by ultrasonic waves with a frequency of 250,000 cycles per second. At pH 6.2, half molecules were formed, and at a pH of 7.2 one-eighth molecules were formed after sonication.

Alpha-amylases are metalloenzymes whose heat stability can be greatly reduced by the removal of calcium (Fischer and Stein, 1960). The alpha-amylase from Bacillus subtilis is a dimer whose two parts are bound together by a single molecule of zinc. The removal of the zinc was

readily accomplished by dialysis against the chelating agent ethylenediaminetetraacetate (EDTA) with no loss of its heat resistant properties. However, after prolonged dialysis and under conditions that were not optimal for the enzyme itself, the calcium was lost as well as the resistance of the enzyme.

Deal, Rutter, and Van Holde (1963b) have described the reversible dissociation of aldolase from rabbit muscle. At pH values 2.9 and below the enzyme dissociated into three subunits of approximately equal molecular weight. They suggested that the native protein, a folded trimer, first became an unfolded trimer, then dissociated into three unfolded monomeric subunits.

Deal et al. (1963a) have studied enolase, alpha-glycerophosphate dehydrogenase, fumerase, lactic dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase in acidic solutions. In all cases except the latter, structural modifications were observed as indicated by changes in sedimentation properties. The lactic dehydrogenase dissociated into unfolded subunits.

Samejima and Shibata (1961) have reported the dissociation of beef liver catalase with formamide and urea. They found that the native catalase was dissociated into one-third size subunits which could either dimerize into two-third size subunits or dissociate again into one-sixth size subunits. Since the catalase contains four iron

molecules, the authors concluded that the iron was distributed unevenly in the native catalase molecule. Furthermore, the catalase molecule dissociated into halves by acid treatment while dissociating into one-third size subunits by alkali treatment.

There are several environmental factors which can contribute to the dissociation of any particular protein molecule, although the degree to which any one of these factors is effective will depend largely on the particular protein involved.

EXPERIMENTAL METHODS

Organism and Medium

The organism used throughout this study was a strain of *B. cereus* which had been obtained from the University of Illinois culture collection in 1955. It was maintained at 4 C in the sporulated form on the surface of nutrient agar slants (Difco Laboratories). Modified G medium (Stewart and Halvorson 1953; and Hashimoto et al., 1960) was used in growing large quantities of spores and consisted of the following materials per liter of solution:

Glucose	4.0 g
$(NH_4)_2SO_4$	4.0 g
Yeast Extract	2.0 g
K_2HPO_4	1.0 g
$MgSO_4$	0.8 g
$CaCl_2$	0.1 g
$MnSO_4 \cdot H_2O$	0.1 g
$ZnSO_4$	0.01 g
$CuSO_4 \cdot 5H_2O$	0.01 g
$FeSO_4 \cdot 7H_2O$	0.001 g

In addition, Dow Corning Antifoam AF emulsion (Dow Corning Corporation) was used as needed. The initial pH

of the medium was 6.6 and needed no further adjustment.

Production of Spores

Cultures of B. cereus were grown in a 100 liter fermentor (Stainless and Steel Products Co.), maintained at 30 C. The contents were stirred at 225 rev/min and aerated at 100 liters/min. In order to obtain sufficient inoculum for the 100 liter fermentor, the following procedure was used. The spores from a storage slant were transferred to a fresh nutrient agar slant which was allowed to incubate for 8 hours. The growth on the slant was transferred to 50 ml of medium contained in a 500 ml dimpled flask and shaken for 2 hours at 30 C. Successive transfers were then made into 4 and then 18 similar flasks using a 20 volume per cent inoculum and incubating with shaking for 2 hours. All of the contents of the 18 flasks were used to inoculate three New Brunswick 3 liter fermentors (New Brunswick Scientific Co.). The contents of the small fermentors were stirred at 450 rev/min and aerated at 5 liters/min for 2 hours. The resulting 9 liters of B. cereus culture were used to inoculate the 100 liter fermentor.

The culture had sporulated and the sporangia had begun to lyse after 22 hours. This lytic process was hastened by raising the temperature in the fermentor to 45 C, decreasing the rate of stirring to 100 rev/min and

discontinuing the air flow. After 1 hour under these conditions, the spores were free of their sporangia and most of the vegetative cells had lysed. The fermentor tank was rapidly cooled and the spores were harvested in a Sharples Super Centrifuge, model A 5-12 (The Sharples Corporation). The spores were washed once in distilled water and stored at -15 C until needed.

Germination of Spores

The yield of glucose dehydrogenase could be greatly increased by breaking germinated spores rather than dormant spores. The germination procedure used was that described by Bach (1963).

A typical germination procedure consisted of heating 400 g (wet wt) of spores in 1 liter of distilled water for 1 hour at 65 C, followed by their suspension in a 3% yeast extract solution (Difco Laboratories). The final germination medium contained 400 g of heat shocked spores suspended in 8 liters of 3% yeast extract. The spore suspension was incubated at 30 C for 1 hour. The extent of germination was ascertained by observing the loss of refractility of spores by means of phase contrast microscopy. The germinated spores were washed once with distilled H_2O and suspended in 1200 ml of 0.1 M phosphate buffer pH 6.8.

Glucose Dehydrogenase Assay

Glucose dehydrogenase is a nicotinamide adenine dinucleotide (NAD) specific enzyme and the reduced form of NAD (NADH) can be assayed spectrophotometrically at a wavelength of 340 millimicrons. A Beckman spectrophotometer, model DU (Beckman Instruments, Inc.), equipped with a Model SR Sargent recorder (E. H. Sargent and Co.) and a Ledland log converter (Ledland Instruments) was used to measure the NADH produced in a 1 ml cuvette containing 800 micromoles of tris buffer pH 8.0 (trishydroxymethylaminomethane), 100 micromoles of glucose, 2 micromoles of NAD and sufficient enzyme preparation and distilled water to make a final volume of 1.0 ml.

Since the activity of glucose dehydrogenase is very temperature dependent, the temperature of the sample chamber of the spectrophotometer was maintained at 25 C by the use of thermospacers and a constant temperature bath.

One unit of enzyme was defined as that amount which caused an optical absorbance change of 0.001 per minute under the above conditions.

Rupture of Germinated Spores

An Eppenbach Colloid Mill (Gifford-Wood Co.) was used to effect the rupture of germinated spores (O'Conner, Doi and Halvorson, 1960). The instrument contained 1200 ml of the spore suspension and 740 g of 110 pavement marking

beads (Minnesota Mining and Manufacturing Co.). The mill was run at 10,000 rev/min with a gap setting of 0.03 inches and the temperature was maintained at 10-15 C by circulating ethanol at -20 C through the cooling coils of the mill. The extent of breakage was observed by phase contrast microscopy.

Partial Purification of Glucose Dehydrogenase

The cell brei from the colloid mill was clarified by centrifugation at 14,600 x g for 1 hour in a Type SS-4 Servall enclosed centrifuge (Ivan Sorvall, Inc.). The supernatant liquid was made 60% saturated with solid ammonium sulfate and the precipitate that developed was removed from the solution be centrifugation and discarded. The glucose dehydrogenase remained in solution. The enzyme was precipitated by increasing the concentration of $(NH_4)_2SO_4$ to 100% saturation in order to effect a concentration of the enzyme. This precipitate was resuspended in a 1/15 volume of 0.05 M imidazole chloride buffer pH 6.5 containing 4 M NaCl. Because the enzyme was heat resistant in high concentrations of NaCl, the preparation was heated to 60-65 C for 30 minutes to effect a purification by differential thermal denaturation. The denatured protein was removed by centrifugation and the enzyme solution was dialyzed overnight against distilled water to reduce the concentration of NaCl. The glucose dehydrogenase was concentrated by "dialysis" against dry Sephadex,

G-25, medium grade (Pharmacia).

Sedimentation Studies

Linear Gradients.--The sedimentation properties of glucose dehydrogenase were determined by the methods of Hogeboom and Kuff (1954) and Martin and Ames (1961). These procedures can be used with relatively impure preparations and utilize the enzymatic activities to locate the position of those proteins under investigation. In the method described by Hogeboom and Kuff (1954) a 0-3% sucrose gradient was used to stabilize the protein boundary. The runs were initiated with the protein distributed throughout the centrifuge tube. Martin and Ames (1961) utilized a 5-20% sucrose gradient with the enzyme layered on the top of the liquid column at the beginning of the sedimentation experiment. Both procedures required the establishment of linear sucrose gradients in the centrifuge tubes.

The preparation of linear gradients was accomplished by using the device shown in Fig. 1. This apparatus, described by Hogeboom and Kuff (1954), was constructed from a lucite block in which were drilled two cylindrical chambers, A and B, connected by a channel. A drain tube was mounted in the bottom of chamber B. The two chambers contained identical solutions except for the concentration of sucrose, with chamber B always containing the solution of higher sucrose concentration. The contents of chamber B

were stirred and progressively diluted as the sucrose solution was conducted to the centrifuge tube. The gradients were formed in 1/2 in. x 2 in. Lusteroid centrifuge tubes.

Sedimentation Conditions.--The centrifuge tubes were placed in a precooled SW-39 swinging bucket rotor which was run at 38,000 RPM (158,000 x g) at 20 C in a model L preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Division). The duration of the individual sedimentation runs was 4-20 hours. At the completion of the experiment, the rotor was allowed to "coast" to a stop. The time taken to reach full speed and the time necessary for the centrifuge to stop was equivalent to 7 minutes at top speed (Hogeboom and Kuff, 1954). The centrifuge tubes were removed and sampled immediately by piercing the bottom of the tube with a hypodermic needle using the device shown in Fig. 2 (Martin and Ames, 1961). Two drops were collected per sample and these were assayed for enzymatic activity. The geometry of the rotor and the volume in the centrifuge tube were utilized in calculating the distance the protein moved under the influence of the centrifugal field.

Theory and Calculation.--The sedimentation coefficient is the velocity of a sedimenting molecule in a unit field and is defined by

$$s = \frac{1}{\omega^2 x} \frac{dx}{dt} \qquad (1)$$

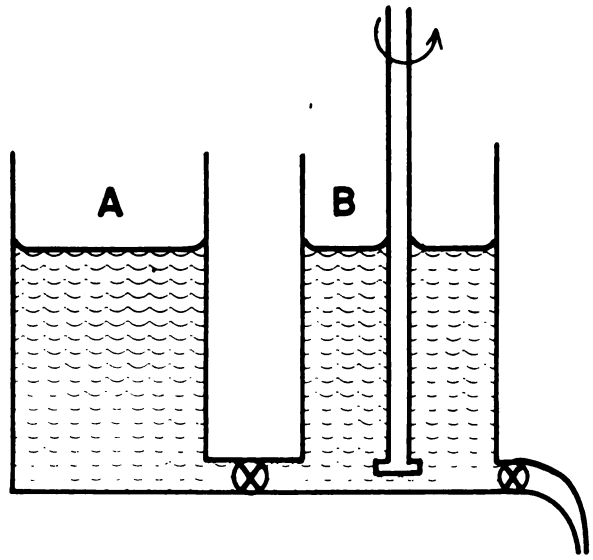

Figure 1. Apparatus for the preparation of linear sucrose gradients. A modification of the device described by Hogeboom and Kuff (1954).

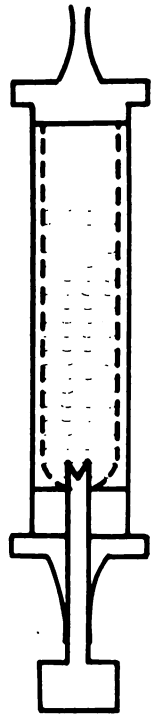

Figure 2. Centrifuge tube sampling device, similar to that described by Martin and Ames (1961).

where ω is the angular velocity in radians/sec

t is the time in seconds, and

x is the distance of the protein boundary from the center of rotation in centimeters.

The unit of sedimentation which is commonly employed is the Svedberg which is equivalent to 10^{-13} seconds. The integrated form of equation (1) is:

$$s = \frac{1}{2_t} \ln \frac{x}{x_o} \qquad (2)$$

where x_o and x are the respective positions of the boundary at time t_o and t. The angular velocity was easily calculated from the rotational velocity of the centrifuge. The movement of the protein boundary down the centrifuge tube was detected by the sampling procedure described above. Utilizing equation (2), it was possible to calculate the sedimentation coefficient of the glucose dehydrogenase.

In order that data derived under various conditions may be compared, it was necessary to correct the sedimentation coefficients to $s_{20,\ w}$, the equivalent sedimentation rate in water at 20 C. The following relationship was used.

$$s_{20,\ w} = \frac{s\eta\ (1 - \bar{V}\rho_w)}{\eta_w\ (1 - \bar{V}\rho)} \qquad (3)$$

where η and η_w are the viscosity of the medium and water at 20 C respectively

$\bar{V}$ is the partial specific volume of the protein

ρ_w the density of water at 20 C

ρ the density of the medium.

The partial specific volume of the glucose dehydrogenase was taken as 0.72 cm^3/g based on the data of Fox and Foster (1957).

The sedimentation velocity of a molecule can also be described in terms of the properties of the molecule.

$$s = \frac{M\ (1 - \bar{V}\rho)}{Nf} \tag{4}$$

where M is the anhydrous molecular weight

N is Avogadro's number and

f is the frictional coefficient of the molecule in solution.

The diffusion coefficient D is given by

$$D = \frac{kT}{f} \tag{5}$$

where k is Planck's constant, the gas constant per molecule and T is the absolute temperature.

On solving equation (4) for molecular weight and substituting for f in terms of the diffusion constant, the following familiar equation for molecular weight is obtained

$$M = \frac{RTs}{D\ (1 - \bar{V}\rho)}. \tag{6}$$

In order to confirm the sedimentation data obtained by the procedures of Hogeboom and Kuff (1954), sedimentation runs were also performed in 5-20% sucrose gradients (Martin and Ames, 1961). In these runs, point to point variation of the viscosity and density down the length of the centrifuge tube had to be taken into account when the sedimentation

values were based on the following considerations.

By combining equations (1) and (3)

$$s_{20,\,w} = \frac{\eta\,(1 - \bar{V}\rho_w)\,dx}{\omega^2\,x\,\eta_w\,(1 - \bar{V}\rho)\,dt} \tag{7}$$

On rearranging terms

$$s_{20,\,w}\,\omega^2\,dt = A\,F(x)\,dx \tag{8}$$

where the constant $A = \dfrac{(1 - \bar{V}\rho_w)}{\eta_w}$ and (9)

$$F(x) = \frac{\eta}{(1 - \bar{V}\rho)x} \tag{10}$$

On integration

$$s_{20,\,w}\,\omega^2\,t = A \int_{x_i}^{x_{i+1}} F(x)dx \tag{11}$$

$$s_{20,\,w} = \frac{A \displaystyle\int_{x_i}^{x_{i+1}} F(x)dx}{\omega^2 t} \tag{12}$$

The term $\displaystyle\int_{x_i}^{x_{i+1}} F(x)dx$ can be evaluated by a trapezoidal approximation (Martin and Ames, 1961).

$$\int_{x_i}^{x_{i+1}} F(x)dx = \sum_{o}^{x_{i+1}} F(x_i)(x_{i+1}) + \frac{1}{2} \sum_{o}^{x_{i+1}} [F(x_{i+1}) - F(x_i)]\,[x_{i+1} - x_i] \tag{13}$$

An example of this calculation is presented in the Appendix.

Density and Viscosity Determinations.--A 25 ml pycnometer was used for the density determinations and an Ostwald viscosimeter was used for the viscosity determinations. When using a pycnometer and a liquid standard (water), the equation for the determination of density is:

$$\rho = \frac{W}{W_{H_2O}} \cdot \rho_{H_2O} \tag{14}$$

where ρ and ρ_{H_2O} are the densities of the solution measured and water respectively and W and W_{H_2O} are the weights of the solution and water in the pycnometer. Similarly, when using an Ostwald viscosimeter the equation for viscosity is:

$$\eta = \frac{t}{t_{H_2O}} \cdot \frac{\rho}{\rho_{H_2O}} \cdot \eta_{H_2O} \tag{15}$$

where η and η_{H_2O} are respectively the viscosities of the solution and water, and t and t_{H_2O} are the times observed for the solution or water to fall from one point to another in the instrument. Density and viscosity determinations were made on the solutions that were used in forming the 0-3% sucrose gradients because over this short range the variation in viscosity was essentially linear. These data are found in the Appendix. The density and viscosity at any point in the centrifuge tube could then be calculated because the gradient was linear. In the 5-20% sucrose gradient, the variation in density was linear but the variation in viscosity was not. The viscosities and densities of buffered 3 M NaCl

solutions containing 0, 3, 5, 12.5 and 20% sucrose were determined and are shown in the Appendix.

RESULTS

The yield of spores from 100 liter cultures of B. cereus was routinely 400 g wet weight. The activity of glucose dehydrogenase in crude preparations extracted from these dormant spores was 10,000 units per gram wet weight. On the other hand, the amount of enzyme extracted from germinated spores was 33,000 units per gram of original weight of spores. This difference in yield can be ascribed, in part, to the greater ease in breaking germinated spores.

The glucose dehydrogenase used in the sedimentation studies was not extensively purified. Its specific activity was 11,500 units per mg of protein, which was a fifty-fold purification over the crude extract. The concentration of enzyme used in the sedimentation runs was 192,000 units per ml. This high concentration was necessary so that reasonable levels of enzyme activity would occur in each fraction collected from the centrifuge tubes.

Since Bach (1963) had found an apparent disaggregation of the enzyme in solutions of NaCl, an attempt was made to separate the aggregated from the disaggregated molecules by gel filtration. Nine hundred milliliters of G-100 Sephadex gel, contained in a 5 x 60 cm column, were used in this study. The buffer system was 0.05 M imidazole

chloride pH 6.5 which was supplemented with 4 M NaCl for some experiments. The columns were run at room temperature because the enzyme was stable. Four milliliters of partially purified enzyme were placed on the column and eluted with either of the above buffers. The results of the experiments with either solvent system were almost identical. Most of the protein contained in the enzyme preparation was eluted prior to the elution of the enzyme. A typical run using 0.05 M imidazole chloride buffer pH 6.5 is shown in Fig. 3.

The method was not sensitive enough to resolve the various states of aggregation of the enzyme. The main protein peak, as measured by 280 mμ absorption, was, however, completely separated from the activity peak. Since it was recognized that future studies of glucose dehydrogenase would necessitate the use of pure enzyme preparations, a more extensive purification than that sufficient for the sedimentation studies was attempted. A column of G-100 Sephadex and a buffer system of 0.05 M imidazole chloride pH 6.5 containing 0.5 M NaCl gave results similar to that seen in Fig. 3. This NaCl concentration was used to insure that the enzyme was heat resistant and that the column could be run at room temperature. Preliminary purification studies showed that a combination of ammonium sulfate fractionation, heat denaturation of contaminating proteins, and gel filtration resulted in an enzyme with a specific activity of 500,000 units/mg or of 90-100% purity. The results are

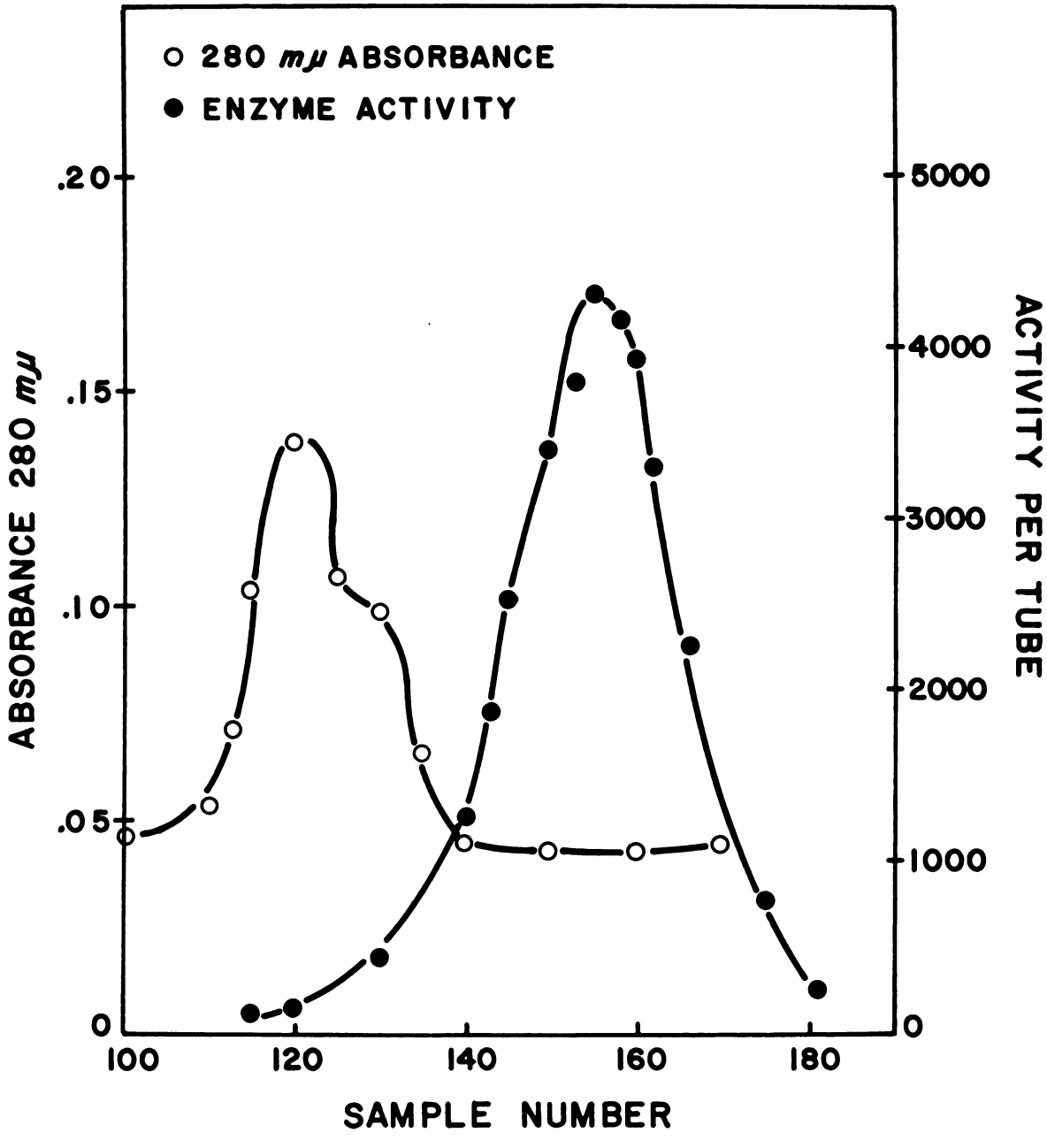

Figure 3. The purification of glucose dehydrogenase by gel filtration. A 900 ml column, which was run at room temperature, contained G-100 Sephadex suspended in and eluted with 0.05 M imidazole chloride buffer pH 6.5. Four milliliters of solution containing 29,000 units/ml were placed on the column and 2 ml fractions were collected.

found in Table 1.

In the course of studying the heat resistance of glucose dehydrogenase, Bach (1963) noted that pH 6.5 was optimal for its thermal stability and that a three hundred-fold difference in the stability of the enzyme occurred between pH 6.5 and 7.5. Furthermore, in 0.05 M imidazole chloride buffer pH 6.5, increases in the sodium ion concentration produced dramatic increases in the thermal stability of glucose dehydrogenase. Fig. 4 is a plot of the relationship between the half-life of the enzyme at 55 C versus the pH of the medium over the range 6.5-7.5. In Fig. 5 is shown the relationship between the half-life of the enzyme at 85 C and the concentration of the sodium chloride contained in 0.05 M imidazole chloride buffer at pH 6.5. The possibility existed that the increase in thermal stability was related to the state of aggregation of glucose dehydrogenase molecules. Bach (1963) had shown that an apparent disaggregation of the enzyme occurred when the sodium chloride concentration of the suspending medium was increased from 0 to 5 M.

Sedimentation studies were made in an attempt to determine the state of aggregation of glucose dehydrogenase in solutions of various sodium chloride and hydrogen ion concentrations. The results of a typical sedimentation experiment are seen in Fig. 6 where the activity per 0.1 ml of individual samples is plotted against the sample number. The sedimentation run was performed at 20 C in 3 M NaCl

Table 1. Summary of the purification results for glucose dehydrogenase from germinated spores of B. cereus.

Procedure	Volume (ml)	Total Activity (units)	Protein (mg)	Activity (units/mg)	Purification	Yield (%)
Initial extract	1845	13,500,000	57,000	235	1	100
$(NH_4)_2SO_4$ fractionation (60-100% sat.)	374	13,450,000	2,240	6,000	26	100
Heat	302	14,000,000	757	18,500	79	104
Sephadex treatment on 4 ml portion	90 (effluent)	180,000	0.36	500,000	2,340	103

dissolved in 0.05 M imidazole chloride buffer pH 6.5. The SW-39 rotor was run at 38,000 RPM for 12 hours. A 0-3% sucrose gradient was used to stabilize the protein boundary. Each sample number represents a given position in the centrifuge tube where 0 and 40 represent the top and bottom of the tube respectively. In the figure there can be seen a zone devoid of enzyme between samples 4 to 8, a zone of increasing enzyme concentration between samples 8 to 20, a concentration plateau region between samples 20 to 32 and finally a zone where the enzyme had been concentrated due to sedimentation

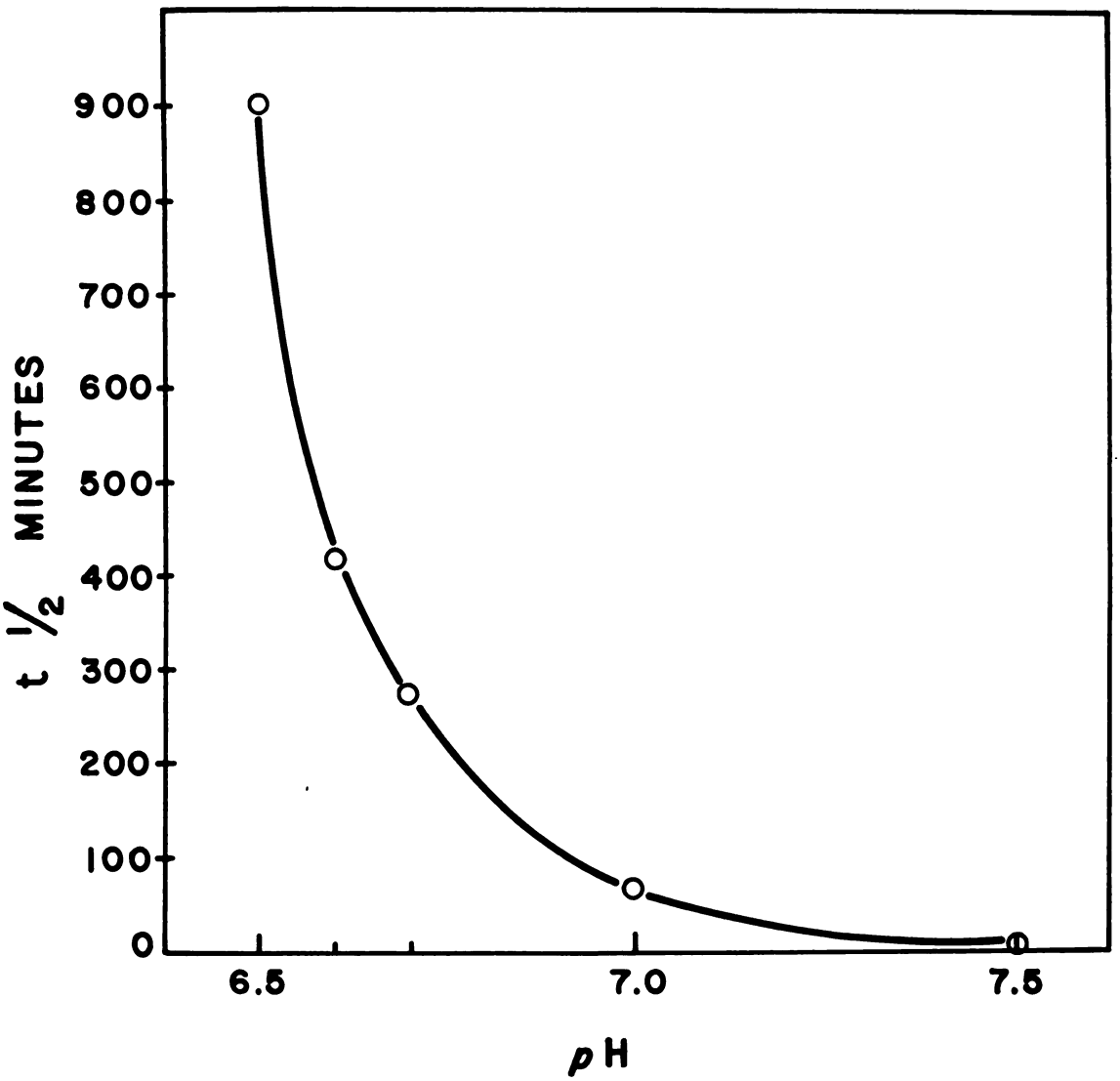

Figure 4. The effect of pH on the half-life of glucose dehydrogenase at 55 C. The enzyme was suspended 0.05 M imidazole chloride buffer at various hydrogen ion concentrations. The values for $t_{1/2}$ were calculated from the original data presented by Bach (1963).

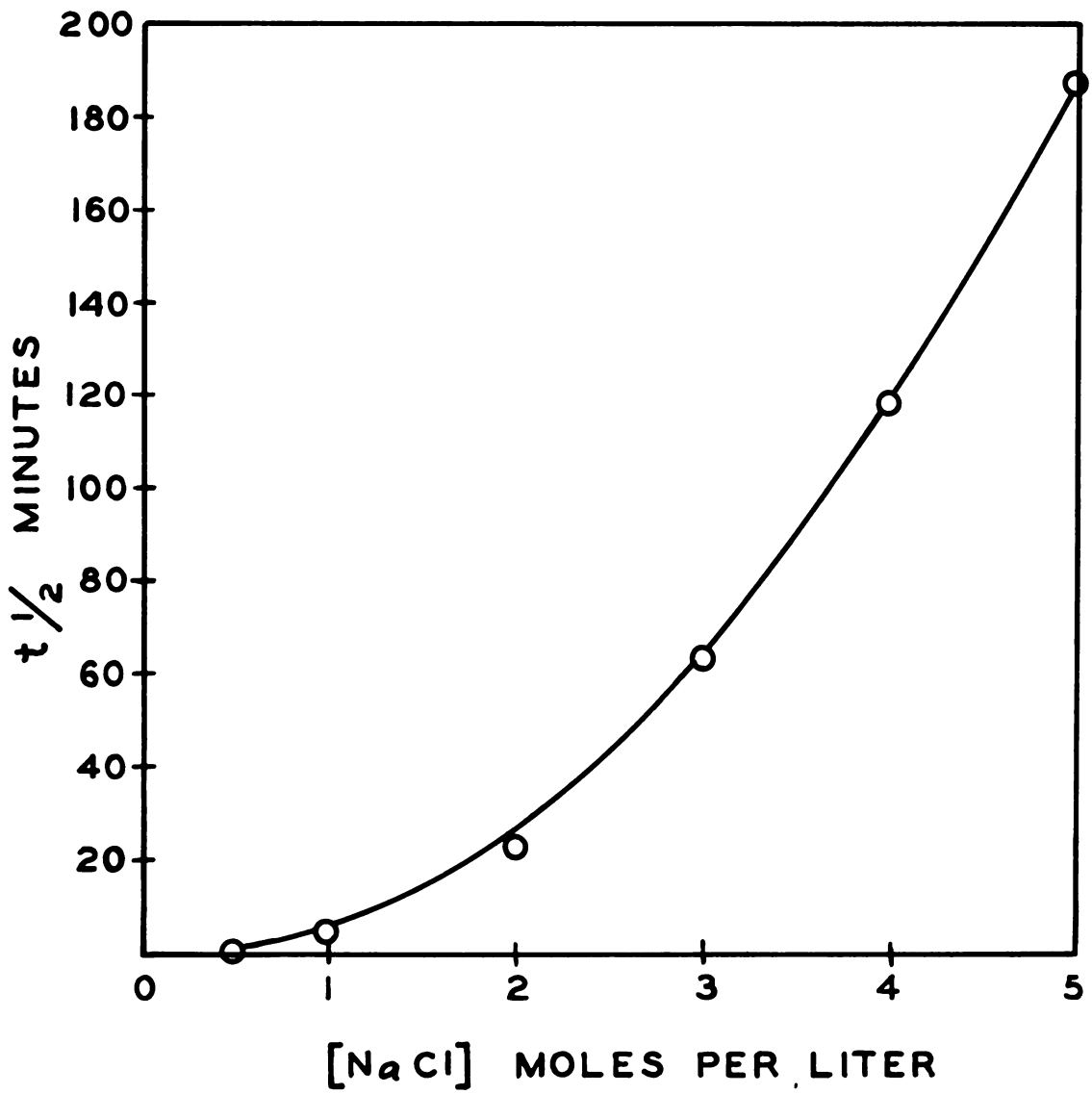

Figure 5. The effect of the NaCl concentration on the half-life of glucose dehydrogenase at 85 C. The enzyme was suspended in 0.05 M imidazole chloride buffer pH 6.5 which contained various concentrations of NaCl. The $t_{1/2}$ values were calculated from the original data presented by Bach (1963).

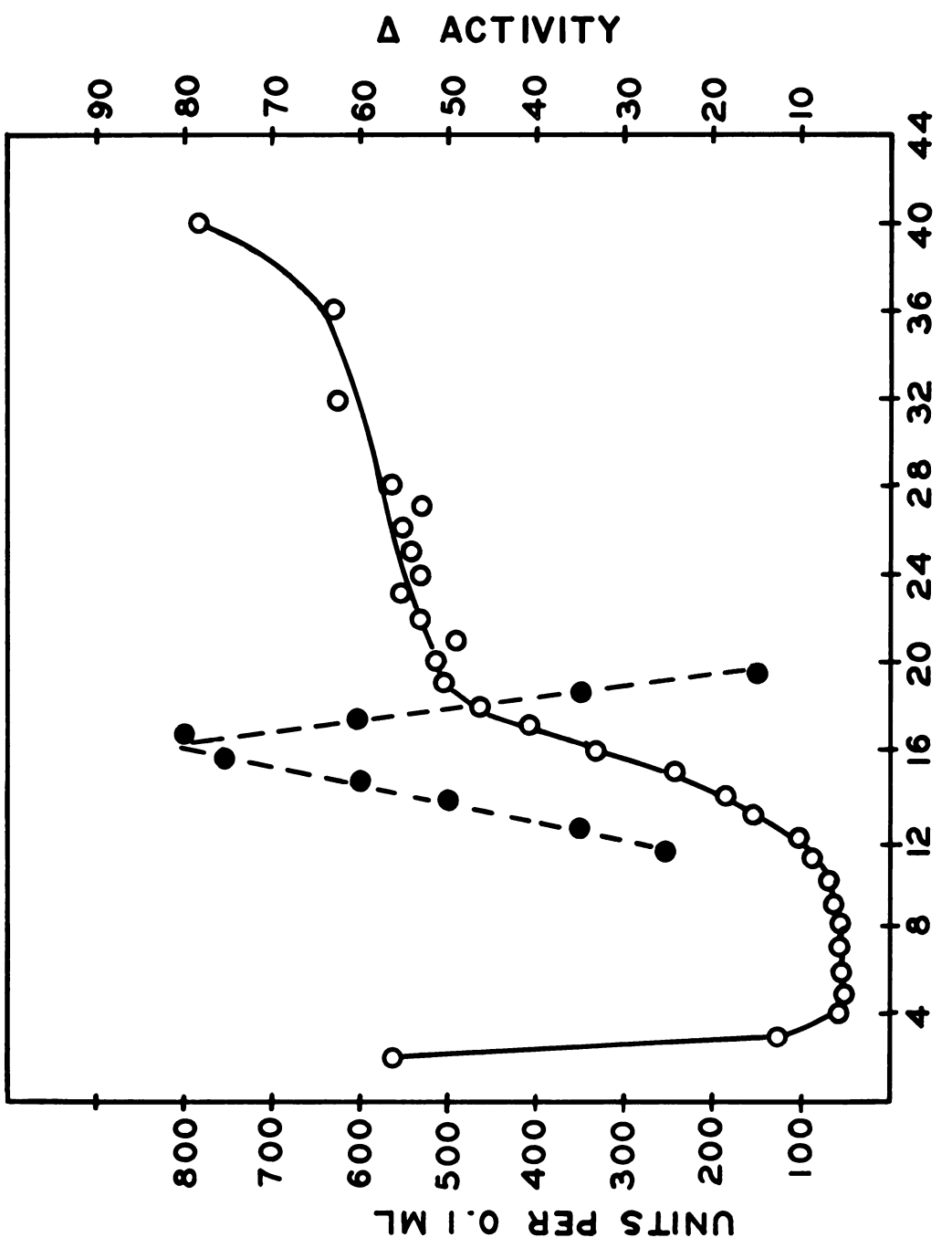

Figure 6. The results of a typical sedimentation experiment by the method of Hogeboom and Kuff (1954). The sedimentation run was performed at 20 C in 0.05 M imidazole chloride buffer containing 3 M NaCl. The protein boundary was stabilized by a linear 0-3% sucrose gradient. The inflection point (dotted line) represents the center of the protein boundary and was determined by plotting the change in activity per sample vs. the sample number.

against the bottom of the tube. It was the zone of increasing enzyme concentration which was of interest in this study. The inflection point which represents the center of the boundary of the sedimenting protein was determined by plotting the change in activity per sample vs. the sample number. The sedimentation constants for glucose dehydrogenase at the various conditions employed were calculated by equation (2). They were then corrected to conditions in water at 20 C by equation (3).

The sedimentation constants for glucose dehydrogenase in 0.05 M imidazole chloride buffer with pH values 6.5, 7.0, 7.5, and 8.0 are shown in Figure 7 and average 8.4 S. The Appendix contains the data from which the points in this curve were calculated. Although there was a slight upward trend in the sedimentation rates with increasing pH values, there was no indication that either aggregation or dis-aggregation of the enzyme occurred.

The effect of various concentrations of sodium chloride on the sedimentation properties of glucose dehydrogenase is shown in Fig. 8. The data from which these points were calculated are presented in the Appendix. In addition to the NaCl, the medium contained 0.05 M imidazole chloride buffer at pH 6.5. The sedimentation constant of the enzyme, in the absence of NaCl, was 8.1 S and this increased to 8.7 S at 1 M NaCl. An over-all decrease in the sedimentation rate to 5.9 S occurred in the concentration range 1-5 M NaCl. Of

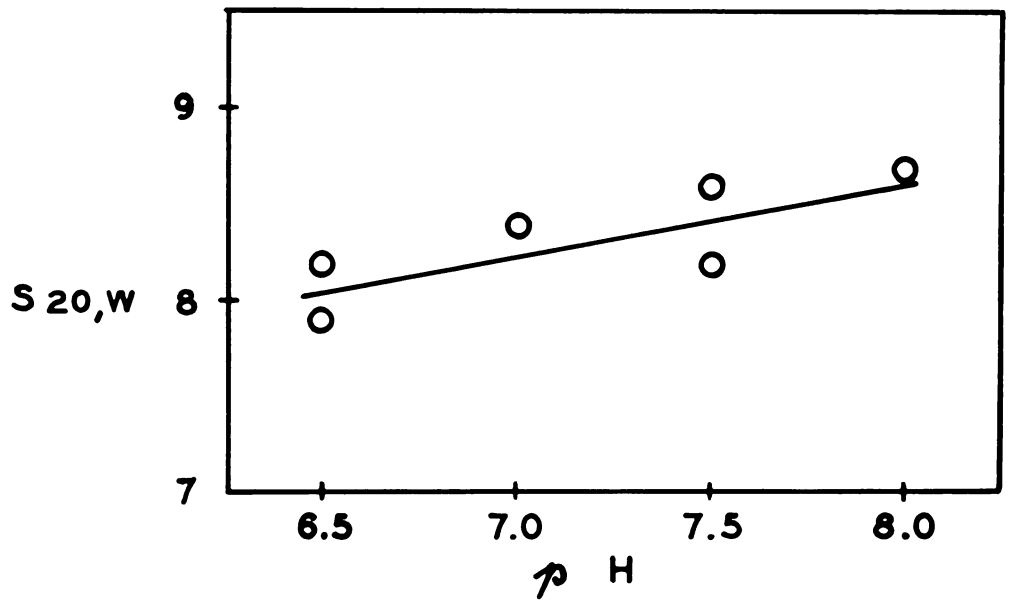

Figure 7. The effect of pH on the sedimentation properties of glucose dehydrogenase. The enzyme was suspended in 0.05 M imidazole chloride buffer at various pH values. The protein boundary was stabilized by a linear 0-3% sucrose gradient.

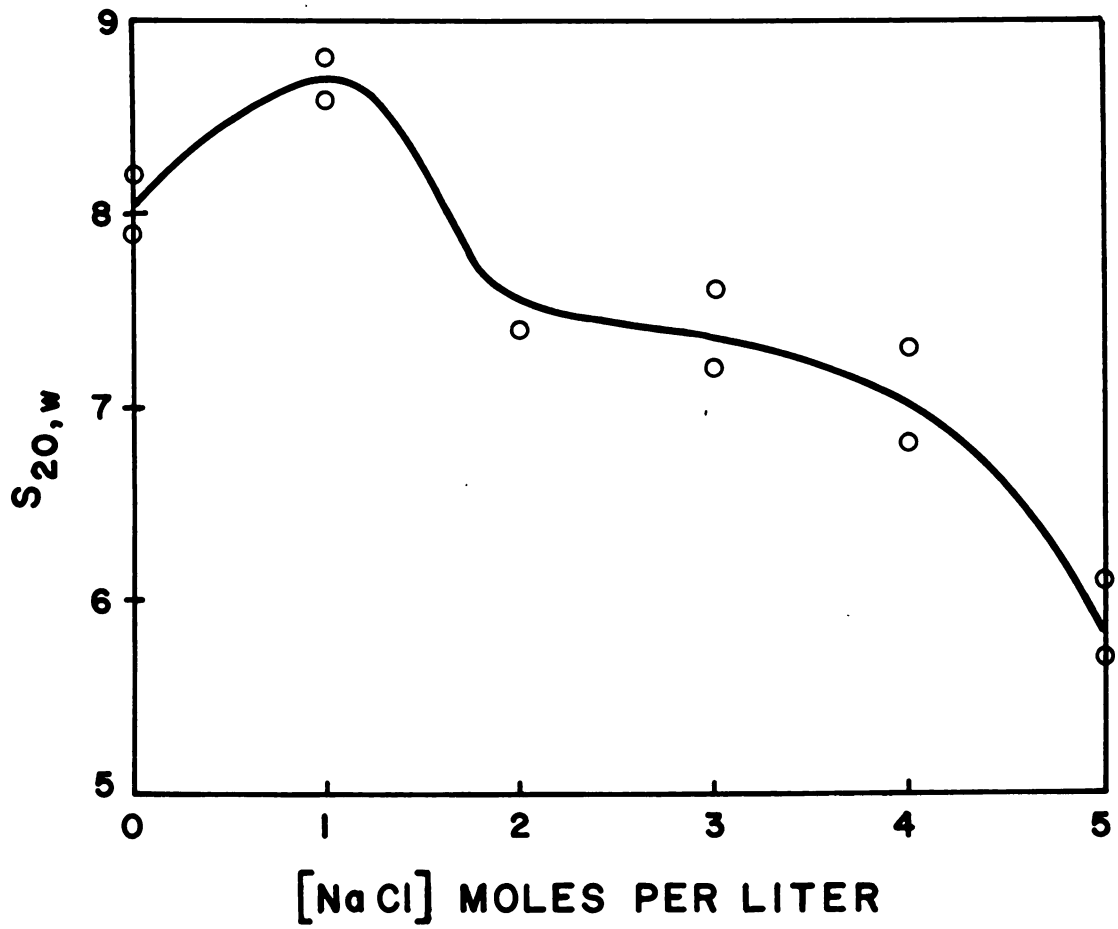

Figure 8. The effect of the NaCl concentration on the sedimentation properties of glucose dehydrogenase. The enzyme was suspended in 0.05 M imidazole chloride buffer pH 6.5 containing various concentrations of NaCl. The protein boundary was stabilized by a linear 0-3% sucrose gradient.

particular interest is the plateau region of 7.4 S which occurs at about 3 M NaCl.

In order to confirm the above findings and to better understand the results obtained with the intermediate NaCl concentrations, sedimentation runs were made using sucrose gradients of 5-20%. The medium contained 3 M NaCl in 0.05 M imidazole chloride buffer and was saturated with respect to sucrose and salt at the most dense portion of the gradient. The results of the experiment are shown in Fig. 9. It should be noticed that two peaks of enzyme activity occur. The $s_{20,w}$ values for these proteins were determined as shown in the Appendix. The larger of the two peaks has a sedimentation rate of 8.2 S, approximately the same as that found for the enzyme in imidazole chloride buffer pH 6.5. The smaller peak moved in the centrifugal field with a sedimentation rate of 7.4 S which was approximately the same as that found for the glucose dehydrogenase in 3 M NaCl solutions.

The molecular weights of the glucose dehydrogenase were determined at 0, 3, and 5 M NaCl concentrations by equation (6), using the diffusion constants obtained at 0 and 5 M NaCl by Bach (1963) and at 3 M NaCl by Sadoff (unpublished). These values, corrected to water at 20 C, were 1.9×10^{-6} cm^2/sec, 2.7×10^{-6} cm^2/sec, and 2.8×10^{-6} cm^2/sec respectively. The molecular weights at 0, 3, and 5 M NaCl were respectively 37,000, 22,000, and 19,000. It is

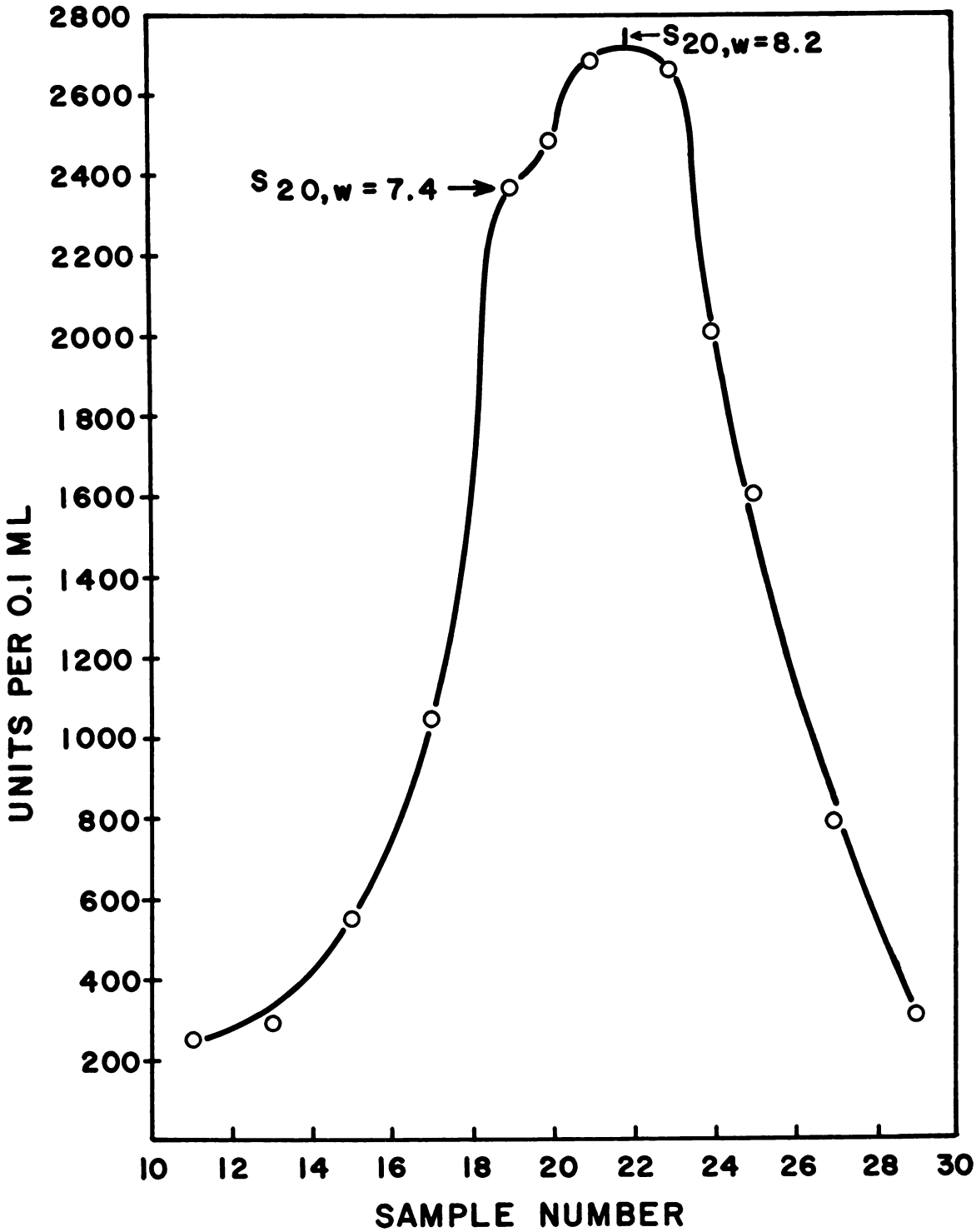

Figure 9. The effect of 3 M NaCl on the sedimentation properties of glucose dehydrogenase. The NaCl was suspended in 0.05 M imidazole chloride buffer pH 6.5. A 5-20% linear sucrose gradient was employed and the enzyme was layered on the top of the fluid column at the beginning of the sedimentation run.

noteworthy that the sedimentation rate decreased from 7.4 S to 5.9 S as the NaCl concentration increases from 3 to 5 M without a change in the molecular weight.

DISCUSSION

The purpose of the research reported in this thesis was to study the relationship between the heat resistance and the state of aggregation of glucose dehydrogenase in solutions of various salt and hydrogen ion concentrations. Gel filtration was used in an attempt to separate the dissociated from the undissociated enzyme. Sephadex G-100 was selected because the enzyme was in the size range that would be "retained" by particles of this gel. Filtration, under the conditions described, was not an adequate means by which to differentiate between the states of aggregation of the enzyme. However, a separation of enzyme and extraneous proteins did occur during the filtration process. This suggested that the purification of glucose dehydrogenase could be readily accomplished by a combination of ammonium sulfate fractionation, differential heat denaturation and gel filtration.

The germination process in spores involves the excretion of DPA, metal ions, and peptides into the surrounding medium with the concomitant loss of the heat resistance of the spores. Even though the germinated spores are heat labile, they contain proteins which are capable of "regaining" heat resistance at pH 6.5 and high

NaCl concentrations. This conclusion is supported by the results of the purification of glucose dehydrogenase. The enzyme, along with other spore proteins, was precipitated by ammonium sulfate in the concentration range 60-100 per cent saturation. Of these proteins, 20-25 per cent remained soluble after the heat treatment used in the enzyme purification. Presumably, their solubility could be taken as a criterion that the proteins remained in their native state. Enzymes in this protein fraction may provide additional models for the study of thermal resistance in spores. The molecular volume of the glucose dehydrogenase must be smaller than that of the other heat resistant proteins because it was retained on the G-100 gel and completely separated from them. This purification technique will permit future studies to be made of the chemical nature of the protein.

The sedimentation studies reported in this thesis were of an exploratory nature and were performed with partially purified preparations in a preparative centrifuge. The centrifuge tubes were uniformly cylindrical in cross section, not sectors as in an analytical instrument, and therefore, the sedimentation values must be considered very close approximations. In the procedure of Hogeboom and Kuff (1954), only the boundary of the most slowly sedimenting glucose dehydrogenase molecules can be determined. If two molecular sizes of the enzyme existed and their boundaries

overlapped, the sedimentation rate obtained was an average value. The partial specific volume, $\bar{V}$, was estimated and errors in this estimate are multiplied approximately three-fold in calculations involving this parameter.

Glucose dehydrogenase does not undergo a change in its state of aggregation at 20 C over the pH range 6.5-8.0 as shown by the constancy of its sedimentation rate in a centrifugal field. The enzyme was very unstable above pH 8 and this limited the pH range investigated. There is no a priori reason to believe that aggregation or disaggregation of the enzyme should occur over this pH range at 20 C even though its heat resistance undergoes a 300-fold change at 55 C.

Bach (1963) indicated, however, that the dissociation of glucose hydrogenase did occur with increasing salt concentrations at pH 6.5, conditions which also enhanced the heat resistance of the enzyme. The extent of the dissociation was determined ultimately by calculating the molecular weights of the enzyme at various salt concentrations using sedimentation-diffusion data. The sedimentation rate of the protein decreased as the NaCl concentration in its environment was increased to 3 M concentration. Approximately 40% of the enzyme existed as a 7.4 S unit (M.W. 22,000) and 60% as an 8.2 S unit (M.W. 38,000) in 3 M NaCl at 20 C, (see Fig. 9). As the sodium chloride concentration was increased to 5 M, the sedimentation rate of glucose dehydrogenase

decreased to 5.9 S with no change in its molecular weight. Undoubtedly, the shape and hydration of the enzyme subunit underwent extensive changes as a result of the increased salt concentration.

The sedimentation runs were performed for convenience at 20 C. However, thermal agitation may play a considerable role in determining the state of aggregation of glucose dehydrogenase under the conditions of the above experiments. Although the preparative ultracentrifuge has a limited temperature range, sedimentation studies have been performed at 5 and 27 C (Sadoff, unpublished). At 5 and 20 C, glucose dehydrogenase has a sedimentation rate of 8.4 S in imidazole chloride buffer pH 6.5. When the buffer system contains 3 M NaCl, the enzyme sediments as a single peak with a rate of 8.6 S at 5 C and 7.2 S at 27 C. The enzyme disaggregates completely in 3 M NaCl as the temperature is increased from 5-27 C.

These data suggest that the extent of dissociation of glucose dehydrogenase is a function of the ionic concentration and temperature of its environment. The possibility exists that at lower salt concentrations and temperatures above 27 C, the enzyme could also exist in a completely disaggregated state. If the subunits of the glucose dehydrogenase are joined by ionic bonds, the function of the salt in producing ion clouds and weakening those bonds is readily appreciated. Thermal agitation could then more readily bring about disaggregation of the protein.

The thermal resistance of glucose dehydrogenase at 85 C increased approximately threefold when the NaCl concentration was increased from 3 to 5 M. That is, its half-life increased from 64 min to 187 min at 85 C. Over this range there was no change in the state of aggregation of the protein but some change in the shape of the subunit must have occurred. Bach (1963) has pointed out that the effect of NaCl on the heat resistance of the protein is a continuous function. That is, the inactivation constant of the denaturation process, k, varies as the reciprocal of the square of the NaCl concentration over the concentration range 0.5 to 5 M. If the effect of NaCl on the protein in the concentration range 0.5-3 M is qualitatively the same as that in the concentration range 3-5 M, the heat resistant form of the enzyme in vitro must be the subunit. Whether this is also true in the intact spore is a matter of conjecture.

In order to achieve levels of heat resistance in vitro equivalent to those in the spore, it is necessary to suspend glucose dehydrogenase in 5 M NaCl solutions. It is unlikely that salt solutions of this concentration ever occur in spores. The bacterial endospore, like all cells, is an organized structure. Its glucose dehydrogenase probably occupies some relatively specific orientation as opposed to the positional randomness that occurs in free solutions of the enzyme. It is possible, that "point concentrations" of salts do occur

in spores and that these are adequate to satisfy the ionic requirements for heat resistance.

SUMMARY

The relationship of the heat resistance of the spore enzyme, glucose dehydrogenase, to its state of aggregation has been investigated in solutions of various salt and hydrogen ion concentrations. Gel filtration was used in an initial attempt to separate the aggregated from the disaggregated enzyme. Although Sephadex G-100 was not suitable for this purpose, it was found that upon filtration through this gel there occurred an excellent separation of glucose dehydrogenase from contaminating proteins. Ninety to one hundred per cent purification of the enzyme was obtained when the protein solution was subjected to $(NH_4)_2SO_4$ fractionation, differential heat denaturation, and gel filtration. The enzyme yield from these procedures approached 100%.

Glucose dehydrogenase has a constant sedimentation rate of 8.4 S at 20 C over the pH range 6.5-8.0. However, a dissociation of the enzyme from molecular weight 37,000 to subunits of 19,000 molecular weight was observed when the NaCl concentration was raised to 3 M. By increasing the salt concentration to 5 M NaCl, a decrease in the sedimentation constant occurred with a corresponding decrease in the diffusion constant. The data indicated that the shape of the protein changed but that its molecular weight was

constant.

The hypothesis was advanced that the property of heat resistance resides in the subunit of glucose dehydrogenase. Furthermore, the disaggregation which produces these subunits occurs under conditions of increased temperature and increased NaCl concentrations.

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APPENDIX

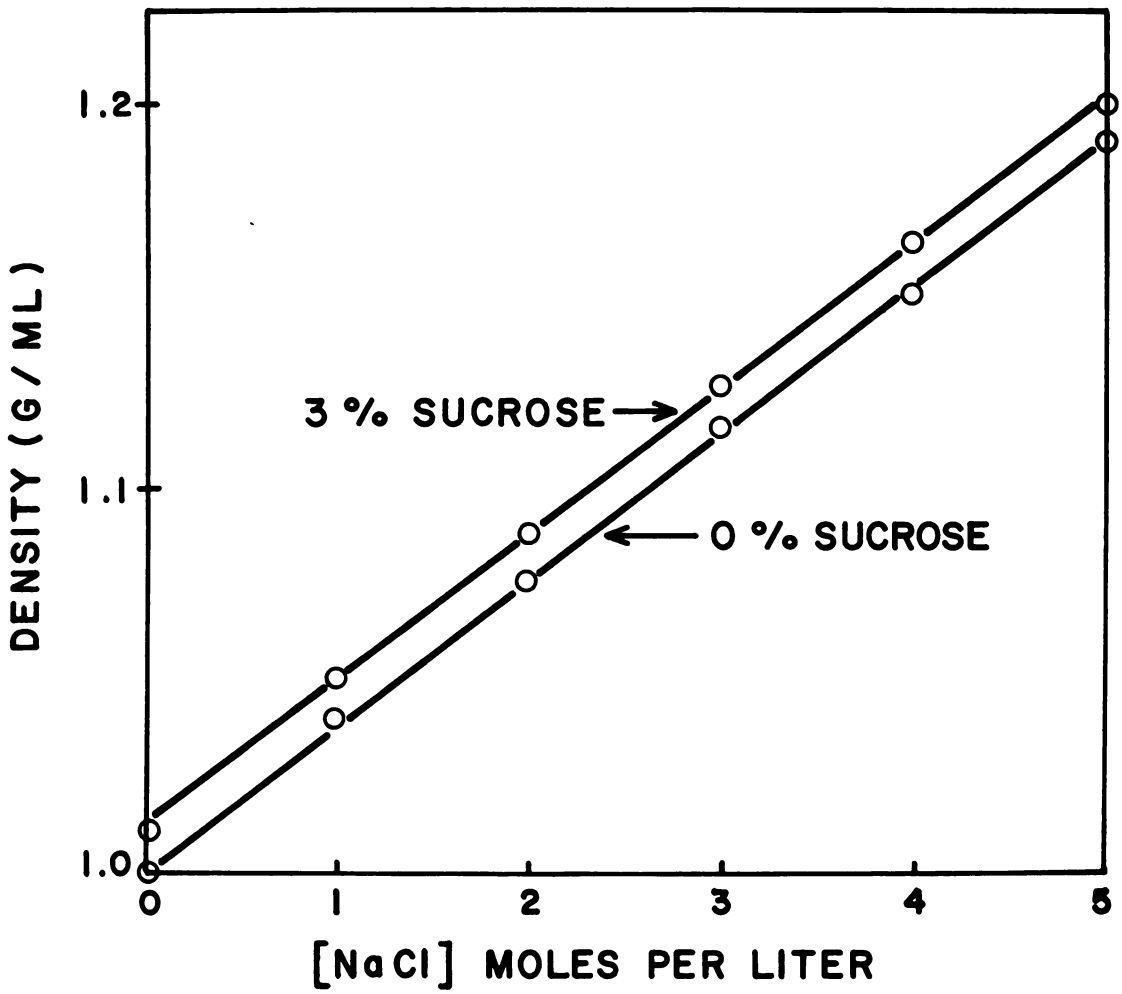

Figure 1. A plot of the minimum and maximum densities as found in the centrifuge tubes vs. the NaCl concentrations. These data were used to calculate the density of the suspending medium, at any point, in centrifugation studies utilizing 0-3% linear sucrose gradients.

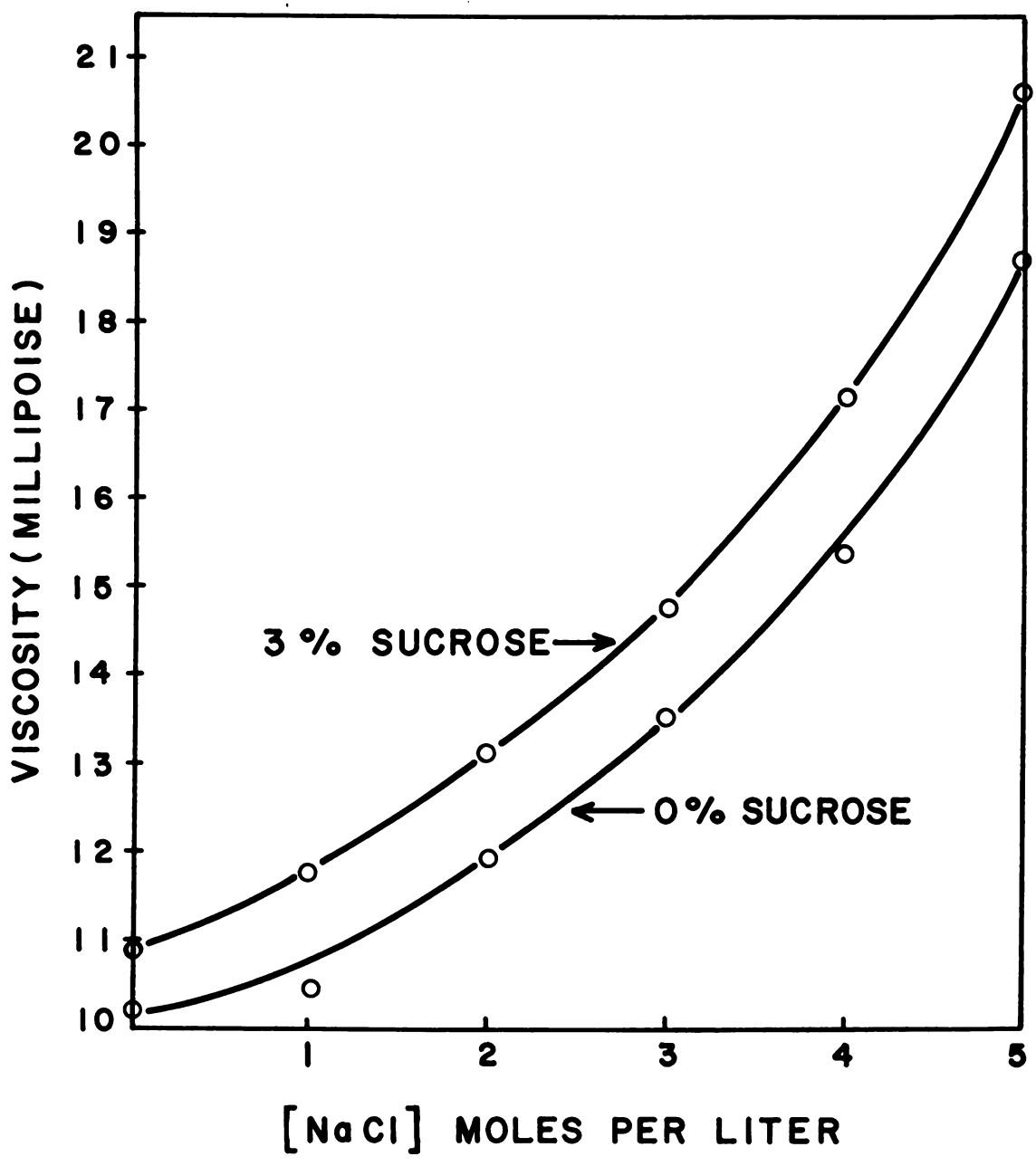

Figure 2. A plot of the minimum and maximum viscosities as found in the centrifuge tubes vs. the NaCl concentrations. These data were used to calculate the viscosity of the suspending medium, at any point, in centrifugation studies utilizing 0-3% linear sucrose gradients.

Appendix Table 1. A summary of the data used for calculating the sedimentation constants of glucose dehydrogenase in 0.05 M imidazole buffer and various hydrogen ion concentrations.*

		pH			
		6.5	7.0	7.5	8.0
Time at top speed (t) in sec x 10^4	a	2.2	1.5	1.5	1.1
	b	1.5		1.5	
Angular velocity (ω) in radians/sec x 10^3	a	4.03	4.07	4.07	4.03
	b	4.03		4.05	
Number of samples	a	30	39	37	37
	b	37		37	
Sample where boundary occurs	a	11.5	10.5	10.5	7.5
	b	9.5		9.5	
Sedimentation constant in given solution at 20 C in Svedbergs	a	7.6	8.1	8.3	8.4
	b	7.9		7.9	
Solvent density (ρ) at boundary in g/cm^3 at 20 C	a	1	1	1	1
	b	1		1	
Solvent viscosity (η) at boundary in centipoise at 20 C	a	1.05	1.04	1.05	1.04
	b	1.05		1.05	
$s_{20,\ w}$ in Svedbergs	a	7.9	8.4	8.6	8.7
	b	8.2		8.2	

*A 0-3% sucrose gradient was used to stabilize the enzyme boundary in the centrifuge tube.

Appendix Table 2. A summary of the data used for calculating the sedimentation constants of glucose dehydrogenase in 0.05 M imidazole buffer pH 6.5 and various NaCl concentrations.*

		[NaCl] Moles/liter					
		0	1	2	3	4	5
Time at top speed (t) in sec x 10^4	a	2.2	2.2	3.7	4.4	5.1	6.2
	b	1.5	2.2		4.4	5.1	6.2
Angular velocity (ω) in radians/sec x 10^3	a	4.03	4.04	4.03	4.05	4.04	4.04
	b	4.03	4.04		4.05	4.04	4.04
Number of samples	a	30	38	46	42	45	49
	b	37	38		42	44	44
Sample where boundary occurs	a	11.5	13.5	19.5	15.0	14.5	10.0
	b	9.5	13.0		16.0	13.0	9.5
Sedimentation constant in given solution at 20 C in Svedbergs	a	7.6	7.0	4.9	3.5	2.8	1.5
	b	7.9	6.8		3.7	2.6	1.6
Solvent density (ρ) at boundary in g/cm^3 at 20 C	a	1	1.04	1.08	1.12	1.16	1.20
	b	1	1.04		1.12	1.16	1.20
Solvent viscosity (η) at boundary in centipoise at 20 C	a	1.05	1.12	1.24	1.40	1.59	1.91
	b	1.05	1.12		1.40	1.59	1.91
$s_{20,\ w}$ in Svedbergs	a	7.9	8.8	7.4	7.2	7.3	5.7
	b	8.2	8.6		7.6	6.8	6.1

*A 0-3% sucrose gradient was used to stabilize the enzyme boundary in the centrifuge tube.

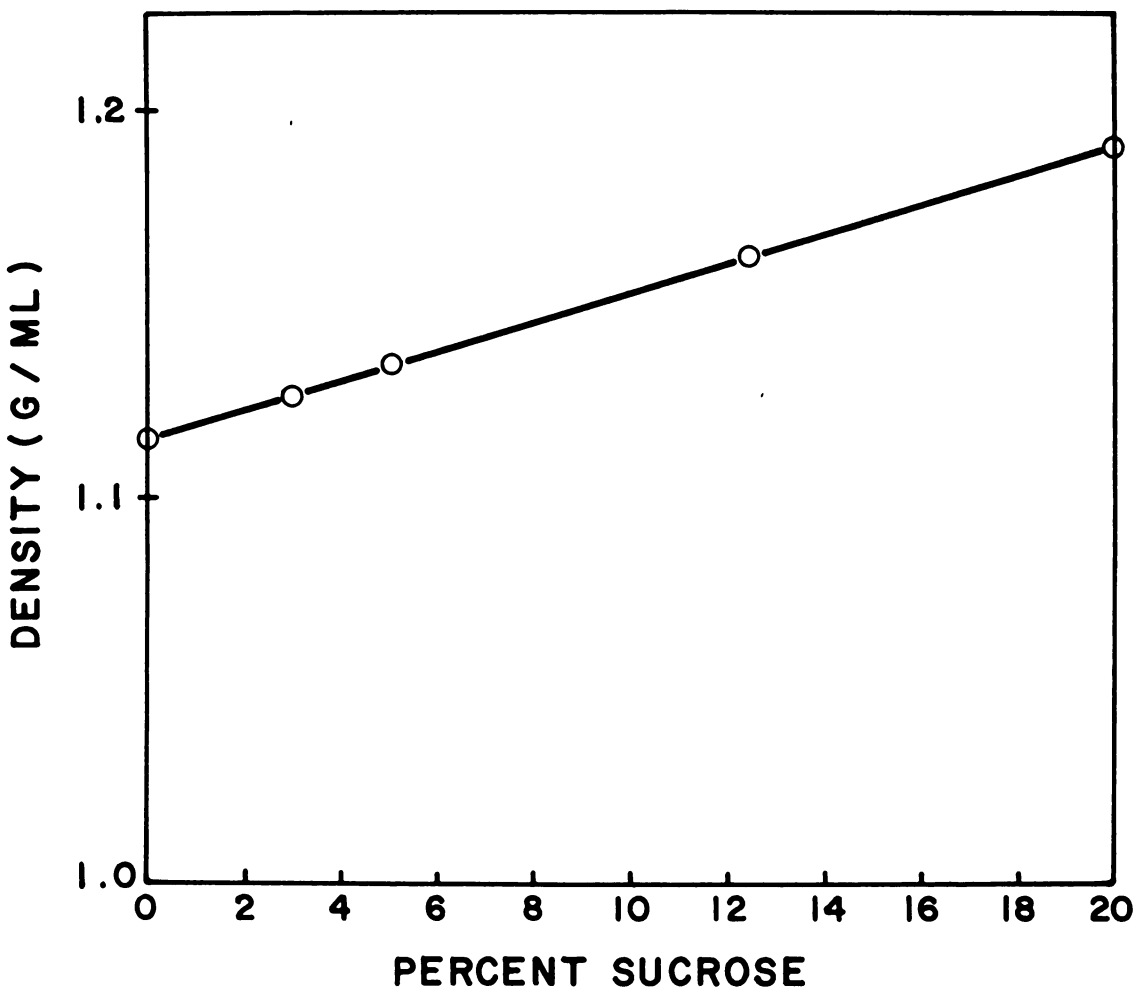

Figure 3. The densities at 20 C of 3 M NaCl solutions dissolved in 0.05 M imidazole chloride buffer pH 6.5 vs. various concentrations of sucrose. These data were used to calculate the density of the suspending medium, at any point, in centrifugation studies utilizing 5-20% linear sucrose gradients.

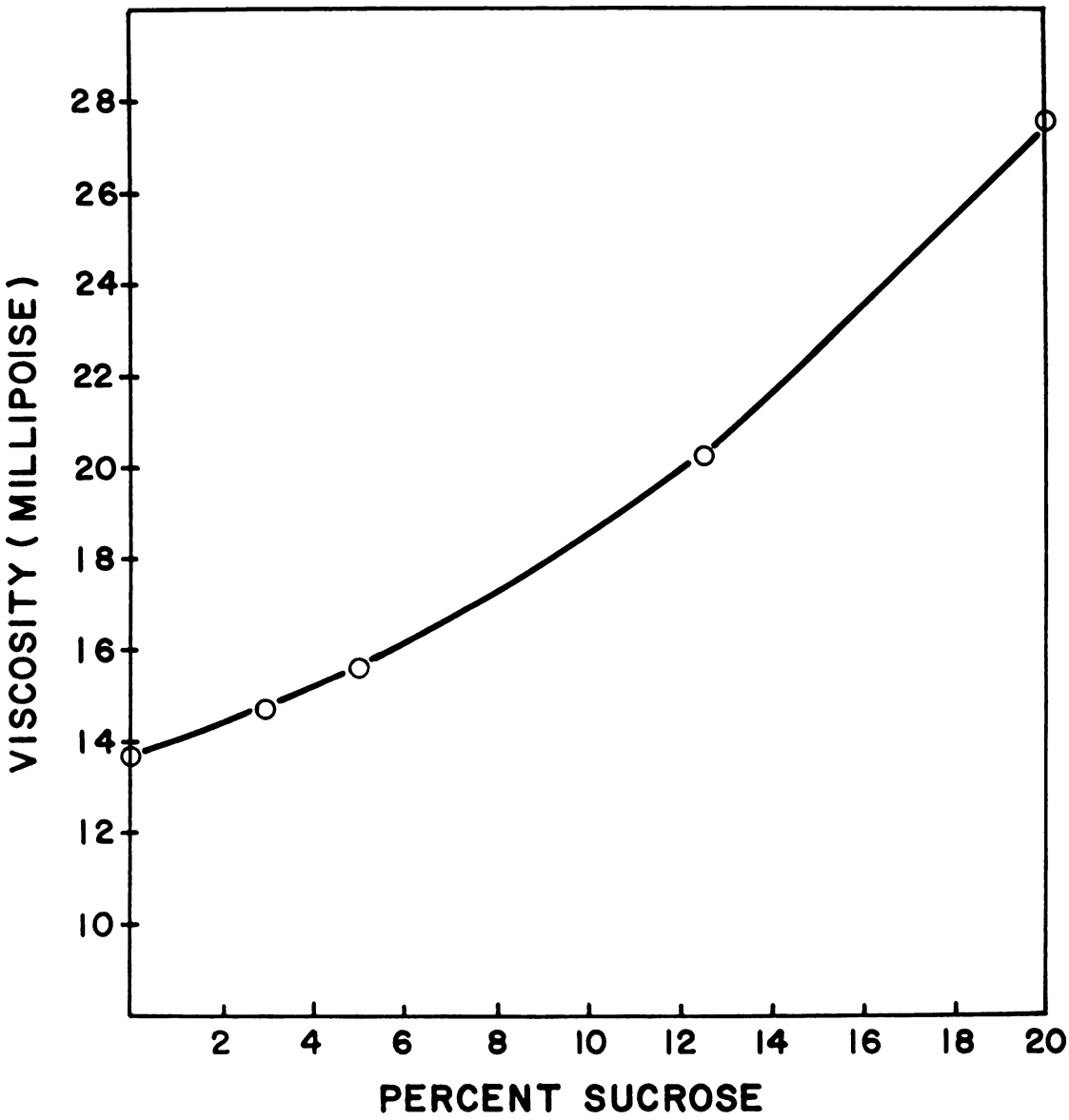

Figure 4. The viscosities at 20 C of 3 M NaCl solutions dissolved in 0.05 M imidazole chloride buffer pH 6.5 vs. various concentrations of sucrose. These data were used to calculate the viscosity of the suspending medium, at any point, in centrifugation studies utilizing 5-20% linear sucrose gradients.

SAMPLE CALCULATION BY THE METHOD OF MARTIN AND AMES (1961)

The enzyme was layered on the top of a 5-20% linear sucrose gradient containing 3 M NaCl dissolved in 0.05 M imidazole chloride buffer pH 6.5. The enzyme peak was found in the 22nd of 44 samples, representing a distance of 7.6 cm from the axis of rotation. At this point the sucrose concentration was 12.5%. In order to determine the sedimentation constant ($s_{20,\ w}$), equation (12) is used:

$$s_{20,\ w} = \frac{A \int_{x_i}^{x_{i+1}} F(x)dx}{\omega^2 t}$$

In order to evaluate the $\int_{x_i}^{x_{i+1}} F(x)dx$ term, 0.2 cm segments (x_i) were taken from the top of the liquid column (5.6 cm) to the position of the enzyme boundary (7.6 cm). At each segment the density (ρ) and viscosity (η) were determined and from these data F(x) was found from equation (10).

$$F(x) \quad \frac{\eta}{(1 - \bar{v}\rho)x_i}$$

Table 3. Data used for the Martin and Ames calculation.

x_i (cm)	Sucrose (%)	ρ (g/cm^3)	η (poise)	F(x)	$F(x_{i+1}) - Fx_i$
5.6	5.00	1.134	.0156	.0152	
5.8	5.75	1.137	.0161	.0153	.0001
6.0	6.50	1.140	.0165	.0153	
6.2	7.25	1.143	.0169	.0154	.0001
6.4	8.00	1.145	.0174	.0154	
6.6	8.75	1.148	.0178	.0155	.0001
6.8	9.50	1.151	.0183	.0157	.0002
7.0	10.25	1.154	.0188	.0159	.0002
7.2	11.00	1.157	.0192	.0160	.0001
7.4	11.75	1.159	.0197	.0161	.0001
7.6	12.50	1.162	.0203	.0164	.0003
				.1722	.0012

The term $\int_{x_i}^{x_{i+1}} F(x)dx$ can then be evaluated by equation (13).

$$\int_{x_i}^{x_{i+1}} F(x)dx = \sum_{x_i}^{x_{i+1}} [F(x_i)(x_{i+1} - x_i)] + \frac{1}{2} \sum_{x_i}^{x_{i+1}} [F(x_{i+1}) - F(x_i)][x_{i+1} - x_i]$$

$$= .1722 \times .2 + \frac{1}{2} (.0012)(.2)$$

$$= .03456$$

The constant, A, in equation (12) is evaluated by the use of equation (9).

$$A = \frac{(1 - \bar{V}\rho w)}{\eta w} = \frac{[1 - (.72)(.998)]}{.01005} = 28$$

Thus, $s_{20,\,w}$ of equation (12) can be found:

$$s_{20,\,w} = \frac{A \int_{x_i}^{x_{i+1}} F(x)\,dx}{2_t}$$

$$= \frac{(28)(.03456)}{(1.62 \times 10^{7})(7.27 \times 10^{4})}$$

$$= 8.2 \times 10^{-13}\ \text{sec}^{-1} = 8.2\ \text{S}$$

ROOM USE ONLY