SOME PROPERTIES OF THE GLUCOSE DEHYDROGENASE FROM SPORES OF *BACILLUS CEREUS*

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ABSTRACT

SOME PROPERTIES OF THE GLUCOSE DEHYDROGENASE FROM SPORES OF BACILLUS CEREUS

by John A. Bach

This research was undertaken in an effort to understand the mechanism of heat resistance in bacterial spores by examining the properties of heat resistant spore proteins. Specifically, this approach involved the purification and characterization of glucose dehydrogenases from sporulating cells and germinated spores of Bacillus cereus. In previous studies these enzymes were identical with respect to their serological and substrate specificities but differed in their levels of thermal stability. However, these differences could no longer be demonstrated in the present investigation. In attempting to account for these discrepancies, the heat resistance of glucose dehydrogenase was examined in both intact and ruptured sporulating cells, dormant spores, and germinated spores. The half-life of the enzyme in each preparation (with the exception of intact dormant spores) was about 5 min at 65 C. The enzyme in dormant spores had a half-life of at least 50 min at 90 C. The discovery was

made that slight changes in solvent properties such as pH and ionic strength profoundly affected the stability of this enzyme.

The half-life at 65 C of the glucose dehydrogenase in a spore extract was increased to about 7 hr by concentrating the extract. Dialysis of this concentrated extract at constant volume against distilled water resulted in a 125 fold decrease in heat stability but all of the original stability was recovered by adding back the lyophilized dialysable material. A glucose-6-phosphate dehydrogenase in these extracts was also stabilized by the dialysable material but not to the same degree.

Glucose dehydrogenase from germinated spores was purified 3,400 fold by a sequence of mechanical extraction, protamine treatment, ammonium sulphate fractionation, and ion exchange chromatography with DEAE cellulose. Some physical-chemical properties of this purified enzyme which might be related to heat resistance were studied in relation to the solvent composition. Heat resistance was found to be strongly dependent on the hydrogen ion concentration, exhibiting a sharp peak at pH 6.5 in 0.05 molar imidazole buffer. The Arrhenius activation constant in this buffer was 17,000 K^{-1}.

High concentrations of certain ionized compounds including NaCl, KCl, Na_2SO_4, $(NH_4)_2SO_4$, and calcium-DPA chelate also increase the spore enzyme heat stability. The increase in stability in NaCl solution is approximately 2nd order with respect to the concentration of NaCl. The effect of $CaCl_2$ was found to be more complex than the NaCl effect. At concentrations up to 0.8 molar, $CaCl_2$ protects the enzyme from heat inactivation while at concentrations above 0.8 molar $CaCl_2$ this effect is reversed.

Purified glucose dehydrogenase is very resistant to guanidine inactivation at 0 C and the presence of 2.5 molar NaCl increases this resistance. The inactivation reaction is approximately 8th order with respect to the guanidine concentration in both low and high ionic strength solvents.

The molecular weight of purified glucose dehydrogenase in a low and high ionic strength solvent was determined by a combination of diffusion and sedimentation measurements. In 0.05 molar, pH 6.5 imidazole buffer, with and without 5 molar NaCl, the molecular weight was approximately 13,000 and 32,000 respectively.

Although it is entirely possible that a highly ionic environment is responsible for the heat resistance of this enzyme in intact spores, the evidence obtained to date is

not sufficient to prove this thesis. The mechanism of the effect of pH and ionic strength on the heat stability of purified glucose dehydrogenase can not be stated with certainty. It appears to involve the strengthening of side chain hydrogen bonds and possibly hydrophobic bonds responsible for maintaining the native configuration of this protein.

SOME PROPERTIES OF THE GLUCOSE DEHYDROGENASE FROM SPORES OF *BACILLUS CEREUS*

By

John Alfred Bach

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Bacterial endospores are stages in a cyclic differentiation process occurring in cells of certain members of the family Bacillaceae. In actively growing cultures, the spores develop within vegetative cells soon after the exponential growth phase and can be recognized microscopically by their resistance to staining and their high refractive index. In some species the outer vegetative cell portion, the sporangium, is lysed by specific enzymes and free spores are released. Spores differ from vegetative cells in their biological and chemical characteristics as well as in their appearance. In particular, they are metabolically dormant and resistant to many chemical and physical agents which are lethal to vegetative cells of the same species. In addition, spores contain high concentrations of sulfhydryl compounds, calcium, and dipicolinic acid (2,6-dicarboxypryidine). Dipicolinic acid or DPA is a unique compound not found in vegetative cells. If dormant spores are placed in fresh nutrient medium, a very rapid loss of calcium, DPA, various spore wall products, and resistance to lethal agents ensues with surprisingly little change in appearance. This process,

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called germination, is followed by more extensive morphological changes as the germinated spores are converted into true vegetative cells to repeat the cycle of cellular differentiation.

Bacterial spores are the most heat stable forms of life known and studies of this property have accounted for a large share of spore research. From an evolutionary point of view, the importance of thermal resistance may be insignificant because conditions that are selective for this degree of resistance occur too infrequently in nature to account for the ubiquity of spore-formers. However, the heat resistance of spores is of considerable practical importance because it must be carefully considered in any sterilization procedure. This is particularly true in the canning of food since Clostridium botulinum, an anaerobic spore-former, produces a very lethal exotoxin.

The thermal death of cells has been generally ascribed to the denaturation of proteins because proteins are both essential to life and are, as a class, the most heat sensitive of cellular components. Considering the heat stability of spores, one might expect most spore proteins to be quite stable in contrast to the proteins of vegetative cells or germinated spores. If this were the case, a comparison of homologous proteins from each source might

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provide some insight into the mechanism of thermal stability at a molecular level and this, in turn, might be related to the heat resistance of the whole organism. Thus the complexities and uncertainties of viability measurements as a measure of heat resistance would be avoided.

The glucose dehydrogenase of Bacillus cereus appeared to be a suitable protein for the study of spore heat resistance. Preliminary work had shown the enzymes from dormant and germinated spores to be similar in catalytic and serologic properties but quite different in their levels of heat resistance. For this reason, a more extensive physical and chemical analysis of the properties of glucose dehydrogenase was planned beginning with the purification of adequate amounts of the two enzymes.

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HISTORICAL REVIEW

A number of theories have been advanced to explain the heat resistance of spores but none have yet been rigorously proved. All have been based on the assumption that high temperatures are lethal because they damage essential proteins and that spore protein is in some way protected from this damage.

Proteins are relatively stable when dried and thus the hypothesis of a reduced internal water content has often been invoked to explain spore heat resistance. In some very early investigations of Bacillus anthracis, Dyrmont (1886) showed that spores contain about 5% more water but twice as much nitrogen as vegetative cells. From the nitrogen to water ratio in each cell type, he concluded that spore protein is less hydrated than vegetative cell protein.

Lewith (1890) attributed spore heat resistance to the existance of a relatively dry interior surrounded by a non-permeable spore coat. However, Virtanen and Pulkki (1933) (as reviewed by Waldham and Halvorson, 1954) investigated the hygroscopic properties of spores and vegetative cells and found no difference in moisture affinity in the range

of the humidities studied. These workers concluded that there was no basis for the hypothesis of a waterproof spore membrane. More recently the hypothesis of an impermeable spore membrane has been completely discounted by the observation of extensive water and solute permeation (Gerhardt and Black, 1961; Black and Gerhardt, 1962; and Murrell, 1961).

The water content of spores and vegetative cells was measured by Henry and Friedman (1937) with particular care given to the removal of extracellular water from the spore packs. During oven drying, vegetative cells of B. megaterium lost 80% of their weight while spores lost 58%. More recently, Ross and Billing (1957) measured the refractive index of wet spores and vegetative cells of several organisms by a combination of phase contrast and interference microscopy. On the basis of these measurements, they reported a water content of about 75% for vegetative cells and about 15% for spores.

The concept of bound water was developed as an explanation for winter hardiness in plants and insects (Gortner, 1930), and the extension of this concept to bacterial spores as an explanation for heat resistance soon followed. Friedman and Henry (1938) examined the bound water content of spores and vegetative cells by a cryoscopic

membrane. More recently the hydrophobic or hy-

spore membrane has been completely disconnected

of extensive water and solute permeation (Gorham

1951; Bisset and Gorham, [illegible]

technique. The results indicated that spores contain twice as much bound water as vegetative cells. However, Waldham and Halvorson (1954) measured the equilibrium vapor pressure of spores and vegetative cells at various moisture contents and found no difference at a water content of up to 0.2 g of water per gram of dry solids. Above this value the vapor pressure of spores increased more rapidly than that of vegetative cells indicating more bound water in vegetative cells than in spores.

Although most of the evidence obtained appears to exclude the possibility of a totally dry and impermeable spore, it does not exclude the possibility of a central dry core. Several observations (Rode and Foster, 1960) are consistent with the hypothesis of a dry core, notably that the central portion of a spore does not stain easily and has a higher refractive index than the rest of the spore. Lewis, Snell and Burr (1960) have suggested that such a core, in essence a vegetative cell, could be kept anhydrous by compressive contraction of the spore cortex. However Murrell (1961), and Black and Gerhardt (1962), using data on spore density and water content, estimated any dry space in spores to be less than 10% of the total volume.

The concept of reduced water content or activity was

not the only one advanced to explain the stabilization of spore protein. True or intrinsically stable protein was proposed by some investigators as a possible mechanism of heat resistance and the properties of several spore enzymes appear to support this hypothesis. For example, a very heat stable adenosine ribosidase, found in the spore coats of Bacillus cereus by Powell and Hunter (1956) was not measurably inactivated at 100 C for 1 hr. The particulate alanine racemase found in spores of B. cereus by Stewart and Halvorson (1953, 1954) is also relatively stable. Less than 10% of the activity of this enzyme is lost during heating at 80 C for 2 hr while, in contrast, the homologous enzyme from vegetative cells is completely inactivated in 15 min at 80 C. Especially interesting is the fact that a soluble, more heat labile enzyme can be produced by sonication of the stable particulate spore enzyme. The possibility exists that the alanine racemase, like the adenosine ribosidase, is located in the spore wall and that the particles are actually spore wall fragments. Both of these enzymes can be assayed directly in intact spores and both retain their heat resistance after the spores have germinated. The heat resistant catalase of B. cereus, first described by Lawrence and Halvorson (1954) is probably another enzyme

of this type since it too can be assayed directly in intact spores.

The concept of protein stabilization by binding to cellular particles or by intermolecular polymerization has been discussed by several other investigators. Militzer, Sonderegger, Tuttle and Georgi (1950) described a tightly bound heat stable cytochrome c which could be extracted from thermophiles, and Koffler (1957) found that flagella from thermophiles were more resistant to depolymerization by heat, urea, acetamide and sodium dodecylsulphate than flagella from mesophiles. Extensive intermolecular polymerization of macromolecules was proposed by Black and Gerhardt (1962) as a mechanism of heat resistance in spores. They described the heat resistant properties of polymerized thiogelatins and cited reports of the high cysteine content of spores (Vinter, 1961) in support of their hypothesis.

Although there is considerable evidence for the existance of intrinsically stable spore protein, there is also some evidence against it. For instance, the adenosine deaminase of B. cereus (Powell and Hunter, 1956) is quite heat stable in intact spores but is much more labile in spore extracts and germinated spores. It is apparent that both intrinsically stable and environmentally stabilized

proteins occur in spores and the relationship of either mechanism to spore heat resistance is uncertain.

It has often been noted that the heat stability of spores is related to their DPA and calcium content. In particular, the coincidence of the appearance of DPA and the appearance of heat stability in sporulating cells (Halvorson, 1957) and the simultaneous loss of heat resistance and the calcium chelate of DPA during germination (Powell, 1953) strongly suggests that DPA and calcium are involved in the heat resistance phenomenon. Various techniques have been devised for altering the concentrations of these materials in spores resulting in the discovery that spore heat resistance is directly related to the concentration of DPA (Halvorson and Howitt, 1961) and calcium (Curran, Brunstetter and Myers, 1943, and Black, Hashimoto and Gerhardt, 1960) in spores. These investigations resulted in a number of theories for the mechanism of protection by these compounds including the stabilization of protein structure (Young, 1959), and the formation of a contractile spore membrane (Lewis, Snell and Burr, 1960, and Lewis, 1961). However, experimental evidence to support these hypotheses has been meager.

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EXPERIMENTAL METHODS

A. Organism and Medium

The organism used throughout this study was a strain of *Bacillus cereus* obtained from the University of Illinois culture collection in 1956. It was maintained primarily in the sporulated form with very infrequent transfers prior to its use in the present investigation.

Large spore crops were grown at 30 C in G medium (Stewart and Halvorson, 1953). This medium contains the following components per liter:

$ZnSO_4$	0.01 g
$CuSO_4$	0.01 g
$CaCl_2$	0.10 g
$FeSO_4 \cdot 7H_2O$	0.001 g
K_2HPO_4	1.0 g
$(NH_4)_2SO_4$	4.0 g
Yeast Extract	2.0 g
$MnSO_4 \cdot H_2O$	0.1 g
$MgSO_4$	0.8 g
Dextrose	4.0 g
Antifoam B	1.0 ml

Organism and Medium

The organism used throughout this study
of **Bacillus** cereus obtained from the University
cultured on Jenner
the source

The pH was approximately 7.0 in each case and did not require adjusting.

Some of the spore crops were grown at 28 C with the dextrose concentration reduced to 3 g/liter. This modification reduced the oxygen demand of the culture and prevented lysis of cells from insufficient aeration.

B. Growth of Spores

Large spore crops were grown in Waldhof type fermenters made from 5 gallon pyrex carboys. An active and fairly synchronous inoculum was obtained by serial transfers through shake flasks and 3 liter New Brunswick fermenters (New Brunswick Scientific Co.). The extent of sporulation was determined by microscopic examination of stained smears. Free spores were harvested in a Sharples continuous centrifuge (The Sharples Specialty Co.) and cleaned by differential centrifugation in a Servall centrifuge, model SS-1 (Ivan Sorvall, Inc.). The clean spores were stored at -20 C until needed.

C. Germination of Spores

Spores were germinated in 3% yeast extract (Baltimore Biological Laboratories) at 30 C after an initial heating in distilled water at 65 C for 1 hr. Complete germination was

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cation reduced the oxygen demand of the culture

dextrose concentration reduced to 3 g/liter. The

Some of the spore crops were blown [illegible]

achieved only if the concentration of spores was no greater than 1 g wet weight per 20 ml of yeast extract solution. Germinated spores are easily distinguished from dormant spores because they stain readily with basic dyes. The germinated spores were washed several times in distilled water and stored at -20 C.

D. Preparation of Cell-free Extracts

Spores were broken in a Servall Omnimixer by suspending them in water or buffer and agitating with number 110 pavement marking beads (Minnesota Mining and Manufacturing Co.). The mixing cup was cooled in crushed ice. The extent of spore breakage was determined by microscopic examination of stained smears. Broken spore suspensions were centrifuged at 32,000 times gravity for 1 hr and the supernatant liquid was retained for study.

E. Glucose Dehydrogenase Assay

Glucose dehydrogenase is a diphosphopyridine (DPN) linked enzyme and can be assayed spectrophotometrically at a wavelength of 340 mμ, the absorption maximum of reduced diphosphopyridine (DPNH). A Beckman spectrophotometer, model DU (Beckman Instruments, Inc.), was used for the measurements. The assay cuvettes contained enzyme, 800

Germinated spores were washed several times in dist.

water and stored at -2) °.

micromoles of pH 8.0 tris buffer (trishydroxymethylaminomethane) 100 micromoles of glucose, 2 micromoles of DPN, and water to 1 ml. Fig. 1, a plot of relative enzyme concentration versus enzyme activity, shows that, within the range of concentrations of enzyme employed, the activity measured reflected the concentration of enzyme protein.

Temperature control of the assay is very important because a 10 C change can cause a 100% change in the activity of this enzyme. For this reason, the spectrophotometer was equipped with thermospacers and a 25 C circulating water bath of 0.1 C sensitivity. The cuvettes were held in the sample housing at least 5 min prior to assay to allow for temperature equilibration. Fig. 2 is a plot of equilibration time versus activity for equal amounts of enzyme. The points are somewhat scattered, indicating an error of about 1%. This degree of error was found consistently for equal samples, perhaps due to temperature fluctuations or perhaps also due to normal pipetting errors.

Under the conditions described above, one unit of enzyme produces an absorption change (commonly referred to as an optical density or O D change) of 0.001 per min.

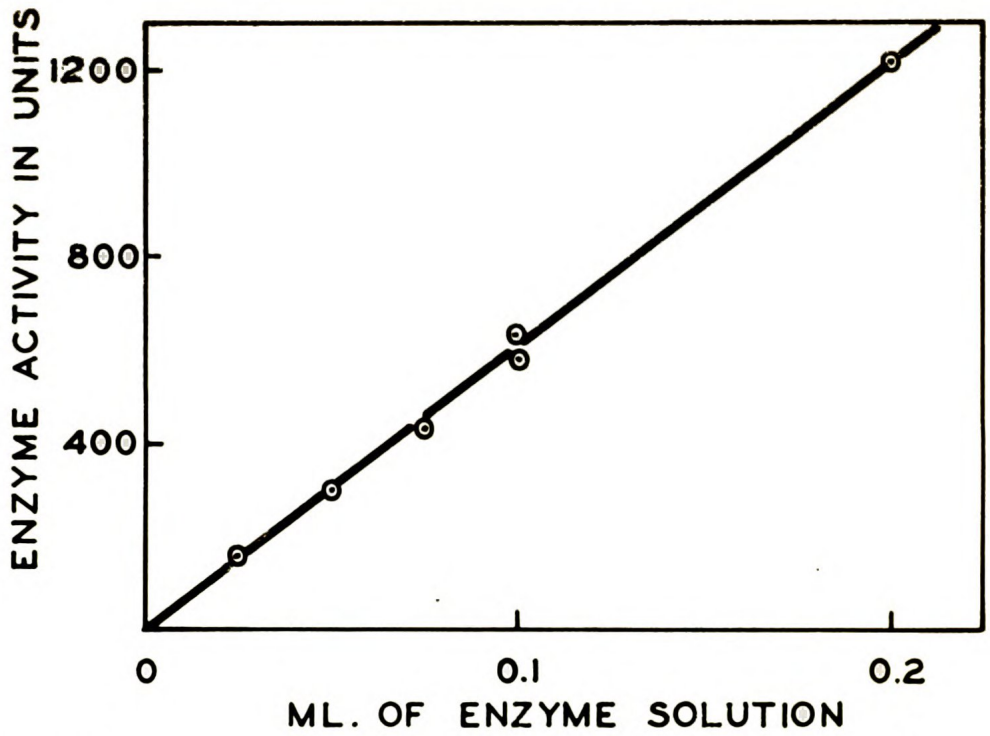

Figure 1. The dependence of glucose dehydrogenase activity on enzyme concentration.

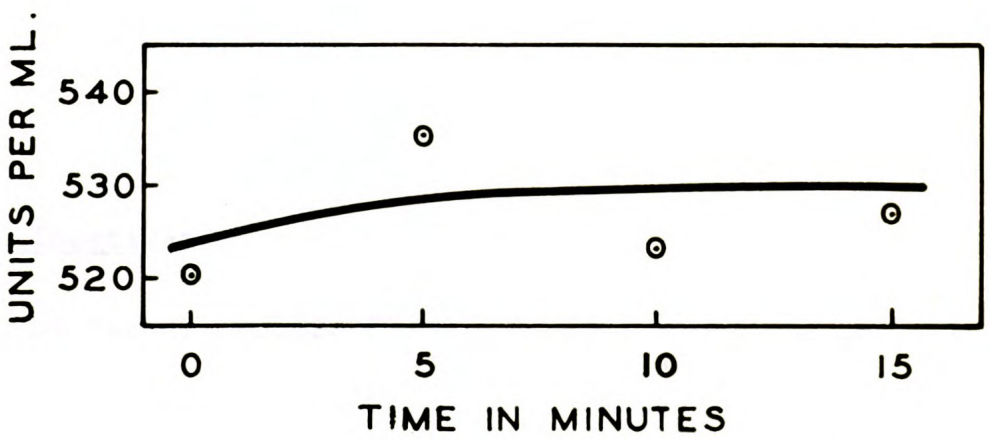

Figure 2. The effect of temperature equilibration time on the activity of equal samples of glucose dehydrogenase.

F. Glucose-6-phosphate Dehydrogenase Assay

This assay was performed in the same manner as the glucose dehydrogenase assay but 0.5 micromoles of TPN and 2 micromoles of glucose-6-phosphate were used in place of DPN and glucose.

G. Protein Determinations

Protein concentrations were determined by the spectrophotometric method of Warburg and Christian (1942). This method is based on the relative absorption at 280 and 260 millimicrons of purified yeast enolase and yeast nucleic acid. In using this method, it is assumed that the proteins being measured have absorption spectra similar to yeast enolase.

H. Heat Inactivation Measurements

The termal inactivation of proteins is a first order reaction. The rate of inactivation is given by the following expression:

$$\frac{-d(E)}{dt} = k(E) \qquad (1)$$

where (E) is the concentration of active enzyme, t is time, and k is the inactivation rate constant. The following integrated equation relates the concentration of active

DPN and glucose.

enzyme at time t to the initial concentration (E_o):

$$2.303 \log \frac{(E_o)}{(E)} = kt \qquad (2)$$

The actual concentration of enzyme in terms of protein need not be known since the change in enzyme activity with time also gives a measure of k as follows:

$$\log \frac{A_o}{A} = kt \qquad \text{or} \qquad (3)$$

$$\log A = -kt + \log A_o \qquad (4)$$

Equation (4) is the familiar form of a linear equation with slope -k and intercept log A_o.

It is characteristic of a first order reaction that a constant fraction of the material present at any moment reacts in a given time interval. For this reason the thermal inactivation rate of an enzyme can be expressed in terms of the time required for 50% inactivation or the half-life. The relationship of half-life or $t_{1/2}$ to k is obtained from equation (4) as follows:

$$\log \frac{A_o}{A_o/2} = kt_{1/2} \qquad \text{or} \qquad (5)$$

$$t_{1/2} = \frac{\log 2}{k} = \frac{.302}{k} \qquad (6)$$

The experimental procedure for obtaining inactivation rates was as follows: small samples of the enzyme solution

(0.05 ml - 0.5 ml) were heated in rubber stoppered 13 x 100 mm pyrex test tubes for various times in a water bath. The temperature of the bath could be controlled to 0.01 C. The tubes were submerged almost to the stopper to prevent concentration changes due to refluxing. After heating, the tubes were cooled rapidly in cold tap water and the enzyme activity was assayed. Inactivation constants were obtained from the slope of a plot of log activity versus time according to equation (4).

The inactivation rates of enzymes in intact spores or cells were obtained in the same manner except that the heated cell suspensions were extracted prior to assay to release the enzymes. A VirTis mixer (The VirTis Co., Inc.) and number 110 glass beads were used for the extractions. Reproducible results could be obtained by this procedure.

RESULTS

Glucose dehydrogenase from 73 g (dry weight) of germinated spores was purified in the manner described by Bach and Sadoff (1962). This modification of the procedure of Doi, Halvorson and Church (1959), consisted of mechanical disintegration of spores, high speed centrifugation (32,000 G) of the extract, precipitation of nucleic acids with protamine sulfate, ammonium sulfate fractionation and DEAE cellulose column chromatography. The enzyme obtained represented a 2,160 fold purification with respect to the dry weight of germinated spores. Attempts to crystallize the enzyme by gradual addition of saturated ammonium sulfate (pH 6.0) were not successful. Instead, an amorphous material of 3,400 fold purity was precipitated. Cellulose acetate electrophoresis of this material in the discontinuous buffer system of Goldberg (1959) yielded one broad dark band and one very thin light band when stained with nigrosin.
The final yield of purified enzyme was 2.5% of the initial activity. The specific activity of the glucose dehydrogenase is 86 micromoles of DPN oxidized per mg of protein per min and its concentration in germinated spores is 0.03% of the dry weight.

As stated earlier, the experimental approach to the present study was to compare the properties of a heat stable glucose dehydrogenase from sporulating cells with those of an homologous heat labile glucose dehydrogenase from germinated spores. The heat inactivation half-lives reported for these enzymes (Bach and Sadoff 1962) were 15 min at 70 C and 1 min at 50 C respectively. However, contrary to expectations, the purified germinated spore enzyme had a half-life of 25 min at 60 C. Approximately the same level of stability was found in samples from various steps throughout purification. Attempts to alter the heat resistance of the enzyme in extracts of germinated spores by varying conditions of growth, germination and extraction were not successful. Finally, the in vivo and in vitro heat stability was measured in sporulating cells, dormant spores and germinated spores. With the exception of intact dormant spores, the enzyme in each case had a half-life between 1 and 10 min at 65 C. However, in intact dormant spores, it had a half-life of at least 60 min at 90 C. The glucose dehydrogenase does not appear to be intrinsically stable at any stage of sporulation but rather appears to be stabilized by conditions in intact dormant spores.

Because of this discovery, the approach to the problem of spore heat resistance was shifted to an analysis of the heat resistance of glucose dehydrogenase in various solvents. An effort was made to reproduce the aqueous environment which must exist in intact dormant spores. Since spores contain a high concentration of calcium, the effect of calcium chloride on the heat stability of glucose dehydrogenase in a spore extract was tested. Solutions of sodium chloride were used as a control of the effect of ionic strength. Both salts increased the enzyme stability at a molar ionic strength of 0.15 but, when the calcium chloride concentration was increased to 1.5 molar, rapid inactivation of the enzyme at room temperature ensued. On the other hand, increasing the NaCl concentration greatly enhanced the heat stability of the enzyme. At a NaCl concentration of 3 molar, the spore enzyme had a half-life of about 8 min at 95 C. The same increase in thermal stability was noted for the glucose dehydrogenase from germinated spores.

Other spore enzymes were tested for the effect of NaCl on their heat stability. Although some enhancement of stability was noted, it was not equivalent to that found for glucose dehydrogenase. For example, the heat resistance of catalase and glucose-6-phosphate dehydrogenase in spore

extracts increased only about 4 fold when 3 molar NaCl was added. A variety of compounds were examined to determine whether the increase in heat resistance when NaCl was added was due to a specific or non-specific effect. Salts with monovalent cations and mono- or di-valent anions such as NaCl, KCl, Na_2SO_4, and $(NH_4)_2SO_4$ at 2 molar concentration and pH 6 increased heat stability as much as 700 fold while salts with divalent cations such as 2 molar $CaCl_2$ and $MgCl_2$ inactivated the enzyme at room temperature. Sucrose, at a concentration of 1.4 molar and pH 6.0, only doubled the heat stability.

Because of the relationship of DPA and calcium-DPA chelate to spore heat resistance, the effect of these compounds on the heat stability of purified glucose dehydrogenase was also determined. Potassium dipicolinate, at a concentration of 0.8 molar and pH of 6.4, increased the heat stability of the enzyme considerably. This effect was enhanced on adding an equal amount of $CaCl_2$ to the system. Presumably a calcium-dipicolonate chelate formed under these conditions. However, when compared under conditions of equal ionic strength and pH, the chelates were no more effective than DPA alone, although chelation did prevent the enzyme inactivation which had been observed with high concentrations of $CaCl_2$.

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whether the increase in heat resistance when MnCl was added

The stability of glucose dehydrogenase in the presence of DPA was particularly meaningful because of the known high DPA content of spores and the extremely stable character of this enzyme in intact spores. It seemed possible that a highly ionic environment was responsible for the heat resistance of this enzyme in intact spores and that the extraction procedure diluted this environment. To test this idea, an extract prepared from 15 g (wet weight) of spores was lyophilized and then resuspended in a small amount of distilled water. After centrifugation to remove insoluble material, about 3 ml of the extract was recovered. The stability of two enzymes in this extract was determined, the glucose dehydrogenase at 65 C and the glucose-6-phosphate dehydrogenase at 45 C. The extract then was dialysed repeatedly against distilled water and the same two enzymes were examined for heat resistance. Finally, the low molecular weight materials which diffused into the distilled water were recovered by evaporation and added back to the dialysed extracts. The heat stability of the enzymes in this "reconstituted" extract was measured. The reconstituted extract contained double the original concentration of dialysable material because about half of the dialysed extract was used for inactivation measurements prior to "reconstitution."

Figs. 3 and 4 clearly show the stabilizing effect of this dialysable material on the two enzymes. The decrease in heat resistance of the glucose dehydrogenase due to dialysis was 125 fold and all of the original resistance was recovered by adding back the dialysable material. The stability of the glucose-6-phosphate dehydrogenase was only decreased 14 fold and the addition of the dialysable material did not restore all of the original heat resistance. When samples of dialysed and undialysed extract were completely dried before heating, the glucose dehydrogenase in both extracts had a half-life of about 50 min at 90 C.

The results described in the preceding paragraph led to a detailed study of the effect of the solvent on the enzyme. The heat stability of the purified enzyme was measured at 55 C in 0.05 molar imidazole buffer at various pH values as shown in Fig. 5. Imidazole was chosen because it buffers well from pH 5.5 to 7.5. In Fig. 6 a plot of the logarithm of the inactivation constants obtained from Fig. 5 versus pH shows that the enzyme stability is at a maximum (minimum k) at pH 6.5 and decreases rapidly on both sides of this value. A similar dependence on pH was observed by Levy and Benaglia (1950) for the thermal denaturation of ricin, and the kinetics of this phenomenon were discussed by

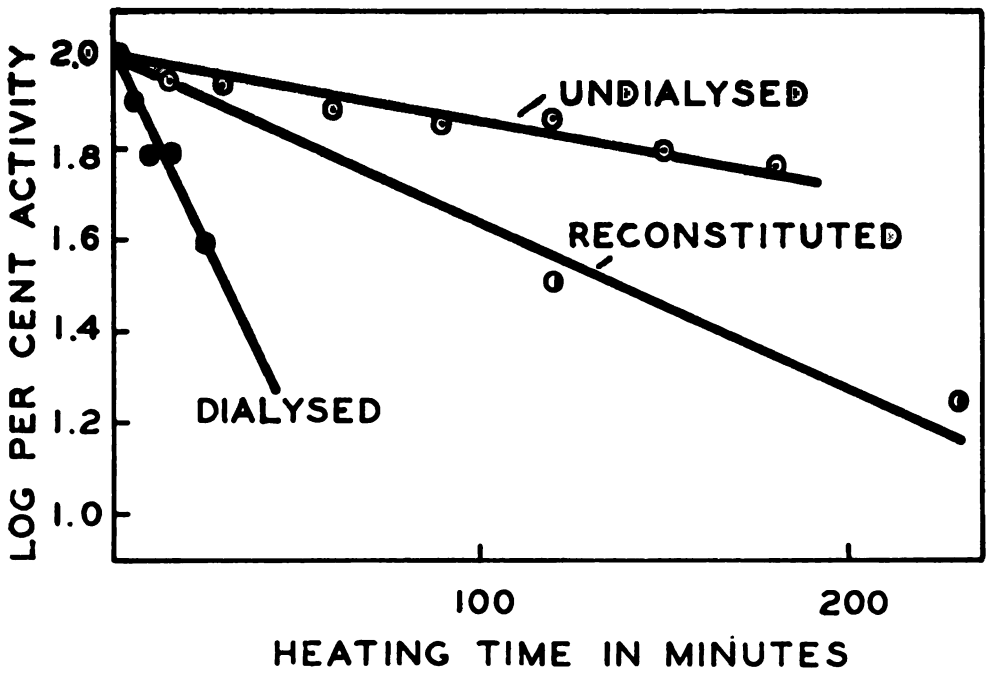

Figure 4. The heat stability of glucose-6-phosphate dehydrogenase in undialysed, dialysed, and reconstituted concentrated spore extract at 45 C.

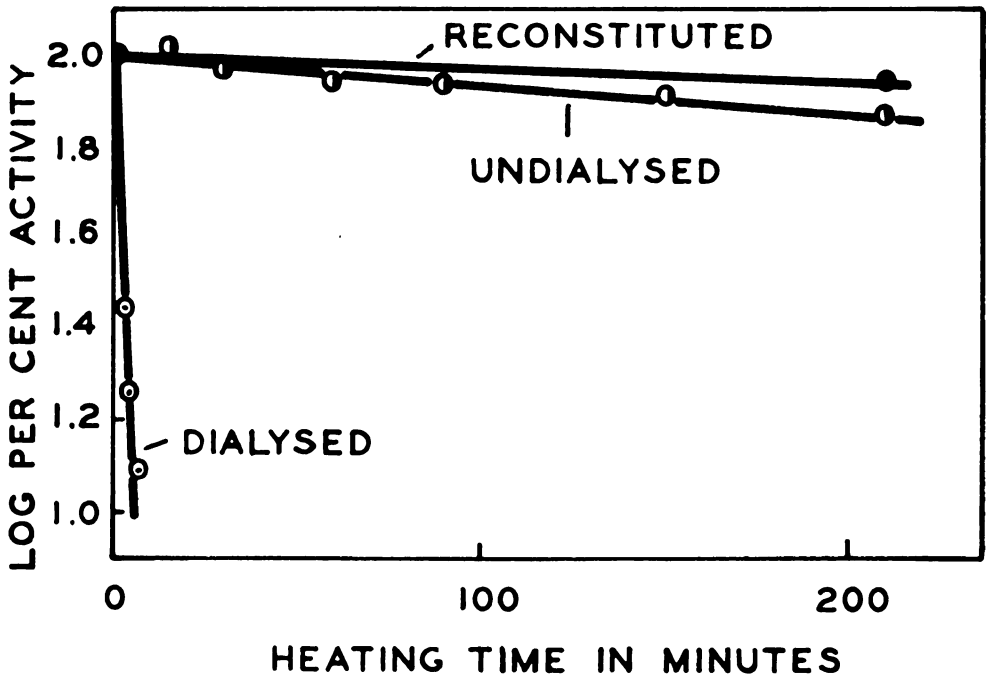

Figure 3. The heat stability of glucose dehydrogenase in undialysed, dialysed, and reconstituted concentrated spore extract at 65 C.

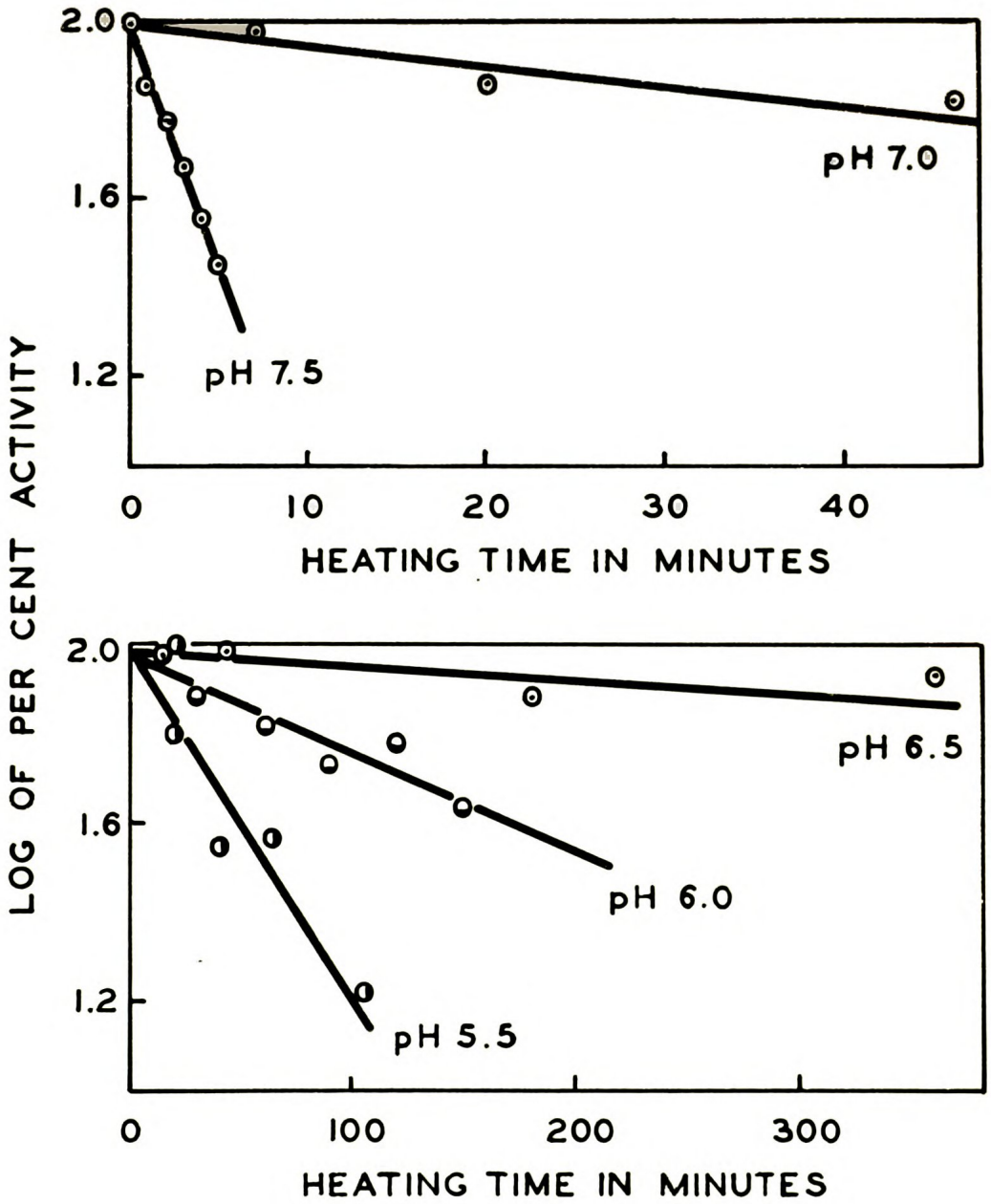

Figure 5. The effect of pH on the heat inactivation of purified glucose dehydrogenase at 55 C.

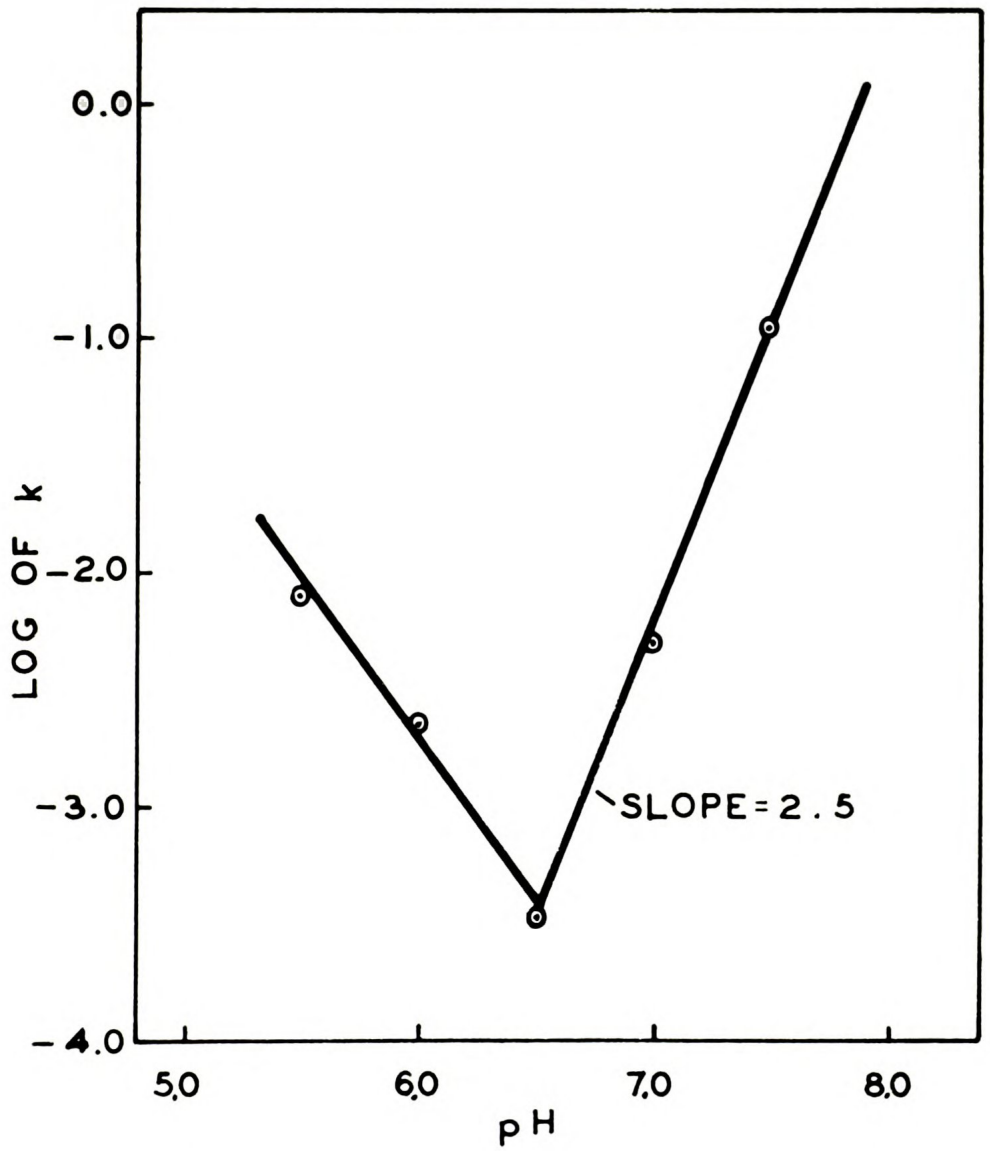

Figure 6. The effect of pH on the heat inactivation of purified glucose dehydrogenase at 55 C.

Scheraga (1961). He attributed the changes in stability to changes in the ionization of groups involved in side-chain hydrogen bonding. Since pH is defined as the negative logarithm of the hydrogen ion concentration (or activity), Fig. 6 is acutally a plot of log k versus log (H^+). This situation is described by the following equations:

$$k = a(H^+)^n \qquad \text{or} \qquad (7)$$

$$\log k = n \log (H^+) + \log a \qquad (8)$$

In equation (8) the slope n indicates the order of the reaction with respect to hydrogen ion concentration. Assuming that side chain hydrogen bonds are being broken during denaturation, the values of the slopes in Fig. 6 are indicative of the number of ionizations involved in the stability changes above and below pH 6.5.

The scatter observed in the heat inactivation data constitutes errors of 20 to 30%; much higher than the usual 1 or 2% sampling errors. These errors suggested that the enzyme was being reversibly denatured and that the effect on the apparent enzyme stability was not being taken into account. To test this idea, samples of enzyme solution at pH 5.5 were heated 15 min at 55 C and assayed immediately and after a 5, 30, and 90 min incubation at 25 C. A 40% increase in enzyme activity occurred during the first 5 min

of incubation and this increased to 60% after 30 min, indicating a reversal of thermal denaturation. On the other hand, the same type of experiment at pH 6.5 gave no increase after an 83 min incubation at 25 C. Apparently the renaturation only occurs at the lower pH values.

Because the heat stability of glucose dehydrogenase is so sensitive to changes in solvent composition, the inactivation constants in various solvents may differ greatly at the same temperature. This necessitates that two separate temperatures be employed to obtain measurable activity differences in a reasonable time interval. Thus, some means of comparing the inactivation constants at different temperatures is useful. The relationship of temperature to inactivation constant was determined empirically by Arrhenius as:

$$k = Ae^{-a/T} \qquad \text{or} \qquad (9)$$

$$\ln k = -a/T + b \qquad (10)$$

The constant a or activation constant is a measure of the temperature dependence of the rate of a reaction and is given by the slope of a plot of log k versus 1/T where T is the absolute temperature. Fig. 7 shows such a plot for the inactivation of purified glucose dehydrogenase in imidazole buffer at pH 6.5. The activation constant obtained from the

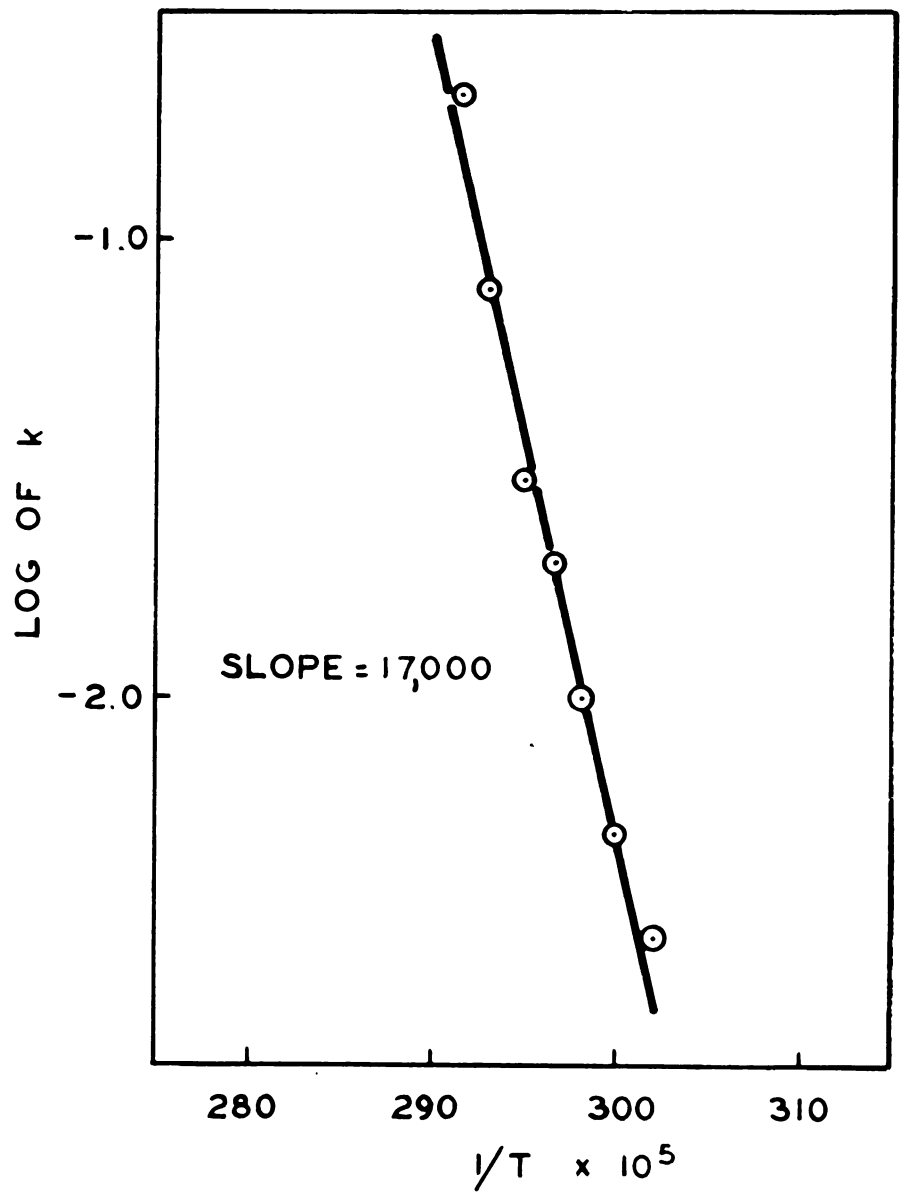

Figure 7. An Arrhenius plot for the inactivation of purified glucose dehydrogenase at various temperatures in 0.05 molar, pH 6.5 imidazole chloride buffer.

slope of this curve is 17,000 $^{o}K^{-1}$. If the activation constant a and the inactivation constant k at a given temperature are known, it follows that the k at any temperature can be calculated from the following equation:

$$\ln k_2 - \ln k_1 = -a \left(\frac{1}{T_2} - \frac{1}{T_1} \right) \qquad (11)$$

Although the Arrhenius equation has been given a fuller interpretation by modern reaction rate theories, the original empirical equation is adequate for the purpose of comparing inactivation constants at different temperatures.

The effect of ionic strength on the thermal resistance of glucose dehydrogenase was studied more quantitatively in an effort to elucidate the mechanism of protection. Enzyme inactivations were performed at 85 C in a pH 6.5, 0.05 molar imidazole chloride buffer with various concentrations of NaCl. In Fig. 8, the log of k is plotted against the log of the NaCl concentration in a manner analogous to the pH dependency curve in Fig. 6. The slope of the curve in Fig. 8 is -2.3 suggesting that the protective reaction involves the binding of two Na^{+} ions or two Cl^{+} ions or two of each. It is of interest that the slopes of Fig. 6 and Fig. 8 are so similar. This could simply be a coincidence or it might indicate a similarity in the mechanism of stabilization by

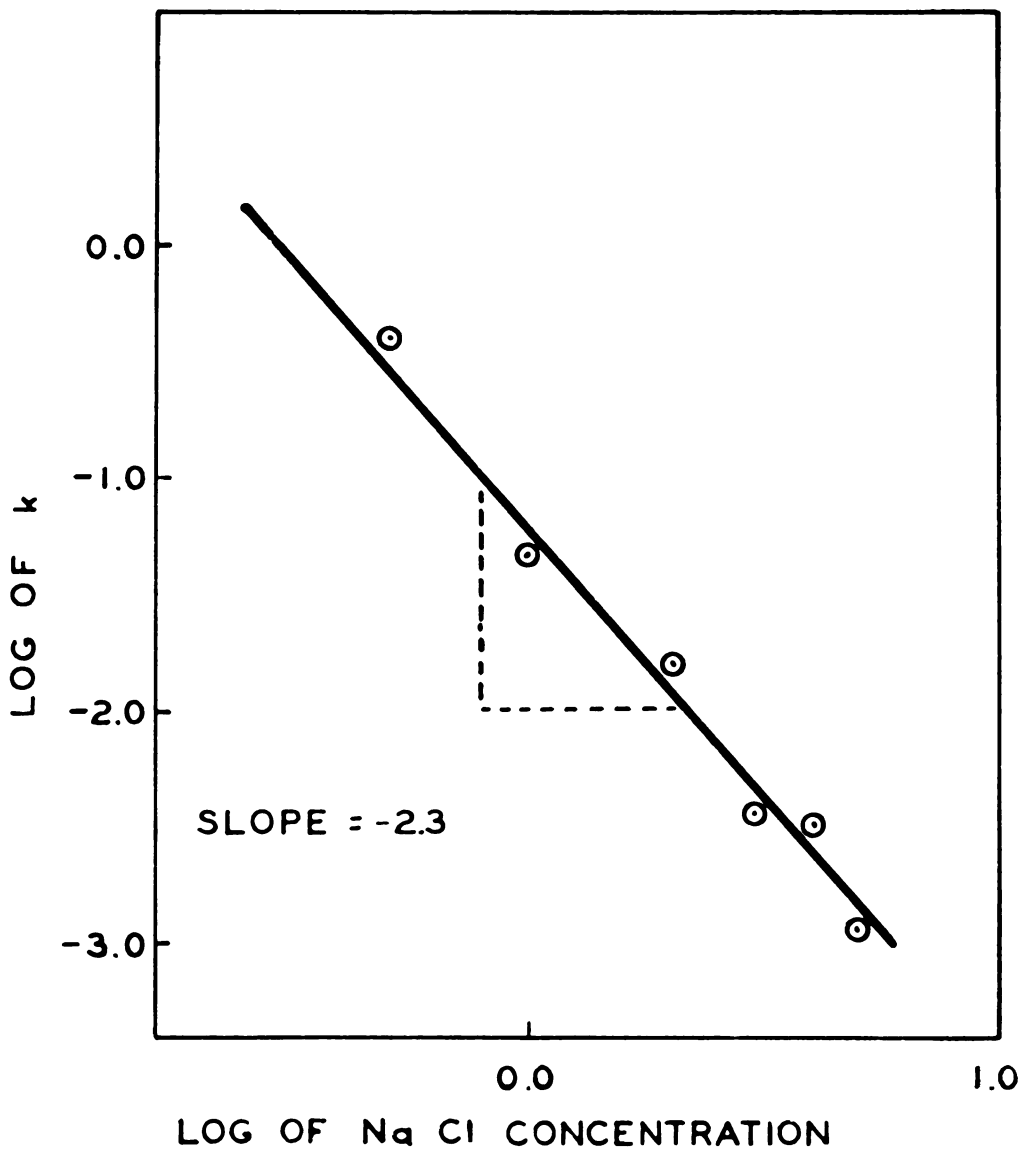

Figure 8. The effect of NaCl concentration on the thermal stability of purified glucose dehydrogenase at 85 C. In addition to the NaCl concentrations indicated, the solvent contained 0.05 molar imidazole chloride buffer at pH 6.5.

changes in hydrogen ion and NaCl concentration. The signs of the slopes are opposite, of course, because the increasing pH values in Fig. 6 correspond to decreasing concentrations of hydrogen ions.

The same type of experiment was performed to determine the effect of the calcium chloride concentration on the heat stability of glucose dehydrogenase. In this case, a pH 6.0, 0.05 molar imidazole buffer was used because this was thought to be the pH of maximum stability at the time this experiment was performed. As seen in Fig. 9, the enzyme is most stable at a $CaCl_2$ concentration of 0.8 molar and loses stability with increasing concentration. It should be noted that the kinetics of the $CaCl_2$ effect are not described by equation (8) since the curves are not linear.

Stewart and Halvorson (1954) and Black and Gerhardt (1962) have suggested that enzymes in spores are stabilized by intermolecular polymerization. To determine if polymerization was responsible for the heat stability of glucose dehydrogenase, the molecular weight of the enzyme was determined in both a low and high ionic strength solvent by a combination of diffusion and sedimentation measurements. A very small amount of glucose dehydrogenase protein was available so the usual methods utilizing refractive index

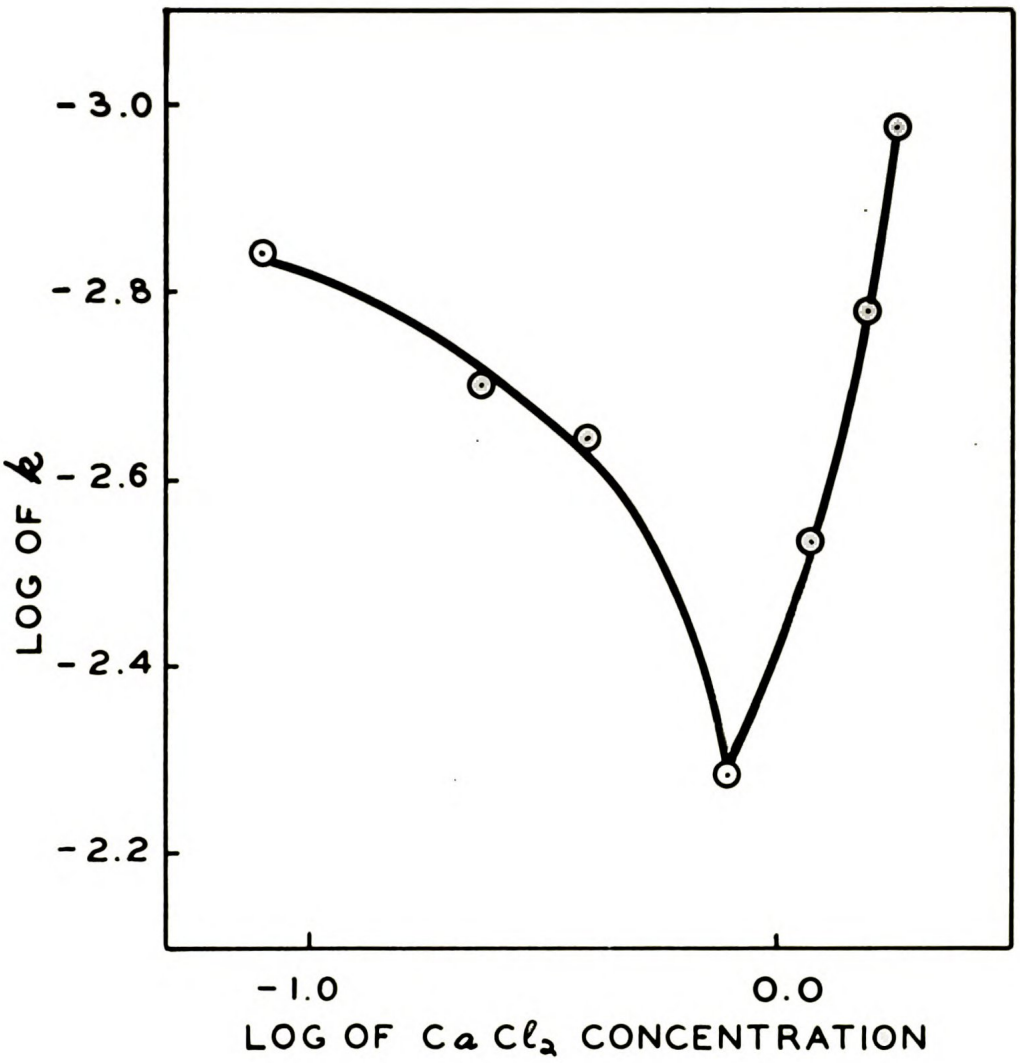

Figure 9. The effect of calcium chloride concentration on the thermal stability of purified glucose dehydrogenase at 75 C. In addition to the $CaCl_2$ concentrations indicated the solvent contained 0.05 molar imidazole chloride buffer at pH 6.0.

measurements could not be used. Instead, methods involving enzymic activity were employed to take advantage of the very sensitive assay for this enzyme.

Diffusion measurements were made in the Stokes cell pictured in Fig. 10. In this device, material at a certain concentration in compartment **A** (lower compartment) is transferred by diffusion through the fritted glass filter into pure solvent in compartment B (upper compartment) under the driving force of a concentration gradient located within the filter. Independent mixing by the magnetic stirring bars maintains a uniform concentration in both compartments but does not disturb the gradient in the fritted glass filter. The entire cell was immersed in a water bath of $\pm$ 0.02 C accuracy, to prevent disturbances from convection and volume changes. The mass transfer in the cell is described by Fick's first law of diffusion as follows:

$$dm = -DA\frac{dc}{dx}\,dt \qquad (12)$$

where m is the mass of material transferred in time t, **A** is the area of the membrane, dc/dx is the concentration gradient across the membrane, and D is the diffusion coefficient characteristic of the material under study. If diffusion continues for an appreciable time, the concentrations of

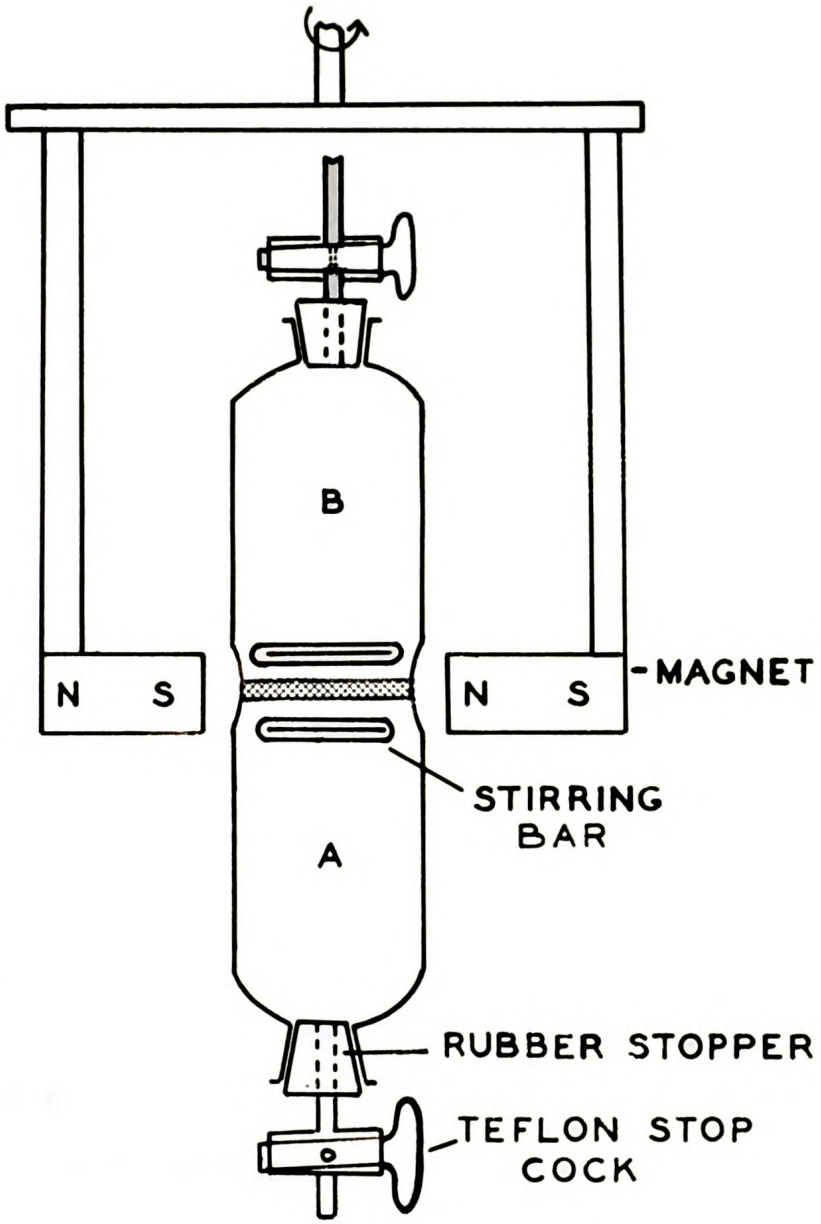

Figure 10. The Stokes diffusion cell.

material in each compartment will change significantly and the integrated form of equation (12) must be used. This is given as:

$$D = \frac{1}{\beta t} \ln \frac{C_\ell^o \, C_u^o}{C_\ell^t \, C_u^t} \qquad (13)$$

where C_ℓ^o and C_u^o are the concentrations of material in the lower and upper compartments respectively at time 0, C_ℓ^t and C_u^t are the concentrations at time t, and β is the cell constant which is equal to:

$$\frac{A}{\ell'} \left[\frac{1}{v_\ell} + \frac{1}{v_u} \right]$$

The symbols v_u and v_ℓ represent the volumes of the upper and lower compartments respectively, and ℓ' is the effective thickness of the porous plate. The compartment volumes were obtained from the weight of water they contained at 25 C divided by the known density of water at this temperature. The upper compartment held 22.84 ml, the lower compartment 23.79 ml, and the fritted filter 0.40 ml. The ratio A/ℓ' or the cell constant β can be determined from equation (13) by using a material with a known diffusion constant. A 0.1 molar solution of KCl in water was used for this purpose. The diffusion constant of this solution at 17.5 C is 1.38 cm^2/day or 1.60×10^{-5} cm^2/sec (Handbook of Chemistry and Physics,

$$D = \frac{1}{\beta t} \ln \frac{C_f^n C_o}{C_o C_o^n} \qquad (13)$$

where C_o and C_o^n are the concentrations of material in the lower and upper compartments respectively at time t, C_f and C_f^n are the concentrations in the [illegible]

40th ed.). Since the cell was calibrated at 15 C, the following relationship between diffusion coefficient and absolute temperature was used to obtain D of KCl at 15 C:

$$D_1 = D_2 \cdot \frac{T_1}{T_2} \cdot \frac{\eta_1}{\eta_2}$$

where η is the viscosity of water and the subscripts 1 and 2 refer to two different experimental temperatures. The diffusion coefficient of 0.1 molar KCl is 1.49×10^{-5} cm^2/sec at 15 C.

The experimental procedure for obtaining diffusion rates was as follows: All solutions were first degassed by boiling at atmospheric pressure or under high vacuum. The entire cell was then filled by suction with the solution under study and placed in the water bath for temperature equilibration. After equilibration the top compartment was emptied, rinsed, and refilled with pure solvent. The magnetic stirring device was set at 120 rpm and diffusion was allowed to proceed for an appropriate period of time. At time t, the solution in the upper compartment was removed and replaced with fresh solvent to begin another period of diffusion. This procedure was repeated until constant values of the diffusion function $\frac{1}{t} \ln \frac{c_\ell^o - c_u^o}{c_\ell^t - c_u^t}$ were obtained; indicating that the concentration gradient dc/dx in the

following relationship between diffusion coefficient and absolute temperature was used to obtain D of NO3 at 15 C:

$$D_1 = D_2 \cdot \frac{T_1}{T_2} \cdot \frac{1}{2}$$

where: 1s the viscosity of water, and the subscripts 1 and 2 refer to two different temperatures. The

membrane had reached a steady state.

Although the solution in the lower compartment could not be sampled while the steady state was being achieved, its concentration could be calculated for each sampling with a knowledge of the original concentration, the concentration in the upper compartment, and the volumes of each compartment. When a steady state transfer was established both compartments were emptied and the concentrations of materials were determined directly.

Fig. 11A illustrates the establishment of a constant diffusion rate for the standard KCl solution. The KCl concentrations were determined by titration with standard $AgNO_3$ using fluorescein as an indicator. A final diffusion function of 1.62×10^{-6} sec^{-1} (with a maximum error of 2%) was obtained from this curve and a cell constant of 0.109 cm^{-2} ($\pm$ 2%) was calculated by the use of equation (13).

Fig. 11B shows the establishment of a constant diffusion rate for glucose dehydrogenase in 0.05 molar, pH 6.5 imidazole buffer, and Fig. 11C shows the same for the enzyme in buffer plus 5 molar NaCl. Enzyme activities rather than true concentrations were used in calculating the diffusion functions. The slopes of the curves are inverted because the membrane was initially filled with solvent

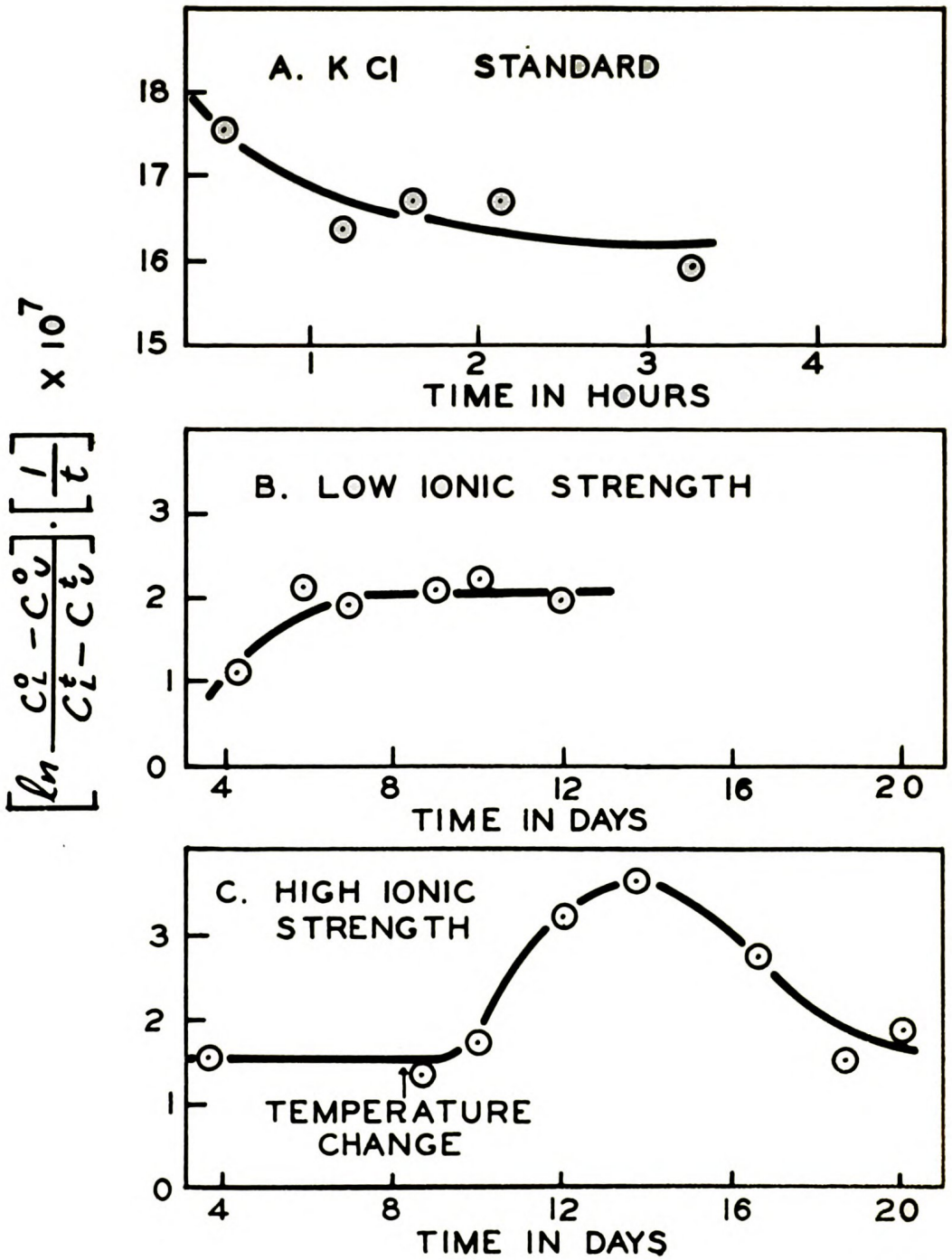

Figure 11. The establishment of a constant diffusion rate in the Stokes cell for 0.1 molar potassium chloride and for glucose dehydrogenase in a low and high ionic strength solvent. The low ionic strength solvent is 0.05 molar, pH 6.5 imidazole chloride buffer and the high ionic strength solvent contains 5 molar NaCl in addition to buffer.

rather than protein solution. Fig. 11B indicates a diffusion function of 2.1×10^{-7} (7% maximum error). This corresponds to a diffusion coefficient D of 1.9×10^{-6} cm^2/sec ($\pm$ 9%) for the enzyme in a low ionic strength solvent. Judging from the curve in Fig. 11C, a steady state gradient was initially established and then destroyed by a temperature increase caused by a faulty thermo-regulator. However, with continued diffusion, the original rate was eventually obtained. The final diffusion function was 1.6×10^{-7} ($\pm$ 20%) which gives a diffusion coefficient of 1.5×10^{-6} cm^2/sec ($\pm$ 22%) for the enzyme in a high ionic strength solvent.

Not all of the material present in the cell initially could be accounted for at the end of the diffusion experiments. Thus, only 93% of the KCl, 85% of the enzyme in low ionic strength solvent and 77% of the enzyme in high ionic strength solvent was accounted for. These losses are too large to be attributed to non-recoverable enzyme in the membrane. They could not be due simply to denaturation either because a control solution of enzyme in the low ionic strength solvent was held for 9 days at 20 C with no detectable loss in activity. However, the porous membrane offers a very large surface for the adsorption or the denaturation of protein. The adsorptive capacity of the membrane was checked by measuring

coefficient D of 1.9×10^{-6} cm^2/sec (+ 3%) for

in a low ionic strength solvent. Judging from

curve in Fig. 11C, a steady state gradient was initi-

established and then destroyed by a temperature increase

caused by a faulty [illegible]. However, when a small

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the decrease in activity of the enzyme in the high ionic strength solution after flowing through the clean porous membrane. The first 1.1 ml lost 25% of the original activity, the next 1.8 ml lost only 2% and the rest of the solution was unaffected. Thus it appears that some adsorption does occur but that the membrane soon becomes saturated. This should not affect the final value of D unless adsoprtion of proteins changes the mechanism of transport across the membrane to something other than pure diffusion.

The diffusion constant alone does not provide enough information for the calculation of a molecular weight but it does provide information about the frictional properties of particles in a given solvent according to the following equation derived by Einstein and Smoluchowski:

$$D = \frac{RT}{Nf} \tag{15}$$

Here R is the gas constant, N is the Avogadro number, and f is the friction coefficient. The friction coefficient also appears in the Svedberg sedimentation equation:

$$s = \frac{M(1-\bar{V}\rho)}{Nf} \tag{16}$$

where s is the sedimentation coefficient, M is the anhydrous molecular weight, ρ is the solution density, and $\bar{V}$ is the partial specific volume of the protein, defined as the

increase in volume (in ml) upon adding a gram of dry protein to an infinitely large volume of solvent. Since it is generally assumed that the frictional forces encountered during diffusion are equivalent to those encountered during sedimentation, equations (15) and (16) can be solved for f and combined to give:

$$M = \frac{RTs}{D(1-\bar{V}\rho)} \tag{17}$$

The sedimentation coefficient is obtained from the rate of movement of the protein in a centrifugal field as follows:

$$\frac{dx}{dt} = s\omega^2 x \tag{18}$$

where x is the radial distance from the center of rotation at time t, and ω is the angular velocity of the rotor. A more useful equation can be obtained by integrating equation (18):

$$x = x_o e^{s\omega^2 t} \qquad \text{or} \tag{19}$$

$$\ln x = \ln x_o + s\omega^2 t \qquad \text{or} \tag{20}$$

$$s = \frac{2.303}{\omega^2 t} \log \frac{x}{x_o} \tag{21}$$

where x_o is the initial distance of the particle from the axis of rotation. In practice, the rate of movement of a single

particle is not followed but rather the rate of movement of the boundary between the sedimenting protein solution and the pure solvent centripetal to it. Thus the position of the solution meniscus is x_o and x is the position of the boundary.

The method of Hogeboom and Kuff (1954) was used to determine the sedimentation coefficient of the glucose dehydrogenase. These authors employed a sucrose density gradient to stabilize the protein-solvent boundary formed during centrifugation and determined the location of the boundary by direct sampling of the centrifuge tube contents. In the experiment to be described a model SW-39 swinging bucket rotor was used in a model L preparative ultracentrifuge (Beckman Instruments, Spinco Div.). The rotor was equipped with a heat radiating collar into which a mercury thermometer was placed during the run.

Linear sucrose density grandients were formed in 6 ml Lusteroid centrifuge tubes with the aid of the double vessel mixing device shown diagrammatically in Fig. 12. For small volumes, this device is most conveniently constructed from a solid block of Lucite by drilling out the vessels and channels. The concentration gradient was obtained by a progressive dilution of the more concentrated solution in vessel A by the less concentrated solution in vessel B as

The method of Hopdoom and Kuff (1954) was used to determine the sedimentation coefficient of the glucose dehydrogenase. These authors employed a sucrose density gradient to stabilize the boundary formed during centrifugation and determined the position of the boundary by the use of the Schlieren optical system. The method involves layering a solution containing protein solution and the pure solvent centripetal to it. Thus the position of the boundary is x_0 and x is the position of the boundary.

the system was emptied into the centrifuge tube. Rapid mixing in vessel A insured uniform dilution. Since the vessels were of equal dimensions, the rate of flow from the system was twice the rate of flow from B to A. This satisfied the requirements for a linear change in the concentration of the solution in vessel A, according to equations derived by Lakshmanan and Lieberman (1954).

The gradient produced by this device was examined experimentally by adding methylene blue to the solution in vessel A. The resulting color gradient was sampled at intervals throughout the tube and the color intensity in the samples was measured at 600 mμ in a Spectronic 20 spectrophotometer (Bausch and Lomb). Samples were removed from the centrifuge tube with the apparatus pictured in Fig. 13. This is essentially the same apparatus used by Hogeboom and Kuff (1954). To avoid contaminating dilute fractions with those of higher concentration, the tube was sampled from top to bottom by displacing the liquid column very slowly with a dense, 65% sucrose solution. Portions of the gradient column were completely removed and their volume measured with a graduated pipette as they flowed through the perforated plate in the sampling cup.

Fig. 14 is a plot of optical density at 600 mμ versus the volume removed from the tube containing the methylene

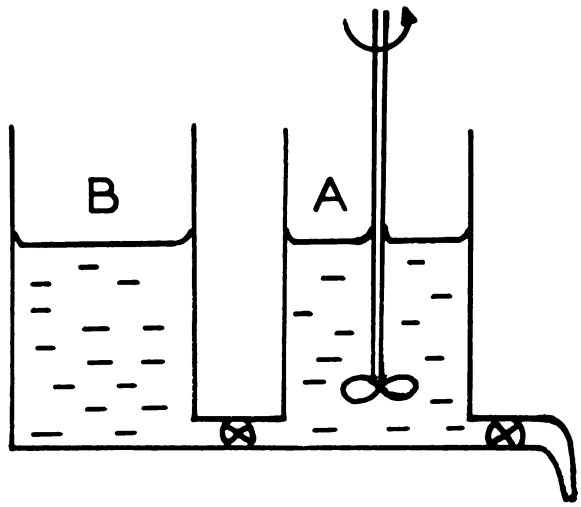

Figure 12. Density gradient forming device.

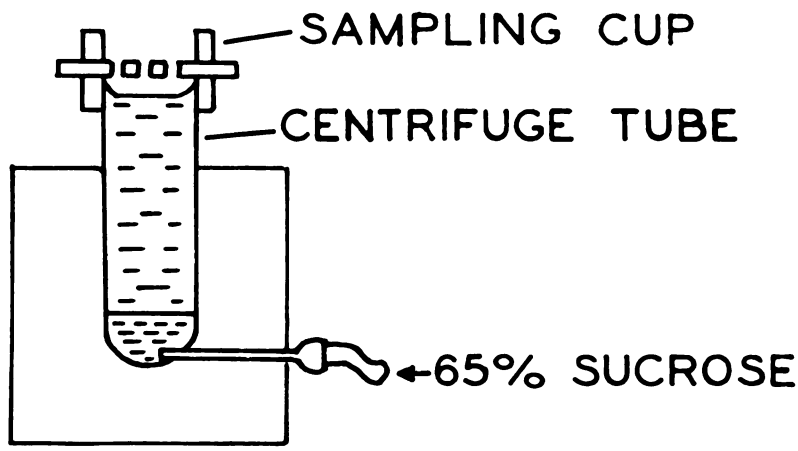

Figure 13. Centrifuge tube sampling device.

blue gradient. The gradient is obviously linear.

The procedure for obtaining sedimentation coefficients for glucose dehydrogenase in low and high ionic strength solvents was as follows: A solution of enzyme in 0.05 molar, pH 6.5 imidazole buffer and a similar solution containing 3% sucrose were mixed in the gradient device to produce a solution with a linear sucrose gradient and a constant concentration of enzyme and buffer. This procedure was also used for the enzyme in a buffer plus 5 molar NaCl solution. Each tube was subjected to a centrifugal field for a known time and sampled in the manner already described. The time and speed during acceleration and deceleration were recorded and expressed in terms of an equivalent time at top speed. This time (7 min in each case) was added to the total time for each run.

The results of the samplings are shown in Fig. 15 as a plot of enzyme activity versus distance from the meniscus. Distances were calculated from the known volume of the fluid column and from the dimensions of the bucket, tube, and rotor (Hogeboom and Kuff, 1954). The positions of the boundaries were obtained from the inflection points of the curves in Fig. 15 and used to calculate sedimentation coefficients according to equation (21). From the sedimentation

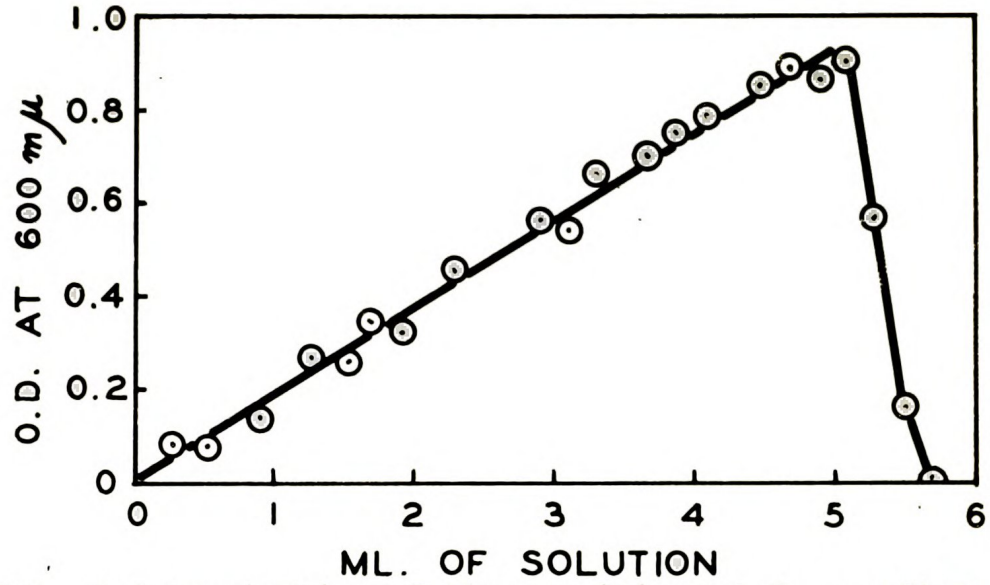

Figure 14. A determination of the precision of the gradient forming and tube sampling devices used in the sedimentation experiments. Optical density at 600 mμ reflects the methylene blue concentration in each sample.

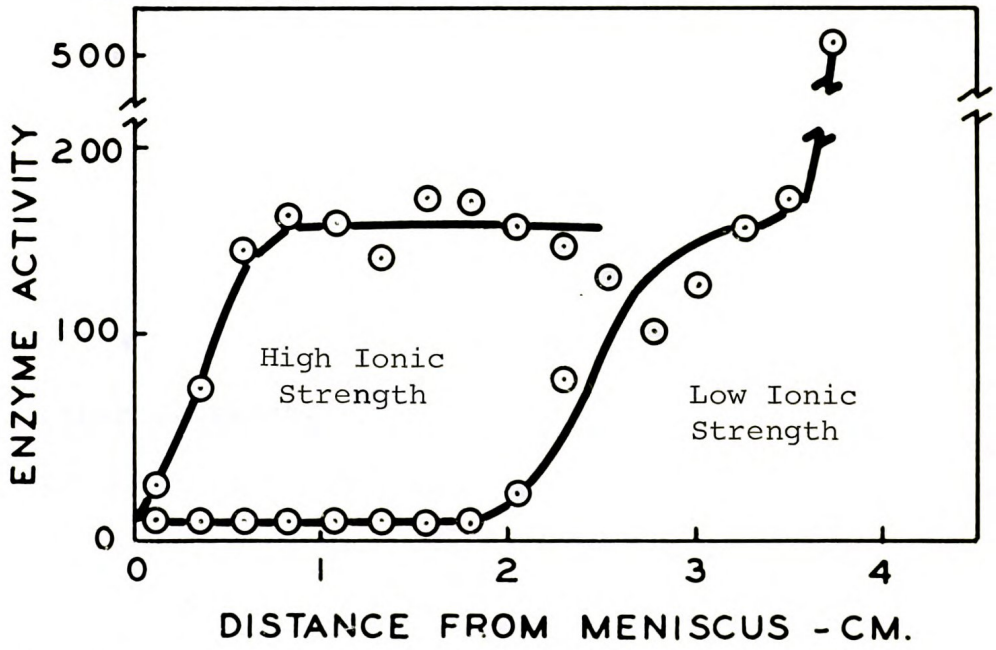

Figure 15. Sedimentation curves for glucose dehydrogenase in low and high ionic strength solvent. The low ionic strength solvent is 0.05 molar, pH 6.5 imidazole chloride buffer and the high ionic strength solvent contains 5 molar NaCl in addition to buffer.

coefficients and the diffusion coefficients, the molecular weights of the enzyme in each solvent were calculated according to equation (17). These data are summarized in Table 1.

The viscosities shown in Table 1 were obtained with an Ostwald viscometer using water as a standard. In this way the characteristics of the viscometer cancel out and solution viscosities can be calculated from the following equation:

$$\eta = \frac{t}{t_{H_2O}} \cdot \frac{\rho}{\rho_{H_2O}} \cdot \eta H_2O \qquad (22)$$

where t is the flow time in the viscometer and ρ is the density of the solution. The subscript H_2O refers to the water standard.

The partial specific volumes were not actually measured but were assumed on the basis of values obtained for most other proteins.

The temperature of the rotor was assumed to be the same as the temperature of the thermometer. The thermometer temperature was 20 C during most of the run with brief fluctuations of $\pm$ 0.2 C.

To obtain D_{20w} the following relationship of the friction coefficient to viscosity was employed:

The viscosities shown in Table I were obtained with an Ostwald viscometer using water as a standard. In the characteristics of the viscosity can

Table 1. A summary of data used for calculating the molecular weight of glucose dehydrogenase in low and high ionic strength solvents.

	Low Ionic Strength ($\tau/2$ = .04)	High Ionic Strength ($\tau/2$ = 5.04)
Distance from axis of rotation to meniscus (x_o) in cm.	5.4 ± 1%	5.4 ± 1%
Distance from axis of rotation to boundary (x) in cm.	7.9 ± 4%	5.8 ± 4%
Time at top speed (t) in sec.	3.40×10^4	3.67×10^4
Angular velocity (ω) in radians/sec.	4.12×10^3	4.12×10^3
Solvent viscosity (η) in poise at 20 C.	1.02×10^{-2}	1.87×10^{-2}
Solvent viscosity with 3% sucrose added (η_s) in poise at 20 C.	1.09×10^{-2}	2.06×10^{-2}
Solvent density (ρ) in g/cm^3 at 20 C.	1.00	1.19
Solvent density with 3% sucrose added (ρ_s) in g/cm^3 at 20 C.	1.01	1.20
Partial specific volume ($\bar{V}$) in ml/g.	0.72 ± 4%	0.72 ± 4%
Absolute temperature (T) in K.	293	293
Diffusion coefficient in solvent (D) in cm^2/sec.	1.9×10^{-6} ± 9%	1.5×10^{-6} ± 22%
Sedimentation coefficient in solvent (s) in sec^{-1}.	6.6×10^{-13} ± 5%	1.2×10^{-13} ± 5%
Diffusion constant in water at 20 C (D_{20w}) in cm^2/sec.	1.9×10^{-6} ± 9%	2.8×10^{-6} ± 22%
Sedimentation constant in water at 20 C (s_{20w}) in sec^{-1}	6.9×10^{-13} ± 5%	4.2×10^{-13} ± 5%
Anhydrous molecular weight (M) in g.	32,000 ± 8,000 (max error)	13,000 ± 5,000 (max error)

$$f = K\eta \qquad (23)$$

where K is a friction coefficient characteristic of the particle alone and η is the solvent viscosity. By combining equation (23) with equation (15), the diffusion coefficient can be related to the solvent viscosity. If one divides this expression for diffusion in water at 20 C by a similar expression for diffusion in a particular solvent at 20 C most of the terms cancel out and the standard form of diffusion coefficient (or, actually, diffusion constant D_{20w}) can be obtained:

$$D_{20w}/D = \eta/\eta_{20w} \qquad \text{or} \qquad (24)$$

$$D_{20w} = \frac{\eta}{\eta_{20w}} \cdot D \qquad (25)$$

The same type of calculation was used to obtain s_{20w} from equation (16). The average viscosity over the distance moved by each boundary was used in these calculations.

It should be noted that the per cent errors included in Table 1 are maximal errors based on the maximal deviations from the best estimate of the data in Figs. 11 and 15, and on the range of values given for the partial specific volumes of various proteins by Fox and Foster (1957). In some cases, the probability of the maximal error may be very low. For example, in Fig. 11B, considerably more confidence

can be placed in the value selected for the diffusion function because more data in the steady state region were obtained. Errors of less than 1% were ignored in the calculations.

It is readily apparent that the glucose dehydrogenase dissociates in high ionic strength into subunits. One would expect the dissociation to yield subunits of 1/2 or 1/4 rather than 12/32 the weight of the molecule in low ionic strength solvent. The unusual ratio of molecular weights observed could result from the high degree of error in these calculations, especially in the assumption that the partial specific volume is not affected by changes in ionic strength. If the value of $\bar{V}$ in high ionic strength solvent was lower than the assumed value, the actual value of M could be somewhat lower than that given in Table 1. It is also possible that the calculated value of M in 5 molar NaCl is a weight average value for a mixture of the low and high molecular weight units. This too would lead to an erroneously high value of M for the subunit.

In spite of the errors involved in the calculation of molecular weights, it is apparent that a depolymerization rather than a polymerization occurs as the ionic strength of the protein solution is increased.

Since it was obvious that the increase in thermal stability in a high ionic strength solvent was not the result of intermolecular polymerization, the possibility of additional intramolecular hydrogen bonding was considered. Guanidine is strongly hydrogen bonded in aqueous solution and, therefore, is capable of rupturing existent hydrogen bonds and forming new ones. This is thought to be the reason for its inactivation of proteins. If increased ionic strength enhances heat resistance by strengthening hydrogen bonds or forming new bonds, it should protect the enzyme from guanidine inactivation. The inactivation rate of the enzyme can be expressed by the following equation:

$$-d(E)/dt = a(G)^n \qquad (26)$$

where the change in enzyme concentration with respect to time is equal to a proportionality constant times the concentration of guanidine raised to a power. The reciprocal of the time required for a given fractional inactivation of the enzyme is also an expression for the inactivation rate:

$$-d(E)/dt = F(1/t_{40\%}) \qquad (27)$$

In the present study the time for 40% inactivation was used. Then:

$$1/t_{40\%} = a(G)^n \qquad (28)$$

By taking the logarithm of each side the equation becomes:

$$\log 1/t_{40} = \log a + n\log(G) \qquad (29)$$

When plotting $\log 1/t_{40}$ versus log (G) the slope of the line indicates the number of moles of guanidine needed to inactivate one mole of enzyme.

The procedure for the guanidine inactivations was as follows: Samples of purified glucose dehydrogenase in 0.05 molar, pH 6.5 imidazole buffer and samples of enzyme in buffer plus 5 molar NaCl were diluted 1:1 with an appropriate concentration of pH 6.5 guanidine. The inactivations were run at 0 C in crushed ice. At various time intervals, 0.1 ml of each enzyme-guanidine mixture was removed, diluted 9-fold immediately, and assayed.

Fig. 16 and 17 are a series of progress curves for the inactivation of glucose dehydrogenase under the conditions described. The numbers at the end of each curve indicate the final concentration of guanidine in the inactivation mixture. It is obvious from these curves that the 2.5 molar NaCl concentration affords some protection for the enzyme from guanidine inactivation. It is also apparent that, even with no salt present, the guanidine inactivation of this enzyme is remarkably slow.

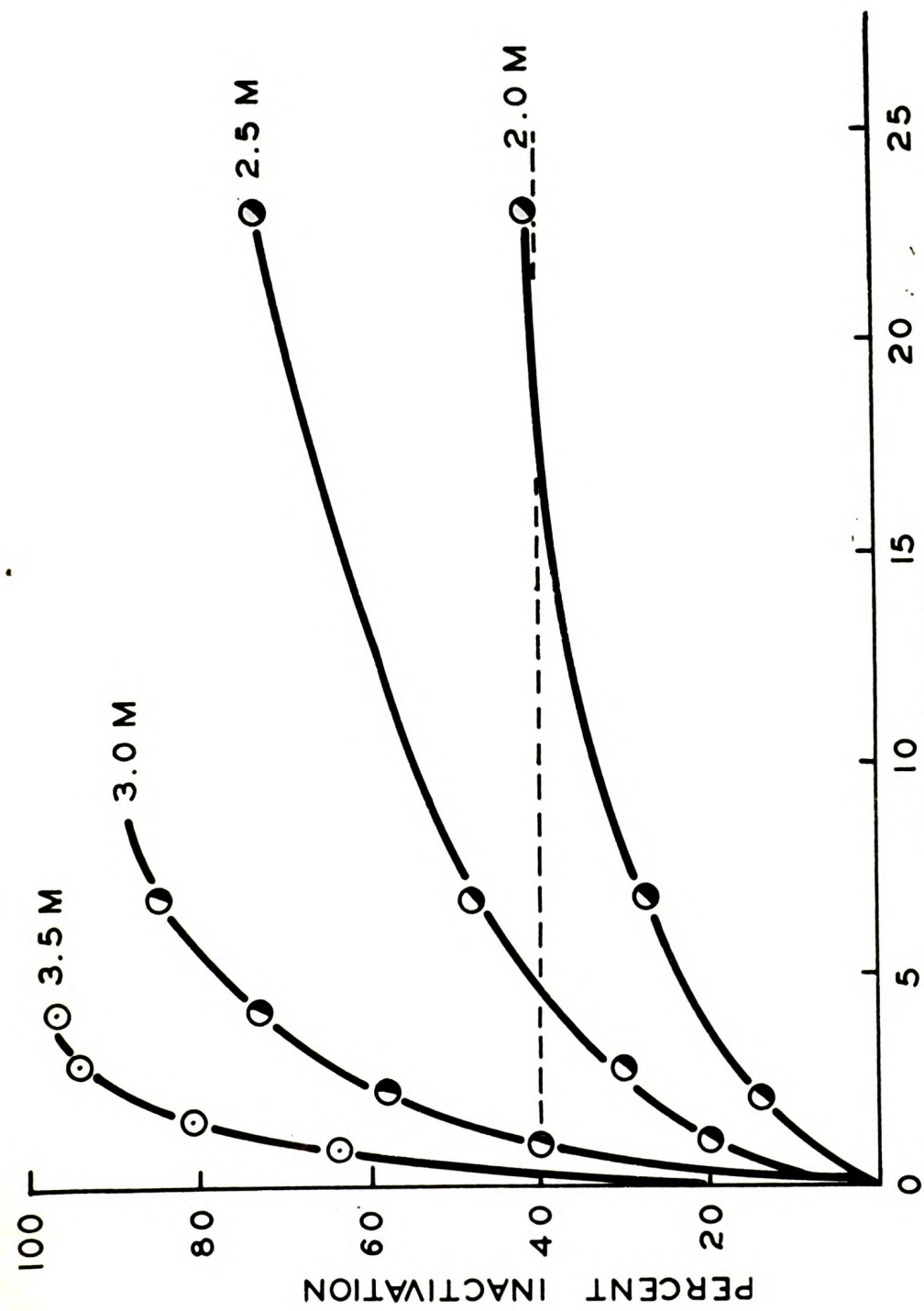

Figure 16. Guanidine inactivation of glucose dehydrogenase in 0.025 molar, pH 6.5 imidazole buffer at 0 C. Guanidine concentrations are indicated at the end of each curve.

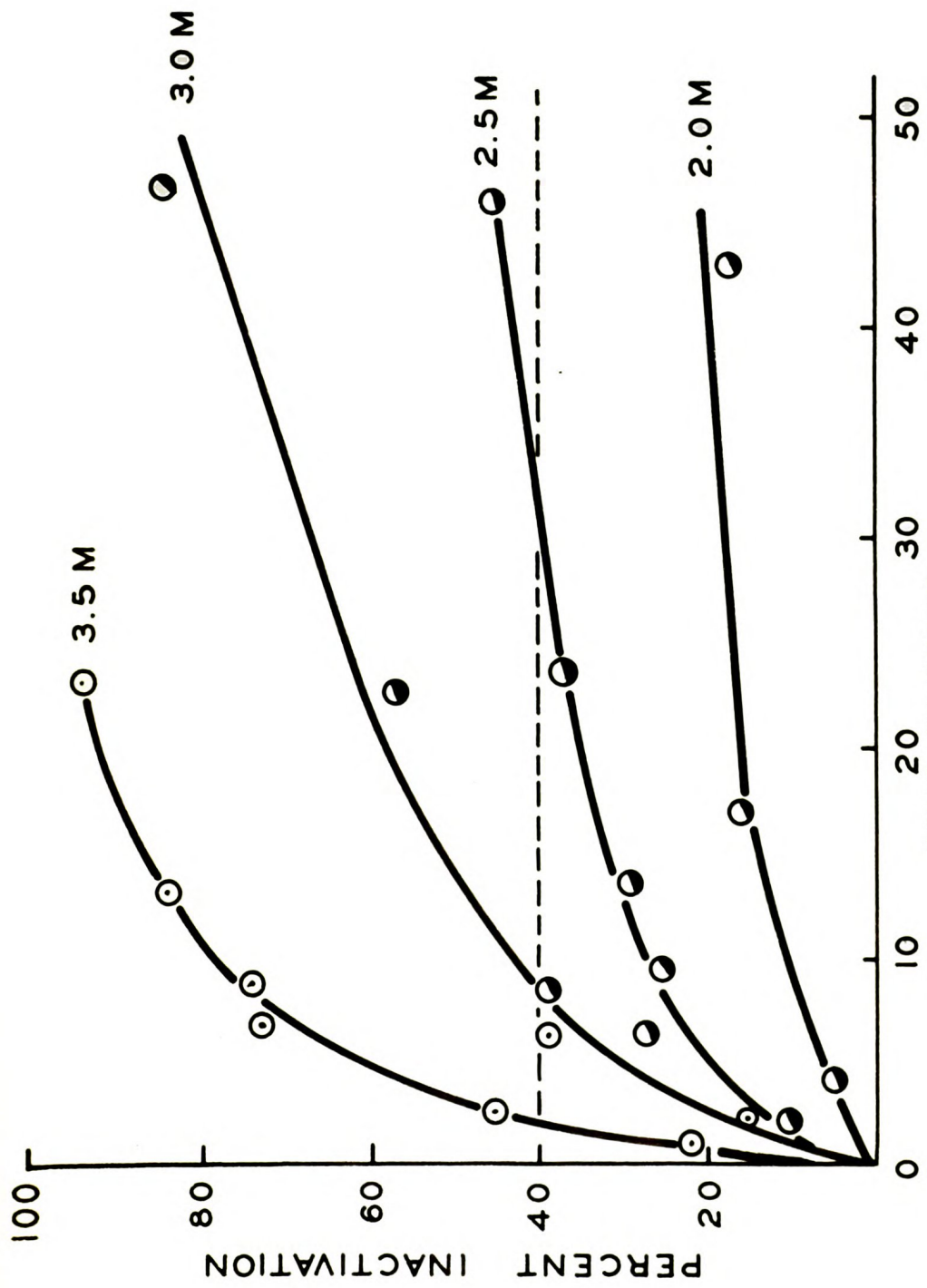

Figure 17. Guanidine inactivation of glucose dehydrogenase in 0.025 molar, pH 6.5 imidazole buffer plus 2.5 molar NaCl at 0 C. Guanidine concentrations are given at the end of each curve.

Fig. 18 is a plot of log $1/t_{40\%}$ versus log of the guanidine concentration according to equation (29). The slopes are the same for the enzyme in both low and high ionic strength medium suggesting that no additional hydrogen bonds are formed due to the high salt concentration; or at least no bonds affecting enzyme activity are formed. The slopes are 8.3 indicating an 8th order reaction with respect to guanidine. In other words, 4 hydrogen bonds must be broken to inactivate one molecule of enzyme assuming that two molecules of guanidine are required to break a hydrogen bond. In interpreting these data it is also assumed that guanidine disrupts only hydrogen bonds. In reality this may not be the case since there has been some evidence that hydrophobic bonds are also weakened by guanidine (Kauzmann, 1959).

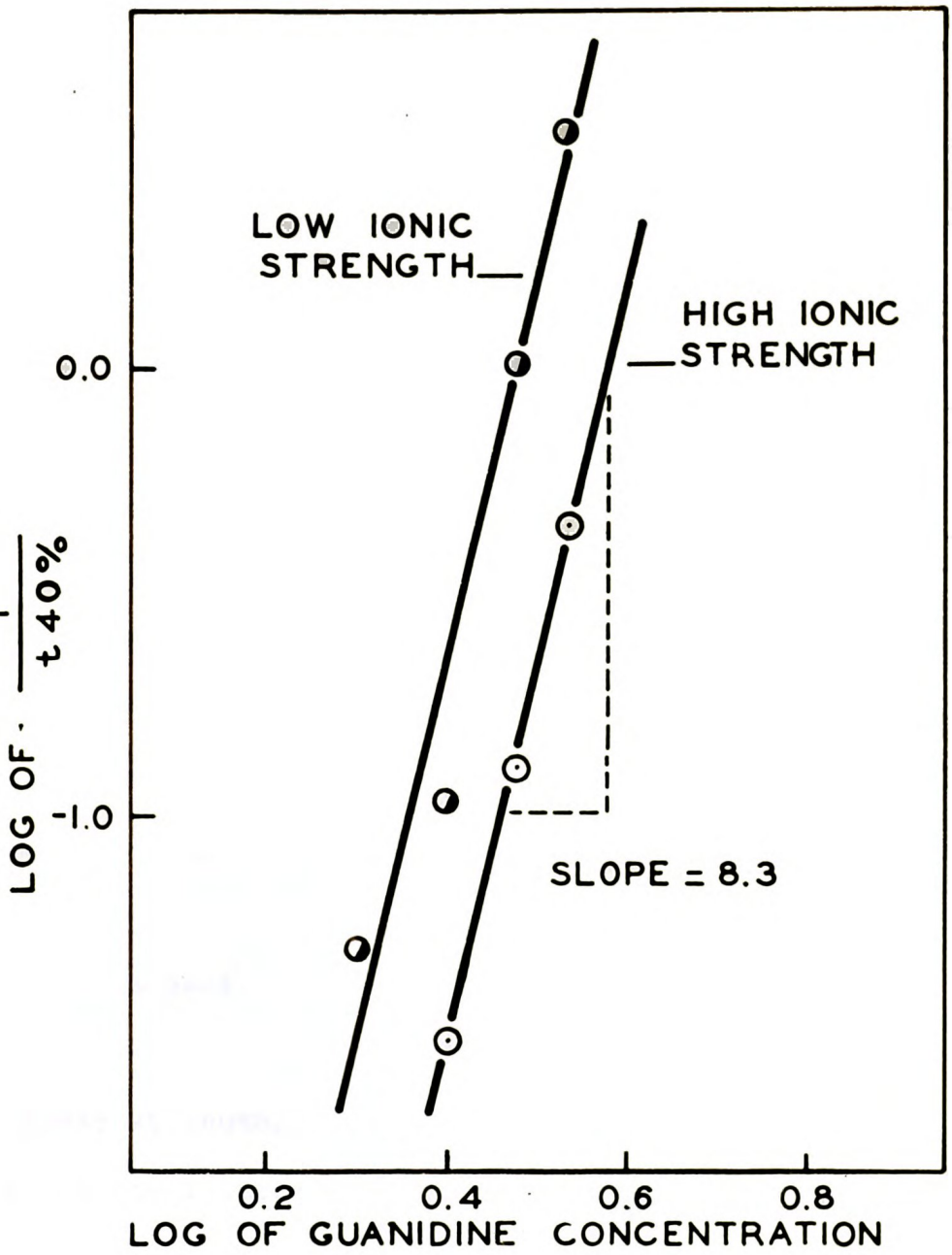

Figure 18. The effect of guanidine concentration on the inactivation of glucose dehydrogenase in a low and high ionic strength solvent at 0 C. Solvent conditions are given in Figs. 16 and 17.

DISCUSSION

The purpose of the research described in this thesis was the isolation of a spore enzyme which could serve as a model for studying spore heat resistance. Information on the factors affecting the heat resistance of this model could be used to formulate a mechanism for the heat resistance of intact spores. Thus the use of a model would permit the investigation of spore heat resistance without the uncertainties inherent in viability measurements. Glucose dehydrogenase is a spore enzyme (Bach, 1961) and is similar to whole spores in some of its properties. For example, this enzyme is extremely heat stable in intact dormant spores, which are themselves heat stable, and relatively heat labile in intact germinated spores. Under the proper conditions of pH and ionic strength, the purified enzyme is as stable <u>in vitro</u> as in the intact spores. Thus the glucose dehydrogenase is a very suitable spore model and should prove to be a powerful tool for studying the mechanism of spore heat resistance.

The possibility that spore macromolecules are protected from thermal denaturation by a highly ionic spore interior

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is very intriguing because it agrees with much of the prevailing data on spore properties. For example, spores accumulate high concentrations of calcium and DPA and thermal stability is directly related to the concentration of these compounds in spores. Also, the loss in spore heat stability during germination is accompanied by a loss in calcium and DPA. The fact that spores are permeable to water and other solutes refutes the hypothesis of a completely anhydrous and therefore heat stable spore, but supports the increased ionic strength concept of stabilization. Although the catalase and glucose-6-phosphate dehydrogenase from spores were only slightly protected from thermal inactivation by increased ionic strength, no final conclusions about their stabilities should be drawn until the effect of pH on these enzymes is studied.

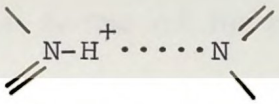
Protein structure can be treated at three different levels of complexity: the primary structure or the sequence of amino acids in the peptide chain; the secondary structure characterized by a regular coiling of the peptide chain into a rigid helix; and the tertiary structure or the folding of the helix into the final three dimensional configuration of the protein molecule. The specificity of the tertiary structure is thought to be responsible for the specificity

of enzyme catalysis and, conversely, the loss of this structure is thought to be responsible for the thermal inactivation of enzymes. If this is true, the effect of various solvents on the thermal stability of glucose dehydrogenase is probably due to effects on the forces which maintain tertiary structure. These forces should therefore be considered in discussing a possible mechanism for the effect of pH and ionic strength on the stability of glucose dehydrogenase.

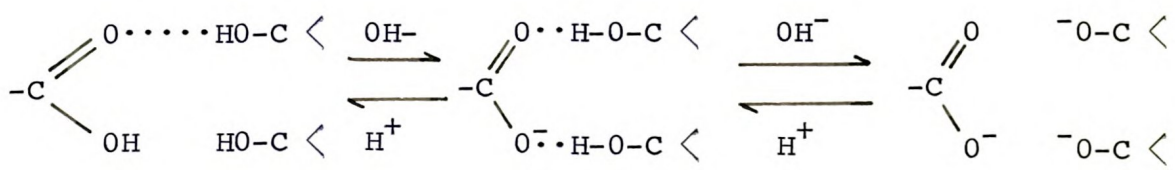
There are ample opportunities for hydrogen bonding between various groups in proteins and this type of bond probably plays a very important role in maintaining protein configuration at both the secondary and tertiary levels. At the secondary level, hydrogen bonding occurs principally between amide nitrogens and carbonyl oxygens of the peptide backbone while, at the tertiary level, the side chain groups of amino acids are involved. If it is assumed for the moment that the thermal inactivation of glucose dehydrogenase results from the rupture of hydrogen bonds, the probable effect of pH and ionic strength on these bonds, and hence on enzyme activity, can be considered.

According to Scheraga (1961) the strength of hydrogen bonds between ionizable amino acid side chain groups is dependent on the state of ionization of the groups and thus on the pH of the solution. If bonding occurs between two

similar groups such as the imid nitrogens of histidine:



the bond would be strongest when half of the imidazolyl groups of the histidine residues were ionized. In view of this conclusion, it is of interest that the pK of the imidazolyl group (6.00) is close to the pH of maximum stability (pH 6.5) of glucose dehydrogenase. However, it is difficult to believe that histidine could be involved because the inactivations were performed in an imidazole buffer. The concentration of imidazole in this solution was 5×10^{-2} molar and the concentration of enzyme was only 3×10^{-8} molar. Thus, one would expect competitive inhibition of any histidine-histidine hydrogen bonds by the imidazole. On the other hand, the pH dependent maximum in stability may result from a combination of the ionization characteristics of several heterologous bonding groups. This might explain the difference in the slope (reaction order) on either side of the minimum of log k seen in Fig. 6. For example, in a cooperative hydrogen bond between a carboxyl acceptor group and a pair of amino or phenolic donor groups, two ionizations can occur at high pH and only one at low pH as follows:



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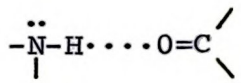
The stabilizing effect of a high NaCl concentration can also be explained in terms of hydrogen bonding. Huggins (1962) discussed the strength of hydrogen bonds of the type:



in terms of the relative distance of the hydrogen from the two bridge-head atoms, nitrogen and oxygen. It appears that the more symmetrical the bond, the stronger it is. If the amide nitrogen is made more electropositive by binding a proton or metal ion, the hydrogen is displaced toward the oxygen, increasing the symmetry and the strength of the bond. The binding of Na ions by the amide nitrogens in proteins should also decrease the rate of inactivation by guanidine, unless the competing protein-guanidine hydrogen bonds are strengthened to the same degree as the protein-protein hydrogen bonds.

The electrostatic bond or salt linkage has also been suggested as a factor in the maintenance of protein configuration. The variation in the stability of glucose dehydrogenase with varying pH could be due to salt linkages. It seems unlikely, though, that bonding of this nature is responsible for the stability of the enzyme in high ionic strength solutions because high concentrations of ionic material tend to rupture salt linkages by forming ion clouds around the bonding charges. On the other hand, the rupture

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of salt linkages between peptide chains could be causing the dissociation of the glucose dehydrogenase molecule in high ionic strength solution.

If glucose dehydrogenase is folded with polarizable and ionizable groups on the surface and with non-polar groups oriented toward the center of the molecule, a competition between extension of the molecule through solvation and contraction of the molecule through hydrophobic bonding would occur. Thus, a decrease in the solvation of the protein should strengthen the existent hydrophobic bonds. A decrease in solvation could occur at some particular pH when the surface charge was at a minimum or perhaps at a favorable balance. It could also occur in high ionic strength solvents because the dense ion clouds surrounding the protein charges would prevent the binding of water molecules. It is of interest in this connection that high concentrations of guanidine may rupture hydrophobic bonds as well as hydrogen bonds (Kauzmann, 1959) especially at low temperatures. It should be noted, also, that the strength of hydrophobic bonds increases, within limits, with increasing temperature.

The rupture of protein hydrogen bonds in aqueous solution probably occurs through an exchange reaction with the hydrogen bonds between water molecules. Thus the

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effect of high salt concentration on enzyme denaturation could be caused by a lowered water activity as well as by direct effects on the strength of bonds in the protein. The very limited effect of concentrated sucrose solutions on the rate of inactivation argues against the idea that lowered water activity is the basis of thermal resistance. However, it should be pointed out that the lowering of water activity by salts is not only due to dilution of the water but is also a consequence of the binding of polarized water molecules by charged ions (Brey, 1958).

The protection of proteins from thermal denaturation by high salt concentrations has been observed by other workers. Harrington and Schellman (1957) (as reviewed by Shooter, 1960) studied the configuration of ribonuclease in solution by a combination of optical rotation, viscosity and UV absorption measurements. They found that high concentrations of LiBr (up to 9.9 molar) prevented the configurational changes observed when this enzyme was heated in water alone. These authors concluded that the addition of LiBr to the solution increases the amount of helical structure in both native and oxidized ribonuclease.

The use of polarimetry or some other non-enzymic method of measuring protein denaturation could be of value

in future studies of the glucose dehydrogenase because it would permit the direct measurement of configurational changes in any solvent and at any pH. With the present enzymic method, only irreversible changes in configuration can be measured unless the changes occur near pH 8. Of course, it may be difficult to correlate the configurational changes with changes in enzymic activity. In any case, the use of physical or chemical methods for examining configurational changes is going to require large quantities of pure glucose dehydrogenase. Perhaps one of the more important contributions of the present study is the information on molecular size and conditions of maximum stability for this protein, since this information may be used to design a more efficient purification procedure.

SUMMARY

Glucose dehydrogenase from Bacillus cereus was examined as a possible enzymic model for the study of spore heat resistance. In vivo studies showed this enzyme to be relatively labile in intact sporulating cells and germinated spores but very stable in intact dormant spores. The heat resistance of both the glucose dehydrogenase and a glucose-6-phosphate dehydrogenase in spore extracts was dependent on the concentration of dialysable material in the extract.

Glucose dehydrogenase from germinated spores was purified 3,400 fold and the effect of various solvents on the properties of this enzyme were investigated in an effort to understand the mechanism of its heat resistance in spores. Heat resistance of this enzyme is strongly dependent on hydrogen ion concentration, exhibiting a sharp peak at pH 6.5 in .05 molar imidazole buffer. The Arrhenius activation constant for this enzyme in the same buffer is 17,000 K^{-1}. High concentrations of certain ionized compounds such as NaCl, KCl, Na_2SO_4, $(NH_4)_2SO_4$, DPA, and calcium-DPA chelate also increase the enzyme heat stability. The increase in stability in NaCl is approximately 2nd order with respect to the concentration of NaCl. The effect of $CaCl_2$ on heat

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resistance was also examined and found to be more complex than the NaCl effect. At concentrations up to 0.8 molar, $CaCl_2$ protects the enzyme from heat denaturation while, at concentrations above 0.8 molar $CaCl_2$, the enzyme was rapidly inactivated.

Purified glucose dehydrogenase is very resistant to guanidine inactivation at 0 C and the presence of 2.5 molar NaCl increases this resistance. The inactivation reaction is 8th order with respect to guanidine concentration and does not change in the presence of the NaCl.

The molecular weight of purified glucose dehydrogenase in a low and high ionic strength solvent was determined by a combination of diffusion and sedimentation measurements. In 0.05 molar, pH 6.5 imidazole buffer, the molecular weight was approximately 32,000 while in buffer plus 5 molar NaCl the molecular weight was approximately 13,000.

Although it is entirely possible that a highly ionic environment is responsible for the heat resistance of this enzyme in intact spores, the evidence obtained to date is not sufficient to prove this thesis. The mechanism of the effect of pH and ionic strength on the heat stability of purified glucose dehydrogenase also cannot be stated with certainty. It appears to involve the strengthening of side-chain hydrogen bonds and possibly hydrophobic bonds responsible

concentrations above 0.8 molar $CaCl_2$, the [illegible] inactivated.

for maintaining the native configuration of this protein.

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