STUDIES OF A HEAT RESISTANT CATALASE FROM CELLS OF BACILLUS CEREUS

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STUDIES OF A HEAT RESISTANT CATALASE FROM CELLS OF *BACILLUS CEREUS*

By

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INTRODUCTION

The Class Schizomycetes contains only two genera that form spores; namely Bacillus and Clostridium. The bacterial endospores, which appear to be highly specialized cells, are characterized by their resistance to heat, deleterious chemicals, ultraviolet light and other unfavorable environmental conditions. Although spores possess little metabolic activity, the only major chemical difference between a spore and a vegetative cell is the relatively large amount of dipicolinic acid (2, 6 dicarboxy pyridine, DPA) contained in the spore. No DPA has, as yet, been found in vegetative cells. Upon germination, the DPA is excreted at the same time that the resistance is lost and certain latent metabolic enzymes once again become active.

If heat resistance is an attribute of a spore, all of the enzymes within the spore which are essential for the germination and outgrowth of the spore are necessarily heat resistant. It should therefore be possible to study the mechanism of heat resistance using isolated spore protein as a model system. The above statement includes the assumption that the protein under study can be maintained in the "same state" as it exists in the spore. In addition, the enzyme should be capable of ready extraction and precise assay. Unfortunately, very few spore enzymes meet these requirements.

In the course of an investigation of the sporulation phenomenon, the presence of a heat stable catalase was noted in vegetative cells of

Bacillus cereus. It was recognized that this enzyme could perhaps serve as the model system described in the previous paragraph. In addition, considerable information is available on bacterial catalase and catalase isolated from other sources.

The object of this thesis is to investigate certain chemical and thermodynamic properties of the vegetative cell heat resistant enzyme and to compare it, where appropriate, to vegetative cell heat labile catalase. The ultimate objective of this work is to arrive at a plausible explanation of the mechanism of heat resistance in bacterial endospores.

Since a final investigation of the physical-chemical properties of the above enzymes is impossible without crystalline material, a purification procedure of the heat stable catalase was also attempted.

HISTORICAL REVIEW

Bacterial catalase was first described by Gottstein[9] in 1893. Very little was done with enzymes from this source until Herbert and Pinsent,[10] in 1948, crystallized bacterial catalase from Micrococcus lysodeikticus. This was the first bacterial enzyme to be crystallized. The above authors used lysozyme to break the cells and partitioned the catalase in an ethanol-chloroform-water system.

The bacterial catalase molecule is composed of four haematin molecules with a total molecular weight of 232,000. This enzyme compares with liver catalase but differs in its relatively high resistance to organic solvents and its instability at low pH. The catalytic activity of the bacterial enzyme is considerably higher than blood or liver catalases. These differences can be attributed to the protein portion of the molecules.

Herbert and Pinsent[10] observed that the catalase amounted to 1-2% of the dry weight of M. lysodeikticus enabling the organism to destroy 35-70 times its own weight of 0.01 M hydrogen peroxide per minute at $0^{\circ}C$. They therefore assumed that the enzyme had some metabolic function in the cell. They estimated that one bacterial cell contained $10\text{-}20 \times 10^3$ enzyme molecules.

Chance[3] found that the four hematin groups of catalase seem to act independently. He found that 1.6 molecules of hydrogen peroxide were bound to each catalase molecule.

Chance and associates,[4] in 1952, formulated the first plausible mechanism of the reaction between catalase and hydrogen peroxide. The first molecule of hydrogen peroxide combines with the hematin iron. The second molecule of hydrogen peroxide reacts with a specific site at or near the hematin-iron-first peroxide group. As a result, a "Michaelis Constant" has no meaning for catalase activity since this is an enzyme mechanism involving consecutive reactions of the substrate molecule with the enzyme and with the enzyme substrate complex.

Lawrence and Halvorson[12] found a heat resistant catalase in spores of B. cereus. This enzyme remained unaffected by heating at 80°C for 30 minutes or 100°C for 5 minutes. They observed that the maximum amount of oxygen was given off after 20-25 minutes on the Warburg manometric apparatus and the total gas given off was proportional to the amount of cell preparation added. The enzyme lost its heat resistance upon being extracted from the spore or upon germination of the spore.

Sadoff, Kools, and Ragheb,[19] in 1959, demonstrated a heat resistant catalase in cell free extracts of sporulating B. cereus. The resistant catalase is formed at an exponential rate during the period when no cell division occurs. These authors showed that the activity of the heat resistant enzyme was not due to the catalase content of the "completed" or viable spores. Approximately 1% of the vegetative cell catalase was found to be heat resistant.

MATERIALS AND METHODS

A. Organism and Medium

The organism used in these studies was Bacillus cereus. It has also been called Bacillus cereus var. terminalis, Bacillus terminalis, and it has been suggested that the name Bacillus cereus strain Illinois be used.[22]

The cells were grown in G medium described by Stewart and Halvorson.[24] The medium consisted of the following components per liter:

$ZnSO_4$	0.00001g.
$CuSO_4$	0.00001g.
$CaCl_2$	0.00001g.
$FeSO_4 \cdot 7H_2O$	0.000001g.
K_2HPO_4	1.0g.
$(NH_4)SO_4$	4.0g.
Yeast Extract	2.0g.
$MnSO_4 \cdot H_2O$	0.1g.
$MgSO_4$	0.8g.
Dextrose	4.0g.

In addition 2 ml of Dow Corning Antifoam B were added to each three liters of medium to control foaming.

B. Production of Large Cell Crops

The heat resistant enzyme constitutes only about 1% of the total catalase or at most 0.01% of the dry weight of the cell. Therefore large cell crops are necessary.

The culture was "carried" on nutrient agar slants held at 4°C but all growth was carried out at 30°C. The inoculum was prepared in 500 ml dimpled flasks, each containing 50 ml of media. A dimpled flask is a 500 ml Erylenmeyer flask with four, half-inch, round depressions equally spaced around the bottom of the flask. The depressions produce turbulence when the contents are shaken thus increasing the aeration of the cells.

In order to produce a uniform, rapidly growing inoculum, the following procedure was employed. An eight hour culture of B. cereus was grown on nutrient agar and then used to inoculate 50 ml of G medium. After four hours, five more flasks of G medium were each inoculated with 10 ml of the previous culture. These were permitted to grow for two hours and used to inoculate 24 more flasks of G medium (20% inoculum). After two hours, eight flasks of inoculum were poured into each of three tanks of a New Brunswick fermentor (New Brunswick Scientific Co.) containing 2700 ml of medium in each tank.

The cells were stirred at 450 rpm and aerated at a rate of 5 liters per minute. The cells were harvested in a Sharples Super Centrifuge (The Sharples Specialty Co.) after seven hours of growth and stored at -15°C. The cells were heated routinely at 63°C for 30 minutes either immediately after harvesting or after storage to destroy any heat labile

catalase activity. The above procedure resulted in 1.7 g dry weight of cells per liter of medium.

C. Preparation of Cell Extracts

Two methods were used to break the cells. The first method employed the high speed mixing of a cell suspension with fine glass beads. This was accomplished in a Servall Omni-mixer (Ivan Sorvall, Inc.) using two parts by weight of Superbrite glass beads size 100 (Minnesota Mining and Manufacturing Co.), two parts of 0.1 M phosphate buffer, pH 7, and one part wet weight of cells. This method was used for the preparation of enzyme for the kinetic studies.

Strange and Dark[26] have described a lytic enzyme which is produced by B. cereus at the time of sporulation. This protein, which they called enzyme V, apparently is concerned with the release of free spores from sporangia. However, it also produces a lysis of vegetative cell walls facilitating the release of the heat resistant catalase.

The lytic enzyme was obtained by growing cells in the same manner as previously described but permitting growth to continue for 16-19 hours, at which time the cells contained intracellular spores. The cells were harvested in the Sharples Super Centrifuge and washed twice with 0.9% saline and once with distilled water.

The cells were suspended in a 50% McIlvaine buffer, pH 5.2. The volume was not critical. After incubation at 37°C for two hours, the resultant mixture was centrifuged leaving a proteinaceous supernate. The entire cell crop produced in seven hours from nine liters of aerated

and agitated G media could be lysed with the lytic enzyme produced by cells in three liters of culture. The lysis occurred in three hours at 56°C.

After breakage of the cells by either method (beads or enzyme), the cell brei was centrifuged and the sedimented material washed with 0.1 M phosphate buffer, pH 7. The combined washings and supernatant liquid were then clarified by a combination of centrifugation and paper filtration. The resulting "solution" was again heated at 68°C for one-half hour and the precipitated proteins were then removed by centrifugation.

The concentration of the enzyme was accomplished by adding saturated ammonium sulfate solution, pH 7, to the enzyme solution until 80% saturation was reached. After centrifugation, the precipated protein which contained the catalase, was suspended in either 0.1 M phosphate buffer, pH 7, or distilled water. The properties of the heat resistant enzyme were studied using enzyme obtained from the above procedure.

The preparation of heat labile catalase was similar to that of the stable enzyme with the exception that the cells or cell free preparations were not heated or concentrated.

D. Catalase Assay

Any assay procedure that titrates hydrogen peroxide can be adapted to measure catalase activity.[1, 2, 5, 8, 15] A summary of various assay procedures is found in Methods of Enzymology[6] and Methods of Biochemical Analysis.[13] In these cases, what is actually taken as the measure of catalase concentration is the first order reaction rate constant for the

inactivation of the enzyme by hydrogen peroxide. When the level of catalase activity is very low and the amount of contaminating material very high, it may be necessary to use a procedure which measures the extent of the decomposition of the substrate. The assay used in this research was described by Lawrence and Halvorson[12] and is a manometric procedure which measures the extent of the decomposition of hydrogen peroxide over a period of twenty-three minutes. Routinely, the Warburg cup contained 0.3 ml of enzyme preparation, 1.0 ml of 0.1 M phosphate buffer, pH 7, and 1.4 ml of water. The side arm contained 0.3 ml of 1.5% hydrogen peroxide. The cups were shaken at 144 oscillations per minute in a water bath at 30°C. One unit of enzyme is defined as one microliter of oxygen evolved per 23 minutes.

E. Thermal Inactivation Studies

Because such striking differences seemed to exist between the thermal stable and thermal labile catalases, studies were made of the inactivation process. Heat inactivation of the enzyme preparations was carried out in glass tubing with a wall thickness of 0.5 mm. Slightly more than 0.3 ml were placed in the tubes and both ends were sealed. The tubes were suspended in a continously agitated water bath. The heat resistant enzyme was heated at 70°, 77°, 80°, 83°, and 85°C for 0-30 minutes. The heat labile enzyme was heated at 51°, 54°, 56°, and 59°C for 0-30 minutes. The inactivation process was stopped by placing the tubes in an ice bath. The remaining catalase activity was assayed and the log of activity was plotted versus the time of heating.

The method of least squares was used to draw the "best line" in the above plots.

F. Optimal pH

A basic characteristic of an enzyme is its optimal activity at a certain pH value. Studies of the optimal pH were conducted with the heat resistant catalase by observing activities in the following buffer systems:

0.05 M Phthalate-sodium hydroxide buffer

pH's- 4.3 and 5.3

0.10 M Acetate buffer

pH's- 4.0 and 5.3

0.05 M Barbital buffer

pH's- 6.3, 6.8, 7.7, and 7.8

0.10 M Phosphate buffer

pH's- 7.0 and 8.0

0.005 M Borax-sodium hydroxide buffer

pH's-9.4 and 10.1

At pH values above 7.5, it was necessary to make corrections in the catalase activities to account for the spontaneous decomposition of hydrogen peroxide.

G. Guanidine Inactivation Studies

The tertiary structure of proteins, which may determine their specificity, is determined in part by their hydrogen bonding. Those agents that hydrogen bond strongly can break hydrogen bonds which

exist in proteins. Schellman[20] and Simpson[21] and others studied hydrogen bond breakage of ovalbumin due to urea and guanidine by observing changes in optical rotation of the protein. They found the reaction to be about 15th order with respect to urea. The protein was inactivated more readily with guanidine than urea since guanidine is a more powerful bonding agent. The relatively high reaction order of guanidine inactivation presents the possibility of stopping the inactivation reaction by dilution. In the present study, neutralized guanidine hydrochloride was used in order to study the inactivation of labile and stable catalase at 0°C. The following molar concentrations of guanidine were used over the indicated time range:

Heat labile enzyme

1.5 M	0-120 seconds
1.75 M	0-90 seconds
2.0 M	0-60 seconds
2.25 M	0-30 seconds
2.5 M	0-60 seconds

Heat stable enzyme

0.5 M	0-120 seconds
1.0 M	0-120 seconds
1.5 M	0-180 seconds
2.0 M	0-120 seconds
2.5 M	0-60 seconds

After the inactivation had proceeded for various periods of time, the solutions were diluted five fold in order to halt the reaction. If the guanidine inactivation were an eighth order reaction, the 5X dilution would result in about a 400,000 reduction in denaturation rate. The enzyme assay was then performed as soon as possible.

H. Enzyme Purification

The catalase preparation resulting from the enzymatic lysis of cells was purified in the following manner. The precipatate which resulted from saturation to 80% with ammonium sulfate was dissolved in 200 ml of distilled water and centrifuged at 15,500 rpm (31,000 X G) for one hour. The pH was adjusted to 5.2 with 0.1 M HCl and again centrifuged at the above speed and time.

A 2% protamine sulfate solution was added in a fine stream to the above supernatant solution at room temperature. The strands of the nucleic acid-protamine precipitate were removed by centrifugation at 11,000 rpm (14,500 X G) for 15 minutes. This process was repeated until the ratio of the light absorption at 280 mu to that at 260 mu was above 0.7. This analysis will be mentioned later.

The pH was again adjusted to 7 and an ammonium sulfate fractionation was performed by adding saturated ammonium sulfate solution, pH 7, with stirring, until the system was 30% saturated with the salt. The precipitate was removed by centrifugation and subsequent fractions at 40, 50, 60, and 80% saturation were obtained. This was done at room temperature. These fractions, after centrifugation, were suspended in a minimal volume of distilled water and the fraction containing the highest amount of activity was saved.

The catalase solution was dialyzed overnight against 0.5% ammonium sulfate solution, pH 7, and again centrifuged at 15,500 rpm for one hour. The resultant solution was treated with neutral saturated ammonium sulfate solution until 90% saturation occurred. After centrifugation at

11,000 rpm, the precipitate was stored at -15°C.

I. Protein Determinations

It was necessary to know the protein content of the various solutions obtained in the course of the purification in order to be able to calculate the specific activities of the enzyme. The initial protein content of the cells could be estimated by assuming that it made up 50% of their dry weight. Two milliliter samples of cell suspensions were heated in an oven at 99°C for three hours and the resulting solid material was weighed and corrected for total dry weight.

The protein contents of cell free extracts were determined either turbidimetrically as trichloroacetic acid precipitates[23] or spectrophotometrically[28] based on their absorption at 280 and 260 mu.

Crystalline lysozyme was used to establish a standard curve in the turbidimetric analysis and readings were taken in a Bausch & Lomb Spectronic "20" at 540 mu. The spectrophotometric protein determination of Warburg and Christian[28] was used when the nucleic acid content of the cell free extracts was less than 10%. Readings were taken in a Beckman DU Spectrophotometer (Beckman Instruments, Inc.).

RESULTS

A. Kinetics of Catalase-Hydrogen Peroxide Reaction

When catalase and hydrogen peroxide react, oxygen is evolved. However, in this interaction the catalase is denatured following first order kinetics. This denaturation results in essentially all of the oxygen in the assay system being released in twenty-three minutes.

During routine assays on both heat labile and heat stable enzymes, it was found that the heat labile enzyme was denatured by hydrogen peroxide following first order kinetics as above while the heat stable enzyme was denatured following second order kinetics. That is, the rate of denaturation was dependent on the catalase concentration raised to the second power. However, upon partial inactivation by heat, guanidine, dialysis against distilled water, or storage of the partially purified enzyme at -15^{o}C for several weeks, the denaturation of heat stable enzyme becomes very close to first order. The plot of the denaturation by hydrogen peroxide of the "native" heat stable enzyme following second order kinetics and the heat labile enzyme following first order kinetics are shown in Figure 1.

The heat stable catalase activity has a very sharp optimum at pH 7 as seen in Figure 2, a plot of enzyme activity versus pH. Therefore, in all assays of this enzyme, one ml of 0.1 M phosphate buffer was added to adjust the assay system to neutrality.

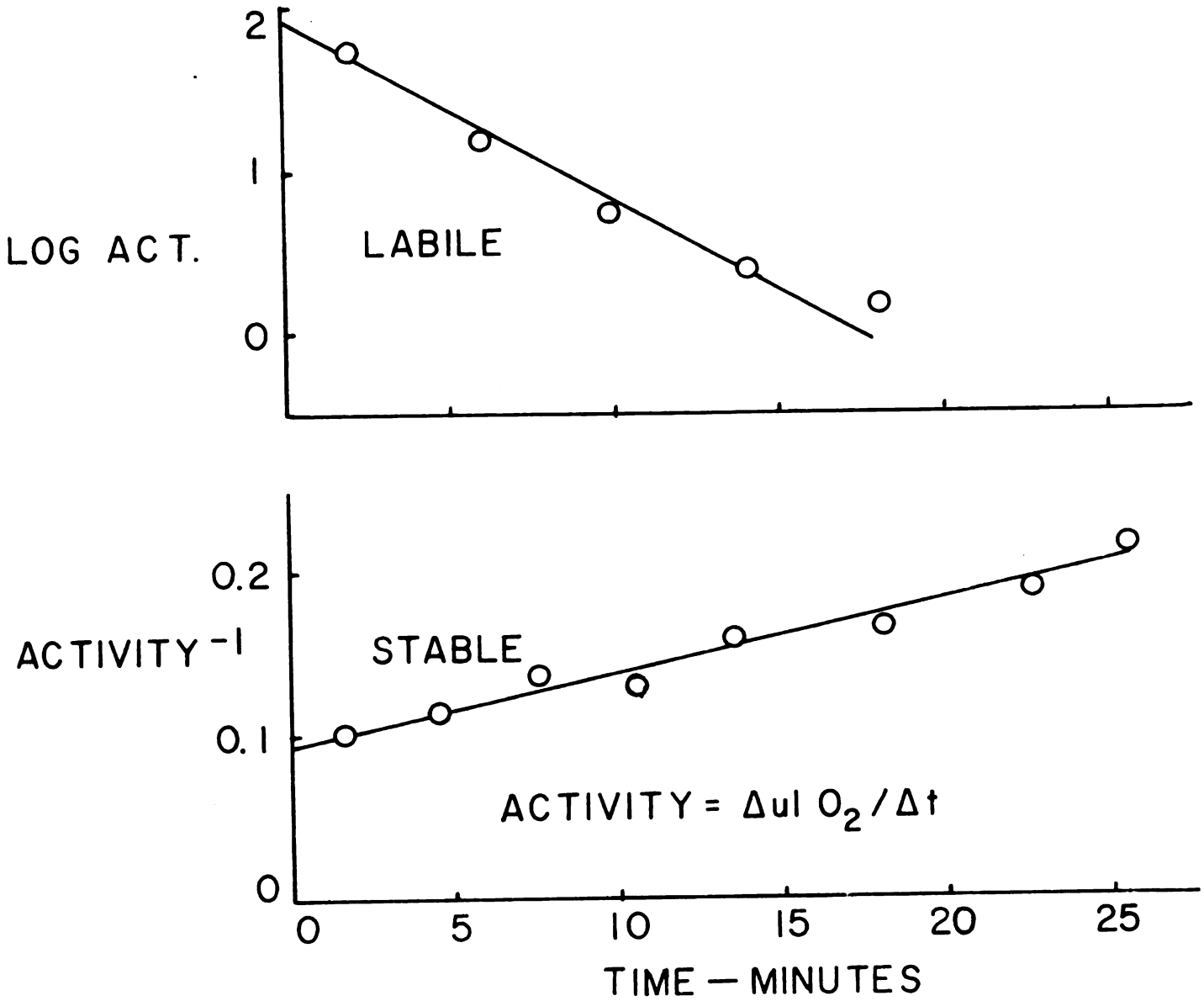

Figure 1: The denaturation of heat labile and heat stable catalase by hydrogen peroxide showing first order and second order kinetics respectively.

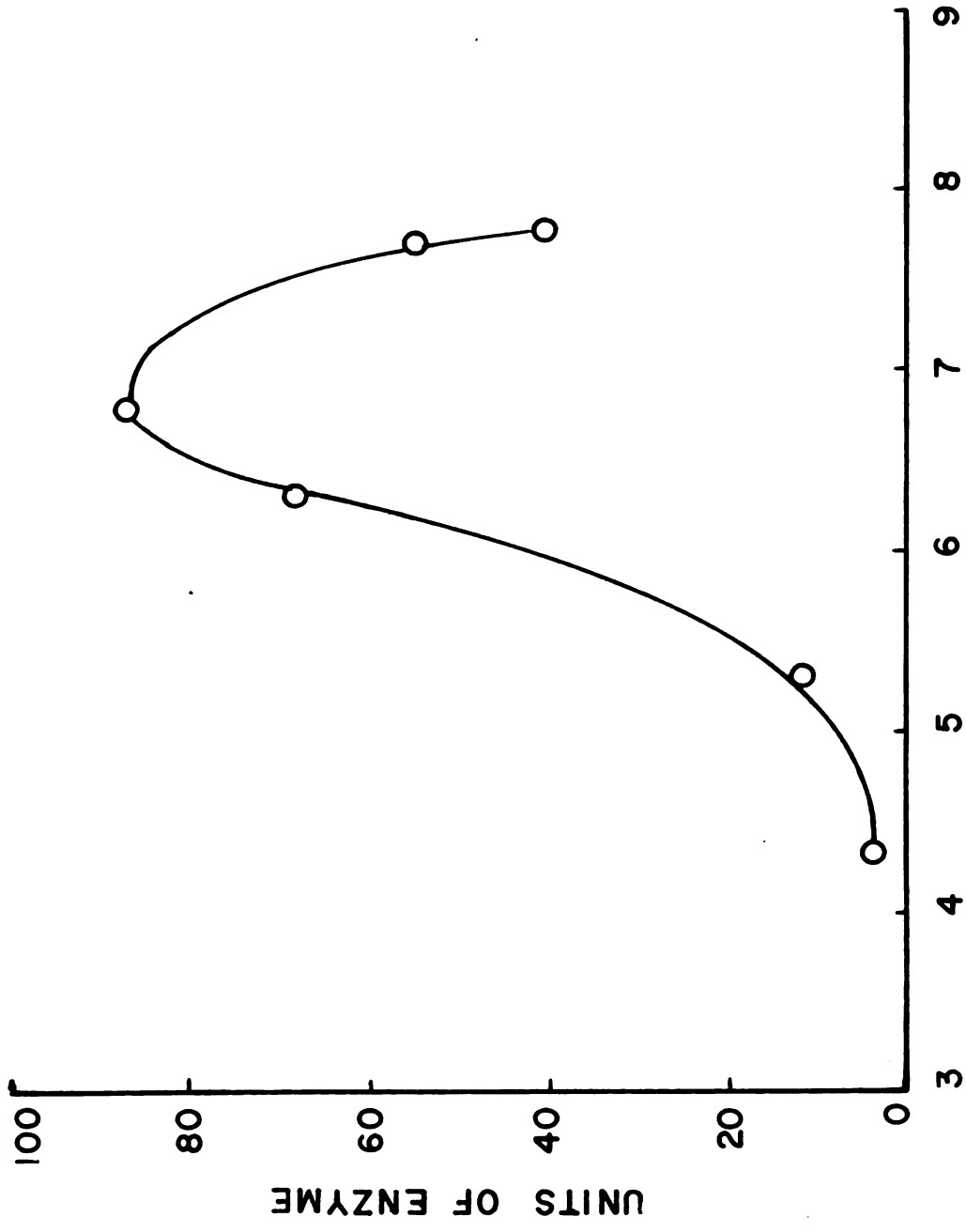

Figure 2: The plot of enzyme activity versus pH demonstrating optimal pH of the heat stable catalase.

B. Preparation of Cell Free Extracts

The breakage of cells by means of the Servall Omni-mixer produced a maximal release of resistant enzyme that was only 50% of that contained in whole cells. Various combinations of cells, beads, and buffer were used as well as various grinding speeds but neither high nor consistent yields of enzyme were obtained.

The lytic method of breaking cells permits the release of as much as 95% of the available heat resistant enzyme. However, in the purification procedure outlined, only 47% of the total enzyme was extracted from the cells, probably due to a partially denatured lytic enzyme.

C. Heat Inactivation Studies

The thermal inactivation of proteins follows first order kinetics. These kinetics would result if the breakage of one bond caused the denaturation of the protein. First order reactions when plotted with parameters log concentration of reactant versus time produce a straight line. Heat inactivation data for extracts of cells containing heat labile and resistant catalase exhibit bimodal characteristics. The higher the inactivation temperature the greater was the negative slope of the straight line when the log of the enzyme activity was plotted against time of heating (Figure 3). It can be seen, therefore, that heat inactivation does follow first order kinetics, and the rate of inactivation is related to the temperature. Only the results of the inactivation at 70° and 80°C are shown but the data corresponding to 77°, 83°, and 85°C were of a similar nature. The heat labile catalase was rapidly destroyed

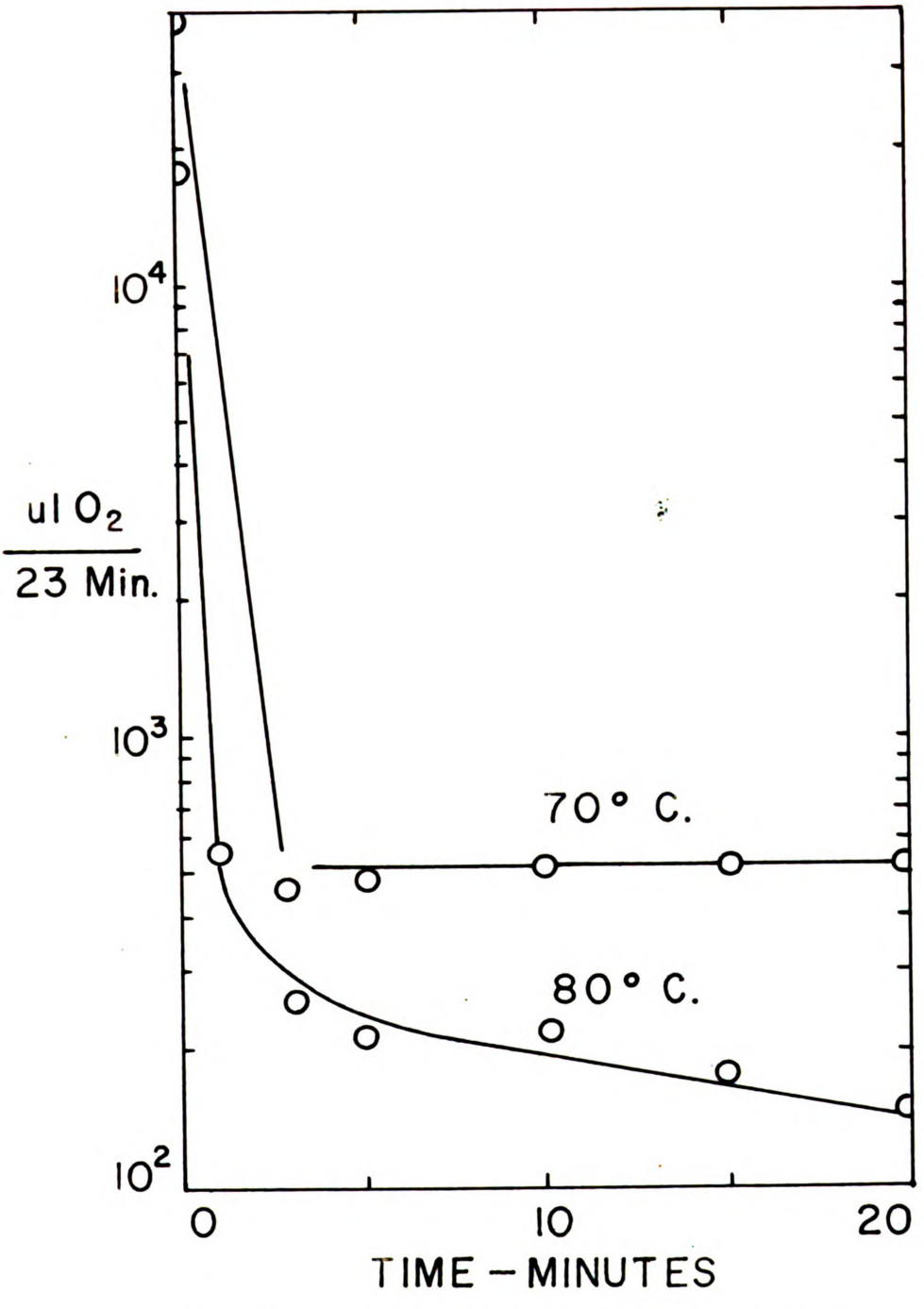

Figure 3: Heat inactivation of the catalase contained in cell free extracts of Bacillus cereus showing bimodal response and first order inactivation kinetics.

at these temperatures. In the study of the denaturation of heat stable and labile catalase, the denaturation followed first order kinetics as expected.

The inactivation constant or "k'" value for each temperature is determined by using the equation:

$$k' = 2.303 \times (\text{Slope of the inactivation curve per second}) \quad (1)$$

This specific reaction rate can be related to the absolute temperature by means of the Arrhenius equation in which it is assumed that there is a constant activation energy for each process.

$$\frac{d \ln k'}{dT} = \frac{A}{RT} \quad (2)$$

where: k' = reaction rate

T = absolute temperature

R = gas constant

A = activation energy

By plotting the log of "k'" for each temperature versus the reciprocal of its corresponding absolute temperature (Figure 4) the activation energy can be calculated. The enthalpy of the activation process, $\Delta H^{\ddagger}$, can be calculated by:

$$\Delta H^{\ddagger} = A - RT \quad (3)$$

The change in entropy of the activation process is found by using the Eyring equation:[11]

$$k' = (kT/h) \exp. (\Delta S^{\ddagger}/R) \exp. (-\Delta H^{\ddagger}/RT) \quad (4)$$

where: k' = inactivation constant

R = gas constant

T = absolute temperature

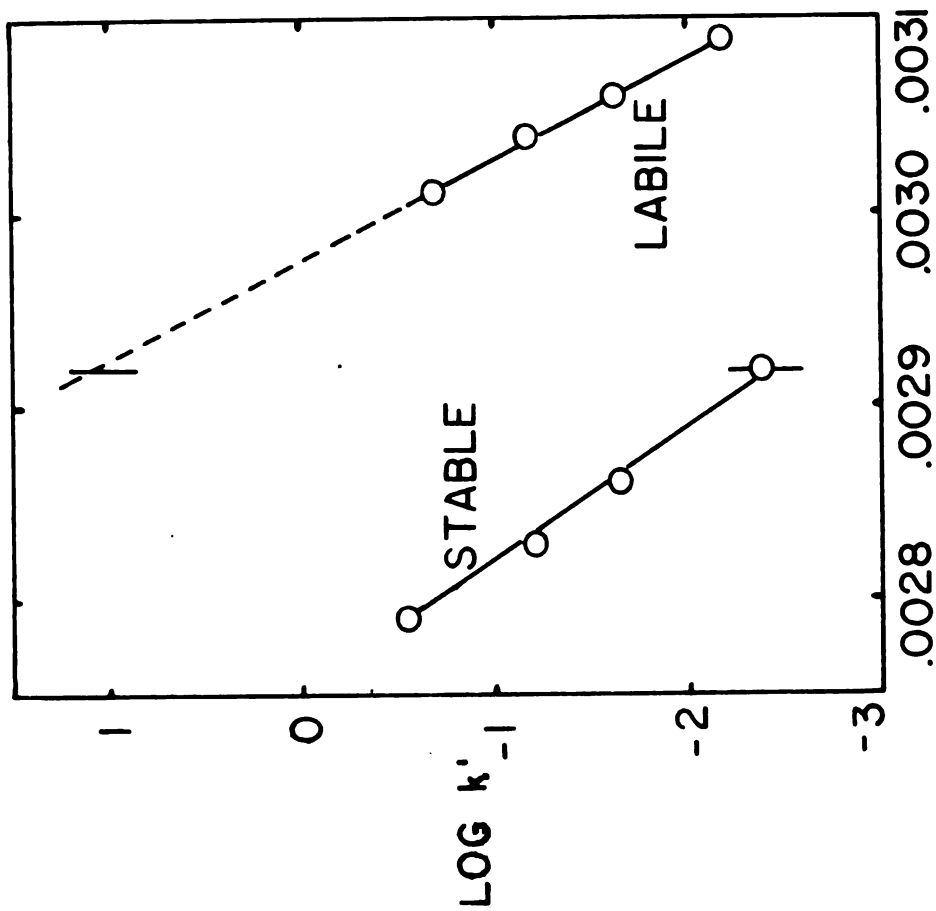

Figure 4: Arrhenius plot of the inactivation data for heat labile and stable catalase.

k = Boltzmann constant

h = Planck's constant

$\Delta S^{\ddagger}$ = change in entropy

$\Delta H^{\ddagger}$ = change in enthalpy

The value of $\Delta H^{\ddagger}$ obtained from equation (3) is substituted into equation (4) and after solving for $\Delta S^{\ddagger}$ it is possible to determine the free energy ($\Delta F^{\ddagger}$) of activation of the denaturation process:

$$\Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \qquad (5)$$

It would be desirable to compare thermodynamic properties of the denaturation of both enzymes at the same temperature. Because it was not possible to measure the denaturation process at the same temperature, the Arrhenius plot for denaturation of the heat labile enzyme was extrapolated to 70°C. The summary of the results of these calculations are found in Table 1.

Table 1: Thermodynamic properties of heat resistant and heat labile catalase when inactivated at 70°C and pH 6.9.

	Stable	Labile
k' sec^{-1}	6.7×10^{-5}	1.7×10^{-1}
$\Delta H^{\ddagger}$ cal.	7.3×10^{4}	8.3×10^{4}
$\Delta S^{\ddagger}$ cal.	134	178
$\Delta F^{\ddagger}$ cal.	2.7×10^{4}	2.2×10^{4}

D. Guanidine Inactivation Studies

The guanidine inactivation was run in an effort to show differences in the tertiary structures of the two proteins. Although there are

marked differences between the two catalases with respect to their inactivation by guanidine, no quantitative comparisons could be made. The amount of inhibition was proportional to the concentration of guanidine in the case of both enzymes. In order to establish the kinetics of the guanidine inactivation, it was necessary to measure the rate of inactivation. However, this was not possible in the case of the heat stable enzyme. Figures 5 and 6 are curves showing respectively the time course of the inactivation by various concentrations of guanidine of the heat labile and heat stable catalase.

The inactivation of the enzyme can be expressed by the equation:

$$-dE/dt = kC_g^n \qquad (6)$$

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 interger "n" must be even since two moles of guanidine are required to break a hydrogen bond in a protein. The reciprocal of the time required for a given inactivation of the catalase is also an expression for the inactivation rate.

$$-dE/dt = F(1/t_{50\%}) \qquad (7)$$

In the present study, the time for 50% inactivation was used.

Then:
$$1/t_{50\%} = kC_g^n \qquad (8)$$

By taking the logarithm of each side of the equation:

$$\log 1/t_{50\%} = \log k + n \log C_g \qquad (9)$$

When plotting $\log 1/t_{50\%}$ versus $\log C_g$, the slope of the line indicates the number of moles of guanidine needed to inactivate one mole of enzyme.

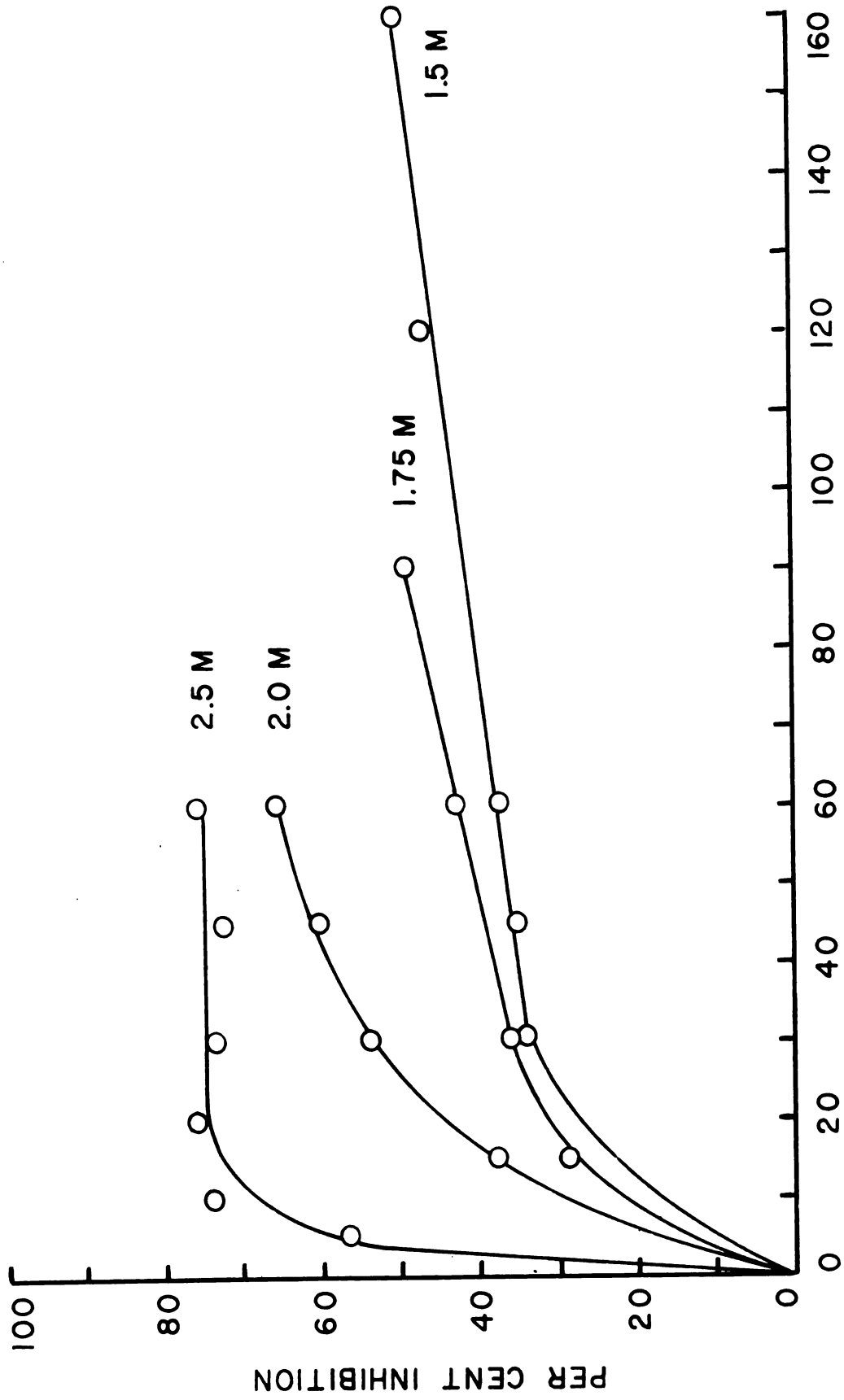

Figure 5: The time course of the inactivation of heat labile catalase by various concentrations of guanidine hydrochloride.

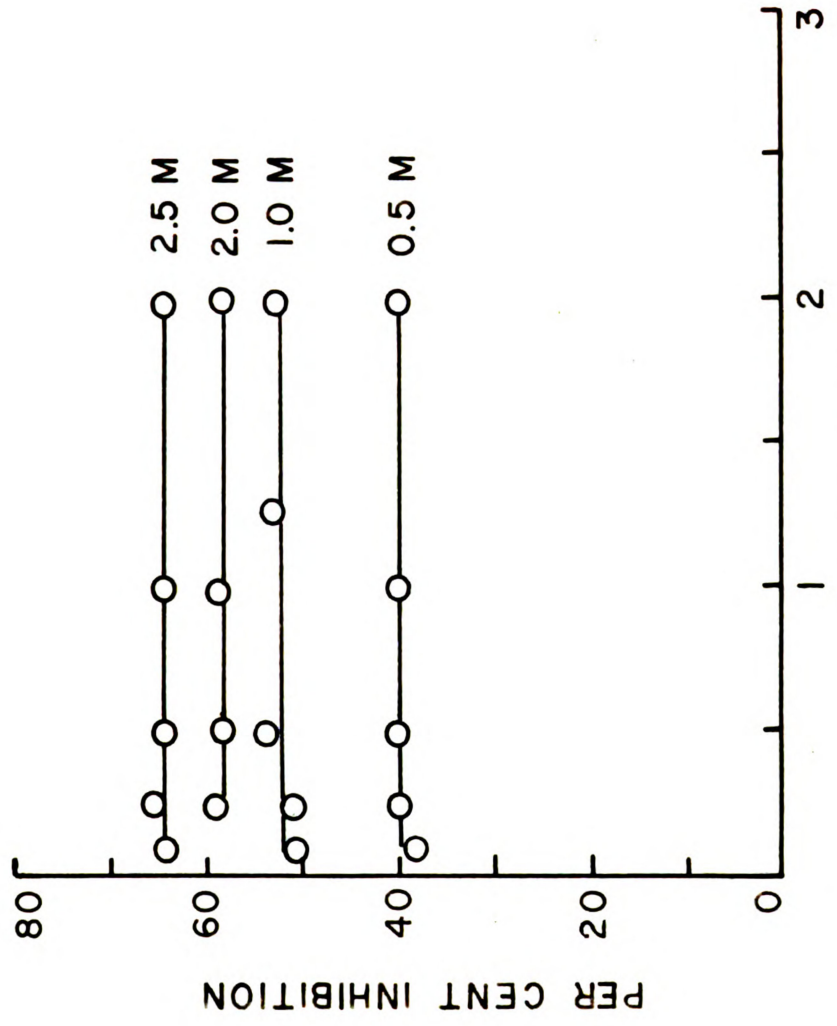

Figure 6: The time course of the inactivation of heat stable catalase by various concentrations of guanidine hydrochloride.

It was found that eight molecules of guanidine were needed to inactivate one molecule of heat labile enzyme.

E. Purification

The initial stage of purification consisted of heating a suspension of B. cereus cells in distilled water at 68°C for 30 minutes which caused a yellow soluble substance to be released. Then, upon centrifugation, the cells packed into a dense uniform pellet which was rather easily resuspended. This was not true if the cells were not heated. There was no catalase activity in the yellow supernatant solution and it was discarded.

It was essential to wash the debris which resulted from the lysis of the bacteria with buffer at pH 7. At pH values below 7, the enzyme was adsorbed strongly to this material.

The supernatant solution from the lysed bacteria was very difficult to centrifuge due to the presence of nucleoproteins. A combination of centrifugation and paper filtration was necessary to clarify the solution. At pH 7, there was no loss of activity due to adsorption of the enzyme on the paper.

The concentration of the enzyme with ammonium sulfate was done to reduce the volume for ease of handling. Better results were obtained using saturated ammonium sulfate solution at pH 7 than solid ammonium sulfate.

The enzyme at this stage of purification was soluble at a pH range of at least 5.0 to 7.0 and the ammonium sulfate precipitate could be dissolved in distilled water at a pH of 6.0. A subsequent centrifugation at 15,500

rpm removed most of the remaining larger particulate matter.

No loss of activity occurred when the pH was lowered to 5.2 and 2% protamine solution was added to the enzyme. The nucleic acid removal was continued until the 280/260 mu ratio was above 0.7. If a large amount of ammonium sulfate was present, the protamine caused an irreversible precipitation of the enzyme.

The ammonium sulfate fractionation resulted in 71% of the activity being precipatated between 40 and 50% saturation. Dialysis of this fraction caused the precipation of more contaminating proteins. It was not possible to dialize against distilled water due to the high loss of enzyme activity. It was found that dialysis against all the salts of the growth media and DPA prevented this loss and it was later found that a 0.5% neutralized ammonium sulfate solution alone could be used to prevent the loss of enzyme activity. The loss of heat resistant activity when dialysis takes place against various salts and DPA is shown in Table 2.

The final heating at 68°C for 20 minutes showed that 3.5% heat labile activity was carried along in the purification procedure or alternatively the enzyme was converted to its labile counterpart.

The summary of the purification results are found in Table 3. Using this procedure, a purification ratio (increase in specific activity) of 80 was obtained.

Table 2: The stability of heat resistant catalase when dialyzed against various salts for 24 hours.

Tank	Contents	Concentration	Percent loss of activity
0*	--------	-------	42
1	$(NH_4)_2SO_4$	0.4%	3
2	K_2HPO_4	0.1%	29
3	$MgSO_4$	0.08%	21
4	$MnSO_4 \cdot H_2O$	0.01%	16
5	$ZnSO_4$	0.001%	34
6	$CuSO_4 \cdot 5H_2O$	0.001%	61
7	$CaCl_2$	0.001%	32
8	$FeSO_4$	0.0001%	34
9	DPA	0.000M	37
10	H_2O	-------	29

* Enzyme preparation in dialysis bag held at 4^oC in air.

Table 3: Summary of the purification of heat stable catalase

	Volume (ml)	Total Protein (g)	Activity (ul/ml)	Specific Activity (act./g)	Purification	Percent Yield
Heated Bacteria	750	20	107,500	5,375	1	100
Lysed Bacteria	2160	2.16	50,400	23,333	4	47
Concentration and Centrifugation	192	0.119	39,040	328,067	61	36
Adjustment of pH to 5.2	184	0.107	37,414	349,664	65	35
Protamine-Ammonium Sulfate Fractionation	14.4	0.077	21,936	284,883	53	20
Dialysis	14.8	0.055	20,375	370,455	69	19
Heating at $68^{\circ}C$ for 20 Minutes	12.8	0.039	16,725	428,846	80	15

DISCUSSION

Following the exponential growth of a culture of B. cereus, there occurs a sequence of events which leads ultimately to the production of spores. While the protein of the "parent" bacterial cell is very susceptible, spore protein is very resistant to thermal denaturation. The overall mechanism by which the cell imparts this heat resistance is unknown but it can be assumed that, at some time in the sporulation process, heat resistant spore protein could exist in the vegetative cell. Recently, Sadoff (unpublished) has shown that the resistant enzyme studied in this investigation is serologically identical with spore protein. This observation lends credence to the above assumption which presents the possibility of studying heat resistance at the enzymatic level in the sporulating cell. The principal advantage of such a study is the use of a model system which is independent of the multitude of factors involved in the viability of the cell. The kinetics of the formation of the heat resistant enzyme can be readily observed and conclusions can be drawn concerning the origin of heat resistant proteins.

The resistant catalase which was investigated was of low concentration and contained impurities which made impractical spectrophotometric or iodometric determinations of the enzyme concentration. Therefore, it was necessary to employ a sensitive and reproducible

manometric assay procedure. The following considerations will show that the total oxygen evolution from peroxide reflects the initial enzyme concentration.

The rate of disappearance of enzyme in the presence of substrate is given by:

$$-dC/dt = k_1 C \qquad (10)$$

where k_1 is the reaction constant for inactivation of catalase by hydrogen peroxide and C is the concentration of catalase. Therefore, the concentration of enzyme at any time is given by:

$$C = C_o e^{-k_1 t} \qquad (11)$$

where C_o is the initial enzyme concentration.

The rate of oxygen evolution from excess hydrogen peroxide in the presence of the enzyme is:

$$dO_2/dt = k_2 C \qquad (12)$$

where k_2 is equal to the turnover number. Therefore:

$$dO_2/dt = k_2 C_o e^{-k_1 t} \qquad (13)$$

$$\int_o^{O_2 Total} dO_2 = \int_o^t k_2 C_o e^{-k_1 t}\, dt \qquad (14)$$

$$O_2 Total = k_2/k_1\ C_o(1-e^{-k_1 t}) \qquad (15)$$

Therefore, when the duration of the assay is held constant, the oxygen evolution is directly related to the initial enzyme concentration. The assay is applicable to the determination of changes in enzyme concentration which occur in various denaturation studies.

The thermal inactivation experiments yielded two important facts. First, the heat resistant catalase was denatured in a manner similar to a protein. This lent support to other observations (cyanide inhibition, autoclaving) that the material in question was really an enzyme. Secondly, it was shown that the thermodynamics of the denaturation process were very similar to those for the thermodenaturation of other proteins.[7, 14] It might be assumed, therefore, that the magnitude of difference between heat resistant proteins as a class and their labile counterparts is no greater than the differences which exist between labile proteins. It should also be expected that differences in primary and tertiary structure will exist between heat labile and heat stable proteins catalyzing the same reactions. The pronounced difference between the tertiary structure of the labile and stable catalases was demonstrated by the results of the guanidine inactivation studies.

The technique, which has been described, could probably identify reactions of at least the sixth order with respect to guanidine. It was shown that the inactivation of labile enzyme was eighth order but the kinetics of inactivation of the thermostable catalase could not be measured. The data are not compatible with zero order kinetics; therefore, the heat stable enzyme undergoes either a second or a fourth order inactivation reaction. The determination of the exact reaction order will have to be found through different techniques, possibly by using urea which also breaks hydrogen bonds.

The ease with which the stable catalase is denatured by hydrogen bond reagents is reflected in its instability in the course of dialysis. The loss of activity which occurred when the enzyme was dialyzed against DPA would tend to indicate that stabilization of the enzyme molecule is not the function of the pyridine compound. It would also indicate that the loss of activity in distilled water is not due to the loss of DPA. Dialysis of the enzyme versus ammonium sulfate resulted in the loss of only 3% of the activity and led to the routine use of a 0.5% ammonium sulfate solution for dialysis.

The pH activity response for the heat stable enzyme is not typical of labile catalase since the activity occurs over too narrow a pH range. Obviously, this is a unique property of the stable enzyme and necessitates the measurement of activity at pH 7.

A purification of the heat resistant enzyme was attempted. The method of Herbert and Pinsent[10] for the purification of bacterial catalase was not applicable in this work. Since their recovery of enzyme was 19%, it was decided that work should be concentrated on a new, more efficient method of purification rather than attempting to adapt their method to the heat stable catalase.

The recovery by the method used was only 15% with an 80 fold purification. However, with modifications the method employed could give better yields. More active lytic enzymes along with a more careful ammonium sulfate fractionation should lead to a 50% yield. It is also evident that at least one or more extensive purification procedures must

be developed before the enzyme can be crystallized.

The occurrence of a heat resistant enzyme in vegetative cells parallels the results of Romig and Wyss[18] who found that ultraviolet resistance in bacilli occurred prior to spore formation. The role of DPA in the above resistance is unknown but it may function in some manner because the cells are undergoing sporulation and only spores contain DPA. It is also possible that another stabilizing factor rather than DPA is important in the functional mechanism imparting heat resistance. The spore coat or a certain peptide found by Powell and co-workers[16, 17, 25, 27] could be involved. Although DPA has been found in large quantities in the spore, no proof of its actual contribution to heat resistance has been found. It is hoped that with the continuation of this work some mechanism of heat resistance can be formulated.

SUMMARY

A heat resistant catalase has been found in vegetative cells of Bacillus cereus by Sadoff, Ragheb, and Kools.[19]

Heat inactivation was found to produce a free energy change of 27,000 calories in comparison to the heat labile enzyme free energy change of 22,000 calories.

Guanidine inactivation of both heat labile and stable enzymes showed that the heat stable enzyme was inactivated instantaneously. The rate of inactivation of the heat labile enzyme could be measured and approximately 8 moles of guanidine were found to inactivate one mole of catalase.

An 80 fold purification of the heat resistant enzyme was accomplished. The cells were broken by means of a lytic enzyme found in B. cereus at a later stage of sporulation than that at which the heat resistant enzyme was found. The primary method of purification was by heating the lysed bacterial solution at 68°C followed by a precipation of nucleic acids and an ammonium sulfate fractionation of the remaining proteins.

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