PHYSICAL STUDIES OF α-AMYLASE FROM *BACILLUS SUBTILIS* AND BARLEY

By

PATRICIO J. RIQUETTI

Bachelor of Science

Jacksonville State University

Jacksonville, Alabama

1969

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
May, 1973

PHYSICAL STUDIES OF α-AMYLASE FROM BACILLUS SUBTILIS AND BARLEY

Thesis Approved:

Earl D Mitchell
Thesis Adviser

H. Thomas Spivey

Billy B. Hudson

Kermit Carraway

N N Durham
Dean of the Graduate College

ACKNOWLEDGEMENTS

I wish to express my appreciation to my research adviser, Dr. Earl D. Mitchell, Associate Professor of Biochemistry at Oklahoma State University, for his guidance, counsel, and encouragement during this research program.

I also am grateful to Dr. H. Olin Spivey for his suggestions and assistance, to Drs. Kermit Carraway and Billy G. Hudson for their interest; also, my fellow lab-coworkers, Mr. Michael Downing and Mr. Ralph Loring for their valuable help. To Ms. Su Wang and Ms. Sarah Koeppe for their technical assistance; to Ms. Nancy Weber for her typing and data preparation, and to Mr. Paul Buck for his help with the computer programs used for calculations. The author would also like to thank Ms. Dixie Jennings for the typing of this thesis.

I am indebted to the Biochemistry Department for financial support and for the use of its facilities.

My special thanks go to Dr. and Mrs. I. M. Boozer, to my parents, Mr. and Mrs. Abdón Riquetti for their financial and moral support, and especially to my wife, Susanne for her patience, understanding, and encouragement during my course of study.

TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	1
II.	LITERATURE REVIEW	3
	Physicochemical Properties of *Bacillus subtilis* α-Amylase	3
	Physicochemical Properties of Barley α-Amylase	7
III.	EXPERIMENTAL METHODS	9
	Sample Preparation for Ultracentrifugation Studies	9
	Determination of Density, Viscosity, and Partial Specific Volume for Sample Solution	10
	Boundary Sedimentation Rates and Molecular Homogeneity	11
	Diffusion Rates by the Height-Area Method	12
	Conventional Sedimentation Equilibrium Method	13
	Meniscus Depletion Sedimentation Equilibrium Method	14
	Gel Filtration Chromatography	21
	Circular Dichroism: Spectropolarimeter Measurements	22
IV.	RESULTS AND DISCUSSION	23
	Bacillus subtilis α-Amylase	23
	Criteria for Enzyme Purity	23
	Sedimentation Coefficient - Concentrations and pH Independence	36
	Diffusion Coefficient	39
	Molecular Weight	39
	Enzyme Activation and Polymerization in the Presence of 1 mM Zinc or Calcium Ions	42
	Effects of Denaturing and Chelating Agents on the Sedimentation Coefficient	48
	Minimum Subunit Molecular Weight	48
	Sodium Dodecyl Sulfate Binding	52
	Gel Permeation Chromatography	55
	Circular Dichroism Studies	55
	Barley α-Amylase	63
	Sedimentation and Diffusion Coefficients	63
	Molecular Weight	70
V.	SUMMARY	71
A SELECTED BIBLIOGRAPHY		73

LIST OF TABLES

Table		Page
I.	Sedimentation Coefficient pH Dependence.	36
II.	Molecular Weight Determination From Sedimentation and Diffusion Data	39
III.	Molecular Weight Determination Data From Conventional Sedimentation Equilibrium Ultracentrifugation.	40
IV.	Molecular Weight Determination Data From Meniscus Depletion Sedimentation Equilibrium Ultracentrifugation.	41
V.	Enzyme Activation by Calcium and Zinc Ions	42
VI.	Calcium and Zinc Effects on the Sedimentation Coefficient. . .	43
VII.	Effects of Denaturing and Chelating Agents on the Sedimentation Coefficient.	49
VIII.	Subunit Molecular Weight in 6 M Guanidine Hydrochloride Meniscus Depletion Sedimentation Equilibrium Ultracentrifugation.	49
IX.	Sodium Dodecyl Sulfate Binding	52
X.	Circular Dichroism	60
XI.	Molecular Weight Determination Data From Meniscus Depletion Sedimentation Equilibrium Ultracentrifugation.	70

LIST OF FIGURES

Figure		Page
1.	Interference Pattern at Equilibrium for a Conventional Sedimentation Equilibrium Ultracentrifugation Run.	16
2.	Interference Patterns, Initial Concentration Determination . . .	18
3.	Interference Pattern at Equilibrium for a Meniscus Depletion Sedimentation Equilibrium Ultracentrifugation Run.	20
4.	Sedimentation Velocity; Schlieren Patterns	25
5.	Diffusion Patterns	27
6.	Diffusion Coefficient Plot	29
7.	Conventional Sedimentation Equilibrium; Plot of Fringe Displacement Versus Radial Distance.	31
8.	Sedimentation Velocity Plot.	33
9.	Meniscus Depletion Sedimentation Equilibrium; Plot of Fringe Displacement Versus Radial Distance.	35
10.	Sedimentation Coefficient; Concentration Dependence.	38
11.	Sedimentation Coefficient; Effects of Calcium Ions	45
12.	Sedimentation Coefficient; Effects of Zinc Ions.	47
13.	Meniscus Depletion Sedimentation Equilibrium; Subunit Molecular Weight	51
14.	Sedimentation Velocity; Binding of Sodium Dodecyl Sulfate. . . .	54
15.	Gel Permeation Chromatography; Molecular Weight.	57
16.	Gel Permeation Chromatography; Subunit Molecular Weight.	59
17.	Circular Dichroism in Various Solvent Media.	62
18.	Sedimentation Coefficient; Concentration Dependence.	65
19.	Sedimentation Velocity Plot.	67

Figure Page

20. Diffusion Coefficient Plot 69

LIST OF PRINCIPAL SYMBOLS AND ABBREVIATIONS

η	Viscosity
λ	Wavelength
ω	Angular velocity in radians per second
π	Constant equal to 3.1416
ρ	Density in grams per cubic centimeter
θ	Ellipticity
$[\theta]$	Molar residue ellipticity
A	Area, usually in square centimeters
c	Concentration
c_m	Concentration at the meniscus
c_b	Concentration at the bottom of the cell
c_o	Initial concentration
D	Diffusion coefficient in square centimeters per second
F	Magnification factor for image enlargement due to camera lens
J	Total fringe shift
j	Fringe shift
M	Molecular weight
R	Universal gas constant; equal to 8.315×10^7 ergs per degree per mole
r	Radial distance in centimeters, corrected for camera lens magnification
r_m	Radial distance to the meniscus
r_b	Radial distance to a point in the plateau region
S	Svedberg; unit of sedimentation rate equal to 10^{-13} seconds

s	Sedimentation coefficient
$s_{20,w}$	Sedimentation coefficient corrected to the value it would have in a solvent with the viscosity and density of water at 20^{o} C
T	Absolute temperature
t	Time
$\bar{v}$	Partial specific volume in cubic centimeters per gram
x	Radial distance measured on photographic plates before correction for camera lens magnification
Δ_x	Radial distance increment
dc/dr	Absolute boundary height of schlieren patterns
y	Vertical distance measured on photographic plates; derivative of concentration with respect to radius; the concentration gradient
Max	Maximum
C	Degrees centigrade
K	Degrees Kelvin
SDS	Sodium dodecyl sulfate
GuHCl	Guanidine hydrochloride
EDTA	Ethylenediaminetetraacetic acid
sch	Schlieren
B. subtilis	Bacillus subtilis

LIST OF EQUATIONS

$$s = \frac{1}{\omega^2 r}\frac{dr}{dt} = \frac{2.303}{60\ \omega^2}\left(\frac{d\ \log\ x}{dt'}\right) \tag{1}$$

t Time in seconds

t' Time in minutes

$$s_{20,w} = s_{obs}\left(\frac{\eta_t}{\eta_{20}}\right)\left(\frac{\eta_{sol}}{\eta_\omega}\right)\left(\frac{1 - \bar{v}\rho_{20,\omega}}{1 - \bar{v}\rho_{t,sol}}\right)$$

$$\rho_{t,sol} \approx \left(\frac{\rho_{sol}}{\rho_\omega}\right)\rho_{t,\omega} \tag{2}$$

s_{obs} Observed sedimentation coefficient

η_t Viscosity of water at t degrees (temperature of centrifuge run)

η_{20} Viscosity of water at 20 degrees

η_{sol} Viscosity of sample solution at known temperature, t'

η_ω Viscosity of water at t' degrees

$\rho_{20,\omega}$ Density of water at 20 degrees

$\rho_{t,sol}$ Density of sample solution at t degrees (temperature of centrifuge run)

$\rho_{t,\omega}$ Density of water at t degrees (temperature of centrifuge run)

$$D_A = \frac{(A_{sch})^2}{\left(\frac{dc}{dr}\right)^2_{max}}\frac{1}{4\pi t} \tag{3}$$

D_A Diffusion coefficient determined by the height-area method

A_{sch} Area under a schlieren peak in cm^2

$$D_{20,\omega} = D_{obs}\left(\frac{293.2}{T}\right)\left(\frac{\eta_t}{\eta_{20}}\right)\left(\frac{\eta_{sol}}{\eta_\omega}\right) \tag{4}$$

D_{obs} Observed diffusion coefficient in cm^2 sec

$D_{20,\omega}$ Diffusion coefficient corrected to the value it would have in a solvent with the viscosity of water at 20

η_t Viscosity of water at t degrees (temperature of diffusion run)

η_{20} Viscosity of water at 20 degrees

η_{sol} Viscosity of sample solution at known temperature, t'

η_ω Viscosity of water at t' degrees

$$M = \frac{RTs}{D(1 - \bar{v}\rho)} \tag{5}$$

$$M = \frac{2RT}{(1 - \bar{v})\omega^2}\,\frac{2.303(d \log c)}{d(r^2)} \tag{6}$$

$$c_m = c_o - \frac{r_b^2(c_b - c_m) - \int_{r_m}^{r_b} r^2\, dc}{r_b^2 - r_m^2} = c_0 - \frac{r_b^2(c_b - c_m) - \Delta j \sum_{r_m}^{r_b} r^2\, dc}{r_b^2 - r_m^2} \tag{7}$$

Δj Increment of fringe shift - usually one fringe

CHAPTER I

INTRODUCTION

The amylases (α-1, 4-glucan-4-glucanohydrolase EC 3.2.1.1.) catalyze the hydrolysis of an α, 1 4-linked glucose polymer by the transfer of a glucosyl residue to water (1). α-amylases hydrolyze the most internal bonds (2) and are then classified as endoenzymes. Bacillus subtilis α-amylase is available in large quantities from culture media and can be easily purified and crystallized (3-9) under conditions that make it very suitable for experimentation.

α-amylase produced by different strains of Bacillus subtilis is classified into two types based on its mode of action on starch: 1) the saccharifying type and 2) the liquifying type (1). This study deals with the latter type.

Inconsistent molecular weights and related parameters for B. subtilis α-amylase have been reported in the literature (10,11,12). Experimentation in this laboratory was conducted to settle the inconsistencies on such values as molecular weights of the native enzyme, sedimentation coefficients for the enzyme in various buffers and divalent ion concentrations (Ca^{++}, Zn^{++}), diffusion coefficient, subunit structure of the enzyme and its secondary structure.

Different amylase forms have been demonstrated in higher plants (13, 14). Greenwood and Milne (13) reported that α-amylases from ungerminated and germinated cereal grains had similar size. Olered and Jonsson (14)

showed that there are two forms of α-amylase in wheat. Schwimmer and Balls (15) prepared a crystalline α-amylase from barley malt and Waldschmidt-Leitz and Sigrist (16) from green malt. Homogeneous barley amylase can be obtained (17); however, homogeneity changes from one preparation to another (18). Molecular weights for barley α-amylase have been reported by Greenwood, et al. (19) and Mitchell (17). Experimentation to confirm the molecular weight, the corrected sedimentation coefficient and the diffusion coefficient for barley α-amylase were conducted.

CHAPTER II

LITERATURE REVIEW

Physicochemical Properties of *Bacillus subtilis* α-Amylase

α-amylases were named by Kuhn (20) because the hydrolytic products possess the α configuration. Meyer, *et al.* (21) accomplished the crystallization of an amylase from *Bacillus subtilis* for the first time. A great deal of information on bacterial and mold amylases is found in the literature (1).

Pure crystalline *B. subtilis* α-amylase has been purified and crystallized by Hagihara (3), Yamamoto (4), Felling, *et al.* (5), Fukumoto, *et al.* (6,7), and Stein, *et al.* (8,9). Bacterial amylase from *Bacillus subtilis* contains no sugar (22), cysteine or cystine (22,23). A characteristic of this enzyme is its relative high content of glutamic and aspartic acids, tryptophan, and tyrosine, and low content of methionine (22,23). Although there are not any striking similarities or differences in amino acid composition among bacterial and mold α-amylases (1), Akabori, *et al.* (22) point out similarities among pancreatic amylase from swine, human salivary amylase, Taka-amylase A, and bacterial amylase from *Bacillus subtilis* which are relatively high in aspartic acid and low in methionine. They also indicated that in view of these results, the amino acid compositions are not responsible for the enzymatic action, but rather, some particular peptide section having a specific amino acid

sequence provides the enzymes with the structural conformation needed for enzymatic activity.

The role of calcium as a stabilizer ion of α-amylase was demonstrated by Wallertein in 1909. B. subtilis α-amylase was reported to be a calcium metallo-enzyme by Fisher, et al. (24), Yamamoto (25), and Sumerwell, et al. (26). More recently Stein, et al. (27) prepared calcium-free B. subtilis α-amylase by EDTA chelation and electrodialysis; full reactivation was accomplished by the reintroduction of calcium. Hsiu, et al. (28) elaborated on the same phenomena and concluded that B. subtilis α-amylase required at least four or more g-atoms of calcium per mole of enzyme for full activity; this requirement was interpreted as a formation of a metal-chelate structure, in which the metal produces intra-molecular cross-links similar in function to disulfide bridges, which confer to the α-amylase molecule the structural rigidity required for effective catalytic activity. Fisher, et al. (24) reported the metal content of crystalline B. subtilis α-amylase as 3.0 g-atom of calcium and 0.5 g-atom of zinc per mole of enzyme. The role of zinc in B. subtilis α-amylase according to Fisher, et al. (24) is the formation of a bridge which holds together two units of enzyme. In 0.05 M calcium acetate, 0.1 M NaCl and 0.01 M EDTA (pH 7.0), each enzyme unit had a molecular weight of 48,900, a sedimentation coefficient of 4.56 S, a diffusion coefficient of 7.98×10^{-7} cm^2/sec and a partial specific volume ($\bar{v}$) of 0.717 calculated from amino acid composition (24).

The following equation describes the mode of zinc action:

$$(\text{protein-Ca}_x) \text{ — Zn — } (\text{protein-Ca}_x) \underset{Zn^{++}}{\overset{\text{Metal Binding Agents}}{\rightleftharpoons}} 2(\text{protein-Ca}_x)^- + Zn^{++}$$

For the dimer which Fisher, et al. (24) call native enzyme, a molecular weight of 96,900 daltons was reported, a $s_{20,w}$ of 6.47 S, a $D_{20,w}$ of 5.72×10^{-7} cm^2/sec and a partial specific volume ($\bar{v}$) of 0.717. Junge, et al. (23) report a monomer molecular weight of 48,700 from ultracentrifugation data and 48,805 as the average molecular weight calculated from all 17 amino acids present; from this data they conclude that it appears very unlikely that the molecules corresponding to this size are constituted of identical subunits, since no common denominator exists for the assumed numbers of residues obtained from the amino acid present in the amylase molecule. Likewise there is no common denominator for the six amino acids which have only one possible integer within the limits of error. The mean calculated molecular weight pertaining to these six amino acids is 49,087 (23). Isemura, et al. (10) reported that the sedimentation coefficient of α-amylase from Bacillus subtilis decreased with dilution. At concentrations of more than 8.0 mg/ml, a single sedimentation peak was observed with a constant sedimentation coefficient of 6.10 S, and a diffusion coefficient of 6.0×10^{-7} cm^2/sec (calculated by the inflection point method). At concentrations less than 5.5 mg/ml they reported a single sedimenting peak deviating somewhat from the Gaussian curve and a sedimentation coefficient which decreased as a function of the protein concentration and the time of sedimentation. However, Fisher, et al. (24) showed that the sedimentation coefficient of the native B. subtilis α-amylase (M.W. 96,900 daltons) does not depend on the enzyme concentration. Isemura, et al. (10) also report that native enzyme exists as a dimer:

$$2 \text{ Monomers} + Zn^{++} \rightleftharpoons \text{Dimer}$$

where the monomer has a molecular weight of 45,000 daltons. Isemura and Kakiuchi (29) reported that the dimerization of bacterial α-amylase (B. subtilis) may result from zinc ions at the sites of histidyl residues located on the surface of the enzyme molecule. Among twelve histidyl residues in a B. subtilis α-amylase molecule, only one or two may be located on the surface and contribute to the association.

A pH change from 6 to 5 was reported as responsible for a decrease of the sedimentation coefficient from 6.2 S to 4.4 S (29); outside this range the $s_{20,w}$ was shown to remain constant. Kakiuchi, et al. (29) concluded that to study the monomer-dimer transformation by sedimentation velocity measurements is unrealistic and that countercurrent distribution by dextran gel filtration is a suitable method for these studies. From gel filtration studies, Kakiuchi, et al. (29) showed that enzyme concentration above 2 mg/ml had two separate components. The faster eluting component corresponding to the dimer showed a sedimentation coefficient of 6.25 S and the slower effluent corresponding to the monomer had a sedimentation coefficient of 4.45 S. However, they pointed out that the sedimenting peaks were not symmetrical. Kakiuchi's group (29) also have shown the independence of the sedimentation coefficient of EDTA treated enzyme on the enzyme concentration and showed experimental results which indicate that B. subtilis α-amylase should be in the monomeric state (molecular weight of 48,200) under the experimental conditions assaying the enzymatic activity; since the enzymatic activity was unchanged in the presence of high concentrations of zinc ions, accordingly, it might not be affected by the change of monomer-dimer transformation. Kakiuchi and Isemura (30) calculated an association velocity constant (k) for the monomer-dimer transformation of 2.2×10^{-9} $(\text{mole/liter})^{-2}$, a

sedimentation coefficient of 6.4 S, and a diffusion coefficient of 5.9 x 10^{-7} cm^2/sec. Sugae (12) reported that B. subtilis α-amylase was inactivated by diazotized sulfanilic acid with the reaction of one tyrosine per 48,000 molecular weight unit. More recently Connellan and Shaw (31) have questioned this interpretation and have shown the loss of one tyrosine per 24,000 molecular weight unit. They consider 24,000 as the chemical minimum molecular weight of B. subtilis α-amylase. Isemura and Kakiuchi (29) reported the α-helical content of the dimer B. subtilis α-amylase as 20%, and 15.5% for photooxidized monomer; both values were calculated from optical rotatory dispersion spectra.

Munier and Drapier (32) conducted intense chromatographic and electrophoretic studies with B. subtilis α-amylase. They reported that this enzyme's mobility changes in relation to the pH of the medium due to a reversible transformation mediated by zinc between a monomeric and dimeric form. The pH regions of dominance for each form are reported as pH 5-6 for the monomer and pH 8-10 for the dimer. In the pH range between 6 and 8, the monomer-dimer transformation takes place; this transformation led them to conclude, that the monomeric form of B. subtilis α-amylase has a net negative charge at those pHs where either form predominates.

Physicochemical Properties of Barley α-Amylase

Very little if anything has been reported in the literature about the physicochemical properties of barley α-amylase. Parameters such as molecular weight, corrected sedimentation coefficient, corrected diffusion coefficient, secondary and quaternary structure of the enzyme have not been established yet. Ultracentrifugation studies, which are

the main concern of this investigation, have been carried out by Mitchell (17) who reports that barley α-amylase has a sedimentation coefficient of 3.92 S. Mitchell (17) also reported an estimated molecular weight of 50,000 daltons; Greenwood and Milne (19) determined the molecular weight of barley α-amylase as 45,000; this value was obtained by gel permeation column chromatography.

CHAPTER III

EXPERIMENTAL METHODS

Sample Preparation for Ultracentrifugation Studies

Crystalline B. subtilis α-amylase was purchased from Sigma Chemical Company, St. Louis, Missouri. The enzyme was weighed to the nearest hundredth of a milligram and dissolved with gentle stirring in the appropriate buffer. The buffer was usually a colorless buffer of low concentration (10^{-2} M) and of 0.1 to 0.2 ionic strength obtained by addition of potassium or sodium chloride in 0.1 molar concentration. A small quantity of 1,1,1-trichloro-2-methyl-2-propanol (10^{-8} M) and phenylmethylsulfonylfluoride (10^{-4} M) were introduced in the buffer systems to inhibit bacterial growth and protease activities. The enzyme solutions were dialyzed against the solvents for 24 hours. The dialyzate was utilized as the reference fluid when double-sector cells were used. Barley α-amylase samples prepared by the method of Mitchell (17) were available in solutions ranging from 0.5 mg/ml to 4 mg/ml (as determined by the method of Lowry (33)). These solutions were handled the same as the B. subtilis enzyme solutions.

Determination of Density, Viscosity, and Partial Specific Volume for Sample Solutions

Density determinations were carried out in pycnometers of 5 and 50 ml volumes. The pycnometers were dried in a vacuum oven, capped, and weighed after equilibrium with room temperature and air moisture had been attained. Then the enzyme solutions were added, and the fitted stopper containing a small capillary hole to allow the escape of air and overflow of liquid was sealed carefully. The filled pycnometers were placed in a constant-temperature bath at 20° C until thermal equilibrium was obtained, taken out, dried and then weighed. Similar procedure was followed when water of known density was the fluid filling the pycnometers. The relative viscosity of the solutions was determined by means of an Ostwald viscometer. Four ml of enzyme solution were placed in the reservoir bulb by means of a pipet. With gentle suction applied at the right-hand limb of the viscometer, the fluid was forced through the capillary into the drainage bulb until the level of the liquid was above the level scratched in the glass tube above the drainage bulb. The negative pressure was then released to permit the flow of the solution since there was atmospheric pressure operating on both sides of the viscometer. The time required for the meniscus to pass between the top engraved line and a similar line engraved in the glass tube below the drainage bulb was recorded. Distilled water was used as the reference fluid and was subjected to identical treatment. The partial specific volume of *B. subtilis* α-amylase was determined from its amino acid composition.

Boundary Sedimentation Rates and Molecular Homogeneity

Sedimentation velocity experiments were carried out for the determination of the sedimentation coefficients for B. subtilis α-amylase and barley α-amylase, and as an indicator of the homogeneity of the sample. Solutions of 0.75, 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 8.00, and 10.00 mg/ml of B. subtilis α-amylase were prepared by weighing dry enzyme and dissolving it in 0.01 M sodium acetate buffer, pH 5.0 with 0.1 M potassium chloride. The colorless solutions were passed through a Millipore filter, 0.45 μm, and dialyzed against the above buffer for 24 hours. A 0.45 ml sample of solution of the stated enzyme concentration was introduced in one sector of a 12 mm double-sector charcoal-filled Epon centerpiece and 0.46 ml of dialyzate was placed in the other sector. The cell assembly included schlieren window holders and sapphire windows. This 12 mm double-sector cell sealed with a torque of 130 inch-pounds and a schlieren counterbalance adjusted to within 0.5 g of the weight of the cell were placed in cell-holder holes one and two of an An-D Rotor, respectively; the rotor was placed in the ultracentrifuge vacuum chamber. A symmetrical mask with 0.75 mm slits at the collimating lens mount was used. The runs were carried out at 59,780 r.p.m. and the temperature was controlled at 20° C. Photographs were usually taken at four or eight minute intervals, with a phase plate angle of 60°. The Kodak Metallographic plates used were exposed for 20 seconds and developed for four minutes in Kodak D-19 developer. Measurements of the photographic images of the schlieren patterns were carried out with a Nikon optical microcomparator, where the position of the boundary was determined and the magnification factor calculated. Barley α-amylase solutions of

concentration 1.60, (three determinations), 1.90, 2.30, 2.70, and 3.50 (two determinations) mg/ml were centrifuged by the same method.

Diffusion Rates by the Height-Area Method

A 4.00 mg/ml quantity of B. subtilis α-amylase solution was prepared by weighing a dry enzyme sample to the nearest hundredth of a milligram and dissolved in and dialyzed against 0.01 M sodium acetate buffer pH with 0.1 M potassium chloride. The solution was then passed through a 0.45 μm Millipore filter to remove undissolved material. A 12 mm double-sector cell was assembled with schlieren window holders and sapphire windows. In one sector of the double-sector synthetic boundary charcoal-filled Epon centerpiece, which completed the cell assembly, 0.15 ml of the enzyme solution was introduced. The other sector was filled completely with the dialyzate. Cell and schlieren centerbalance adjusted to within 0.5 grams of each other were placed in an An-D aluminum rotor. The symmetrical mask with 0.75 mm was inserted at the collimating lens mount. The rotor, secured to the drive shaft in the vacuum chamber of the ultracentrifuge, was accelerated to 4,908 r.p.m. The metallographic plates were exposed for 20 seconds at four minute intervals with a phase angle of 60^{o}. The photographs were developed in Kodak D-19 developer and read on the microcomparator. A characteristic of this method is the transfer of the dialyzate from one sector of the cell to the other via the capillary connection. The transfer was made at 12,590 r.p.m., and the first picture was taken when the menisci in the two sectors were at the same level. Several solutions of barley α-amylase with concentrations between 2.0 and 3.0 mg/ml were treated in identical form.

Conventional Sedimentation Equilibrium Method

B. subtilis α-amylase was weighed and dissolved in 0.01 M sodium acetate buffer (pH 5.0) with 0.1 M potassium chloride. The final concentration of the solution ranged from 0.75 to 2.0 mg/ml (several runs). A 12 mm double-sector cell with interference window holders, sapphire windows, and with a double-sector, capillary synthetic boundary, charcoal-filled Epon centerpiece was assembled and closed tightly with a torque of 130 inch-pounds. A 0.03 ml volume of FC-43 fluorocarbon oil (3M Company, Minneapolis, Minnesota) was introduced with a Hamilton syringe into one sector of the cell followed by 0.12 ml of sample solution which had been previously dialyzed against the solvent buffer. In the remaining sector, 0.17 ml of the dialyzate provided the reference fluid. The double-sector cell and an interference counterbalance adjusted to within 0.5 g of each other were placed in an An-D Rotor (without tilting the cell to prevent transfer of fluid from one sector to the other) and they were aligned carefully before securing the rotor in the Model-E chamber. A symmetrical condensing lens mask with 0.75 mm slits was used on the collimating lens mount. Several runs were made and the speeds were between 12,000 and 14,000 r.p.m. The first interference photo was taken at the selected operating speed. Then the rotor was accelerated to approximately 1.5 times the operating speed and the progress of the run was followed by observing the schlieren patterns through the ultracentrifuge viewer. After 195 minutes the boundary left the base line. The distribution of enzyme along the cell was 20 times more concentrated at the bottom half of the cell than at the top half. At this time the over-speed period was over and the speed was reduced to the selected equilibrium speed. Equilibrium, attained at this operating speed, was confirmed from analysis of the

spectroscopic plates (Figure 1) which showed less than 0.03 fringe shift between two pictures taken at the assumed equilibrium time and 4 hours later. The temperature indicated by the RTIC unit was recorded along with the number of counts registered by the revolution counter during a set period of time (usually 30 minutes). Then the run was stopped. To determine the initial concentration a synthetic boundary run with controlled temperature was made by reintroducing the cell to the rotor with new sample dialyzate in the reference sector filling it completely. The rotor was accelerated to 9.945 r.p.m. All photographs were taken at four minute intervals (Figure 2). Barley α-amylase samples which were available as solutions of concentrations between 0.5 and 0.7 mg/ml as determined by Lowry, et al. (33) were treated in a similar manner.

Meniscus Depletion Sedimentation Equilibrium Method

B. subtilis α-amylase (0.4-0.8 mg/ml) in 0.1 M acetate buffer, 0.1 M potassium chloride, and pH 5.0 was used for this determination. A 12 mm double-sector cell with interference window holders, sapphire windows, and a double-sector charcoal-filled Epon centerpiece was assembled and sealed tightly to avoid leakage. One sector of the cell was filled with 0.01 ml of FC-43 fluorocarbon oil and 0.12 ml of enzyme solution (protein concentration 0.5 mg/ml). As before the other sector was filled with 0.13 ml of the dialyzate. Speeds between 27,000 and 30,000 r.p.m. were maintained (several runs) for 16 hours until equilibrium was attained and this was confirmed by analysis of the spectroscopic plates (Figure 3). The photographs were read with a Nikon microcomparator and a base line correction was also recorded. (To calculate the average operating

Figure 1. Interference Pattern at Equilibrium for a Conventional Sedimentation Equilibrium Ultracentifugation Run

B. subtilis α-amylase concentration was 2.0 mg/ml. Equilibrium was attained after 22 hours of centrifugation at 12,590 r.p.m. and temperature of 292.01° K.

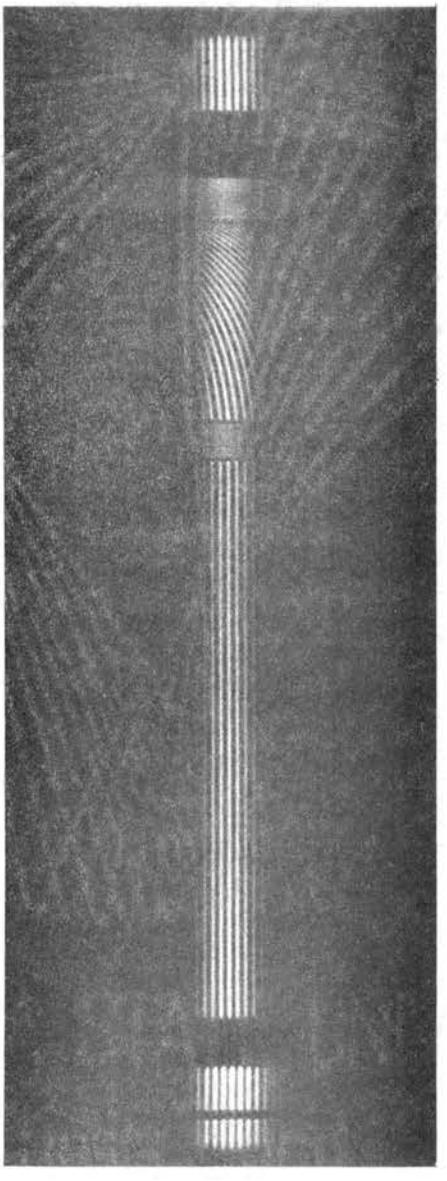


Figure 2. Interference Patterns, Initial Concentration Determination

Synthetic boundary run at 9.945 r.p.m. The dialyzate in the reference sector was replenished and pictures taken at four minute intervals. Initial concentration was determined by extrapolation to zero time. The top picture was taken at run speed, and the bottom picture was taken after 27 minutes of run time.

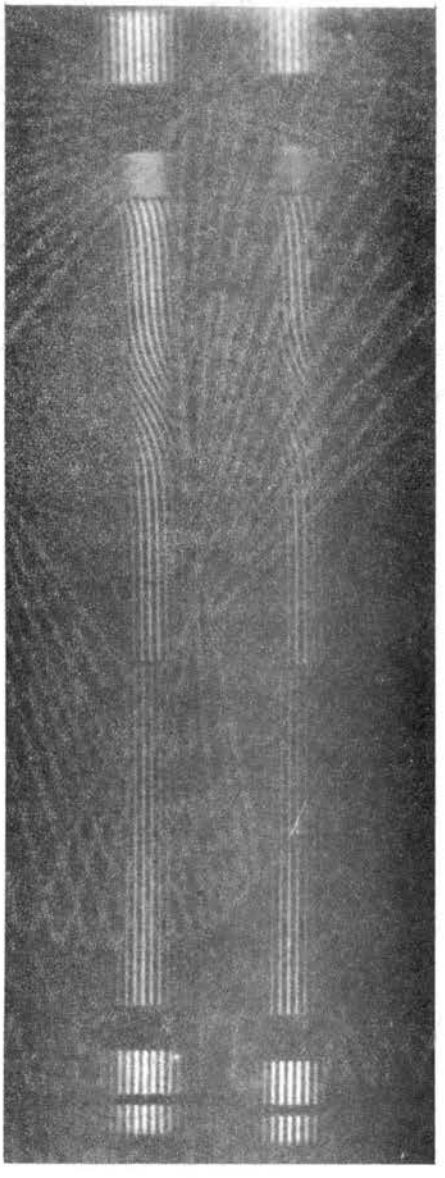
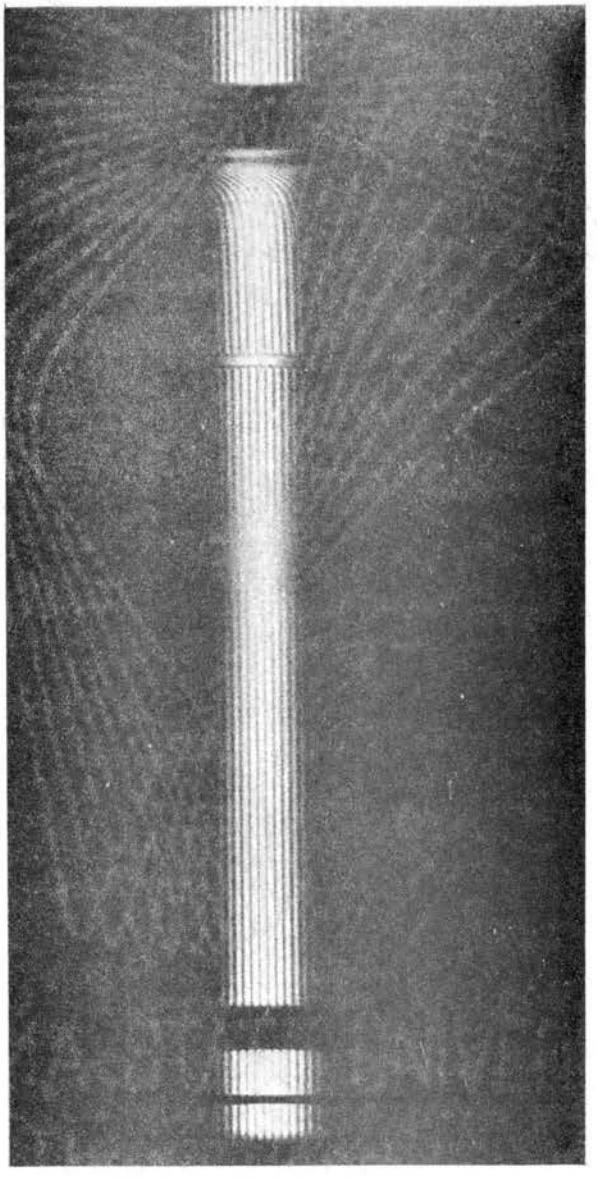


Figure 3. Interference Pattern at Equilibrium for a Meniscus Depletion Sedimentation Equilibrium Ultracentrifugation Run

B. subtilis α-amylase concentration was 0.5 mg/ml. Equilibrium was attained after 10 hours of centrifugation at 29,501 r.p.m. and a temperature of 292.86° K.



speed, the record of counts per 30 minutes was obtained from the revolution counter.)

Gel Filtration Chromatography

A B. subtilis α-amylase solution (0.1 ml, 100 μg of enzyme) was applied to a Bio-Gel P-100 column (0.6 cm x 110 cm), equilibrated with 0.05 M Tris in 0.1 M KCl (pH 8.0), and eluted with the same buffer at a flow rate of 2.0 ml/hr. Fractions of 0.2-0.3 ml were collected. The column was calibrated with Bovine Serum Albumin (BSA), β-Lactoglobulin, and α-Lactalbumin - molecular weights, 69,000, 36,500, and 14,500, respectively. Blue Dextran was used to measure the void volume.

A second experiment was carried out to determine the subunit molecular weight for B. subtilis α-amylase. A solution (0.2 ml, 200 μg of enzyme) was applied to a Sepharose 4-B column (1.5 x 84 cm) that had been equilibrated with 6 M guanidine hydrochloride. The column was eluted with the above solvent and fractions ranging from 1.0 to 1.1 grams were collected with a flow rate of 2.3 ml/hr. Prior to sample application the column was permitted to flow for two days. The column was standardized with BSA, Ovalbumin, chymotrypsinogen, and cytochrome C - molecular weight 69,000, 43,000, 25,700, and 12,400, respectively. Blue Dextran 2000 was used to measure the void volume and DNP-alanine was used as a marker to determine the total volume accessible to the solvent.

Circular Dichroism: Spectropolarimeter Measurements

The Cary 61 Spectropolarimeter was used for circular dichroism measurements. B. subtilis α-amylase samples were dissolved and dialyzed with the appropriate buffer solution; these solutions were passed through a 0.45 μm Millipore filter to remove undissolved material and dust. Final enzyme concentrations were in the range of 200 to 400 μg per milliliter and were determined by absorption at 280 nm using $E_1^{1\%}$ of 25.3 (27) (which was verified experimentally). In order to obtain CD measurements of maximum signal-to-noise ratio, the concentration of the enzyme solutions was diluted with dialyzate to give 0.87 absorbance units (14 per cent transmission) at 222 nm. Circular dichroism spectra were obtained at 20° under constant flow of nitrogen in cylindrical 1 mm pathlength fused-silica cells and the spectra were recorded at 2.24 nm/sec scan with a constant spectral bandwidth of 2 nm.

CHAPTER IV

RESULTS AND DISCUSSION

Bacillus subtilis α-Amylase

Criteria for Enzyme Purity

Enzyme samples utilized in this work showed a high degree of homogeneity. The schlieren patterns observed during sedimentation and diffusion runs were symmetrical, and they corresponded to Gaussian distribution curves (Figures 4 and 5). Furthermore, the plot of the ratio of the square of the area under these curves to the square of the maximum height versus the time of diffusion was linear (Figure 6). This linearity is a good criteria for purity since both parameters (area and height) are sensitive to impurities in the enzyme sample (35). The linearity of the plots of the logarithm of enzyme distribution along the centrifugal field versus the square of their radial distance in the field, and the linearity of the plots of the logarithm of the boundary position versus time of sedimentation were also diagnostic of purity of the enzyme (36). In these studies, such linear plots were reproducible at various centrifuge speeds and enzyme concentrations (Figure 7, 8, and 9). Finally, polyacrylamide gel electrophoresis chromatography showed a single protein band (37).

Figure 4. Sedimentation Velocity; Schlieren Patterns

Schlieren patterns corresponding to a concentration of 4.0 mg/ml of Bacillus subtilis α-amylase. Sedimentation proceeding from left to right at 59,780 r.p.m. and 20° C. The first frame shows the position of boundary after sixteen minutes of sedimentation time. Subsequent frames are taken at eight minute intervals.

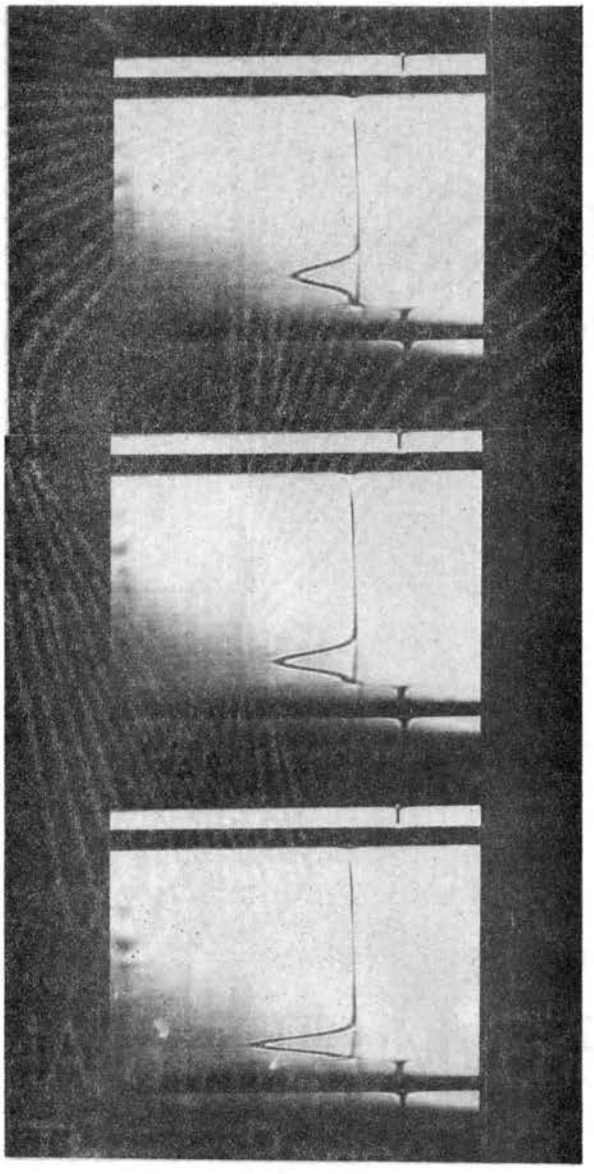
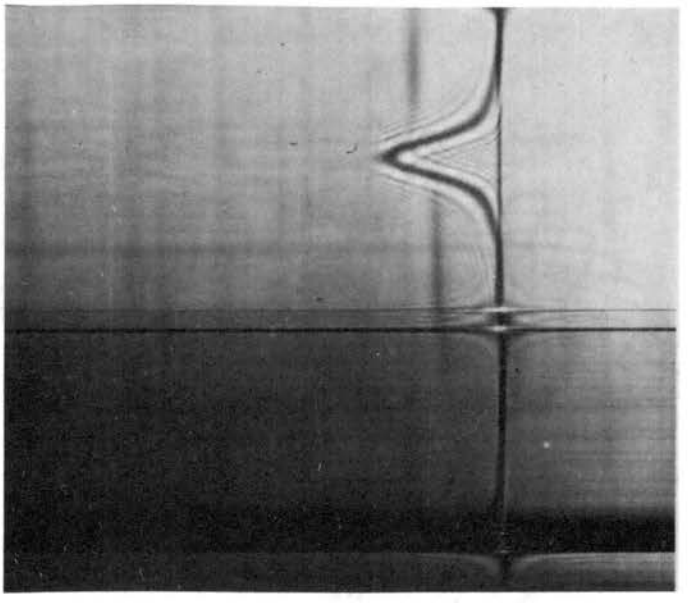


Figure 5. Diffusion Patterns

Schlieren patterns produced by Bacillus subtilis α-amylase at a concentration of 4.0 mg/ml in a diffusion coefficient experiment. Frames from left to right correspond to 24 and 32 minutes of diffusion time, respectively. 4,908 r.p.m. and 29° C.



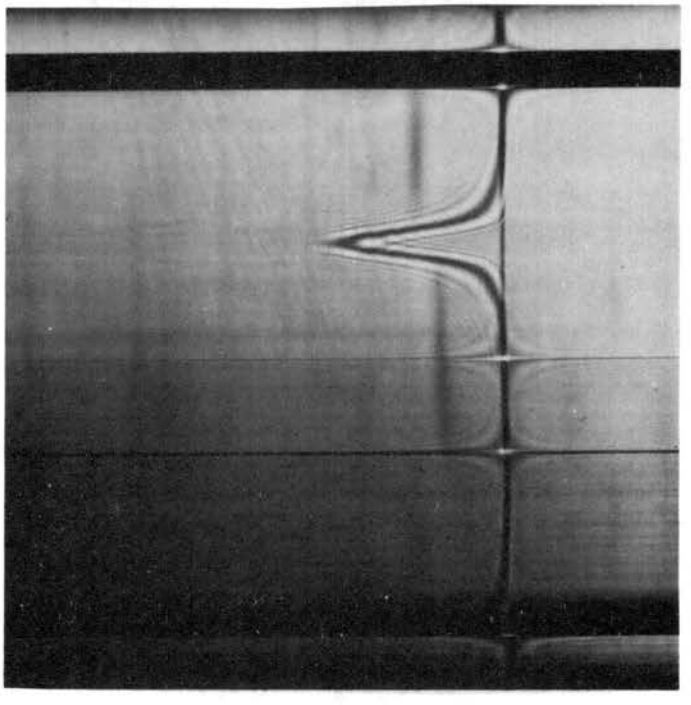


Figure 6. Diffusion Coefficient Plot

Plot of $A^2_{sch}/(dc/dr)^2_{max}$ vs. time of diffusion in seconds is linear for homogeneous solutions. B. subtilis α-amylase concentration was 3.0 mg/ml. Synthetic boundary run at 4,908 r.p.m. and 20° C.

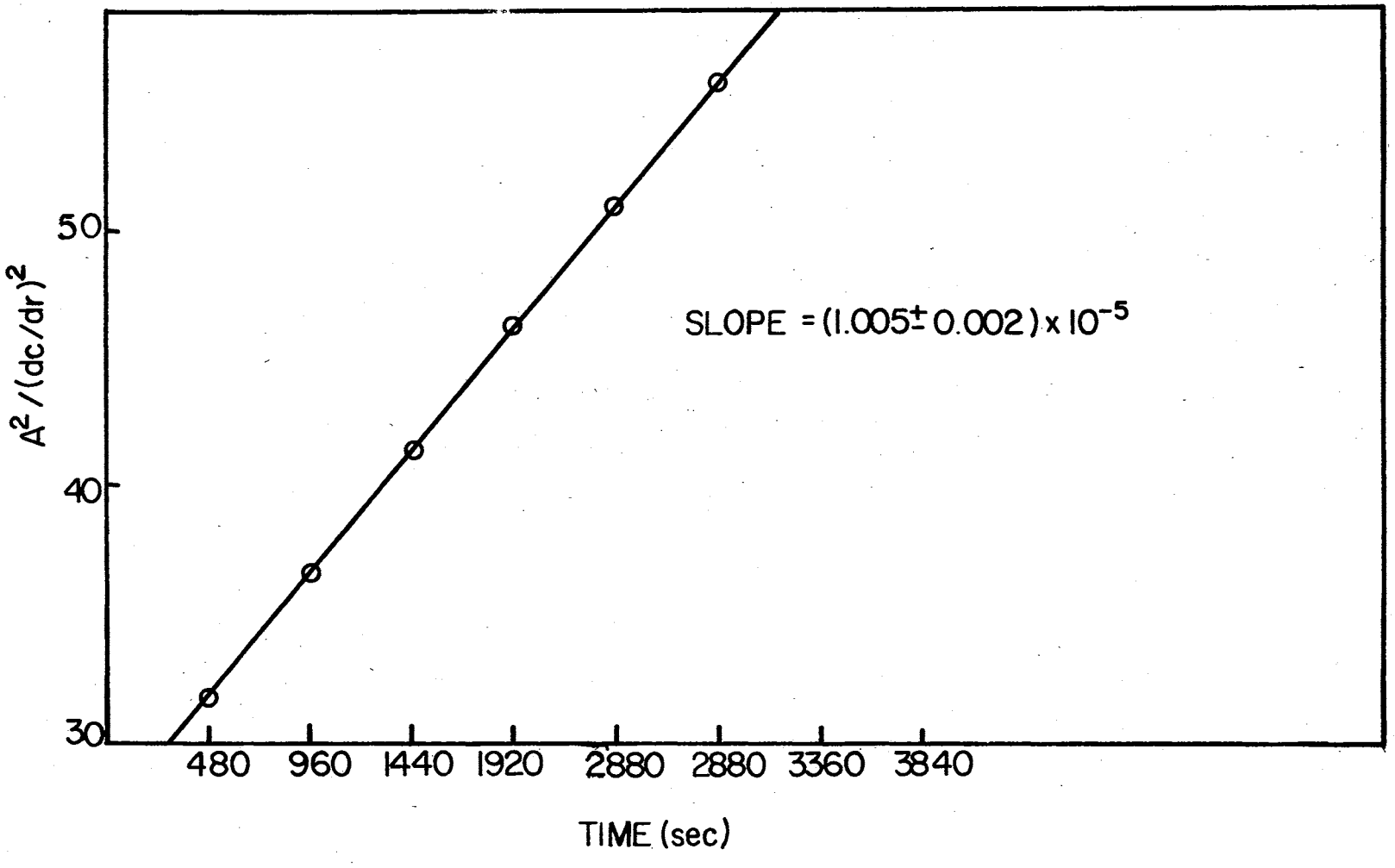

50
40
30
$A^2/(dc/dr)^2$
SLOPE = $(1.005 \pm 0.002) \times 10^{-5}$
480
960
1440
1920
2880
2880
3360
3840
TIME (sec)

Figure 7. Conventional Sedimentation Equilibrium, Plot of Fringe Displacement Versus Radial Distance

Typical log c versus r^2 plot used for the analysis of conventional sedimentation equilibrium data for the molecular weight determination of Bacillus subtilis α-amylase. For a homogeneous, ideal solute, the plot is a straight line. Enzyme concentration was 2.0 mg/ml. Centrifuge speed and temperature were 12,590 r.p.m. and 292.01° K, respectively. Duration of run was 22 hours.

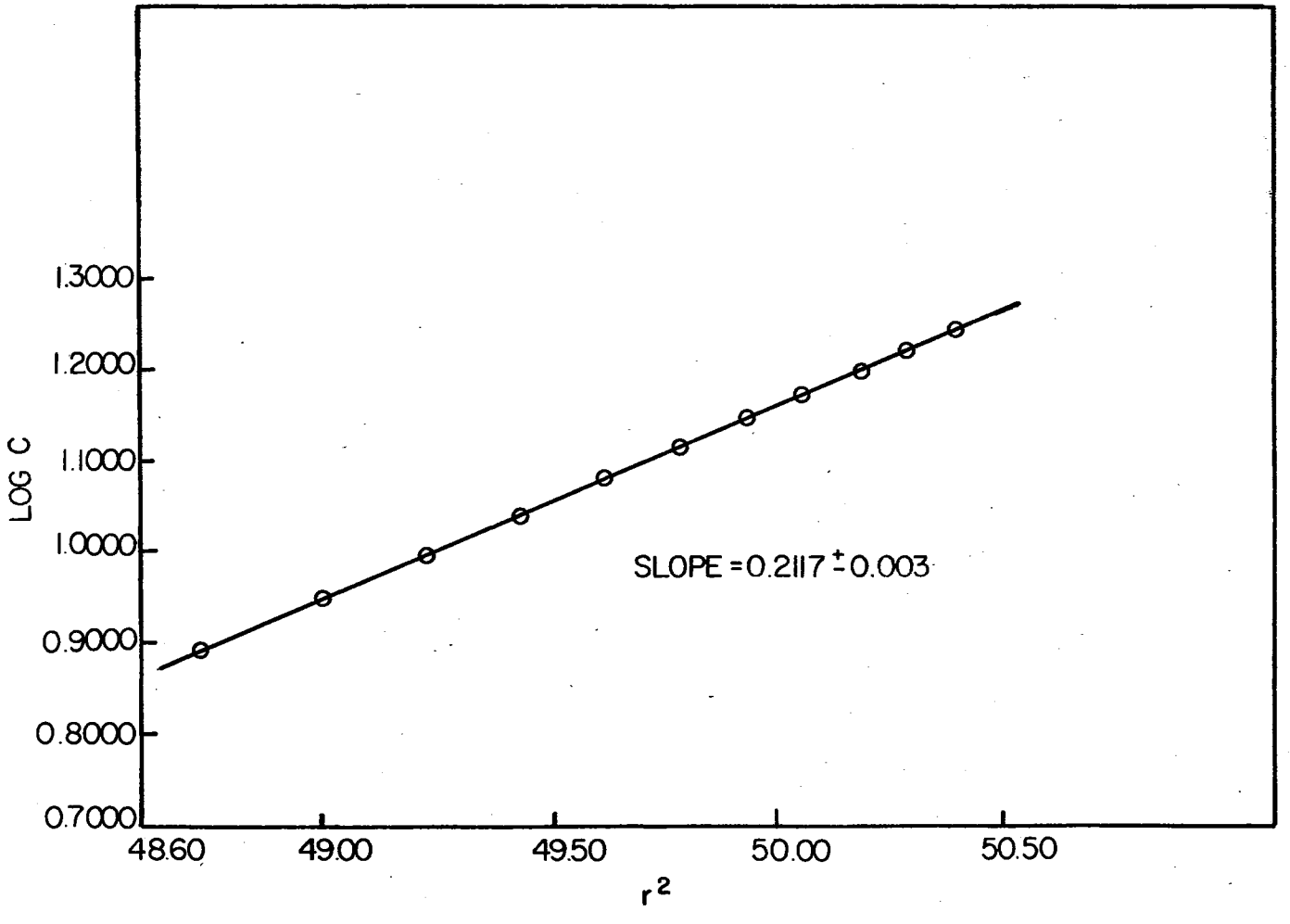

1.3000
1.2000
1.1000
1.0000
0.9000
0.8000
0.7000
LOG C
SLOPE = 0.2117 ± 0.003
48.60
49.00
49.50
50.00
50.50
r²

Figure 8. Sedimentation Velocity Plot

Plot of log x versus time in minutes for B. subtilis α-amylase. Enzyme concentration was 2.0 mg/ml and sedimentation was carried out at 59,780 r.p.m. and 20° C.

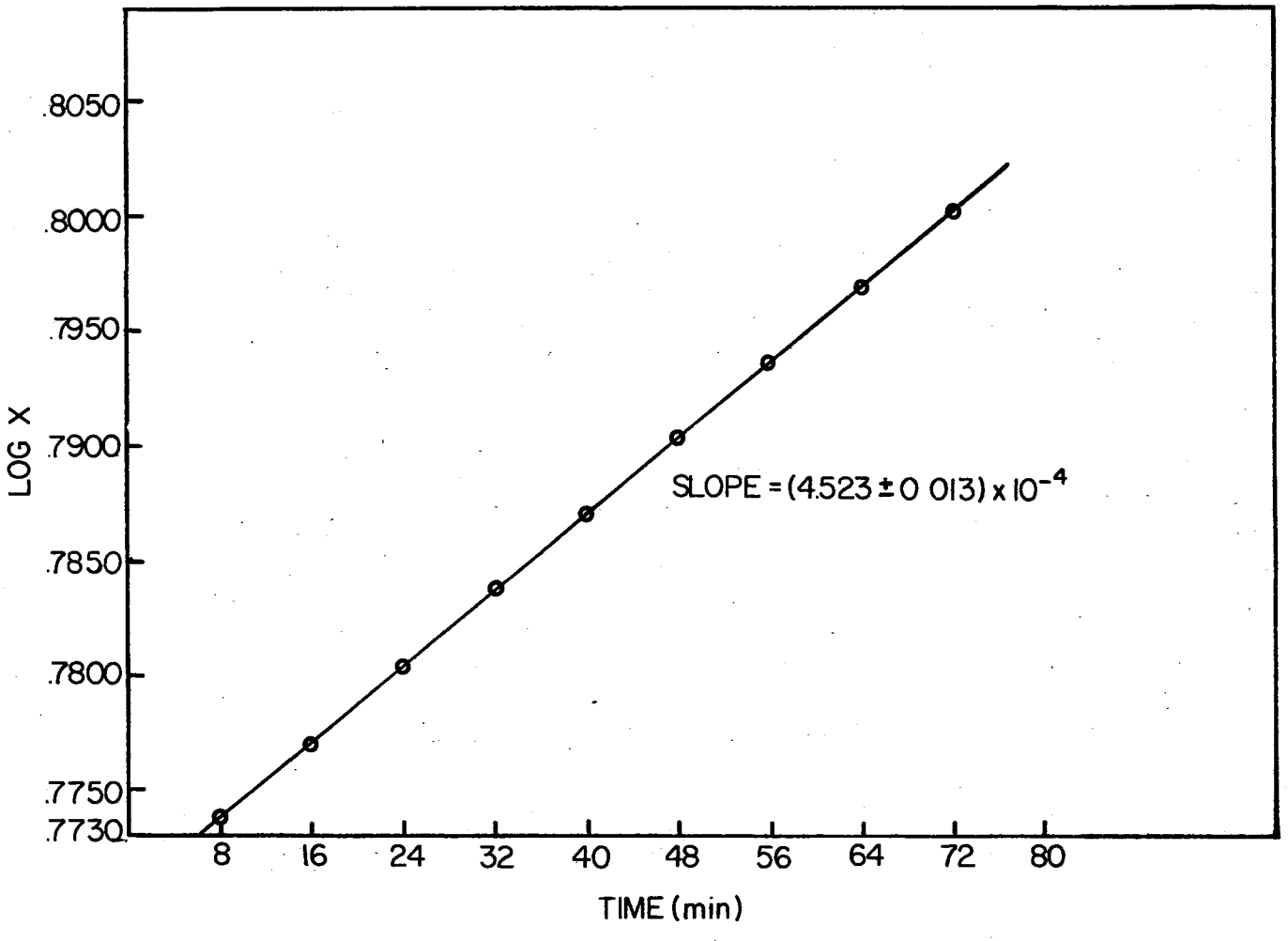
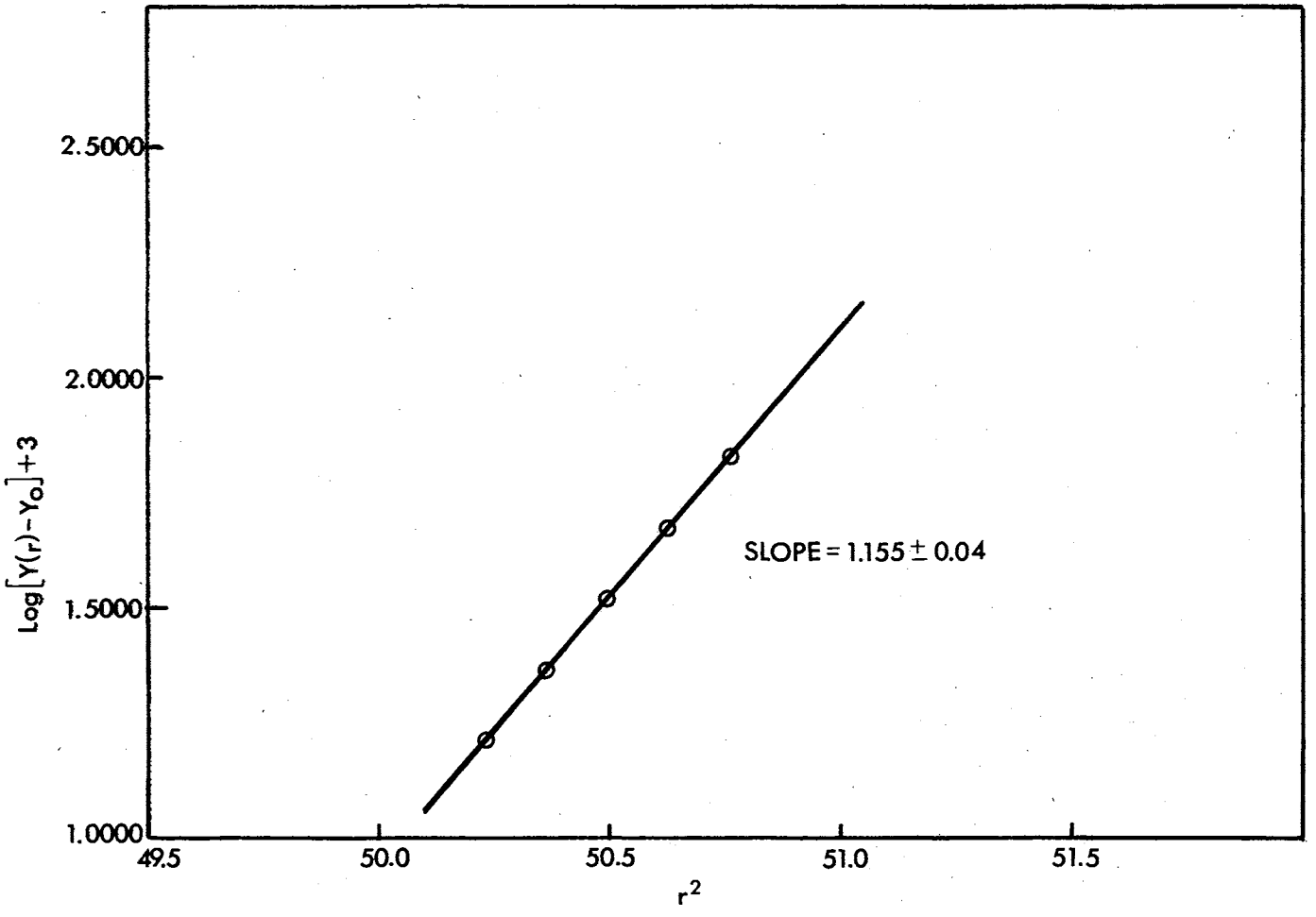

.8050
.8000
.7950
.7900
.7850
.7800
.7750
.7730
LOG X
SLOPE = (4.523 ± 0 013) x 10^{-4}
8
16
24
32
40
48
56
64
72
80
TIME (min)

Figure 9. Meniscus Depletion Sedimentation Equilibrium; Plot of Fringe Displacement Versus Radial Distance

Log $((Y_r)-(Y_o)) + 3$ versus r^2 plot used for the analysis of meniscus depletion sedimentation equilibrium data for Bacillus subtilis α-amylase. For a homogeneous sample, this plot is a straight line. Enzyme concentration was 0.5 mg/ml and sedimentation was carried out at 29,501 r.p.m. and 292.86° K. Duration of run was 10 hours.


2.5000
2.0000
1.5000
1.0000
Log[Y(r)-Yo]+3
49.5
50.0
50.5
51.0
51.5
r2
SLOPE = 1.155 ± 0.04

Sedimentation Coefficient - Concentrations and pH Independence

The corrected sedimentation coefficient determined for B. subtilis α-amylase was 4.44 S. This value was obtained by graphically extrapolating to zero concentration the plot of observed individual sedimentation coeffcients versus their corresponding enzyme concentrations (Figure 10). Observed sedimentation coefficients were calculated by means of Equation One (List of Equations). A similar value, 4.46 S, was obtained for the $s_{20,w}$ when the observed sedimentation coefficient of an enzyme solution of 4.0 mg/ml was corrected for density and viscosity relative to a water-enzyme solution at 20° C by means of Equation Two (List of Equations). The relative density (ρ) of enzyme solutions in 0.01 M sodium acetate, 0.1 M KCl buffer was 1.00405 g/ml; whereas the relative viscosity (μ) determined by the Ostwald method was 1.0019. Contrary to the findings of Iseumura, et al. (10) the sedimentation coefficient here determined showed no concentration dependence; Fisher, et al. (24) agrees with this finding. Furthermore, the sedimentation coefficient was found to be pH independent (Table I).

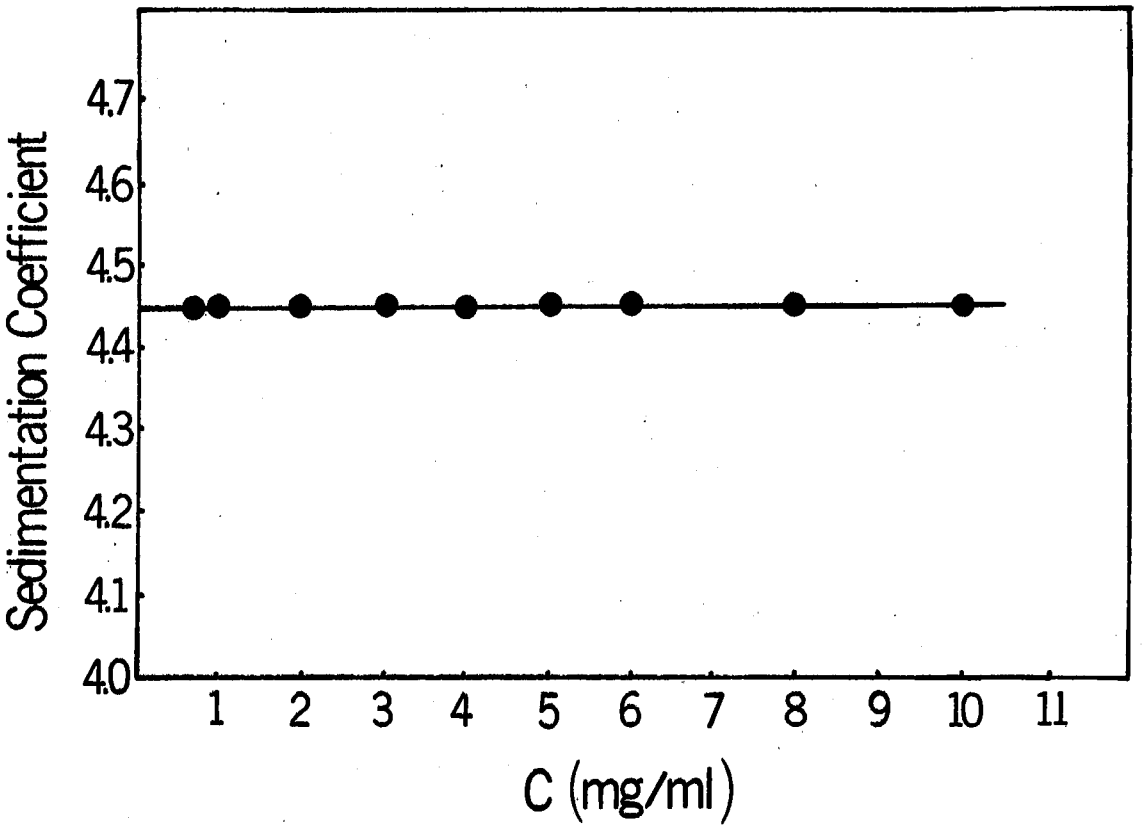
TABLE I

SEDIMENTATION COEFFICIENT pH DEPENDENCE

pH	Observed Sedimentation Coefficient(s)
5	4.44
7	4.45
8	4.50
9	4.42
12	4.46

Figure 10. Sedimentation Coefficient; Concentration Dependence

Concentration dependence of the sedimentation coefficient for Bacillus subtilis α-amylase. Individual runs were made at 59,780 r.p.m. and 20° C. $s^{0}_{20,w}$ was determined by extrapolation to zero enzyme concentration.


Sedimentation Coefficient
4.7
4.6
4.5
4.4
4.3
4.2
4.1
4.0
1
2
3
4
5
6
7
8
9
10
11
C (mg/ml)

Diffusion Coefficient

The observed diffusion coefficient calculated for the enzyme by means of Equation Three (List of Equations) was 8.00×10^{-7} cm^2/sec. After correcting for density and viscosity relative to water solutions at 20° C utilizing Equation Four the diffusion coefficient value of 8.00×10^{-7} cm^2/sec was unchanged.

Molecular Weight

The molecular weight of native *B. subtilis* α-amylase calculated from the relationship between the corrected sedimentation coefficient and the corrected diffusion coefficient (Equation Five, List of Equations) was 48,400 daltons. A value of 48,535 daltons was determined from observed parameters corresponding to an enzyme solution in 0.01 M sodium acetate buffer (pH 5.0) of concentration of 4.0 mg/ml (Table II).

TABLE II

MOLECULAR WEIGHT DETERMINATION FROM SEDIMENTATION AND DIFFUSION DATA

c(mg/ml)	$s_{20,w}$	D	M.W.
0.0	4.44	8.00×10^{-7}	48,400
4.0	4.46	8.00×10^{-7}	48,500

Conventional sedimentation equilibrium ultracentrifugation results

are listed in Table III.

TABLE III

MOLECULAR WEIGHT DETERMINATION DATA FROM CONVENTIONAL SEDIMENTATION EQUILIBRIUM ULTRACENTRIFUGATION

Trial No.[1]	Enzyme Concentration (mg/ml)	C_o^z fringes	Duration of run (hours)	$\omega^2 \times 10^6$	Slope of log c vs. r^2	Temperature ^oK	M.W.[3] daltons
1	2.0	7.73	22	1.746	0.211	292.01	48,400
2	1.8	8.10	24	1.971	0.254	203.00	48,450
3	2.2	7.51	24	1.393	0.201	291.80	48,350

[1]Molecular weight average from three trials = 48,400 $\pm$ 50.

[2]Initial concentration values were calculated by extrapolation to zero time from the plots of the fringe number versus the time of run in minutes.

[3]Molecular weights determined with a partial specific value of 0.717 (38).

Meniscus depletion equilibrium ultracentrifugation yielded results listed in Table IV.

TABLE IV

MOLECULAR WEIGHT DETERMINATION DATA FROM MENISCUS DEPLETION SEDIMENTATION EQUILIBRIUM ULTRACENTRIFUGATION

Trial No.[1]	Enzyme Concentration (mg/ml)	$\omega^2 \times 10^6$	Duration of run (hours)	Slope of $\log((Y_r)-(Y_o))+3$ vs. r^2	Temperature ^oK	M.W. daltons
1	0.5	9.540	10	1.155	292.86	48,500
2	0.8	8.415	12	1.012	292.86	48,100
3	0.4	9.531	10	1.142	293.00	48,000

[1]Molecular weight average from three trials = 48,200.

From Tables II, III, and IV, it is concluded that Bacillus subtilis α-amylase has a molecular weight of 48,400 daltons when calculated from sedimentation and diffusion corrected data; this value increases by 100 daltons when observed values for sedimentation and diffusion are used; such a small variation (100 daltons) reaffirms the independence of these parameters from the enzyme concentration. From conventional sedimentation equilibrium data the enzyme's molecular weight is 48,400 ± 500 daltons; a similar value, 48,200 ± 200 daltons is obtained from meniscus depletion ultracentrifugation. The above molecular weight values in the absence of added Ca^{++} or Zn^{++} should be considered as the actual molecular weights for B. subtilis α-amylase since several runs at different enzyme concentrations and ultracentrifuge speeds gave results deviating from each other by only one percent.

Enzyme Activation and Polymerization in the Presence of 1 mM Zinc or Calcium Ions

Values reported for the molecular weight of B. subtilis α-amylase in the presence of Ca^{++} ions are in the order of 96,000 daltons (10,24). These values correspond to a dimer with subunits of molecular weights in the order of 48,000 daltons bridged by a zinc atom (24). Isemura, et al. (10) and Fisher (24) recognized the functionality of Ca^{++} ions as an enzyme activator only; but our experimentation carried out in the presence of 10^{-3} M Ca^{++} or Zn^{++} ions, disclosed that Ca^{++} and Zn^{++} ions not only activated the enzyme (Table V) but also polymerized it. This phenomena was evident on the values of the sedimentation coefficients calculated in the presence of both ions at pH values 5, 7, and 9 (Table VI).

TABLE V

ENZYME ACTIVATION BY CALCIUM AND ZINC IONS

Enzyme Addition	Assay pH	Relative Activity[1]
Ca^{++} pH 5.0	5.0	3
	7.0	2
Ca^{++} pH 7.0	5.0	3
	7.0	2
Ca^{++} pH 9.0	5.0	3
	7.0	2
Zn^{++} pH 5.0	5.0	1.5
	7.0	1.0
Zn^{++} pH 7.0	5.0	3
	7.0	2
EDTA pH 11.8	5.0	0
	7.0	0
Native Enzyme	5.0	1
pH 5.0	7.0	1

[1]Activity determined by the method of Rindernecht, et al. (38).

TABLE VI

CALCIUM AND ZINC EFFECTS ON THE SEDIMENTATION COEFFICIENT

$c = 10^{-3}$ M	Observed Sedimentation Coefficient Values		
	(pH 5.0)	(pH 7.0)	(pH 9.0)
Ca^{++}	4.44 S	6.50 S	5.30 S
	MW ≃ 48,500	MW ≃ 96,000	MW ≃ 72,000
	(pH 5.0)	(pH 7.0)	(pH 9.0)
Zn^{++}	5.49 S	7.46 S	
	MW ≃ 72,000	MW $\geq$ 96,000	[1]

[1]At this pH zinc precipitated from solution.

The nature of the polymerization for Ca^{++} appeared to be different from that of Zn^{++} (Figure 11). In the presence of both ions the sedimentation coefficient became pH dependent. At low pH (5.0) the enzyme did not dimerize with calcium but did so with zinc; the corresponding sedimentation coefficients were 4.44 S and 5.49 S, respectively. A single symmetrical sedimenting peak was observed with Ca^{++} and two peaks appeared when Zn^{++} was added. At pH 7.0 the S values were 6.50 S in the presence of Ca^{++} and 7.46 S in the presence of Zn^{++}. The schlieren patterns at this pH resulted in two distinguishable peaks with Zn^{++} and a single skew peak with Ca^{++}. When the pH of the solutions was increased to 9, zinc hydroxide precipitated from the solution which made the determination of parameters impossible at this pH, but the Ca^{++} containing solution showed a single symmetrical peak with a sedimentation coefficient of 5.30 S (Figures 11 and 12).

Figure 11. Sedimentation Coefficient; Effects of Calcium Ions

Effects of 10^{-3} M Ca^{++} ion on the sedimentation coefficient of B. subtilis α-amylase. Left frame (pH 5.0) shows single symmetrical peak. Middle frame (pH 7.0) shows shoulder. Right frame (pH 9.0) shows again a single symmetrical peak. Sedimentation carried out at 59,780 r.p.m., 20^{o} C and an enzyme concentration of 5.0 mg/ml.

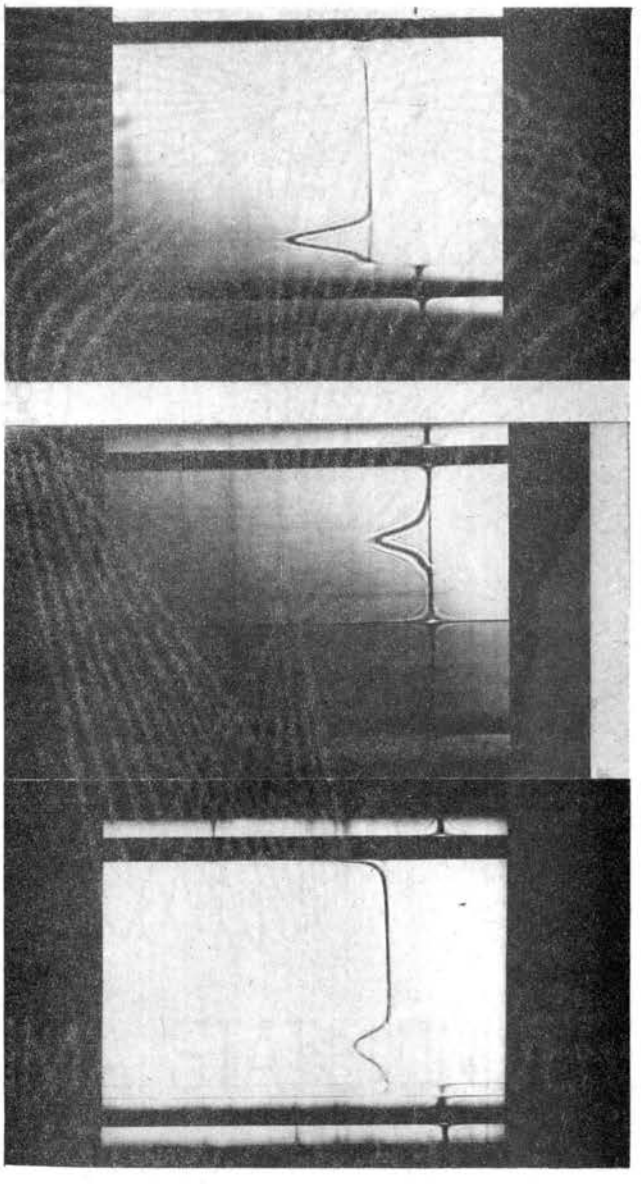
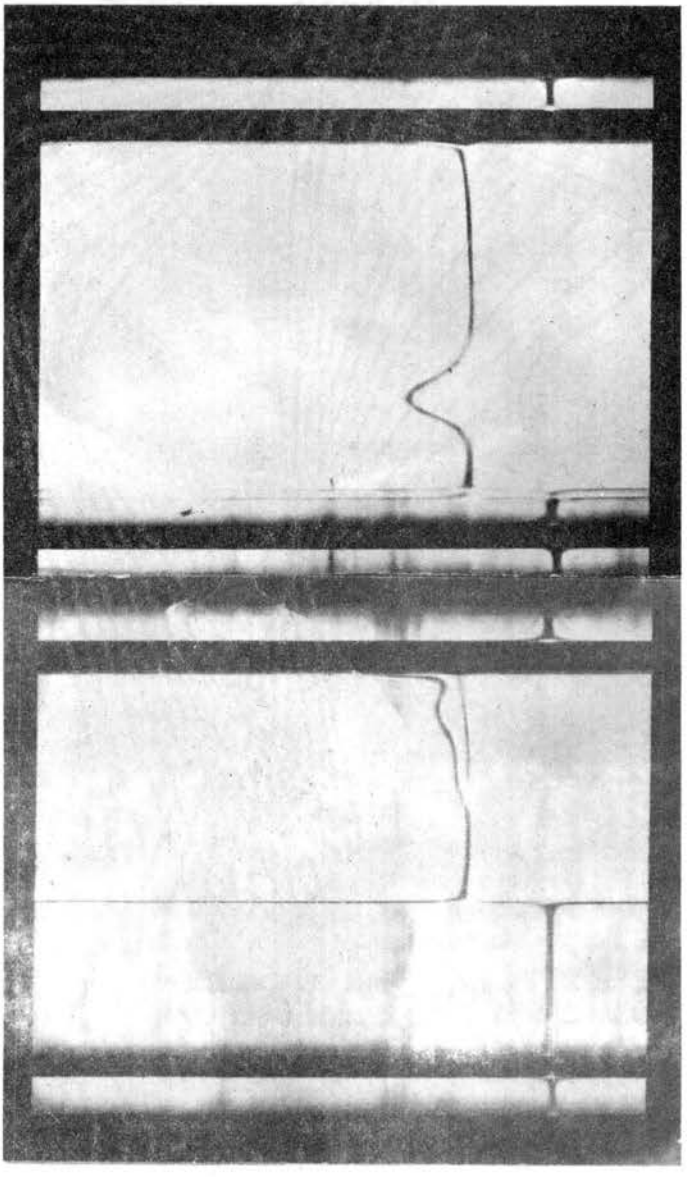


Figure 12. Sedimentation Coefficient; Effects of Zinc Ions

Effects of 10^{-3} M Zn^{++} ion on the sedimentation coefficient of B. subtilis α-amylase. Left frame (pH 5.0) shows two sedimenting peaks. Right frame (pH 7.0) shows an apparent single peak somewhat non-symmetric. Centrifugation speed was 59,780 r.p.m.; temperature was 20° C and enzyme concentration was 4.0 mg/ml.



The decreased value for the sedimentation coefficient from 6.50 S at pH 7.0 to 5.30 S at pH 9.0 indicated that dimerization of the enzyme was complete at pH 9.0. The apparent higher sedimentation coefficient value at pH 7.0 was due to the Johnson-Ogston effect where one form of enzyme piles up behind a faster sedimenting form resulting in an acceleration in the migration of the molecules of the latter.

Effects of Denaturing and Chelating Agents on the Sedimentation Coefficient

When EDTA (0.05 M) was introduced in the solutions at pH 5.0, the apparent sedimentation coefficient decreased from 4.44 S to 3.56 S, indicating that this chelating agent removed metal ions which were already present in the crystalline enzyme.

In the presence of denaturing agents such as 6 M guanidine hydrochloride and the detergent SDS (1%), loss of native structure was expected. The information obtained from the sedimentation coefficient value determined in 6 M guanidine hydrochloride (3.57 $\pm$ 0.02 S) indicated that the enzyme dissociated, whereas, sodium dodecyl sulfate seemed not to denature the enzyme to any noticeable degree; the sedimentation coefficient determined in 1% SDS was 4.48 S which is identical to the $s_{20,w}$ (4.44 S) (Table VII).

Minimum Subunit Molecular Weight

Dissociation into subunits in the presence of 6 M guanidine hydrochloride was confirmed by equilibrium ultracentrifugation from which a subunit molecular weight of 22,900 daltons was calculated, using a reduced partial specific volume ($\bar{v}$) of 0.707 (39) (Table VIII).

TABLE VII

EFFECTS OF DENATURING AND CHELATING AGENTS
ON THE SEDIMENTATION COEFFICIENT

	Enzyme Concentration g/ml	S
EDTA (0.05 M)	6.0	3.56
SDS (1%)	6.7	4.48
Gu HCl (6 M)	6.0	3.57[1]

[1]Values corrected for density (1.145 g/ml) and viscosity (1.1612) of 6 M guanidine hydrochloride solution.

TABLE VIII

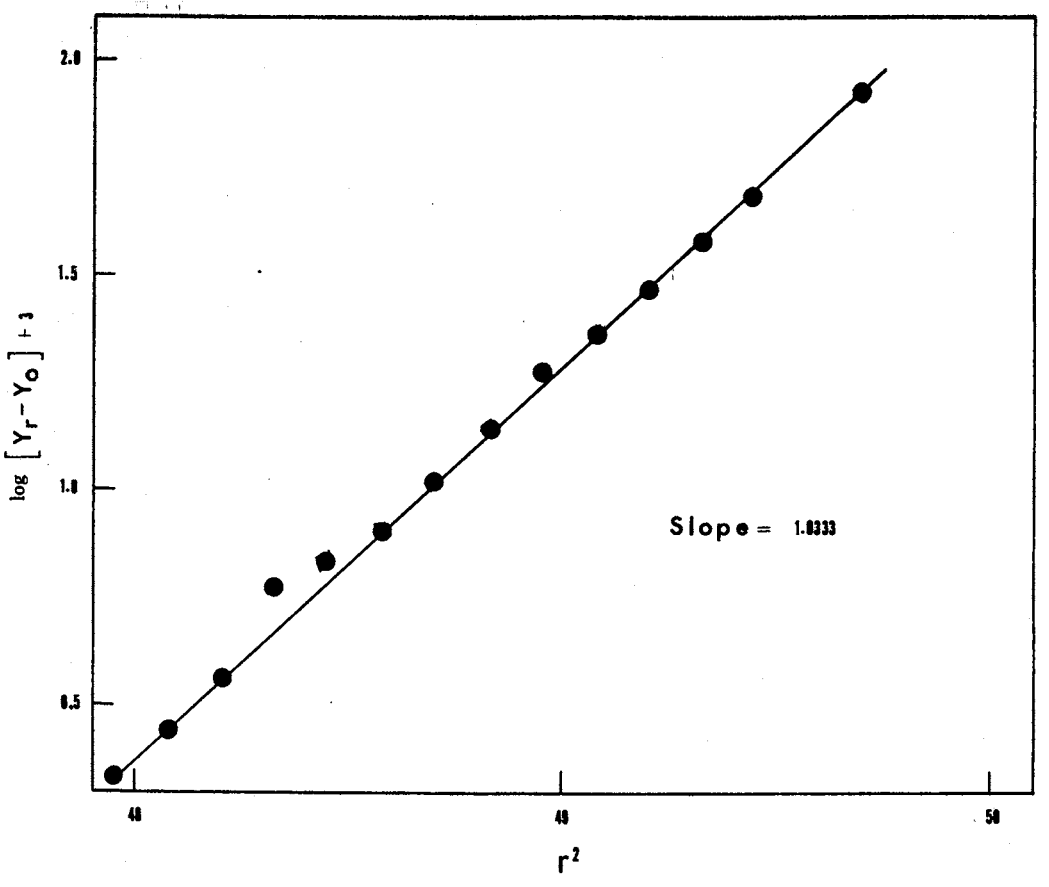
SUBUNIT MOLECULAR WEIGHT IN 6 M GUANIDINE HYDROCHLORIDE
MENISCUS DEPLETION SEDIMENTATION
EQUILIBRIUM ULTRACENTRIFUGATION

$\omega^2 \times 10^7$	Enzyme Concentration (mg/ml)	Slope[1] of $\log[Y(r)-Y_0)]+3$ vs. r^2	Temperature °K	Duration of run (hours)	M.W. daltons
2.8217	0.5	1.0333	293	50	22,925

[1]Calculated from Figure 13.

Figure 13. Meniscus Depletion Sedimentation Equilibrium; Subunit Molecular Weight

B. subtilis α-amylase concentration of 0.5 mg/ml, and run made at 25,000 r.p.m. and 293° K. Equilibrium readied after 50 hours.


2.0
1.5
1.0
0.5
log [Yr - Yo] + 1
Slope = 1.0333
48
49
50
r²

Sodium Dodecyl Sulfate Binding

Sodium dodecyl sulfate was found to bind to B. subtilis α-amylase in a ratio of 0.29 grams of detergent to one gram of protein. The fraction of SDS bound to the enzyme was calculated by means of sedimentation velocity ultracentrifugation. The amount of SDS bound to the protein was determined according to the method used by Nelson (40). Figure 14 shows the schlieren pattern of such a run. The SDS micelle is the slower moving peak. The results are summarized in Table IX.

TABLE IX

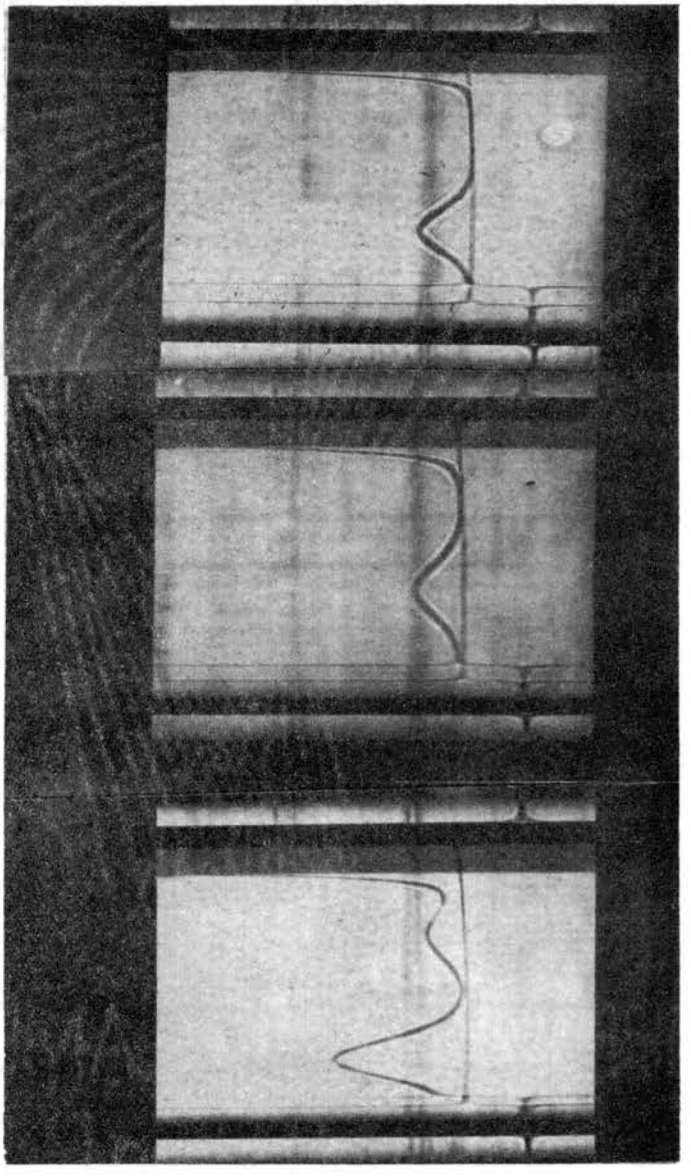
SODIUM DODECYL SULFATE BINDING

Time of Dialysis (hours)	Enzyme Concentration (g/ml)	% SDS Bound	g SDS Bound per g of Protein
12	0.067	0.111	1.66×10^{-3}
24	0.067	19.55	0.29
168	0.067	19.55	0.29

An interesting feature resulting from the failure of the B. subtilis α-amylase to bind sufficient detergent to obtain a charge to mass ratio similar to that of standard proteins, was the anomalous behavior of the enzyme on SDS-acrylamide gel electrophoresis (37). Similar behavior has been shown for erythrocyte membrane glycoproteins (41,42,43). Furthermore, Nelson (40) has found that other proteins have variable binding

Figure 14. Sedimentation Velocity; Binding of Sodium Dodecyl Sulfate

SDS binding to B. subtilis α-amylase. Left frame shows a large SDS micelle peak and a smaller peak corresponding to the enzyme-SDS complex. Middle frame shows the same SDS micelle peak after the enzyme-SDS complex peak has completely sedimented to the bottom of the cell (234 minutes). Right frame shows a single peak corresponding to the SDS micelle of a separate run where no enzyme was present (sedimentation time 234 minutes). Sedimentation from left to right. Enzyme concentration was 6.6 mg/ml. Sedimentation carried out at 59,780 r.p.m. and 20° C.



capacities for dodecyl sulfate; B. subtilis α-amylase therefore, belongs to this category.

Gel Permeation Chromatography

To estimate the molecular weights of the native and dissociated enzyme, gel permeation chromatography was also used. From a Bio-gel P-100 column eluted with aqueous buffers, the native enzyme yielded a molecular weight of 48,000 ± 500 as a single, symmetrical peak when compared to a series of standard proteins (Figure 15). The subunit molecular weight of B. subtilis α-amylase as determined by chromatography on a Sepharose 4-B column eluted with 6 M guanidine hydrochloride according to the method of Fish, et al. (44) was 24,000 daltons (Figure 16). These results were consistent with those obtained previously from ultracentrifugation studies.

Circular Dichroism Studies

Circular dichroism studies carried out on enzyme solutions of different composition and pH gave some interesting results which are summarized in Table X.

Enzyme solutions in aqueous buffers of pHs ranging from 5 to 10 produced no change in profile of the spectrum and the α-helical content calculated at 222 nm. The CD spectrum showed an ellipticity minimum at 209 nm, a shoulder at 222 nm, and a cross-over point at 202 nm (Figure 17B).

When SDS was introduced into the system there was no appreciable change in the spectrum. This observation was to be expected since the enzyme was shown not to bind SDS. The characteristic spectrum of a dodecyl sulfate denatured protein was observed for the amylase only after

Figure 15. Gel Permeation Chromatography; Molecular Weight

Determination of molecular weight of native B. subtilis α-amylase by gel permeation chromatography. Bio-Gel P-100 column (0.6 x 100 cm) was eluted with 0.05 M Tris in 0.1 M KCl (pH 8.6).

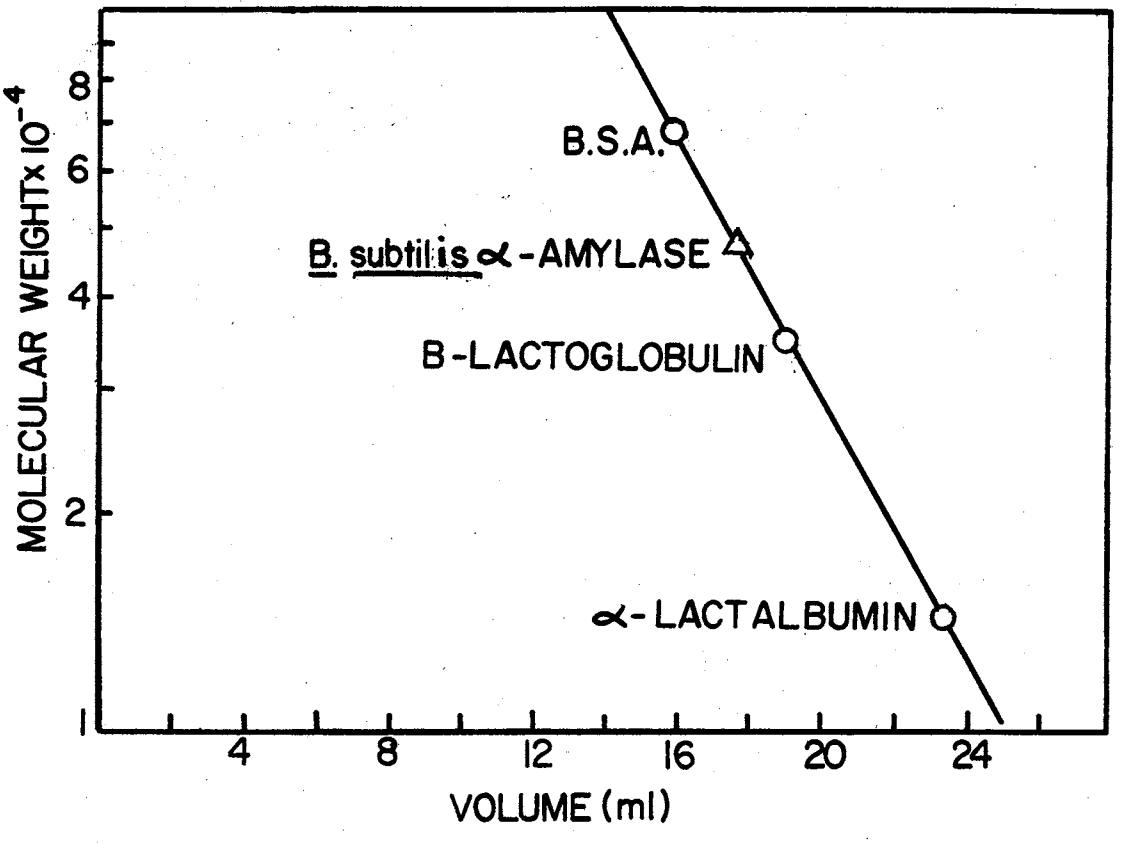

MOLECULAR WEIGHT x 10⁻⁴
8
6
4
2
1
B.S.A.
B. subtilis α-AMYLASE
B-LACTOGLOBULIN
α-LACTALBUMIN
4
8
12
16
20
24
VOLUME (ml)

Figure 16. Gel Permeation Chromatography; Subunit Molecular Weight

Determination of the minimum subunit molecular weight of B. subtilis α-amylase. Gel permeation carried out on a Sepharose 4-B column (1.2 x 90 cm) and eluted with 6 M guanidine hydrochloride, pH 6.5.

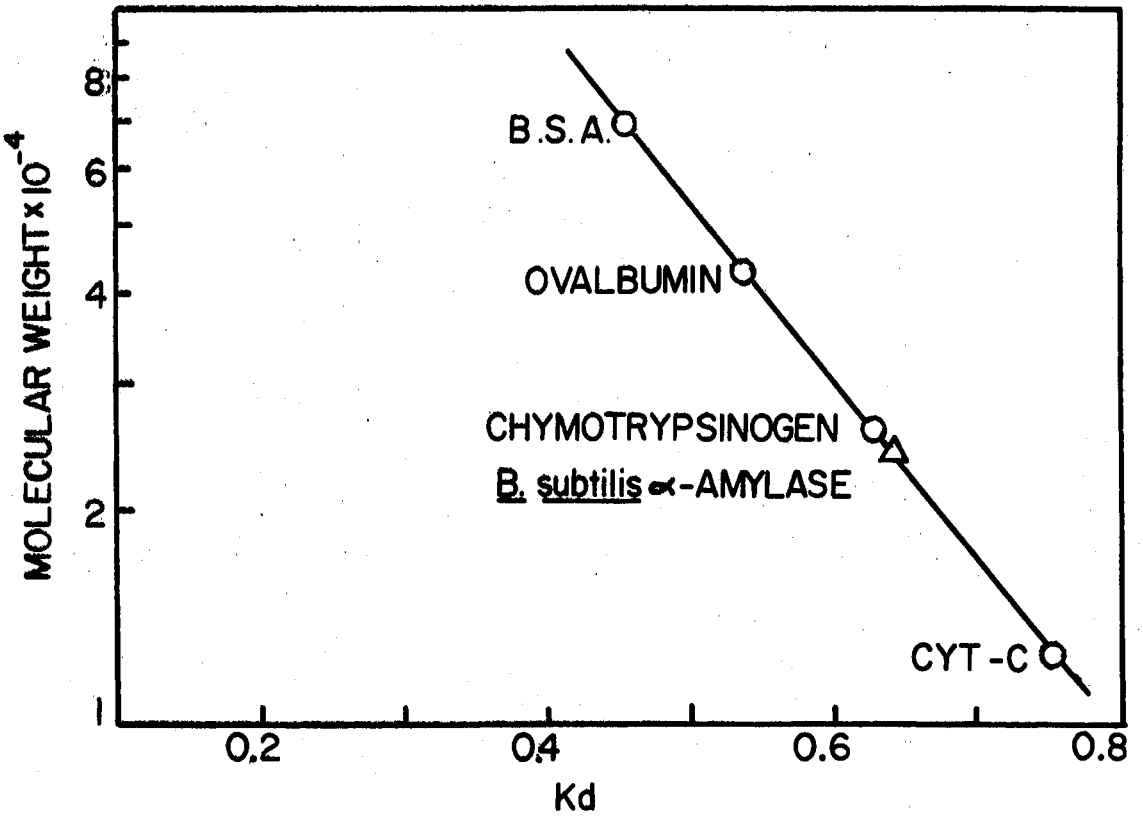

MOLECULAR WEIGHT x 10⁻⁴
8
6
4
2
1
B.S.A.
OVALBUMIN
CHYMOTRYPSINOGEN
B. subtilis α-AMYLASE
CYT-C
0.2
0.4
0.6
0.8
Kd

TABLE X

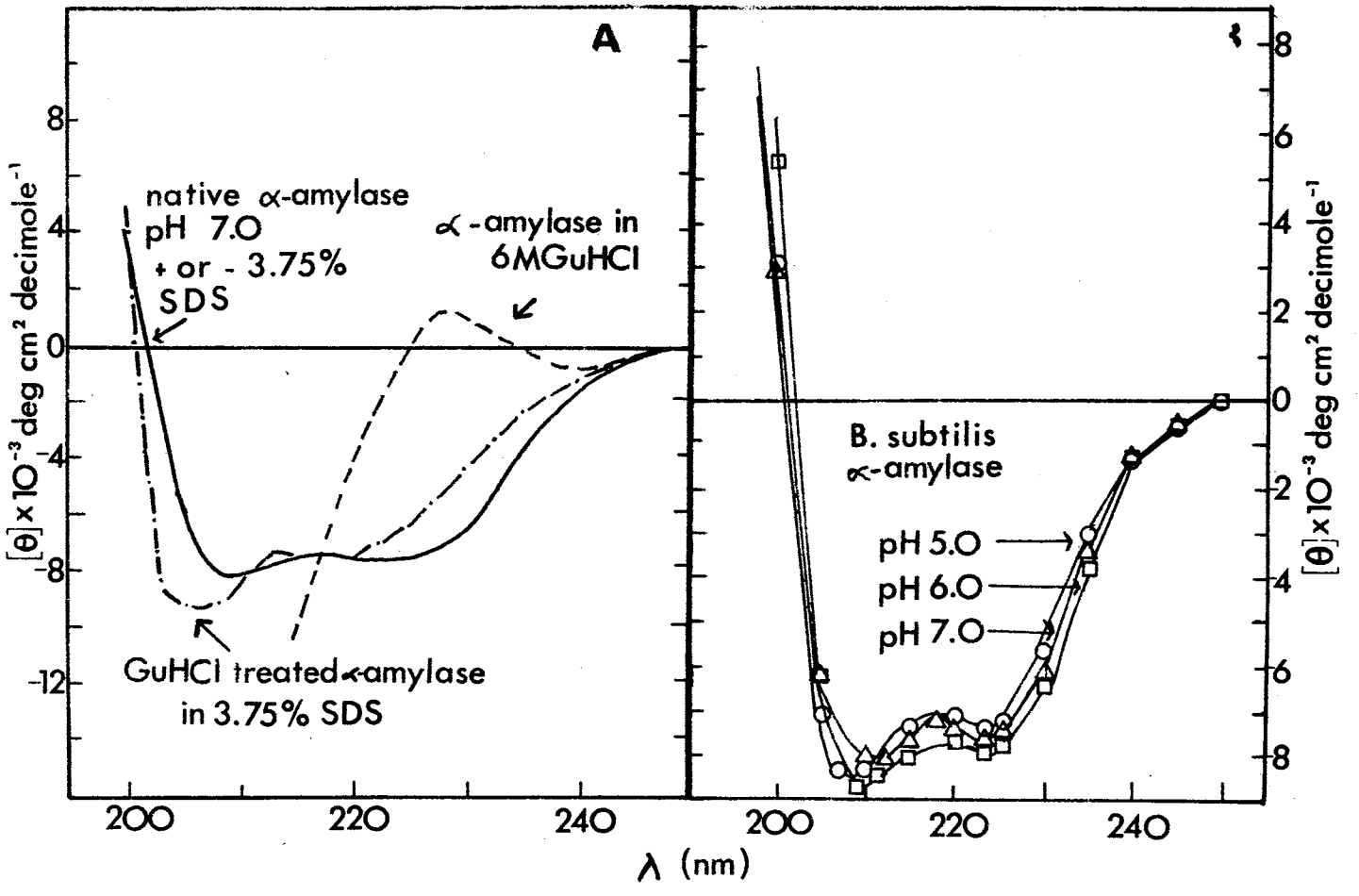
CIRCULAR DICHROISM

B. subtilis α-amylase Solution	pH	C(μg/ml)	$[\theta]^{222} \times 10^{-3}$ ($\frac{deg\ cm^2}{decimol}$)	[1]Fh(%)
0.01 M Sodium Acetate, 0.1 M KCl	5.0	281	-7.45	16.86
0.01 M Sodium Acetate, 0.1 M KCl	6.0	340	-7.51	17.06
0.01 M Sodium Acetate, 0.1 M KCl	7.0	277	-7.68	17.62
0.01 M Potassium Phosphate 0.1 M KCl	6.0	366	-7.70	18.02
0.01 M Potassium Phosphate, 0.1 M KCl	7.0	206	-7.54	17.16
0.01 M Potassium Phosphate, 0.1 M KCl	8.0	200	-7.53	17.13
0.01 M Potassium Phosphate, 0.1 M KCl	9.0	278	-7.61	17.39
0.01 M Potassium Phosphate, 0.1 M KCl	10.0	235	-7.63	17.46
6 M Guanidine HCl, 0.1% MeSH	6.5	200	-2.33	0.00
0.01 M Phosphate, 3.72 mM SDS, 0.1% MeSH	7.0	162	-7.52	17.10
6 M Guanidine HCl, 0.1% MeSH, GuHCl removed by exaustive dialysis, SDS (3.72 mM) introduced in system	6.5	200	-7.19	16.00
0.01 M Phosphate, 3.72 mM SDS, 0.1% MeSH, heated to 60° C for 10 minutes	7.0	238	-6.28	13.00

[1]Percent α-helix calculated by method of Chen and Yang (45).

Figure 17. Circular Dichroism in Various Solvent Media

A. Circular dichroism spectra of native, dodecyl sulfate solubilized and denatured α-amylase. Native enzyme and enzyme dissolved in 3.75% dodecyl sulfate give identical spectra (——). α-amylase in 6 M guanidine hydrochloride (---). α-amylase in dodecyl sulfate after treatment with 6 M guanidine hydrochloride (- - -). B. Circular dichroism of B. subtilis α-amylase at different pH values. pH 5 (O-O), pH 6 (-), and pH 7 (Δ-Δ).


A
native α-amylase
pH 7.0
+ or - 3.75%
SDS
α-amylase in
6MGuHCl
GuHCl treated α-amylase
in 3.75% SDS
B. subtilis
α-amylase
pH 5.0
pH 6.0
pH 7.0
[θ] x 10⁻³ deg cm² decimole⁻¹
λ (nm)
200
220
240
8
4
0
-4
-8
-12
6
2

it had been denatured with 6 M guanidine hydrochloride before the treatment with dodecyl sulfate. The spectrum in 6 M guanidine hydrochloride alone was characteristic of a "random" structure (Figure 17A). Therefore, it was concluded that the enzyme retains its native structure in aqueous buffers of increasing pHs whether in the presence of SDS or not. Similar observations were tentatively put forward in our discussion of the independence of sedimentation coefficient on pH in aqueous media. In 6 M guanidine hydrochloride, the enzyme is completely denatured and only then is it accessible to SDS binding.

Circular dichroism spectroscopy thus confirms our ultracentrifugation studies on the enzyme, and since the enzyme is in a random coil configuration when dissolved in 6 M guanidine hydrochloride, the values determined for the molecular weight in this media should represent the minimum subunit molecular weight.

Barley α-Amylase

Sedimentation and Diffusion Coefficients

The sedimentation coefficient for barley α-amylase, $s_{20,w}$, was 4.17 $\pm$ 0.04 S (Figure 18). Some concentration dependence was observed within a concentration range of 1.0 mg/ml to 3.5 mg/ml.

A value of 9.7 $\pm$ 0.97 x 10^{-7} cm^2/sec was noted as the observed diffusion coefficient; when corrected for density, viscosity, and temperature of the solvent media, this value remained unchanged.

The sedimenting and diffusing peaks were somewhat non-symmetrical; although a single peak gave linear plots of log x versus time (Figure 19), diffusion data showed some scatter of points in the plot of the rate of diffusion versus the time of the run (Figure 20).

Figure 18. Sedimentation Coefficient; Concentration Dependence

Concentration dependence of the sedimentation coefficient for barley α-amylase. Individual runs were made at 59,780 r.p.m. and 20° C; $s_{20,w}$ was determined by extrapolation to zero enzyme concentration.

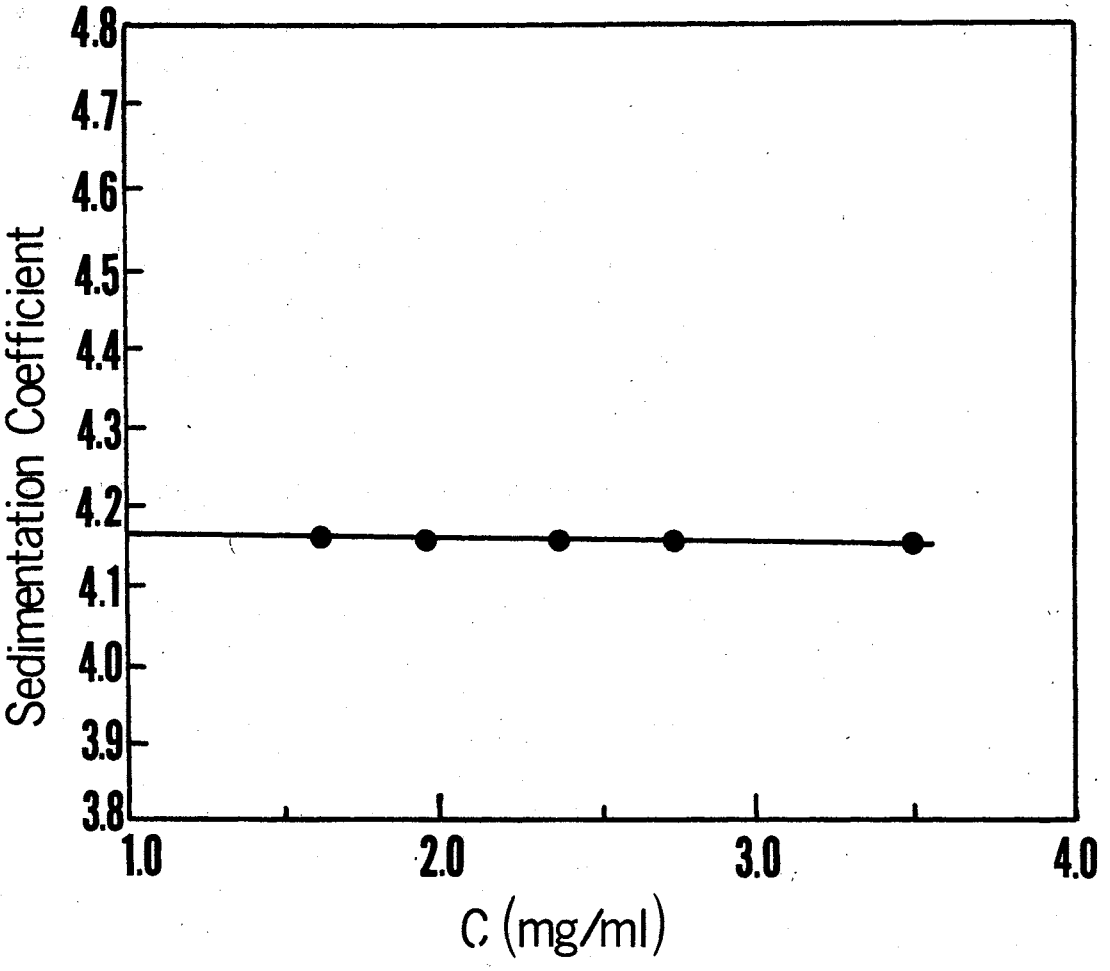

Sedimentation Coefficient
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
4.0
3.9
3.8
1.0
2.0
3.0
4.0
C (mg/ml)

Figure 19. Sedimentation Velocity Plot

Plot of the logarithm of the boundary position versus the time of sedimentation (log x versus t) for barley α-amylase. Sedimentation carried out at 59,780 r.p.m. and 20° C.

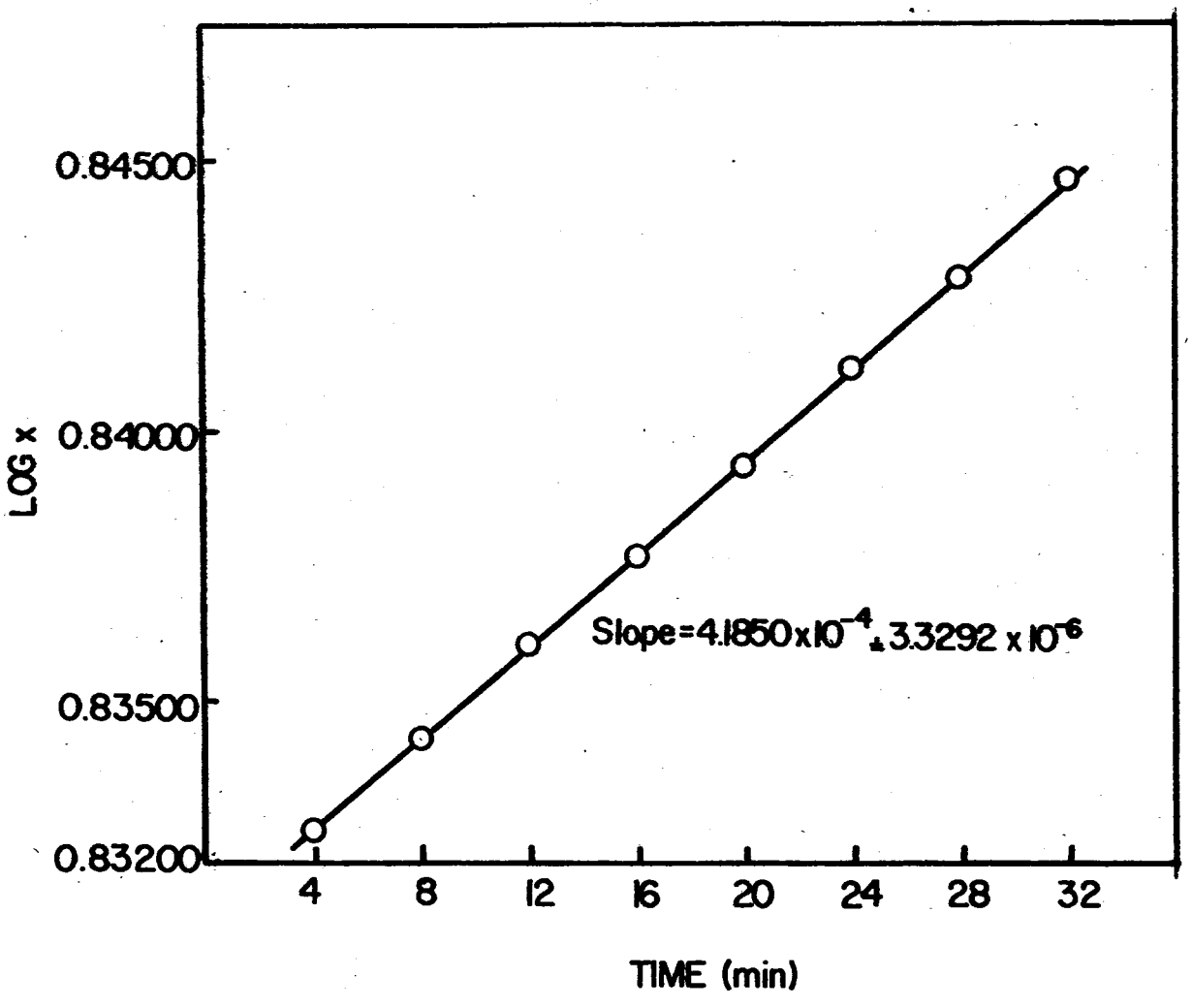
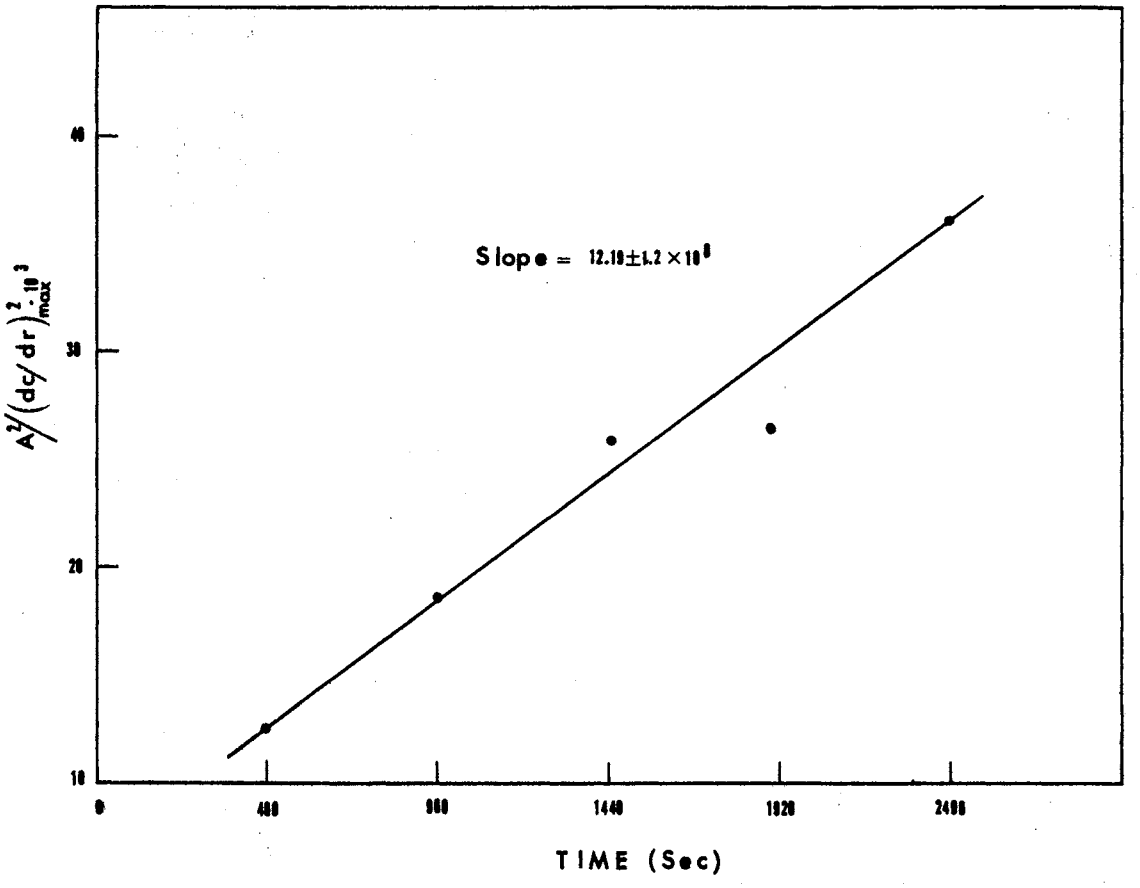

0.84500
0.84000
0.83500
0.83200
LOG x
Slope=4.1850x10⁻⁴±3.3292 x 10⁻⁶
4
8
12
16
20
24
28
32
TIME (min)

Figure 20. Diffusion Coefficient Plot

Plot of $A_{sch}^2/(dc/dr)_{max}^2$ versus time of diffusion in seconds should be linear. For barley α-amylase scatter of data points is evident in this plot. The calculated standard deviation for all points from the least squares best fit line was 10%.


Slope = 12.18±1.2 ×10⁸
40
30
20
10
0
480
960
1440
1920
2400
TIME (Sec)
A²/(dc/dr)²max·10³

Molecular Weight

A molecular weight of 40,700 daltons was calculated for barley α-amylase by means of the Svedberg equation (Equation Five, List of Equations). The results of sedimentation equilibrium ultracentrifugation carried out by the meniscus depletion method are shown in Table XI.

TABLE XI

MOLECULAR WEIGHT DETERMINATION DATA FROM MENISCUS DEPLETION SEDIMENTATION EQUILIBRIUM ULTRACENTRIFUGATION

Trial No.	Enzyme Concentration (mg/ml)	$\omega^2 \times 10^6$	Duration of run (hours)	Slope of $\log[(Yr)-(Y_0)]+3$ vs. time	Temperature ^oK	M.W. daltons (app.)
1	0.5	9.54	11	0.874	290.1	40,000
2	0.7	9.54	12	0.867	292.4	39,500

The molecular weight values of barley α-amylase were 40,700 and 40,000 by means of the Svedberg equation and equilibrium ultracentrifugation, respectively, and should be considered approximate since with both methods an assumed value of 0.740 for the partial specific value was utilized for calculation purposes.

CHAPTER V

SUMMARY

From the symmetry of the schlieren patterns of sedimentation velocity data and from the linearity of the plots of the concentration versus the square of radial distance equilibrium data of different speeds and concentrations, it was concluded that the enzyme B. subtilis α-amylase available for this study was homogeneous.

Neither the sedimentation coefficient nor the diffusion coefficient were concentration or pH dependent. The values determined for these parameters were 4.44 S and 8.00×10^{-7} cm^2/sec, respectively. The molecular weight determined from these parameters (48,400 daltons) was in agreement with those values obtained from high and low speed sedimentation equilibrium ultracentrifugation, 48,400 and 48,200, respectively. Gel filtration chromatography carried out as an independent method, based on considerably different physical factors, for the determination of the molecular weight of B. subtilis α-amylase, resulted in a value of 48,000 ± 500 daltons.

Thus, data from several independent methods corroborate a molecular weight of 48,000 daltons for the enzyme in the absence of added zinc or calcium.

Circular dichroism spectra indicated that 6 M guanidine hydrochloride denatures the enzyme completely. The molecular weight determined for the enzyme subunits by sedimentation equilibrium

ultracentrifugation and gel filtration chromatography was 24,000 daltons.

In the presence of the detergent sodium dodecyl sulfate, the enzyme retains its native configuration and SDS does not bind to the enzyme in the empirical ratio of 1.4 g of SDS per 1.0 g of protein.

Calcium and zinc ions present in concentrations in the order of 10^{-3} M produce not only enzyme activation but also polymerization to dimers and higher forms.

Although barley α-amylase appeared completely homogeneous in gel electrophoresis, it was found to contain low molecular weight impurities. This was evident from the asymmetry of the schlieren patterns from sedimentation velocity runs.

The sedimentation coefficient for barley α-amylase was 4.17 S and the diffusion coefficient had a value of 9.70×10^{-7} cm^2/sec. The approximate molecular weight of barley α-amylase calculated from sedimentation and diffusion data was 40,700 daltons. Meniscus depletion sedimentation equilibrium yielded an approximate molecular weight of 40,000 daltons.

A SELECTED BIBLIOGRAPHY

(1) Boyer, Paul D. "The Enzymes", Vol. V, p. 140, Academic Press, New York and London, 1971.

(2) Greenwood, C. T. and E. A. Milne. (1968) Advan. Carbohydrate Chem. 23, 281.

(3) Hagihara, B. (1951) Proc. Japan. Acad. 27, 346.

(4) Yamamoto, T. (1955) Bull. Agr. Chem. Soc. Japan 19, 121.

(5) Felling, J., E. A. Stein, and E. H. Fisher. (1957) Helv. Chim. Acta 40, 529.

(6) Fucumoto, J., Y. Yamamoto, and T. Ichikawa. (1951) Proc. Japan Acad. 27, 352.

(7) Fucumoto, J. and S. Okada. (1963) J. Ferment. Technol. 41, 427.

(8) Stein, E. A. and E. H. Fisher. (1961) Biochem. Prep. 8, 27.

(9) Stein, E. A. and E. H. Fisher. (1958) J. Biol. Chem. 232, 867.

(10) Isemura, T., K. Kakiuchi, and H. Eto. (1960) J. Biochem. (Tokyo) 47, 548.

(11) Isemura, T. and K. Kakiuchi. (1962) J. Biochem. (Tokyo) 51, 385.

(12) Sugae, K. (1959) J. Biochem. (Tokyo) 48, 790.

(13) Greenwood, C. T. and E. A. Milne. (1968) Stärke 4, 101.

(14) Olered, R. and J. Jönsson. (1970) Sci. Food Agric. 321, 385.

(15) Schwimmer, S. and A. K. Balls. (1948-9) J. Biol. Chem. 176, 465; 179, 1063.

(16) Waldschmidt-Leitz, E. and C. Sigrist. (1969) Z. Physiol. Chem. 350, 889.

(17) Mitchell, E. D. (1972) Phytochemistry 11, 1673.

(18) Mitchell, E. D. Unpublished Results.

(19) Greenwood, C. T. and E. A. Milne. (1968) Stärke 20, 139.

(20) Kuhn, R. (1925) Ann. Chem. 1, 443.

(21) Meyer, K. H., M. Fuld, and P. Bernfeld. (1947) Experientia 3, 411.

(22) Akabori, S., Y. Okada, S. Fujiwara, and K. Sugae. (1956) J. Biochem. (Tokyo) 43, 741.

(23) Junge, J. M., E. A. Stein, H. Neurath, and E. H. Fisher. (1956) J. Biol. Chem. 234, 556.

(24) Fisher, E. H., W. N. Sumerwell, J. M. Junge, and E. A. Stein. "Proceedings of the VIIIth Symposium of the IVth International Congress of Biochemistry, Vienna", Pergamon Press, London, 1958.

(25) Yamamoto, T. (1956) Bull. Agric. Chem. Soc. (Japan) 20, 188.

(26) Sumerwell, W. N., E. A. Stein, and E. H. Fisher. "Abstracts, Northwest Regional Meetings, American Chemical Society", Spokane, Washington, June 13-14, 1957.

(27) Stein, E. A., J. Hsiu, and E. H. Fisher. (1964) Biochemistry 3, 56.

(28) Hsiu, J., E. H. Fisher, and E. A. Stein. (1964) Biochemistry 3, 61.

(29) Kakiuchi, K., S. Kato, A. Imanishi, and T. Iseumura. (1964) J. Biochem. (Tokyo) 55, 102.

(30) Kakiuchi, K. and T. Iseumura. (1966) J. Biochem. (Tokyo) 60, 72.

(31) Connellan, M. J. and D. C. Shaw. (1970) J. Biol. Chem. 245, 2845.

(32) Munier, R. L. and A. Drapier. (1970) Chromatographia 3, 478.

(33) Lowry, O. H., A. C. Rosebrough, A. C. Farr, and R. J. Randall. (1951) J. Biol. Chem. 193, 265.

(34) Moore, S. and W. H. Stein. (1963) Methods Enzym. 6, 819.

(35) Gosting, L. J. (1956) Advan. Protein Chem. 11, 429.

(36) Yphantis, D. A. (1964) Biochemistry 3, 297.

(37) Mitchell, E. D., P. Riquetti, R. Loring, and K. Carraway. (1973) Biochim. Biophys. Acta 295, 314.

(38) Rinderknecht, H., P. Wilding, and B. J. Haverback. (1967) Experientia 23, 805.

(39) Reisler, E. and H. Eisenberg. (1969) Biochemistry 8, 4572.

(40) Nelson, C. A. (1971) J. Biol. Chem. 246, 3895.

(41) Kobylka, A., A. Khettry, B. C. Shin, and K. L. Carraway. (1972) Arch. Biochem. Biophys. 148, 475.

(42) Segrest, J. P., R. L. Jackson, E. P. Andrews, and V. T. Marchesi. (1971) Biochem. Biophys. Res. Commun. 44, 390.

(43) Bretscher, M. S. (1971) Nature 231, 229.

(44) Fish, W. W., K. G. Mann, and C. Tanford. (1969) J. Biol. Chem. 244, 4989.

(45) Chen, Y. H. and J. T. Yang. (1971) Biochem. Biophys. Res. Commun. 44, 1285.

VITA

Patricio J. Riquetti

Candidate for the Degree of

Master of Science

Thesis: PHYSICAL STUDIES OF α-AMYLASE FROM BACILLUS SUBTILIS AND BARLEY

Major Field: Biochemistry

Biographical:

Personal Data: Born in Cuenca, Ecuador, South America, June 10, 1946; the son of Mr. and Ms. Abdón Riquetti-A.

Education: Attended public schools of Anniston, Alabama; received the Bachelor of Science degree from Jacksonville State University, Jacksonville, Alabama, in 1969 with majors in Chemistry and Mathematics; completed requirements for Master of Science degree in May, 1973.

Professional Experience: Served as Research Assistant in Biochemistry at Oklahoma State University from 1970 to 1972; became a staff member in September, 1972.