ELECTROPHORETIC AND MOLECULAR WEIGHT
STUDIES OF BARLEY PEROXIDASE

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Chemistry
by
Parsegh Ananian

August, 1974
The thesis of Parsegh Ananian is approved:

Committee Chairman

California State University, Northridge
August, 1974
To my mother and my wife

who made this possible and worthwhile
ACKNOWLEDGEMENT

I wish to express my appreciation to my research director, Dr. Margaret Holzer, for the aid and encouragement she gave over the course of this work.

I would like to express my sincere gratitude to Dr. Henry Klostergaard and Dr. Girair Nazarian for the encouragement and for the many hours of patient assistance which they willingly gave when writing this manuscript.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF TABLES AND FIGURES</th>
<th>vii</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Theory</td>
<td>7</td>
</tr>
<tr>
<td>Diffusion Coefficient by Gel Chromatography</td>
<td>7</td>
</tr>
<tr>
<td>Sedimentation Coefficient from Sedimentation Velocity</td>
<td>11</td>
</tr>
</tbody>
</table>

Chapter

I  EXTRACTION AND SALT PRECIPITATION OF BARLEY PEROXIDASE | 15 |
   A. Materials | 15 |
   B. Methods | 15 |
   Preparation of the enzyme extract | 15 |
   Determination of peroxidase activity | 18 |
   Study of ammonium sulfate precipitation | 19 |
   C. Results | 21 |

II ELECTROPHORETIC STUDIES | 25 |
   A. Materials | 25 |
   B. Methods | 25 |
   C. Results and Discussion | 26 |

III MOLECULAR WEIGHT STUDIES | 32 |
   A. Materials | 32 |
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td></td>
</tr>
<tr>
<td>B. Methods</td>
<td>32</td>
</tr>
<tr>
<td>C. Results</td>
<td>38</td>
</tr>
<tr>
<td>D. Discussion</td>
<td>49</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>51</td>
</tr>
</tbody>
</table>
LIST OF TABLES AND FIGURES

Table

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Dependence of Enzyme Activity on Relative Enzyme Concentration</td>
</tr>
<tr>
<td>II</td>
<td>Peroxidase Activity Remaining in Solution in the Presence of Increasing Concentration of Ammonium Sulfate</td>
</tr>
<tr>
<td>III</td>
<td>Proteins used in Gel Chromatography Experiments</td>
</tr>
<tr>
<td>VI</td>
<td>Results of Sedimentation Experiments</td>
</tr>
</tbody>
</table>

Figure

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Plot of elution volume, V, against mol. wt. (on a logarithmic scale) for proteins on a Sephadex G-200 column at pH 7.5.</td>
</tr>
<tr>
<td>2</td>
<td>Plot of absorbance at 400 μm versus relative enzyme activity of barley peroxidase in 0.05 M phosphate buffer pH 6.1</td>
</tr>
<tr>
<td>3</td>
<td>Plot of absorbance (activity) remaining in solution at 400 μm after ammonium sulfate precipitation, versus percent w/w of final ammonium sulfate concentration</td>
</tr>
<tr>
<td>4</td>
<td>Electrophoretic patterns of barley peroxidases on untreated paper</td>
</tr>
<tr>
<td>5</td>
<td>Electrophoretic pattern of barley peroxidases on positive paper</td>
</tr>
<tr>
<td>6</td>
<td>Electrophoretic pattern of barley peroxidases on bovine serum albumin-treated paper</td>
</tr>
<tr>
<td>7</td>
<td>Experimental set up for fractionation</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>8</td>
<td>Elution pattern (obtained from the column of gel chromatography using Sephadex G 100) of barley peroxidase in 0.05 M phosphate buffer pH 6.1</td>
</tr>
<tr>
<td>9</td>
<td>Plot of elution volume, $V$, against diffusion coefficient (on a logarithmic scale) for proteins on a Sephadex G-100 column at pH 6.1.</td>
</tr>
<tr>
<td>10</td>
<td>Plot of absorbance (enzyme activity) per gram of solution fraction versus the cumulative weight of fractions</td>
</tr>
<tr>
<td>11</td>
<td>Beckman ultracentrifuge rotor No. 40 specifications</td>
</tr>
</tbody>
</table>
ABSTRACT

ELECTROPHORETIC AND MOLECULAR WEIGHT STUDIES OF BARLEY PEROXIDASE

by

Parsegh Ananian

Master of Science in Chemistry

July, 1974

A peroxidase, obtained from barley (White Naked Atlas) treated with giberellic acid, was analyzed electroforetically using a Durrum cell and bovine serum albumin-treated paper; resolution into six bands (four of which are cathodic at pH 6.1) was obtained in 14 hours as determined with benzidine and guaiacol sprays. This simple, and quick method of analysis seems adequate for screening in studies involving stresses of various kind applied to a plant system.

For the same peroxidase the diffusion coefficient was determined by gel chromatography (Sephadex) and the sedimentation coefficient was obtained using a preparative ultracentrifuge and a special sampling device. These coefficients together with the Svedberg relation lead to a molecular weight of about 34,000.
INTRODUCTION

Enzymes are biological catalysts that accelerate the reactions which occur in the living organism. Many of these reactions are not only accelerated by enzymes but would not occur at sufficient rates at body temperature in their absence. Although enzymes become intimately involved in the reactions they catalyze, they emerge essentially unchanged at the end of the reaction. Certain enzymes are solely protein such as pepsin and trypsin. However, other enzymes have been found to consist of a non-protein part plus the protein and are therefore called conjugated proteins. If the non-protein portion is an organic moiety and is readily separated from the enzyme, this fragment is called a coenzyme. It is called a prosthetic group if it is firmly attached to the protein portion of the enzyme.

Photosynthesis, respiration and biological oxidation, fermentations, synthesis of the many organic compounds required for growth, performance of mechanical and osmotic work are among the processes in which enzymes take part.

Isozymes are enzymes encountered within a single type of organism, which will assume the same enzymic function. These molecular forms are variants of the same
protein, and thus structurally related. They can often be separated electrophoretically because of slight differences in the pI of the protein molecule (1).

Peroxidases are a class of enzymes which utilize a peroxide to oxidize organic substances in a plant. Among the peroxidases we find some which exist in isozymic form. In plants there exists an enzyme system comprising an oxygenase which activates oxygen to produce a peroxide and a peroxidase which utilizes the peroxide to oxidize organic substances.

The essential characteristic of biological oxidation is the removal or transfer of electrons from the substrate molecule to an appropriate acceptor,

\[ \text{SH}_2 + A_{\text{red}} \xrightarrow{\text{oxid}} \text{AH}_2 + S \]

S stands for substrate, A for acceptor.

If during oxidation, hydrogen is removed from the substrate then the enzyme is called dehydrogenase. When oxygen molecules serve as the electron acceptor the enzymes have been called oxidases. Enzymes catalyzing the activation of oxygen and the subsequent incorporation of either one or two atoms of oxygen per molecule of various substrates are called oxygenases. Peroxidases catalyze reactions with molecular oxygen by forming reactive free radicals during its peroxidatic activity (2); as enzymes
they are absolutely specific for peroxide as substrate. They can be regarded as oxygenases only in the broadest sense of the term that includes any enzymic system promoting reactions with oxygen. By the narrow definition, according to which an oxygenase is an enzyme activating molecular oxygen for reaction with another substrate, a peroxidase is not an oxygenase (2).

Peroxidases contain ferriprotoporphyrin (haematin) as the prosthetic group. The action of these enzymes is different from that of dehydrogenases in that the prosthetic group is involved directly in the activation of the substrate. The enzymes thus bring about the oxidation of various substances by peroxides. Peroxidases oxidize a wide range of materials, including phenolic substances, cytochrome c, nitrite, leucomalachite green but not \( \text{H}_2\text{O}_2 \) itself (1).

The motives for studying isozymes are diverse. Biologists have used isozymes to study the genes involved in enzyme synthesis. Genetic studies have indicated that glucose-6-phosphate dehydrogenase polymorphism in wild type strains of \textit{D. melanogaster} is controlled by a gene located on the X-chromosome (3). Genetic studies also indicated that phosphogluconate dehydrogenase from strains of \textit{Drosophila} is controlled by a gene on the X-chromosome (4). Biochemists and physiologists look upon different physio-chemical properties of isozymes as a
means to study the regulation of cell metabolism. Evidence has been presented which indicates a possible relationship between the multiple molecular forms of hexokinase and diabetes (5). Peroxidase isozyme formation in germinating barley seeds, the number and the amount produced varies according to experimental conditions. An increased peroxidase activity was found when barley seeds were irradiated with ultraviolet light (6). Decreased production of peroxidase activity was found for the PZHR variety of barley during germination under the influence of gibberellic acid whereas no change in peroxidase activity was found for the Wisa breuna variety (7). Peroxidase activity is found to be higher during colder months in barley and wheat (8). More peroxidase isozymes have been found in ultraviolet irradiated than in unirradiated barley seeds (9). Barley varieties which are more resistant to powdery mildew were shown to have higher peroxidase content (10). Ceratocystic infection in sweet potatoes was shown to alter electrophoretic patterns of peroxidase isozymes. Changes were observed in the protein and isozymic patterns of susceptible bean leaves after infection by the bean rust fungus (12).

The present work started in conjunction with studies by Highkin and Wensel (13) on the effect of temperature and gibberellic acid concentration on the production of peroxidase isozymes in barley endosperm. In order
to study barley peroxidase isozymes we needed a genetically pure barley and a method to produce the enzyme in high yield. Since both of these requirements were satisfied in the Highkin-Wensel approach, we adopted their method without change.

Our work deals with electrophoretic and molecular weight studies of barley peroxidase. A literature survey indicated that electrophoretic studies of peroxidases from different plant species have been conducted with gel electrophoresis. Six peroxidase components have been demonstrated after starch gel electrophoresis of corn-leaf sheath preparations (14). Using starch gel electrophoresis, Klapper and Hackett (15) found six components in commercial preparations of horse radish peroxidase and stated that the heterogeneity depended on charge, rather than molecular size. Ockerse (16), using starch gel electrophoresis, has shown that young stem sections of dwarf peas grown in light yielded at least seven peroxidase isozymes. In addition, characteristic peroxidase isozymic patterns have been described in the root nodules of various leguminous plants, the most outstanding species differences being between *Vicia sativa* and *Galega officinalis* (17). We attempted paper electrophoretic separation of barley peroxidase although proteins are known to adsorb strongly to the paper support which may make it impossible to observe substances with lower mobilities (18).
Recognition of the isozymic nature of certain proteins becomes much easier if the overall molecular structure and the approximate molecular weight is known (19). The method of estimating molecular weights from gel filtration data alone has the disadvantage in common with molecular weight estimations by velocity sedimentation alone. One experimental quantity is being used to solve an equation containing two parameters of unknown magnitude (20).

The essential features of the procedure for molecular weight estimation based on gel filtration data alone are first: calibration of the gel column by determining the elution volumes of a series of proteins of known molecular weight, M; second: estimation of elution volume, $V_e$, for the protein under investigation; and third: conversion of elution volume, $V_e$, to molecular weight on the basis of the calibration curve, plotted in the form $V_e$ versus log M (21, 22).

This work deals in estimating the molecular weight of barley peroxidase by a combination of data from gel filtration and sedimentation experiments (23, 24). The superiority of this procedure over the method of estimating molecular weight by gel filtration alone is demonstrated by the ability to distinguish between the glycoproteins and carbohydrate free globular proteins when elution volume is plotted against diffusion.
coefficient instead of molecular weight (23).

Theory

Diffusion Coefficient by Gel Chromatography (25)

If a very small volume (compared to the bed volume) of solution containing a protein is introduced at the top of a column containing a gel and then followed by a flow of solvent, it is found (26) that the elution volume, $V_e$, of solvent which passes through the column between introduction of the sample and subsequent emergence of its maximum concentration, can be described by the equation

$$V_e = V_o + JV_i$$  \[1\]

where

- $J$ = the partition coefficient
- $V_o$ = the void volume (volume of solvent between gel particles)
- $V_i$ = the internal volume (volume of stationary solvent phase)

The following logarithmic relation between molecular weight and partition coefficient for proteins has been found to hold true (25) in many gel systems

$$J = -A \log M + B$$  \[2\]

where $A$ and $B$ are empirical constants and $M$ is the
molecular weight.

For spherical molecules or random coils, the molecular weight can be expressed in terms of some power, \( p \), of molecular radius, \( a \) (25),

\[
M = K a^p
\]  \[[3]\]

where \( K \) is a geometrical factor.


\[
J = -A' \log a + B'
\]  \[[4]\]

where \( A' = p \cdot A \) and \( B' = B - A \log K \).

A thorough investigation of the above relationships has been carried out by P. Andrews in a series of studies (21, 22) aimed at evaluation of the validity of molecular weight determination using gel chromatography. A representative set of experimental data is shown in Figure 1 for Sephadex G-200 gel. Here the elution volume, \( V_e \), has been correlated with molecular weight \( M \). This correlation can be derived from equations [1] and [2], giving

\[
V_e = -A'' \log M + \text{terms not involving } M,
\]

where \( A'' = V_1 A \).

This relationship holds only over the central portion of the curve (Figure 1). It was found that
Figure 1. Plot of elution volume, $V_e$, against mol. wt. (on a logarithmic scale) for proteins on a Sephadex G-200 column at pH 7.5. After P. Andrews (22).
asymmetric molecules and those containing carbohydrate
deviate significantly from the curve generated from the
elution volumes of the normal proteins (27).

The behavior of all molecular species could be
better correlated by a curve relating elution volume, $V_e$,
to diffusion coefficient. The diffusion coefficient is
calculated from the molecular Stokes radius, $a$, by means
of the Einstein relation (28),

$$D = \frac{RT}{N\eta f} = \frac{RT}{N6\pi\eta a} \quad [6]$$

where

- $R$ = the gas constant,
- $T$ = the absolute temperature,
- $N$ = Avogadro number,
- $\eta$ = viscosity,
- $a$ = the Stokes radius,
- $f$ = frictional coefficient.

Substituting the value of $a = \frac{RT}{N6\pi\eta D}$ from
equation [6] and the value of $J = \frac{V_e - V_o}{V_i}$ from equation
[1] in equation [4], we get:

$$V_e = A' V_i \log D + \text{terms not involving } D \quad [7]$$

Therefore according to equation [7], if $V_e$ is plotted
versus log D, we get a straight line with a slope equal to \( a' v_i \).

**Sedimentation Coefficient from Sedimentation Velocity (29)**

A molecule in a solution when acted on by a centrifugal field will experience three forces.

First, centrifugal force, \( F_c = m \omega^2 r \),

where

- \( m \) = the mass of the molecule
- \( \omega \) = the angular velocity in radians per second
- \( r \) = the distance from the center of rotation.

Second, buoyant force, \( F_b = -m_0 \omega^2 r \).

It is equal to the force exerted on the mass, \( m_0 \) of displaced solution. We can write \( m_0 = m \overline{V}/\rho \).

where

- \( \overline{V} \) = partial specific volume of the molecule,
- \( m \overline{V} \) = volume of the molecule,
- \( \rho \) = the density of solution.

The minus sign is due to the direction of the force, which is opposite to the centrifugal force.

Third, the drag force, \( F_d = -fv \),

where \( v \) = velocity of the molecule.
The relationship of these three forces is that their sum is zero.

\[ F_c + F_b + F_d = 0 \]

Substituting their values, we get:

\[ m \omega^2 r - m \omega^2 r \bar{\rho} - f v = 0 \]

Factoring and multiplying by \( N \) (Avogadro number) we get:

\[ M \omega^2 r (1-\bar{\rho}) = f v N. \]

Separating the molecular parameters, we get:

\[ \frac{M (1-\bar{\rho})}{Nf} = \frac{v}{\omega^2 r} = S = \text{sedimentation coefficient} \] \[ \text{[8]} \]

Therefore, the sedimentation coefficient, \( S \), of a molecular species is proportional to its molecular weight and the buoyancy factor \((1-\bar{\rho})\), and inversely proportional to the frictional coefficient \( f \).

The velocity of the particles in a centrifugal field can be expressed as \( \frac{dr}{dt} \), the rate of change of distance from the center of rotation. \( v = \frac{dr}{dt} = S \omega^2 r \), which can be written as \( \frac{dr}{r} = S \omega^2 dt \). Integrating in the limits of \( t_1 \) and \( t_2 \), we get:
\[
\int_{r_1}^{r_2} \frac{dr}{r} = \int_{t_1}^{t_2} S w^2 dt
\]

\[\ln \frac{r_2}{r_1} = S w^2 (t_2 - t_1)\]

\[S = \frac{1}{w^2(t_2 - t_1)} \ln \frac{r_2}{r_1}\]  \[\text{[9]}\]

Knowing experimentally the values of \(w\), \(t_1\), \(t_2\), \(r_1\), and \(r_2\), \(S\) can be calculated.

If the diffusion coefficient of the protein were zero, the boundary between solution and solvent would remain infinitely sharp. For actual proteins, diffusion spreads the boundary during sedimentation. Especially in the case of small molecular weight proteins with large diffusion coefficients and small sedimentation coefficients, a clear boundary will not be formed.

In the relationship \(j = S w^2 rc - D \frac{dc}{dr}\) \[\text{(29)},\]

\(j\) = the flow at any point in a system in a centrifugal field of force at any instant,

\(S\) = sedimentation coefficient,

\(r\) = distance of the point from the center of rotation,
\( c = \) the concentration,
\( D = \) diffusion coefficient,
\( \omega = \) angular velocity in radians per second.

This equation gives us a complete description of the processes occurring in an ultracentrifuge cell. The first term of the equation corresponds to transport by sedimentation, and the second term transport in the opposite direction by diffusion.

When centrifugation is started, concentration is everywhere uniform, \( \frac{dc}{dr} = 0 \) and only the first term \( S \omega^2 rc \), is important. Once the protein starts to move, it creates a boundary creating a concentration gradient, \( \frac{dc}{dr} \); then the diffusion term becomes important with subsequent broadening of the boundary. If the centrifugation were stopped \((\omega = 0)\) the boundary would stop but continue to spread with time as in a simple diffusion experiment.
Chapter 1

EXTRACTION AND SALT PRECIPITATION OF BARLEY PEROXIDASE

A. Materials

The barley variety, White Naked Atlas, was used in the extraction of barley peroxidase enzyme because it represents a genetically stable and homogeneous population which can easily be studied (11). It was graciously supplied by Prof. C. W. Shalter, University of California, Davis.

Giberellic acid (grade III) was from Sigma Chemical Co. Streptomycin, B grade, was from Calbiochem. Guaiacol and Benzidine were from Matheson, Coleman and Bell Co. Chemicals otherwise specified were commercially available reagent grade.

B. Methods

Preparation of the enzyme extract.

White Naked Atlas barley seeds were dehulled by hand. By means of a sharp knife, the endosperm part was separated (approximately half to 3/4 of the whole seed) from the rest of the seed.

The endosperm (100g) was sterilized by soaking the seed halves in one percent NaOCl for 20 minutes at room temperature. After decanting the solution, traces
of NaOCl were removed by washing in sterile water (deionized water boiled for 20 minutes, and cooled) until the wash water no longer gave a positive reaction with KI-starch paper.

The washed and sterilized seed halves were then transferred to an erlenmeyer flask and covered with 250 ml of $10^{-5}$ M gibberellic acid solution prepared by dissolving 3.8 mg of the potassium salt of the acid in one liter of sterile water to which 50 mg of streptomycin had been added. The flask containing the incubation mixture was stoppered and maintained at 25°C in a constant temperature bath for 18 hours.

Next, the incubating solution was decanted and the seed halves were homogenized in a chilled Waring blender with 250 ml of 0.05 M phosphate buffer pH 6.1 at 8-10°C. To the homogenate 20 mg of a streptomycin was added and the homogenate was left in the refrigerator for 24 hours.

The homogenate, a thick slurry, was subjected to centrifugation for 30 minutes in the cold (1-2°C) at 27,000 g in a Sorvall model RC-2 refrigerated centrifuge.

The residue in the centrifuge tube was discarded. To the supernatant liquid (now approximately 220 ml) 44 g of ammonium sulfate was added to make 20 percent w/v*

solution. During ammonium sulfate addition, the solution was continuously stirred by a magnetic stirrer. Next, the solution was stoppered and left at 3-4°C overnight for the more complete precipitation of proteins. Then the solution was subjected to centrifugation for 30 minutes at 27,000 g in the refrigerated centrifuge, and the supernatant liquid, which contained most of the enzyme activity,* was decanted. To this solution a second 44 g* of ammonium sulfate was added gradually with constant stirring and allowed to remain in the cold (3-4°C) for 5-6 hours. At this point the enzyme activity was precipitated into a brownish mass, which was separated from the solution by centrifugation as above and resuspended in 25-30 ml cold (8-10°C) 0.05 M phosphate buffer, pH 6.1 as a brown slurry.

The slurry was transferred to a dialyzing tube of 1-1/2 inches in diameter, and dialyzed against 300 ml 0.05 M phosphate buffer at pH 6.1 for 20 hours in the cold (3-4°C). The dialyzing buffer was replaced every eight hours. After dialysis, the solution contained some solid residue which was removed by centrifugation for 30 minutes in the Sorvall refrigerated centrifuge at 27,000 g. The final solution, amounting to approximately 30 ml, was transferred to a vial and stored in the freezer.

Determination of peroxidase activity

Peroxidase activity was determined using a modification of the techniques of N. Jermyn and R. Thomas (30).

Color developing mixture was prepared by mixing equal volumes of saturated aqueous guaiacol solution and 0.1 M hydrogen peroxide solution. Saturated guaiacol solution was prepared and stored in the cold (3-4°C) and the cold hydrogen peroxide solution was freshly prepared.

To each of several test tubes containing 0.5-1 ml of the enzyme extract (in 0.05 M phosphate buffer pH 6.1), 3 ml of the color developing mixture was added. The tubes were shaken to mix, then immersed in a water bath kept at 38°C for 15 minutes. Next the tubes were chilled in ice-water mixture for 5 minutes. To each tube, 2 ml of 0.5 N potassium hydroxide solution was added and the reaction mixtures were allowed to stand for one hour* at room temperature. Then the absorbance of each was read at 400 μm with a Spectronic model 20 spectrophotometer.

The potassium hydroxide solution both destroys the enzyme and dissolves the reddish brown precipitate of tetraguaiacol formed by the reaction. The color changes to a yellowish green solution with maximum absorbance at

* The absorbance reaches its maximum value in one hour.
400 m\textmu L. The yellowish green color is stable for 24 hours (30).

One ml of the enzyme extract was diluted to 50 ml with 0.05 M phosphate buffer, pH 6.1. The diluted enzyme solution was further diluted, such that a series of enzyme solutions were prepared with varying concentrations. See Table I. One ml samples of each of these diluted enzyme solutions were taken and peroxidase activities were determined as explained above.

**Study of ammonium sulfate precipitation.**

This study was made in order to find an optimum range of ammonium sulfate concentrations, so that most of the enzyme activity is precipitated, thus eliminating the unwanted proteins which precipitate outside this range.

Phosphate buffer 0.05 M, pH 6.1, was used to prepare the following ammonium sulfate concentrations. To 100 ml of the buffer solution 10 g of ammonium sulfate was added and the solution was labeled 10 percent. Following this method, 10 percent, 15 percent, 20 percent, 25 percent, 30 percent, 35 percent, 40 percent, 45 percent, and 50 percent solutions were prepared.

To 10 ml of each of the above solutions, one ml of the enzyme extract was added, mixed well, stoppered and left in the cold (3-4°C) overnight. Next the above solutions were subjected to centrifugation in the
## TABLE I

Dependence of Enzyme Activity on Relative Enzyme Concentration

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume of Enzyme Solution</th>
<th>Volume of Buffer</th>
<th>Relative Concentration</th>
<th>Absorbance (average)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>0</td>
<td>100%</td>
<td>1.157</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>2</td>
<td>90%</td>
<td>1.060</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>4</td>
<td>80%</td>
<td>.958</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>6</td>
<td>70%</td>
<td>.825</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>8</td>
<td>60%</td>
<td>.750</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>10</td>
<td>50%</td>
<td>.623</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>12</td>
<td>40%</td>
<td>.512</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>14</td>
<td>30%</td>
<td>.407</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>16</td>
<td>20%</td>
<td>.283</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>18</td>
<td>10%</td>
<td>.177</td>
</tr>
</tbody>
</table>
Sorvall RC-2 refrigerated centrifuge for 30 minutes at 27,000 g. From the clear supernatant solutions, two ml aliquots were taken and transferred to a 1/4 inch diameter dialysis tubing, and dialyzed for 20 hours against 200 ml of 0.05 M phosphate buffer, pH 6.1 in the cold (3-4°C). The buffer solution was replaced every eight hours.

After dialysis, the contents of the tubings were quantitatively transferred to 25 ml volumetric flasks and were made up to volume with the buffer solution. One ml aliquots of these solutions were used for quantitative peroxidase activity determinations as outlined above.

C. Results

There was a linear relationship when relative barley peroxidase enzyme activities were plotted against absorbance measured at 400 nm up to an absorbance value of 1.1. See Figure 2.

The results of the ammonium sulfate precipitation are presented in Table II and Figure 3. It can be seen from Figure 3 that most of the enzyme activity was precipitated in the range between 15 percent to 32 percent ammonium sulfate w/w in solution.
Figure 2. Plot of absorbance at 400 μm versus relative enzyme activity of barley peroxidase in 0.05 M phosphate buffer pH 6.1.
<table>
<thead>
<tr>
<th>Ammonium Sulfate Solutions*</th>
<th>Initial Concentration of Ammonium Sulfate w/w</th>
<th>Density of Solution at 4°C g/ml</th>
<th>Final Concentration of Solutions w/w</th>
<th>Enzyme Activity Absorbance at 400 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>9.1%</td>
<td>1.0515</td>
<td>8.3%</td>
<td>.345</td>
</tr>
<tr>
<td>15%</td>
<td>13.0%</td>
<td>1.0750</td>
<td>11.9%</td>
<td>.345</td>
</tr>
<tr>
<td>20%</td>
<td>16.7%</td>
<td>1.0980</td>
<td>15.3%</td>
<td>.345</td>
</tr>
<tr>
<td>25%</td>
<td>20.0%</td>
<td>1.1154</td>
<td>18.4%</td>
<td>.284</td>
</tr>
<tr>
<td>30%</td>
<td>23.1%</td>
<td>1.1326</td>
<td>21.2%</td>
<td>.243</td>
</tr>
<tr>
<td>35%</td>
<td>26.0%</td>
<td>1.1496</td>
<td>23.9%</td>
<td>.180</td>
</tr>
<tr>
<td>40%</td>
<td>28.6%</td>
<td>1.1660</td>
<td>26.3%</td>
<td>.135</td>
</tr>
<tr>
<td>45%</td>
<td>31.0%</td>
<td>1.1777</td>
<td>28.6%</td>
<td>.060</td>
</tr>
<tr>
<td>50%</td>
<td>33.3%</td>
<td>1.1890</td>
<td>30.7%</td>
<td>.015</td>
</tr>
</tbody>
</table>

Figure 3. Plot of absorbance (activity) remaining in solution at 400 μm after ammonium sulfate precipitation, versus percent w/w of final ammonium sulfate concentration.
Chapter II
ELECTROPHORETIC STUDIES

A. Materials

A Durrum type apparatus (Beckman - Spinco II) was used as the electrophoretic cell using 0.05 M phosphate buffer, pH 6.1.

Electrophoresis was normally carried out on Beckman-Spinco No. 300-546, 3.0 x 30.6 cm paper strips. Bovine serum albumin used in treating the electrophoretic papers was from Sigma Chemical Co., Girard's "P" reagent used in forming positive papers was from Matheson, Coleman and Bell Co. The chemicals otherwise specified were commercially available reagent grade.

B. Methods

Preparation of barley peroxidase enzyme extract was as explained in Chapter I, Methods.

The technique for producing positive filter paper strips was by the method of M. A. Jermyn and R. Thomas (30). The periodic oxidation was done at 20°C in the dark for 30 minutes before treatment with Girard "P" reagent.

Electrophoresis was also conducted on paper strips which were soaked with cold (3-4°C) 0.2 percent bovine serum albumin in 0.05 M phosphate buffer solution,
pH 6.1, before applying the enzyme extract. At the center of the paper strips, 20-30\% of the extract was applied with a micropipet.

The spraying reagent, benzidine hydrogen peroxide solution, and guaiacol hydrogen peroxide solution were prepared by the method of M. A. Jermyn and R. Thomas (30), who used them for detection on paper of peroxidase from horseradish, fig latex, and other plant peroxidases.

The filter paper electrophoresis was normally carried out at 2-5°C with a constant current of 15 m.A. After electrophoresis the paper strips were dried on paper towels in the refrigerator then double sprayed, first with cold guaiacol-hydrogen peroxide solution and then with cold benzidine-hydrogen peroxide solution (30).

C. Results and Discussion

Working with untreated paper strips, the electrophoretic patterns were as shown in Figure 4.

It is obvious that the peroxidase components move in the first four hours, then the pattern remains immobile, and gradually fades away due to inactivation of the enzyme. There are two distinct bands which move towards the cathode and one band that is stationary.

When working with positive papers, the mobilities did not increase. The only observed
Figure 4. Electrophoretic patterns of barley peroxidases on untreated paper. Electrophoresis was carried out in 0.05 M phosphate buffer, pH 6.1 with a constant current of 15 m.A. (a) 4 hours. (b) 8 hours. (c) 16 hours.
difference between the two patterns was that due to transformation of cathodic electro-osmotic flow (untreated paper) to anodic electro-osmotic flow (positive papers) (30). There was a significant shift of the whole pattern towards the anode (See Figure 5).

With prolonged (16 hours or more) electrophoresis the peroxidase was inactivated both on positive as well as on untreated papers. The pattern was hardly perceptible after 24 hours on untreated paper and after 16 hours on positive papers.

Electrophoretic paper treated with serum albumin gave six bands, four of which are cathodic and one anodic (See Figure 6). The band seen at the starting line occupying an area corresponding to the same position as the original extract may be due to the precipitation of non-enzyme material on the paper followed by secondary adsorption of the enzyme (30).

The resolution into different components was better by this treatment than when the electrophoresis was conducted on untreated or positive papers, because the electrophoresis could be conducted longer without appreciable loss of peroxidase activity. Treating the paper with serum albumin not only protected the enzyme from inactivation but also increased the mobilities of these components.

In contrast to our results. R. Nilson and T.
Figure 5. Electrophoretic pattern of barley peroxidases on positive paper. Electrophoresis was carried out in 0.05 M phosphate buffer, pH 6.1 with a constant current of 15 mA, for 8 hours.
Figure 6. Electrophoretic pattern of barley peroxidases on bovine serum albumin-treated paper. Electrophoresis was carried out in 0.05 M phosphate buffer, pH 6.1 at 2-5°C with 15 m.A. constant current for 14 hours.
Hermelin (31) using starch gel electrophoresis found 12 peroxidase components from each of 12 varieties of barley at the milk-ripe stage of the endosperm.

The difference in our findings is probably due to higher peroxidase content of the milk-ripe stage of the endosperm and also the inhibitory action of the giberellic acid. No attempt was made to duplicate our results with starch or polyacrylamide gels.

The above method can replace starch gel electrophoresis of peroxidases. It is simple, fast and reliable, requiring minimum equipment and technique. Furthermore, by the use of a Durrum-type cell, eight electrophoresis experiments can be run simultaneously. This can enable the biologist to correlate the relative concentrations of enzyme components with environmental conditions on a routine basis.
Chapter III

MOLECULAR WEIGHT STUDIES

A. Materials

Sephadex G100, particle size 10-40μ was from Pharmacia Chemical Co. Ribonuclease was from Worthington Biochemical Corp. (RAF 7JA). Trypsin (2X crystallized), pepsin (P-6875) and bovine serum albumin were from Sigma Chemical Co. Horseradish Peroxidase and hemoglobin (bovine) were from Worthington Biochemical Corp.

Spectrophotometric measurements were done using Beckman DU spectrophotometer. In sedimentation velocity experiments a Beckman model L ultracentrifuge with rotor number 40 was used. The centrifuge tubes were Beckman No. 302235 cellulose nitrate, size 5/8" x 3".

B. Methods

Gel chromatography column was prepared in a vertical glass tube, internal diameter 2 cm, filled with Sephadex G100, suspended in 0.05 M phosphate buffer, pH 6.1, containing 0.1 M KCl, following the method described by P. Andrews (21, 22). The bed height was 51-52 cm.

Barley peroxidase extract was prepared as described in Chapter II. Horseradish peroxidase solution was prepared by dissolving 0.7 mg of the enzyme in cold (1-4°C) 0.05 M phosphate buffer pH 6.1 and the volume
made up to 25 ml.

In gel chromatography experiments, 1 ml of the above peroxidase solution was applied to the top of the column. For other proteins, 2-5 mg of each were dissolved in one ml of the buffer solution, and then applied to the top of the column.

The gel chromatography experiments were conducted at room temperature 22±2°C. Proteins were estimated spectrophotometrically by using the whole of each effluent fraction, in a quartz cuvet 1/2 cm light path. The wavelength was selected as in Table III. For barley peroxidase and horseradish peroxidase, peroxidase activities were estimated according to the procedure described in Chapter II, by using 0.2-0.3 ml samples.

The effluent volume, $V_e$, corresponding to maximum concentration of a protein was estimated by extrapolation to the nearest 0.5 ml from a plot of effluent volume, $V_e$, versus absorbance as described in P. Andrews (21, 22).

Sedimentation experiments were conducted with barley and horseradish peroxidase only. For sedimentation experiments 3 ml of the horseradish peroxidase solution (0.7 mg of horseradish peroxidase in 25 ml of buffer solution) was diluted with cold buffer solution to 25 ml. For barley peroxidase, the solution was prepared by diluting 3 ml of the enzyme extract (prepared as
### TABLE III. Proteins Used in Gel Chromatography Experiments

<table>
<thead>
<tr>
<th>Protein</th>
<th>Elution Volume* $V_e$ in ml</th>
<th>$10^{-3} \times$ Mol. Wt.</th>
<th>Reference</th>
<th>$10^7 \times$ Diffusion Coefficient $D_{20}$ (cm$^2$/sec)</th>
<th>Reference</th>
<th>Method of Estimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribonuclease (bovine)</td>
<td>99.0</td>
<td>13.7</td>
<td>33</td>
<td>10.2 - 13.10</td>
<td>39, 41</td>
<td>E at 278 mµ</td>
</tr>
<tr>
<td>Trypsin</td>
<td>97.5</td>
<td>23.2</td>
<td>38</td>
<td>10.95</td>
<td>41</td>
<td>280 mµ</td>
</tr>
<tr>
<td>Pepsin</td>
<td>82.0</td>
<td>36.0</td>
<td>37</td>
<td>9.3</td>
<td>41</td>
<td>280 mµ</td>
</tr>
<tr>
<td>Rennin</td>
<td>72.0</td>
<td>34.4</td>
<td>35</td>
<td>7.9</td>
<td>41</td>
<td>278 mµ</td>
</tr>
<tr>
<td>Horseradish Peroxidase</td>
<td>57.5</td>
<td>40.0</td>
<td>36</td>
<td>7.05</td>
<td>40</td>
<td>Enzyme activity</td>
</tr>
<tr>
<td>Haemoglobin (bovine)</td>
<td>58.0</td>
<td>64.5</td>
<td>34</td>
<td>6.7</td>
<td>41</td>
<td>400 mµ</td>
</tr>
<tr>
<td>Serum Albumin (bovine)</td>
<td>50.0</td>
<td>65 - 70</td>
<td>32</td>
<td>5.9</td>
<td>32</td>
<td>280 mµ</td>
</tr>
<tr>
<td>Barley Peroxidase</td>
<td>65.5</td>
<td></td>
<td>Estimated</td>
<td>7.6</td>
<td></td>
<td>Enzyme activity</td>
</tr>
</tbody>
</table>

* From gel chromatography experiments reported in this work.
described in Chapter II) to 25 ml with cold buffer solution.

The centrifuge tubes were filled after the aluminum caps were put on, the solution (horseradish peroxidase or barley peroxidase) being slowly introduced through the screw hole in the cap with a syringe. Centrifugation was carried out at 1-2°C. The filled centrifuge tubes were always placed in the same position in the rotor. The rotor was kept in the cold when not in use. Centrifugation runs were conducted for three hours and for five hours at 40,000 rpm. At the end of each run, the rotor was allowed to come to rest without brakes and also the refrigeration unit was turned off to minimize any turbulence due to vibrations.

After centrifugation, the contents of the centrifuge tubes were fractionated without disturbing the concentration gradient formed by the centrifugal field. The fractionation set-up is shown in Figure 7. In this method, the centrifuge tube was not removed from the rotor. The cap screw was removed and by means of a hypodermic needle, layers of the solution 0.5 mm thick were drawn from the top by applying very mild suction with a buret attachment to a syringe tube, which could be snapped off for transfer of solution, and replaced again for obtaining the next sample.

In Figure 7, "A" is the rotor, Beckman 40, which
Figure 7. Experimental set up for fractionation.

A = Rotor
B = Vernier Caliper
C = 2 ml Syringe Tube
D = 2 inch Hypodermic Needle
E = 50 ml Buret
F = Rubber Stopper
holds the centrifuge tube; "B" is a vernier caliper, the purpose of which is to enable one to remove a constant volume of the solution by plunging the hypodermic needle to a constant depth (0.5-1 mm) below the surface of the solution; "C" is a 2 ml syringe tube (without its plunger) which is attached to the hypodermic needle. The top of "C" is connected to a 50 ml buret "E" by 3 mm rubber tubing, 150 cm long; "D" is a 2 inch hypodermic needle attached to the central movable portion of the vernier caliper.

Procedure for a run was as follows. The stand which holds the vernier caliper was moved until the tip of the hypodermic needle was at the center of the screw hole of the centrifuge tube and parallel to its axis (the vernier caliper is attached to the stand, making an angle of 26° with the vertical). Next, the buret which was filled with water was adjusted to give a rate of flow, 1 drop in 2-3 seconds. In the meantime, the hypodermic needle was slowly lowered in the tube 0.5 mm at a time until liquid started to flow in "C". The end of suction was known from bubbling of air sucked through the liquid in "C".

To transfer the liquid fraction, "C" was removed from the hypodermic needle and transferred to the test tube while the suction was on. The suction was stopped by removing the rubber stopper "F" and the
collected solution was allowed to flow into the test tube. Final clinging droplets were forced out by a small hand blower.

The rubber stopper "$F$" was replaced on "$C$", which was replaced on the hypodermic needle. For the next aliquot, the plunger was lowered another 0.5-1 mm to draw the next aliquot and so on.

The volumes of the aliquots were determined by weighing. Before fractionation, the assay tubes were weighed to the nearest 0.001 g on an analytical balance. They were reweighed after transfer of solution; knowing the density of the solution and assuming it to remain constant, volume was calculated.

The assay tubes containing the aliquots were transferred to a rack immersed in ice water mixture and left there until fractionation was over. Peroxidase activities of the aliquots were measured, as explained in Chapter II.

Knowing the weight of the aliquot and its activity in absorbance units, the absorbance per gram of solution was calculated and a curve was drawn by plotting absorbance per gram values versus total weight of solution.

C. Results

The results of the gel chromatography experiments are summarized in Table III.
Figure 8 shows the elution pattern obtained for barley peroxidase using gel chromatography with a Sephadex G-100 column. The location of the maximum of the curve gives the value of $V_e = 65.5$ ml for the barley peroxidase. All the peroxidase components observed in electrophoretic experiments were eluted as a single peak and the resolution of different peaks was difficult.

Figure 9 shows the correlation of elution volume, $V_e$, and $\log D_{20}$, where $D_{20}$ is the diffusion coefficient of the protein of 20°C. The linear relationship obtained is in agreement with the previously derived equation [7]. At the value $V_e = 65.5$ ml, we read off the corresponding value of the diffusion coefficient of barley peroxidase $D_{20} = 7.60 \times 10^{-7}$ cm$^2$/sec.

The sedimentation studies were conducted to measure the sedimentation coefficients of both barley and horseradish peroxidase in the buffer solution.

The sedimentation rate was followed by plotting absorbance per gram of solution fraction versus cumulative weight* (the volume of the aliquots were determined from their weights). Curves similar to Figure 10 were obtained. Additional data points were taken to define the curve near the inflection point. The distance moved

* Cumulative weight was obtained by adding half the weight of the aliquot to the previous total weight of solution.
Figure 8. Elution pattern (obtained from the column of gel chromatography using Sephadex G 100) of barley peroxidase in 0.05 M phosphate buffer pH 6.1.
Figure 9. Plot of elution volume, $V_e$, against diffusion coefficient (on a logarithmic scale) for proteins on a Sephadex G-100 column at pH 6.1.
Figure 10. Plot of absorbance (enzyme activity) per gram of solution fraction versus the cumulative weight of fractions.
by the enzymes (the inflection point of the curve in Figure 10) perpendicular to the axis of rotation (See Figure 11) can be calculated by the relation:

\[ h = \frac{4W \sin \theta}{\pi d^2 \rho} \]

where

- \( h \) = the perpendicular distance moved by the enzyme,
- \( W \) = cumulative weight of solution in grams,
- \( d \) = diameter of the centrifuge tube \((d = 1.60 \text{ cm})\),
- \( \rho \) = density of solution which is 1.004 g/cm\(^3\), and
- \( \theta \) = the angle the axis of the centrifuge tube makes with the vertical, \( \theta = 26^\circ \).

The results obtained for horseradish peroxidase and barley peroxidase are summarized in Table IV for three and five hours of centrifugation at 40,000 rpm.

The perpendicular distance of point A from B is AC (See Figure 11),

\[ AC = AB \times \cos 26^\circ = 0.80 \times 0.900 = 0.72 \text{ cm} \]

AB is the radius of the centrifuge tube. See Figure 10. The perpendicular distance of A from the axis of rotation is

\[ 3.80 + 0.72 = 4.52 \text{ cm} \]

Sedimentation coefficients are calculated according to
Figure 11. Beckman ultracentrifuge rotor No. 40 Specifications (44).
TABLE IV: Results of Sedimentation Experiments

<table>
<thead>
<tr>
<th></th>
<th>Three hours</th>
<th>Five hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cumulative</td>
<td>Cumulative</td>
</tr>
<tr>
<td></td>
<td>Weight</td>
<td>Weight</td>
</tr>
<tr>
<td></td>
<td>at Inflection</td>
<td>at Inflection</td>
</tr>
<tr>
<td></td>
<td>Point</td>
<td>Point</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>horseradish peroxidase</td>
<td>1.160 g</td>
<td>1.975 g</td>
</tr>
<tr>
<td></td>
<td>0.252 cm</td>
<td>0.429 cm</td>
</tr>
<tr>
<td>barley peroxidase</td>
<td>1.085 g</td>
<td>1.830 g</td>
</tr>
<tr>
<td></td>
<td>0.235 cm</td>
<td>0.398 cm</td>
</tr>
</tbody>
</table>
the previously derived equation [9].

\[ S = \frac{1}{W^2(t_2 - t_1)} \times 2.303 \log \frac{r_b(t_2)}{r_b(t_1)} \]  

For the angular velocity in radians sec\(^{-1}\), we have

\[ 2\ T (rpm/60) = 2\ T (40,000/60). \]

For \(r_b(t_2)\) and \(r_b(t_1)\), the perpendicular distances of the boundary from the axis of rotation at times \(t_2\) and \(t_1\), respectively, we have

<table>
<thead>
<tr>
<th></th>
<th>(t_2 - t_1)</th>
<th>(r_b(t_2))</th>
<th>(r_b(t_1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horseradish</td>
<td>3-0 hours</td>
<td>(4.52 + 0.252) cm</td>
<td>4.52 cm</td>
</tr>
<tr>
<td>peroxidase</td>
<td>5-0 hours</td>
<td>(4.52 + 0.429) cm</td>
<td>4.52 cm</td>
</tr>
<tr>
<td></td>
<td>5-3 hours</td>
<td>(4.52 + 0.429) cm</td>
<td>(4.52 + 0.252) cm</td>
</tr>
<tr>
<td>Barley</td>
<td>3-0 hours</td>
<td>(4.52 + 0.235) cm</td>
<td>4.52 cm</td>
</tr>
<tr>
<td>peroxidase</td>
<td>5-0 hours</td>
<td>(4.52 + 0.398) cm</td>
<td>4.52 cm</td>
</tr>
<tr>
<td></td>
<td>5-3 hours</td>
<td>(4.52 + 0.398) cm</td>
<td>(4.52 + 0.235) cm</td>
</tr>
</tbody>
</table>

Using 5-3 hours values of \(r_b(t_2)\) and \(r_b(t_1)\), the sedimentation coefficient for horseradish peroxidase is
found to be $2.90 \times 10^{-13}$ sec, and that of barley peroxidase is $2.68 \times 10^{-13}$ sec.

Having found the experimental values of both $D$ and $S$, we are now in position to calculate the molecular weight of barley peroxidase by means of the Svedberg relation. From equations [8] and [6], we have

$$S = \frac{M(1-\bar{\nu}/\rho)}{Nf} \quad \text{and} \quad D = \frac{RT}{Nf}$$

Dividing one by the other gives

$$\frac{S}{D} = \frac{M(1-\bar{\nu}/\rho)}{RT}$$

which is the well-known Svedberg relation (29).

Assuming the partial specific volume of horseradish peroxidase is equal to the partial specific volume of barley peroxidase (both sedimentation and diffusion coefficients are close to each other), we can write:

$$\frac{S_{20,w}^{(HPR)}}{D_{20,w}^{(HPR)}} = \frac{M_{HPR}}{M_{BPR}}$$

where

$HPR = \text{Horseradish peroxidase}$

$BPR = \text{Barley peroxidase}$
The diffusion coefficients measured by gel chromatography are \( D_{20,w} \) values (in water at 20°C). The sedimentation coefficients measured by the above method are in the buffer solution (0.05 M phosphate buffer) at a temperature of 5-10°C. However, using equation [8] together with the Stokes (29) friction coefficient, it can be shown that

\[
S_{T,b} = \frac{(1-V/\rho)_{T,b}}{(1-V/\rho)_{20,w}} \eta_{20,w}^{T,b} \eta_{T,b} \eta_{20,w} S_{20,w}
\]

where \( S_{T,b} \) is the sedimentation coefficient in the buffer solution at temperature \( T \). Combining equations [10] and [11], we obtain

\[
\frac{S_{T,b}^{(HPR)}}{D_{20,w}^{(HPR)}} = \frac{S_{T,b}^{(BPR)}}{D_{20,w}^{(BPR)}} = \frac{M_{HPR}}{M_{BPR}}
\]

The sedimentation coefficients of both horseradish peroxidase and barley peroxidase were measured at the same temperature and in the same buffer solution.

Substituting our measured values of \( S_{T,b} \) (BPR), \( S_{T,b} \) (HPR) and \( D_{20,w} \) (BPR) together with the values of \( D_{20,w} \) (HPR) and \( M_{HPR} \) from Table III, we get
\[ M_{\text{BPR}} = 40,000 \times \frac{2.68}{7.60} \times \frac{7.05}{2.90} = 34,300 \]

D. Discussion

P. Andrews in his studies (21, 22) tried to evaluate the general validity of direct molecular weight determination of proteins using gel chromatography. The logarithm of molecular weight of proteins versus elution volume, \( V_e \), is graphed in Figure 1. It can be seen that a linear relationship holds only over the central portion of the curve. It has been found that highly asymmetric molecules and those containing carbohydrate deviate from the linear relationship (27).

From inspection of the Svedberg relation

\[ M = \frac{RTS_{20}}{D_{20} (1-\bar{\nu}/\rho)} \]

it can be seen that the molecular weight is dependent on three parameters, namely, sedimentation coefficient, diffusion coefficient and partial specific volume of the protein. However, the determination of molecular weight by either the density gradient centrifugation technique (43) or the gel chromatography method (21, 22) involves direct experimental measurement of only one of these parameters while estimating the other two indirectly on the basis of empirical methods. Combination of the two
experimental approaches yielding $S$ and $D$ allows a much more reliable estimation of molecular weight since the third parameter, partial specific volume, varies over quite a narrow range (0.71 - 0.75) for most proteins (24).

With some minor changes in the design of the model L ultracentrifuge, we believe that the above method should give accuracy comparable to that of the analytical ultracentrifuge. The following additions to the design of the model L ultracentrifuge will greatly simplify similar determinations:

(1) A system for locking the rotor after centrifugation, so as to be able to fractionate the contents of the centrifuge tube, without removing the rotor from the centrifuge.

(2) A cylindrical metallic centrifuge tube, with known dimensions, which can be calibrated with proteins of known sedimentation coefficients.

(3) Temperature-controlling and measuring assembly so as to set the desired temperature during a sedimentation experiment.
REFERENCES


